STUDIES ON ROUS SARCOMA VIRUS
REVERSE TRANSCRIPTASE BIOGENESIS

By

© HYO-SUNG RO, B.Sc.

A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree
Doctor of Philosophy

McMaster University
November 1986

STUDIES ON ROUS SARCOMA VIRUS REVERSE TRANSCRIPTASE BIOGENESIS

DOCTOR OF PHILOSOPHY (1986)
(Biochemistry)

McMaster University
Hamilton, Ontario

TITLE: Studies on Rous Sarcoma Virus Reverse-Transcriptase Biogenesis

AUTHOR: Hyo-Sung Ro, B.Sc. (McMaster University)

SUPERVISOR: Dr. Hara P. Ghosh (Chairman)

NUMBER OF PAGES: xix, 281

ABSTRACT

The purpose of this thesis was to investigate the biosynthesis and function of reverse transcriptase in relation to the assembly and maturation of RNA tumor viruses. The assembly and maturation of RNA tumor viruses were examined by biochemical characterization of a conditional lethal mutant of Rous sarcoma virus (RSV). The functional aspects of reverse transcriptase was investigated by recombinant DNA methods.

A temperature-sensitive coordinate mutant tsLA83 of Prague B (PR-B) strain of RSV at the nonpermissive temperature (41°C) produces noninfectious virus particles (NI-LA83) which contained only 3% of the reverse transcriptase activity present in infectious virions. Analyses of [^{35}S] methionine labveled NI-LA83 showed the presence of all of the viral proteins except reverse transcriptase. Pulse-chase analyses of the virus specified proteins in cells infected with LA83 or PR-B showed that the gag and glycoprotein precursors, $Pr76^{gag}$ and $gPr92^{env}$, respectively, were processed at both 35°C and 41°C. The reverse transcriptase precursor, $Pr180^{gag-pol}$, however, was not processed in LA83-infected cells at 41°C. In contrast, cells infected with LA83 or PR-B at 35°C as well as with PR-B at 41°C

showed normal cleavage of $Pr180^{gag-pol}$. A shiftdown of LA83-infected cells at 41°C to the permissive temperature, 35°C, resulted in the normal processing of $Pr180^{gag-pol}$ and production of infectious virus containing reverse transcriptase. Electron microscopic analysis showed that at 41°C cells infected with LA83 showed a large number of budding structures but fewer released particles. A shiftdown from 41 to 35°C resulted in an increase of virus particles with a concomitant decrease in budding structures suggesting that the processing of reverse transcriptase precursor is related to virion assembly.

The mechanism of synthesis of reverse transcriptase was investigated. Immunoprecipitation experiments have revealed possible intermediates in $Pr180^{gag-pol}$ processing. These are 130,000- and 150,000-dalton intracellular proteins ($Pr130^{gag-pol}$ and $Pr150^{gag-pol}$) that contain antigenic determinants of reverse transcriptase and gag proteins. $Pr130^{gag-pol}$ contains antigenic determinants of reverse-transcriptase and p15, $Pr150^{gag-pol}$ contains all the gag-proteins, but not the complete sequence of reverse transcriptase. Immuno-complexes of $Pr180^{gag-pol}$ molecules with anti-reverse transcriptase antiserum were tested for _in-vitro_ cleavage in the presence of detergent-disrupted virus (a source of p15, the viral protease). The results identified two possible cleavage intermediates (Pr130 and Pr70). A precursor-product relationship has been demonstrated between

$Pr180^{gag-pol}$ and $Pr130^{gag-pol}$, although a direct precursor-product relationship could not be demonstrated between $Pr180^{gag-pol}$ and $Pr150^{gag-pol}$. A possible biosynthetic scheme of reverse transcriptase was proposed.

In an attempt to establish the structural and functional relationships of the reverse transcriptase molecule, site directed mutagenesis on molecularly cloned RSV proviral DNA was undertaken. Expression of the reverse transcriptase gene in both bacteria and avian cell cultures was studied. E. coli harboring the recombinant plasmids containing proviral DNAs from different strains of RSV synthesized a protein of 65,000 molecular weight (p65) which is immunoprecipitated with anti-reverse transcriptase antiserum. The RNA dependent-DNA polymerase activities from E. coli containing the recombinant plasmids were about 30-fold higher than the background activity present in E. coli containing pBR322. The RNA dependent-DNA polymerase activity was specifically neutralized by the antibody against the reverse transcriptase. A possible translational initiation site was suggested to be an internal methionine residue, which is located at residue number 295 or 297 from the amino-terminus of the mature reverse transcriptase molecule. The amino-terminal one-third of the reverse-transcriptase molecule thus appears to be dispensable for the polymerase activity. The presence of a promoter-like sequence in the reverse transcriptase gene which is involved

in the transcription of the polymerase gene for synthesis of reverse transcriptase activity in _E. coli_ has also been suggested.

Biological activity of the site directed mutagenized viral DNAs were tested by transfection into chick embryo-fibroblast cells. Deletion near the 5'-end region of the pol gene resulted in inefficient production of virus due to possible functional defect in the truncated reverse transcriptase molecules of inefficient processing of the precursor molecules. However, the cells were transformed. Deletion near the 3'-end region of the pol gene resulted in inefficient production of virus and the cells were not transformed. These results suggest that the pp32 DNA binding protein, which is encoded from the 3'-end region of the pol gene, or the carboxy terminal moiety of the β subunit, or both, are required in the life cycle of avian retroviruses.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation and respect to my supervisor, Dr. Hara P. Ghosh, for his continuous support and guidance throughout the course of this work.

I thank the members of my supervisory committee, Dr. Phil Branton, Dr. Dennis McCalla, Dr. Lud Prevec, and Dr. Jim Smiley for helpful advice and criticisms.

I wish to thank Dr. Peter K. Vogt for gifts of the viruses. I am grateful to Dr. Hermann Oppermann, Dr. Takis Pappas, Dr. Danni Bolognesi, Dr. Duane Grandgenett, Dr. Bart Sefton, and Dr. R.C. Huebner for gifts of antisera, and Dr. R.V. Guntaka, Dr. J.T. Parsons, Dr. H.E. Varmus, Dr. E. Stavnezer, and Dr. M.B. Dworkin for providing recombinant plasmids.

I would also like to express my thanks to the members of the Ghosh laboratory, both past and present, for stimulating discussions and advice, and their companionship for making my stay a pleasant one.

I especially wish to thank my parents and sister for their prayers and encouragement, whose efforts, perhaps more than my own, have made the attainment of this goal a reality.

The financial support of the ontario Ministry of Colleges and University, and McMaster University is gratefully acknowledged.

TABLE OF CONTENTS

	Page
List of Figures	x
List of Tables	xv
List of Abbreviation	xvi
Introduction	1
Materials and Methods	36
Results:	79
Section 3.1: Biochemical Characterization of a Temperature Sensitive Mutant of Rous Sarcoma Virus Defective in Processing of the Reverse Transcriptase Precursor.	79
Section 3.2: Processing of Reverse Transcriptase Precursor.	155
Section 3.3: Expression of Reverse Transcriptase Gene in _E. coli_.	167
Discussion	238
Summary and Conclusions	260
Bibliography	266

LIST OF FIGURES

Figure		Page
1.1	The Life Cycle of Retroviruses.	2
1.2	Replication and Expression of Retrovirus Genes.	5
1.3	Structures of Genomic and Viral Specific mRNAs and Biogenesis of RSV Proteins.	9
1.4	Structure and Processing of the RSV gag Precursor Polyprotein.	11
1.5	Structure and Processing of the Precursor to ASLV Reverse Transcriptase.	19
1.6	Maturation of ASLV Envelope Glycoproteins.	22
3.1.1	Sucrose density gradient of [^{3}H] uridine-labeled particles of PR-B and LA83 grown at 35 and 41°C.	81
3.1.2	Electron microscopy of LA83-infected chick-embryo fibroblasts at the permissive and nonpermissive temperatures.	83
3.1.3	Autoradiogram of SDS-polyacrylamide gel containing electrophoretically separated proteins of purified viruses.	87
3.1.4	Autoradiogram of SDS-polyacrylamide gel containing electrophoretically separated reverse-transcriptase subunits.	87
3.1.5	Reverse transcriptase activity associated with purified NI-LA83 particles.	90
3.1.6	*In vitro* cleavage analyses of the high molecular weight proteins from NI-LA83 particles.	92
3.1.7	*In vitro* cleavage analyses of the high molecular weight proteins from LA83-infected cells.	92

Figure		Page
3.1.8	Tryptic-peptide analyses of the virion associated high molecular weight proteins.	96
3.1.9	Phosphoproteins of PR-B, LA83, and NI-LA83.	98
3.1.10	Glycoproteins of PR-B, LA83, and NI-LA83.	101
3.1.11	Agarose gel electrophoretic analysis of RNAs of NI-LA83, LA83, and PR-B.	103
3.1.12	Polyacrylamide gel analysis of [^{35}S] methionine labeled intracellular viral proteins immunoprecipitated with anti-RSV antiserum.	106
3.1.13	Polyacrylamide gel analysis of [^{35}S] methionine labeled intracellular viral proteins immunoprecipitated with anti-gp antiserum.	108
3.1.14	Polyacrylamide gel analysis of [^{35}S] methionine labeled intracellular viral proteins immunoprecipitated with anti-p27 antiserum.	110
3.1.15	Polyacrylamide gel analysis of [^{35}S] methionine labeled intracellular viral proteins immunoprecipitated with anti-pol antiserum.	112
3.1.16	Two-dimensional tryptic-peptide analyses of $Pr76^{gag}$ and the mature gag proteins.	115
3.1.17	Immunological characterization of Pr180.	117
3.1.18	Two-dimensional tryptic-peptide fingerprinting analyses of Pr180 with Pr76 and reverse-transcriptase subunits.	119
3.1.19	Two-dimensional tryptic-peptide fingerprinting analyses of Pr180 from LA83- and PR-B-infected cells.	122
3.1.20	Autoradiogram of intracellular phosphoproteins.	124
3.1.21	Polyacrylamide gel analyses of [^{35}S] methionine labeled $pp60^{src}$.	128

Figure		Page
3.1.22	The protein kinase activity induced by LA83.	132
3.1.23	Temperature-sensitivity of the protein kinase activity induced by LA83.	135
3.1.24	Polyacrylamide gel analyses of [^{35}S] methionine labeled viral proteins from pulse-chase experiments immunoprecipitated with anti-RSV antiserum.	138
3.1.25	Polyacrylamide gel analyses of [^{35}S] methionine labeled viral proteins from pulse-chase experiments immunoprecipitated with anti-gp antiserum.	140
3.1.26	Accumulation of Pr180 in LA83-infected cells at the nonpermissive temperature.	142
3.1.27	Disappearance of precursor molecules in RSV-infected cells.	144
3.1.28	Processing of Pr180 in LA83-infected cells after shiftdown to the permissive temperature.	148
3.1.29	Appearance of reverse transcriptase subunits in virus particles.	152
3.2.1	Immunological characterization of Pr130 and Pr70.	156
3.2.2	Two-dimensional tryptic-peptide fingerprinting analyses of Pr150.	159
3.2.3	*In vitro* processing of the reverse transcriptase precursors.	162
3.2.4	(A) The rate of disappearance of Pr180 after the *in vitro* cleavage. (B) The relative amounts of putative intermediates Pr130 and Pr70 and product p27 after the *in vitro* cleavage.	164

Figure		Page
3.2.5	Proposed structures of the precursor and intermediate molecules and proposed scheme for the processing of the precursor molecules to their mature reverse transcriptase.	166
3.3.1	Nucleotide sequence of the pol gene of PR-C-RSV and restriction map of PR-A-RSV DNA.	176
3.3.2	Schematic representation of RSV proviral DNA recombinant plasmids and proposed transcription strategy.	177
3.3.3	Autoradiogram of Dot Blot Assay with _E. coli_ RNAs.	178
3.3.4	Autoradiogram of SDS-PAGE of the gag-related proteins synthesized in _E. coli_.	181
3.3.5	Autoradiogram of SDS-PAGE of the pol-related proteins synthesized in _E. coli_.	184
3.3.6	Construction of a RSV proviral DNA recombinant plasmid with specific deletion at the pol gene.	187
3.3.7	Autoradiogram of two-dimensional gel electrophoresis analyses of the total proteins synthesized in _E. coli_.	189
3.3.8	Autoradiogram of two-dimensional gel electrophoresis analyses of the pol-related proteins synthesized in _E. coli_.	191
3.3.9	Nucleotide sequence of the putative initiation site in the pol gene.	194
3.3.10	Rate of degradation of [^{3}H] poly (rA):poly (dT) by RNase H present in _E. coli_ harboring pBR322 or pATV-8.	203
3.3.11	Agarose gel electrophoresis of the pSAL-102 mutants deleted at the Kpn I site of the pol gene.	204

Figure | Page

3.3.12 Restriction enzyme analyses of the pSAL-102 mutants deleted at the Kpn I site of the pol gene. 206

3.3.13A Subcloning of the polymerase gene into the expression vector pER103. 211

3.3.13B Restriction enzyme analyses of pERΔPOL. 209

3.3.14 Histogram of the polymerase activity induced in E. coli harboring pERΔPOL. 213

3.1.15 Autoradiogram of SDS-PAGE of pol-related proteins synthesized in E. coli harboring pER POL. 215

3.1.16 Subcloning of the pol-specific fragment into pBR322. 219

3.3.17 Comparison of the apparent promoter sequence in the pol gene with model promoter sequences. 221

3.3.18 Structures of the ^{32}P-labeled probe and the putative pol-transcripts from E. coli and RSV-infected cells. 224

3.3.19 Nuclease-S1 mapping of the pol-transcripts. 226

3.3.20 Polyacrylamide gel analyses of the viral proteins from transfected chick embryo fibroblast cells. 233

3.3.21 Polyacrylamide gel analyses of the intracellular viral proteins from transfected chick-embryo fibroblast cells. 235

LIST OF TABLES

Table		Page
I	Avian retrovirus precursor polyproteins and virion proteins.	13
II	Approximate half-life of RSV precursor polypeptides.	150
III	DNA polymerase activities of E. coli harboring different recombinant plasmids.	196
IV	Effect of ribonuclease on the DNA polymerase activity of E. coli cell lysates containing different RSV recombinant plasmids.	198
V	Characterization of the polymerase products.	200
VI	RNase H activity of E. coli harboring different recombinant plasmids.	201
VII	DNA polymerase activities of E. coli harboring pSAL-102 derivative mutants.	207
VIII	DNA polymerase activities of E. coli harboring pol-specific recombinant plasmids.	217
IX	Inhibition of DNA polymerase activity of pBRPOL-62 by the monospecific antibody to a synthetic peptide of the avian retrovirus pp32 and the β DNA polymerase subunit (anti-synthetic pol-peptide antiserum).	228

LIST OF ABBREVIATIONS

A	adenine or adenosine
A_{280nm} unit	An amount of material that has an absorbance of 1.0 at 280nm when dissolved in 1 ml water and measured with a 1 cm light path.
AEV	avian erythroblastosis virus
ASLV	avian sarcoma and leukemia virus
ASV	avian sarcoma virus
ATP	adenosine 5' triphosphate
bp	base pair
BSA	Bovine Serum Albumin
C	cytosine
CEF cells	Chick Embryo Fibroblase Cells
ChS	Chick serum
COOH	carboxyl-terminal
cpm	count per min.
CS	Calf serum
Da	dalton
dATP	deoxyadenosine 5' triphosphate
dCTP	deoxycytosine 5' triphosphate
DFBS	dialyzed fetal bovine serum
dGTP	deoxyguanosine 5' triphosphate
DMS	dimethylsulfate
DMSO	dimethyl sulfoxide

DNA	deoxyribonucleic acid
cDNA	complementary DNA
DNase	deoxyribonuclease
dT	deoxythymidine
DTT	dithiothreitol
<u>E. coli</u>	Escherichia coli
EDTA	ethylene diaminetetraacetic acid
EGTA	ethylene glycol bis (β-aminoethylether)-N,N,N',N' tetraacetic acid
FBS	fetal bovine serum
G	guanine or guanosine
GM	growth medium
GTP	guanosine 5' triphosphate
HEPES	N-2-hydroxyethyl peperazine-N'-2-ethane-sulfonic acid
HGD medium	high-glucose Dulbecco medium
HICS	heat-inactivated chicken serum
HZ	hydrazine
IPTG	isopropylthio-β-galactoside
kb	kilobase pair
KIU	KalliKrein Inactivating Units
LTR	long terminal repeat
MC29 virus	myelocytomatosis-29 virus
MEM	minimum essential medium
MLV	murine leukemia virus
MO-MLV	Moloney-murine leukemia virus
MMTV	murine mammary tumor virus

moi	multiplicity of infection
MSV	murine sarcoma virus
NBCS	new born calf serum
NH_2	amino-terminal
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
PPO	2,5-diphenyloxazole
rA	Adenosine
RBS	ribosome binding site
RNA	ribonucleic acid
RNase A	ribonuclease A
RNase H	ribonuclease specific for DNA:RNA hybrid
mRNA	messenger ribonucleic acid
tRNA	transfer ribonucleic acid
$tRNA^{Trp}$	transfer ribonucleic acid specific for tryptophan
RNP	ribonucleoprotein
RSV	Rous sarcoma virus
S	Svedberg unit (A sedimentation coefficient of 1×10^{-13} seconds)
SDS	sodium dodecyl sulfate
T	thymine or thymidine
$t_{\frac{1}{2}}$	half-life
TCA	trichloroacetic acid
TBR serum	tumor bearing rabbit serum

TEMED	N,N,N',N'-tetramethylethylene diamine
TLC	thin layer chromatography
TLE	thin layer electrophoresis
TPB	tryptose phosphate broth
TPCK	tolylsulfonyl phenylalanyl chloromethyl ketone
Tris	Tris (hydroxymethyl) aminomethane
ts	temperature-sensitive
U	uracil or uridine
VSV	vesicular stomatitis virus
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactose

INTRODUCTION

The history of RNA tumor viruses, or retroviruses, exemplifies how classical virology has developed into considerations of viral genetics and molecular biology, and that this change has been concomitant with a gradual shift of attention from fowl to rodent, to primate and human models. It also shows how a coordination of scientific efforts has brought us to the brink of understanding the role of retroviruses in human cancer.

A comprehensive review of retroviruses would exceed the scope of the thesis, and certain areas have received only a brief treatment. The introduction below describes the molecular structure and life cycle of Rous sarcoma virus, the prototype of retroviruses, as they pertain to biological phenomena.

RNA tumor viruses or retroviruses are RNA containing viruses which cause tumors in susceptible host animals and are also able to transform certain cells in tissue culture. Retroviruses contain RNA as their genomic material with sizes varying from 5 to 10 kilobases (kb) in length. The RNA virion exists as two identical RNA subunits with several viral coded structural proteins and at least one enzyme capable of copying the genomic RNA into DNA molecules ("reverse transcriptase").

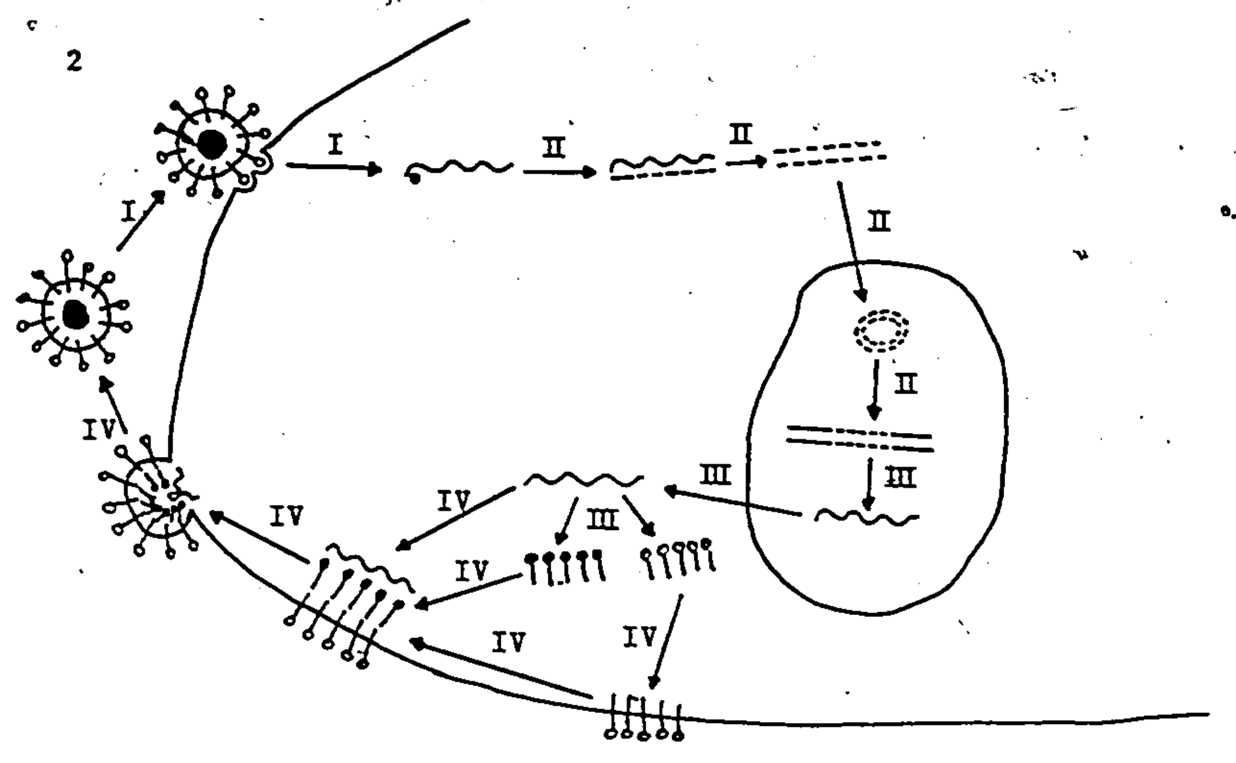


Figure 1.1 The Life Cycle of Retroviruses.

(I) Initiation of the infection; (II) replication and formation of the proviral DNA; (III) expression of viral genes; and (IV) assembly and maturation of viral particles. Symbols: ~~~, genomic or messenger RNAs; •, the virion associated reverse transcriptase; -----, intermediate DNAs, ═══, host chromosomal DNA; ⫯, the envelope glycoproteins; ⫯, the internal structural proteins. The primary polyproteins and the mature proteins are not distinguished.

I. First Stage - Infection

The life cycle of retroviruses can be grouped into four major stages (Fig. 1.1). First, infection is initiated by attachment of virus particles onto susceptible host cells by binding to their cellular receptors present on the cell surface. Retroviruses are enveloped particles about 90-100 nm in diameter with two virus-coded glycoproteins (gp37 and gp85) present on the viral membrane which are responsible for the recognition. They form as spikes on the surface of the virus particle; gp37 is anchored in the envelope membrane and gp85 forms as a knob which binds to gp37 through disulfide bonds (Bolognesi et al. 1972; Leamnson and Halpern 1976; Pauli et al. 1978; Montelaro et al. 1978). In the early stage of infection the envelope of the virus particle fuses with the cellular membrane, and the core particle consisting of the genomic RNA and the virion associated reverse transcriptase enters into the cytoplasm.

II. Second Stage - Replication of Retroviruses

In the second stage, a double-stranded DNA intermediate is synthesized by the virion associated reverse-transcriptase with the viral RNA genome as a template.

Initially, the first strand of DNA (termed the minus strand and complementary to the viral genome) is synthesized after initiation utilizing a specific tRNA molecule as a primer (Faras et al.. 1973). It was demonstrated that the synthesis of plus-strand DNA (the same polarity as the viral genome) is also initiated with a RNA primer (Smith et al. 1984). Reverse transcriptase contains both synthetic and degradative activities. The former activity includes RNA-dependent DNA polymerase and DNA-dependent DNA polymerase. The degradative activity is characterized by a DNA endonuclease activity that has been proposed to act during the integration of viral DNA into the host cell's genome (Golomb and Grandgenett 1979). It has the ability to degrade the RNA moiety of RNA-DNA hybrids (RNase H) and is thought to degrade genomic RNA after it has served as a template for the synthesis of the minus-strand DNA (Watson et al. 1979). Recently, it was demonstrated that the RNase H activity present in the reverse transcriptase molecule was responsible for the formation of a primer for the synthesis of positive-strands, and presumably the same activity is used for specific removal of the intact primer by cutting at the junction between the RNA and DNA moieties of newly synthesized positive-strand DNA (Champoux et al. 1984). It was indicated earlier that the RNase H activity can also remove the tRNA primer from the minus-strand DNA (Omer and Faras 1982).

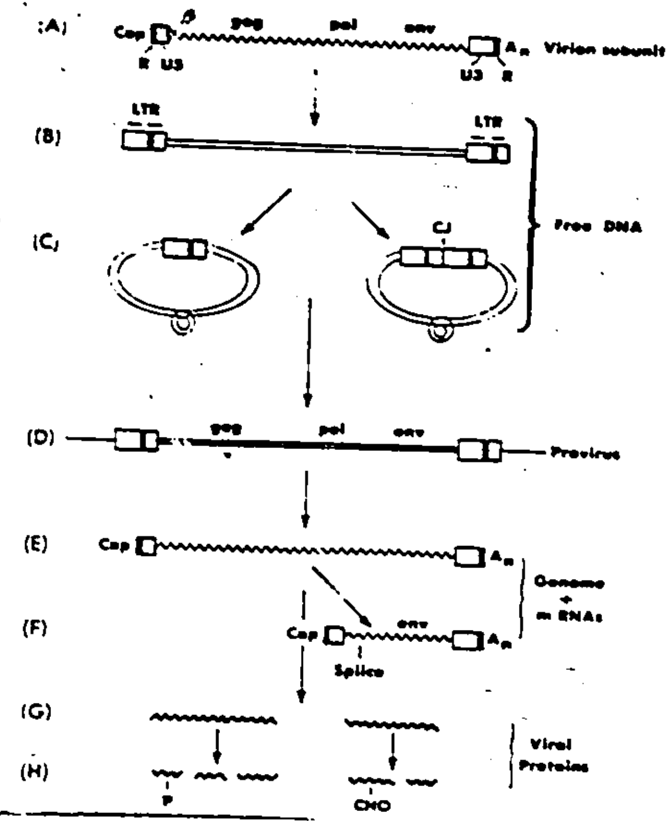


Figure 1.2 Replication and Expression of Retrovirus Genes.

(A) One of the two identical subunits of a typical, replication-competent viral RNA genome with its major structural and genetic features as illustrated in Fig. 1.3. (B) The primary product of reverse transcription, linear duplex DNA, with its long terminal repeats (LTRs) composed of U#, R, and U5. (C) The major forms of closed circular DNA, with one or two copies of the LTR. (D) The integrated form of viral DNA, the provirus. (E,F) Genomic and mRNAs, derived from the primary transcript by capping, polyadenylation, and (in the case of subgenomic mRNA) splicing; the site at which the 5' and 3' domains of subunit RNA are joined to form env mRNA is indicated in F. (G,H) The polyproteins synthesized from viral mRNAs and their mature products after cleavage and, in some cases, glycosylation (CHO) or phosphorylation (P). An infrequent splicing event, removing only a few nucleotides, is believed to generate an mRNA for the polyprotein precursor for reverse transcriptase; expression of the pol gene is not illustrated here. This figure is directly from Varmus and Levine (1983).

In infected cells, both linear and circular forms of double-stranded DNA intermediates were detected (Fig. 1.2). Initially, the viral DNA is found in the cytoplasm of the host cell and subsequently in the nucleus (Guntaka et al. 1976; Fritsch and Temin 1977; Shank et al. 1978a,b), where integration into host cell DNA occurs, resulting in the formation of the provirus (Khoury and Hanafusa 1976; Varmus et al. 1976; Battula and Temin 1978). Very little is known about the mechanism of the integration reaction. The reaction is efficient, in that viral DNA can become established in every cell of an infected population; and it is specific, in that recombination always occurs at a specific site on the viral genome, near the tips of the viral long terminal repeat (LTR) sequences (Hughes et al. 1978) so that the provirus is colinear with the genomic RNA. LTR sequences are generated from sequences at both ends of the viral RNA during DNA synthesis (Fig. 1.2). It was demonstrated that circular DNA with two tandem LTRs is a precursor to the provirus (Panganiban and Temin 1984). It was demonstrated earlier that the endonuclease associated with the reverse transcriptase molecule is responsible for selective cleavage in the LTR sequence (Duyk et al. 1983). More recently, the avian retrovirus pp32 protein, which possesses a DNA nicking activity (Grandgenett et al. 1978), was shown to be preferentially bound to the promoter region of LTR sequence (Knaus et al.

1984). The pp32 protein shares peptide sequences with the β subunit, but not the α subunit, of the avian retrovirus reverse transcriptase (Schiff and Grandgenett 1978). This protein is derived from the carboxyl terminus of the β subunit *in vivo* (Eisenman et al., 1980c; Copeland et al. 1980). The pp32 protein was shown to be bound to the most conserved region of avian retrovirus LTR sequences (Misra et al. 1982), thus leading to the suggestion that this protein may be involved in either replication, integration, or possibly transcription of viral DNA. In fact, the conserved LTR sequences of retrovirus DNA are apparently involved in the integration process (Shoemaker et al. 1980; Shimotohno et al. 1980; Swanstrom et al. 1981). It was more directly demonstrated recently that the terminal nucleotides at either end of the LTR sequences of the avian retrovirus are required for integration (Panganiban and Temin, 1983). The target site in the host DNA is locally random (Hughes et al. 1981), although regional specificity has not been ruled out.

III. Third Stage - Expression of Viral Genes

Productive infection by the retroviruses requires the expression of the integrated proviral DNA. Transcription of the proviral DNA by RNA polymerase II in host cells is a highly efficient event. Viral RNA transcribed from

between 1 and 20 copies of proviral DNA may comprise as much as 20 % of the polyadenylated RNA of infected cells (Weiss et al. 1982). At least three species of virus-specific mRNA molecules are produced in cells infected with non-defective avian sarcoma viruses (RSV) (Fig. 1.3): (i) a 3.3 kb (21S) RNA that serves as mRNA for $pp60^{src}$, the protein responsible for the mediation of cell transformation (Brugge et al. 1978); (ii) a 5.4 kb (28S) RNA that serves as mRNA for the precursor to the virion envelope proteins (Pawson et al. 1977, 1980); and (iii) a 9.3 kb (35S) RNA that serves as mRNA for the nonglycosylated viral structural protein precursor, $Pr76^{gag}$ (Von der Helm and Duesberg, 1975; Pawson et al. 1976). The structure of the mRNA for the precursor to the reverse transcriptase $Pr180^{gag-pol}$ has not yet been established, but information derived from the sequence of RSV (Prague C strain) genome RNA suggests the possibility that it is coded for by an approximately 9.3 kb RNA in which the termination codon for $Pr76^{gag}$ is removed by splicing (Schwartz et al. 1983). Beside these RNA species, additional 9.3 kb RNA molecules are packaged into virions as dimeric 70S complexes (Mangel et al. 1974). It is not yet clear whether the 9.3 kb RNA that is packaged is identical to the 9.3 kb mRNA.

RSV has been studied extensively as the prototype of the retroviruses. The major class of proteins in RSV

Figure 1.3 Structures of Genomic and Viral Specific mRNAs and Biogenesis of RSV Proteins.

Genomic RNA derived from the primary transcript by capping ($m^7G_{ppp}Gm$) at 5' end and polyadenylation (A_{200}) at 3' end. Four viral genes are located in the middle of the genome: gag, coding for internal structure proteins; pol, coding for reverse transcriptase; env, coding for the envelope glycoproteins; src, coding for a transforming-specific protein. Genome size viral mRNAs are responsible for the synthesis of the polyprotein precursor $Pr76^{gag}$. It is believed that synthesis of the gag-pol fusion protein could be achieved by occasional ribosomal frameshifting during translation. The subgenomic size mRNAs responsible for the synthesis of envelope glycoproteins precursor and the transforming protein are produced by splicing events. The leader sequences in the subgenomic mRNAs are not shown.

5′ $m^7G_{ppp}G_m$ gag pol env src c (A)$_{200}$ 3′

35S 5′ gag pol env src c (A)$_{200}$ 3′

→

↓

$Pr76^{gag}$

↓

P19 P10 P27 P12 P15

→

↓

$Pr180^{gag\text{-}pol}$

?↓

pol-β → α & pp32

28S 5′ env src c (A)$_{200}$ 3′

→

↓

$Pr60^{env}$

↓ glycosylation

$_{g}Pr95^{env}$

↓

gp85-s-s-gp37

21S 5′ src c (A)$_{200}$ 3′

→

↓

$pp60^{src}$

hp

Pr76^{gag} NH$_2$ p19 p10 p27 p12 ↓ p15 COOH

vp vp

Pr66 or Pr60 NH$_2$ p19 p10 ↓ p27 ↓ p12 p15 COOH Pr16

?

p23 NH$_2$ p19 ↓ p10 p27 p12 p15

p19 p10

Figure 1.4 Structure and Processing of the RSV gag Precursor Polyprotein.

Pr76^{gag} is cleaved to produce at least two primary intermediates (Pr66/Pr60 and Pr16) by a putative host-cell protease (hp). Pr16 is further processed to p15, a putative viral protease (vp), which acts on the other intermediates to generate the secondary intermediate (p23) and two mature gag proteins (p27 and p12). The p23 polypeptide is further processed by an unknown protease to generate p19 and p10. These cleavage events are intracellular, although low levels of p23 can be detected in virions. This figure is derived from Weiss et al. (1982).

consists of the five internal structural proteins p27, p19, p15, p12, and p10. They account for about 80 % of the total viral protein and form the internal structure that is detected in thin sections by electron microscopy. During assembly these polypeptides are processed proteolytically from a single large precursor polyprotein ($Pr76^{gag}$) that is encoded by the gag gene (Eisenman and Vogt, 1978). Several presumed cleavage intermediates were detected in this process (Fig. 1.4): Pr16, Pr60, Pr66 and Pr32. Apparently, Pr16, which is derived from the carboxyl terminal region of $Pr76^{gag}$ molecule, is the first cleavage intermediate to be processed to p15 protein. The other cleavage intermediates Pr60 and Pr66 are processed to the rest of the gag proteins (p19, p10, p27 and p12). The intermediate Pr32, which appears transiently during a chase period, is probably an alternative cleavage intermediate which may be processed to p19 protein (the amino-terminal portion of $Pr76^{gag}$ molecule). In mature virions, p19 is associated with viral envelope (Montelaro et al. 1978; Pepinsky and Vogt, 1979); p10 is within the space between the membrane and core (Pepinsky and Vogt, 1983); p27 forms the core shell (Bolognesi et al. 1973; Stromberg et al. 1974); and p12 is within the core associated with viral RNA in a ribonucleoprotein complex (Bolognesi et al. 1973; Davis and Rueckert, 1972). The location of p15 is not clearly known (Table I).

Table I. Avian retrovirus precursor polyproteins and virion proteins.

Protein	Gene	Function	Localization
Pr76gag	gag	precursor to virion internal structural proteins	cell cytoplasm, probably associated with inner surface of plasma membrane
Pr66gag, Pr60gag	gag	presumed intermediates in Pr76gag cleavage	probably associated with plasma membrane
p27gag	gag	subunits of the core shell	virion core
p19gag pp19gag	gag	may be involved in RNA processing and packaging	associated with genomic RNA in the core, also found outside core structure, possibly bound to lipid
p15gag	gag	protease involved in cleavage of gag-protein precursors; may also cleave the β subunit of reverse transcriptase to generate α	between the virion core and the inner envelope
p12gag	gag	may be involved in virion RNA packaging and folding	RNP complex within virion core
p10gag	gag	unknown	possibly virion membrane
p23gag	gag	unknown	unknown
Pr180gag-pol	pol	precursor to reverse transcriptase	cell cytoplasm, probably associated with the plasma membrane
Pr130gag-pol	pol	probably an intermediate in Pr180 processing	probably associated with plasma membrane
αβ Subunits	pol	transcription of genomic RNA	virion core
p32pol	pol	unknown	virion core
P63env	env	aminoterminal signal sequence allows insertion of nascent polypeptide into endoplasmic reticulum	probably associated as a nascent chain with the rough endoplasmic reticulum
P57env	env	polypeptide backbone of env-precursor polyprotein	rough endoplasmic reticulum
Pr90env	env	precursor to envelope glycoproteins gp85 and gp37	cell and virion membranes
gp85env	env	host range; neutralization; interference; subgroup specificity	virion envelope; knob structure directly associated with gp37
gp37env	env	may anchor gp85 to membrane	virion envelope; spike structure directly associated with membrane and gp85

This table is directly from Weiss et al. (1982).

In addition to acting as a matrix protein, p19 interacts with genomic RNA. A small fraction of the ca. 2,000 p19 molecules is reported to be associated with genomic RNA in virions (Sen and Todaro, 1977). _In vitro_, purified p19 binds with high affinity to its genomic RNA but not to other types of RNA (Leis et al. 1978). These observations have led to models in which p19 regulates diverse activities such as splicing (Leis et al. 1980), RNA packaging (Darlix and Spahr, 1982), and translation (Dalix and Spahr, 1982). Thus p19 is potentially a key component in gene expression and assembly of the virus. More recently, it was demonstrated that the viral protein p12 is by far the major protein linked to the RNA after ultra violet irradiation of the virus (Meric et al. 1984) and not protein p19 as reported by Sen and Todaro (1977). Even though p12 is an abundant protein in the virion, little is known about its biological role in virus replication. The polynucleotide-binding properties of the p12 protein were analysed and it was found that the protein preferentially binds to single-stranded polynucleotides. The intack p12 protein exists in two RNA-binding states such that conversion from one state to the other is regulated by its degree of phosphorylation, and this regulation may be responsible for its release from the viral RNA during reverse transcription after viral infection of cells (Leis and Jentoft, 1983).

It was postulated that the role of the p15 protein in the life cycle of RSV was to process the polyprotein precursor $Pr76^{gag}$, in which p15 possesses a proteolytic activity specific for gag proteins (Von der Helm 1977; Dittmar and Moelling 1978; Vogt et al. 1979). Defective viruses with a deletion in the p15 coding region have indicated that the absence of p15 from the polyprotein precursor $Pr76^{gag}$ contributes to their inability to be processed, and in temperature sensitive mutant viruses infected cells, $Pr76^{gag}$ cleaves aberrantly and produces abnormal virions at the nonpermissive temperature (Bister et al. 1977; Hayman et al. 1979; Rettenmier et al. 1979a; Eisenman et al. 1980b; Rohrschneider et al. 1976).

Two possible mechanisms could be considered for the activity of p15 in the processing of $Pr76^{gag}$. First, p15 is cleaved from $Pr76^{gag}$ by an autoproteolysis reaction such as has been previously reported in other virus systems (Aliperti and Schlesinger 1978; Pelham 1978, 1979). This postulation contradicts the observations that $Pr76^{gag}$ synthesized in an in vitro cell-free translational system remain stable indefinitely unless exogenous p15 is added (Von der Helm 1977). Furthermore $Pr76^{gag}$ molecules synthesized in RSV-transformed mammalian cells do not cleave to generate virion proteins or viral particles (Eisenman et al. 1975). An alternative mechanism would be the possible

involvement of a host-specific protease. This possibility was demonstrated by the fusion experiment in which $Pr76^{gag}$ was cleaved when the RSV-transformed mammalian cell lines were fused with permissive chick cells (Eisenman et al. 1975); however, one can not rule out the possibility that specific compartmentation and conformation of $Pr76^{gag}$ molecules are necessary in order to achieve proper processing.

Reverse transcriptase is encoded by the pol gene and synthesized in RSV-infected cells as a polyprotein ($Pr180^{gag-pol}$) containing the polymerase and the precursor of the gag structural proteins $Pr76^{gag}$ (Fig. 1.3) (Opperman et al. 1977; Paterson et al. 1977; Hayman 1978; Rettenmier et al. 1979b). The β subunit of reverse transcriptase is formed by cleavage of $Pr180^{gag-pol}$ (Eisenman et al. 1980a; Ro and Ghosh 1984). The mechanism of synthesis of $Pr180^{gag-pol}$ is suggested to involve a spliced mRNA species (Berget et al. 1977; Chow et al. 1980) which contains a small deletion near the gag and pol junction sequences. The nucleotide sequence analysis of RSV indicates that the coding sequences for gag and pol are on different reading frames, and suggests that splicing may occur in such a way that the reading frame for the gag coding sequence is continued to the reading frame coding for pol gene products; furthermore, consensus RNA splicing sequences appear to bracket the region just preceding the carboxyl terminus

of $Pr76^{gag}$ and the amino terminus of reverse transcriptase (Schwartz et al. 1983). The cDNA sequence data also confirmed the amino terminal amino acid sequence of reverse transcriptase (Copeland et al. 1980). These observations suggest the possible existence of two populations of similar sized mRNA species. The detection of this putative spliced mRNA coding for $Pr180^{gag-pol}$ would be difficult due to its low abundance in infected cells and its similar size to the mRNA coding for $Pr76^{gag}$. An alternative mechanism for the synthesis of $Pr180^{gag-pol}$ is the frameshifting model. Synthesis of the gag-pol fusion protein could be achieved by occasional ribosomal frameshifting during translation. The frameshifting model was supported by recent evidence (Jacks and Varmus 1985). Synthesis of both gag protein and gag-pol fusion protein was successfully achieved in a cell-free rabbit reticulocyte translation system programmed by a single RNA species that was synthesized from cloned RSV DNA with a bacteriophage RNA polymerase. Furthermore, the ratio of two protein products was similar to that previously observed in infected cells. These results strongly favor the frameshifting model over the RNA splicing event.

The mature reverse transcriptase isolated from purified viral particles consists of two polypeptide chains - the α and β subunits, which have molecular weights of

68,000 and 91,000, respectively (Gibson and Verma 1974; Rho et al. 1975). The α subunit is formed from the β subunit by proteolytic cleavage, which releases a 32,000-dalton protein, pp32 (Gibson and Verma 1974; Rho et al. 1975). The major molecular heterodimer species of α and β subunits possesses all the enzymatic activities, namely, RNA-dependent and DNA-dependent DNA polymerase, RNase H and DNA endonuclease. Polymerase and RNase H activities are also present in the α subunit and the β chain (Kacian et al. 1971; Verma 1977). Endonuclease activity is also associated with the pp32 protein but not with the α subunit (Golomb and Grandgenett 1979; Schiff and Grandgenett 1978). This suggests that the active site for the endonuclease activity is in the carboxyl-terminal domain of the β subunits as defined by pp32.

The exact mechanism by which the mature reverse-transcriptase is formed in infected cells is not completely known. It is clear, however, the processing of $Pr180^{gag-pol}$ occurs concomitantly or follows immediately after the virus release, since no mature reverse transcriptase molecules were detected inside the cells (Eisenman et al. 1980a; Ro and Ghosh 1984). Eisenman et al. (1980a) have observed a reverse transcriptase-related protein with molecular weight of 130,000 in RSV infected cells ($Pr130^{gag-pol}$).

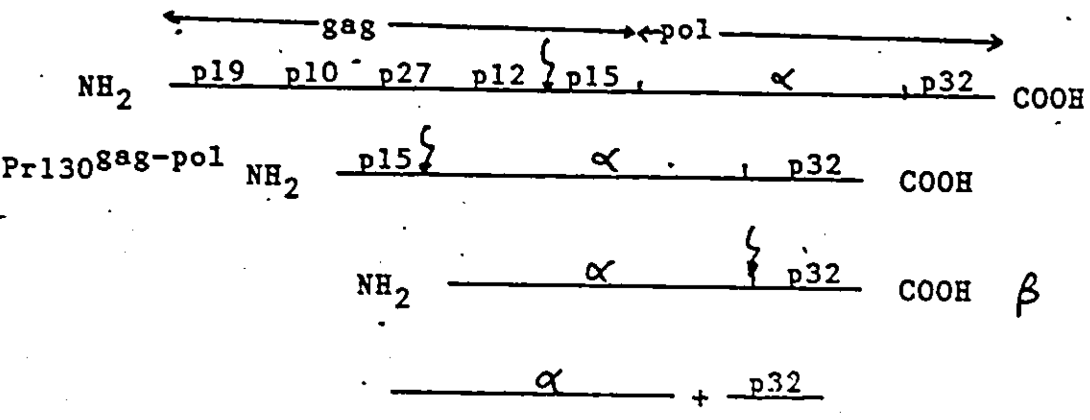


Figure 1.5 Structure and Processing of the Precursor to ASLV Reverse Transcriptase.

$Pr180^{gag-pol}$, the 180,000-dalton product of the gag and pol genes, is first cleaved within the gag polypeptide region between p12 and p15. This cleavage generates $Pr130^{gag-pol}$, which is further processed by removal of the p15 segment to generate the β subunit of reverse-transcriptase. Removal of the carboxyterminal region of β results in production of the α subunit and the p32 endonuclease. Cleavage of Pr180 and Pr130 occurs within the infected cell, whereas cleavage of $\beta \longrightarrow \alpha$ + p32 occurs within the virion. This figure is reproduced directly from Weiss et al. (1982).

$Pr130^{gag-pol}$ contains the antigenic determinant for p15 and reverse transcriptase sequences; furthermore, the peptide make-up of $Pr130^{gag-pol}$ was established by tryptic peptide mapping and was shown to possess peptides of both p15 and reverse transcriptase. However, they were not able to demonstrate unequivocally whether $Pr130^{gag-pol}$ is a cleavage product of $Pr180^{gag-pol}$ or a primary translation product. In spite of this, they postulated a plausible processing scheme, which assumed $Pr130^{gag-pol}$ as the cleavage intermediate from $Pr180^{gag-pol}$ (Fig. 1.5).

The structural proteins of RSV include two viral glycoproteins, gp85 and gp37 (for nomenclature see August et al. 1974). Earlier studies suggested that gp37 is anchored in the lipid membrane whereas gp85 is attached to the virus mainly through disulphide bonding to gp37 and can be released by treatment of intact virus with reducing agents (Bolognesi et al. 1972; Leamnson and Halpern 1976; Pauli et al. 1978; Montelaro et al. 1978). The membrane localization of gp37 could be confirmed from the amino acid sequence of the viral glycoproteins derived from the nucleic acid sequence (Schwartz et al. 1983; Hunter et al. 1983a).

The env gene of RSV which codes for the two viral glycoproteins gives rise to a glycosylated precursor poly-

protein, $gPr92^{env}$, which contains the polypeptides of both gp85 and gp37 (England et al. 1977; Moelling and Hayami 1977). The env gene product contains 16 glycosylation sites (Schwartz et al. 1983; Hunter et al. 1983a). Processing involves, in addition to proteolytic cleavage, conversion of the high-mannose oligosaccharides (MW 2200) found in $gPr92^{env}$ to larger (MW > 3000), complex, sialidated oligosaccharides which make up the bulk of the oligosaccharides found in gp85 and gp37 (Bosch et al. 1982; Hunt and wright 1981). $gPr92^{env}$ is the major glycoprotein species found in the infected cells (Buchhagen and Hanafusa 1978; Bosch et al. 1982), and processing to mature glycoprotein occurs intracellularly, most likely in internal smooth membranes and not at the cell surface (Hayman 1978; Bosch et al. 1982).

The unglycosylated primary env-gene product has been detected by _in vitro_ cell-free translation and in infected cells treated with a glycosylation-inhibitor. The primary env-gene product was synthesized in RSV-infected cells which are treated with tunicamycin or 2-deoxyglucose, inhibitors of initial glycosylation events. Its molecular weight was estimated to be 57,000 ($P57^{env}$), and it contains antigenic determinants and tryptic peptide patterns of the envelope glycoproteins (Diggelmann 1979; Stohrer and Hunter 1979). It was well established previously that

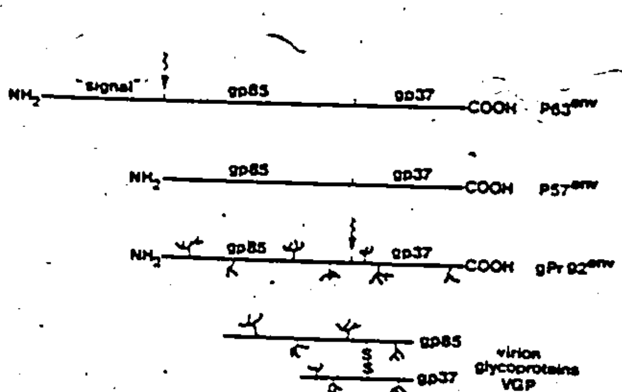


Figure 1.6 Maturation of ASLV Envelope Glycoproteins.

The polypeptide designated P63^{env} is believed to be that synthesized by cell-free translation of env mRNA. Its <u>in vivo</u> counterpart is probably cleaved as a nascent chain after insertion into the endoplasmic reticulum. Cleavage at the site indicated by the arrow would remove the 64-amino-acid hydrophobic "signal" sequence. P57^{env} is the proposed structure of a protein observed <u>in vivo</u> after treatment of infected cells with inhibitors of glycosylation. Although the signal peptide is believed to have been cleaved from P57^{env}, suggesting membrane insertion, further processing does not appear to occur. In pulse-labeled ASLV-infected cells, gPr92^{env} is readily detected and is presumed to be derived from P63^{env} after membrane insertion, removal of the signal sequence, and glycosylation. Cleavage, disulfide-bond formation, and further glycosylation of gPr92^{env} generate the viral glycoproteins (VGP). This figure is reproduced directly from Weiss et al. (1982).

secretory and membrane proteins are synthesized initially with a leader sequence or signal sequence, attached to the amino-terminal end of nascent chain (Milstein et al. 1972; Blobel and Dobberstein 1975). The signal sequence is cleaved shortly after passing through the endoplasmic reticulum. The possible existence of a signal sequence in the primary env-gene product was tested by in vitro cell-free translation experiments. A precursor polypeptide ($P63^{env}$) was immunoprecipitated with anti-env antibody from the in vitro reaction mixture and it was believed to contain the signal sequence. The polypeptide $P57^{env}$ could then be generated in vivo from $P63^{env}$ by the cleavage of the signal sequence (Pawson et al. 1977, 1980; Purchio et al. 1977). The presence of a signal sequence was later confirmed by the nucleotide and amino acid sequence analyses, in which a putative signal sequence coding region was present that just preceded the start of the mature gp85 amino acid sequence (Hunter et al. 1983a; Schwartz et al. 1983). The possible processing of the ASLV glycoprotein is summarized in Figure 1.6.

IV. Fourth Stage - Assembly and Maturation of RSV

Many enveloped viruses leave the infected cells by budding from the plasma membrane. The available evidence

indicate that the formation of a core structure by assembly of viral components near the budding site occurs prior to release of virus particles. The intracellular processes involved in the correct assembly of viral components is not clearly known. It was suggested that the processing of viral polyproteins and the interactions between viral components are necessary for, or trigger, viral assembly and budding. These interactions may also determine the structure and stability of the virus particles. Much supporting evidence was presented for the importance of proper synthesis and processing of gag-related polyproteins in the production of mature virus particles (Eisenman et al. 1975, 1978; Rohrschneider et al. 1976; Bister et al. 1977; Hayman et al. 1979). Virus maturation was also affected in cells infected with a temperature-sensitive mutant defective in processing of the reverse transcriptase precursor polyprotein ($Pr180^{gag-pol}$) at the nonpermissive temperature (Rø and Ghosh 1984). The viral production was reduced by 70% and the released viruses were noninfectious. These results suggest that the processing of reverse transcriptase precursor is related to virion assembly.

Several studies of the interactions of viral components indicated that p19 is a membrane associated protein and interacts with gp37 (Montelaro et al. 1978; Pepinsky and Vogt 1979; Pepinsky et al. 1980; Gebhardt et al. 1984),

lipid (Pepinsky and Vogt 1979; Gebhardt et al. 1984), and the viral RNA (Darlix and Spahr 1982; Gebhardt et al. 1984). These close associations with the components, which are utilized in virus formation, strongly indicates that p19 plays an important role, not only in determining the structure and stability of the virus particles, but in virus assembly, maturation and budding. As mentioned earlier, p19 is found at the amino-terminus of $Pr76^{gag}$, the precursor for the gag proteins. A model concerning the mechanisms of virus assembly and budding was postulated (Bolognesi et al. 1978; Gebhardt et al. 1984). The primary triggering mechanism is the association of p19, as part of the precursor, to the budding site at the membrane where the viral glycoproteins are concentrated. The p19 polypeptide then associates with the viral RNA and trigger its packaging at the site of assembly. Bolognesi et al. (1978) have postulated that gag and possibly also gag-pol polyproteins are organized under the plasma membrane, and are subsequently cleaved to individual structural proteins which are organized in concentric shells. It seems that the cleavage may occur just prior to budding and the mature proteins localize and interact with specific components to become properly assembled molecules. The polypeptide p19, as part of the precursor, could act as a carrier for the other gag proteins to the budding site. It is interesting to note that a mutant with a lesion in p19 is defective in virus assembly

(Rohrschneider et al. 1976). However, it is not clear how glycoproteins are involved in virus assembly. Virus particles lacking glycoprotein are produced from cells treated with tunicamycin (Diggelmann 1979; Stohrer and Hunter 1979). Furthermore, virus particles lacking reverse-transcriptase (Hanafusa et al. 1972; Eisenman et al. 1980a; Ramsay and Hayman 1980; Ro and Ghosh 1984) or genomic RNA (Levin et al. 1974; Linial et al. 1978) were also obtained from cells infected with defective virus. It should now be possible using DNA recombinant techniques and site-directed mutagenesis to modify specific regions of the viral proteins to investigate their role in viral assembly.

Viral genomic RNAs, transcribed from the integrated provirus DNAs by cellular polymerase II, are specifically packaged into a virus particle. This specific packaging of the genomic RNA, presumably through the specific interaction between viral proteins and viral genomic RNAs, occurs at the cell surface (Weiss et al. 1982; Gebhardt et al. 1984). The sequences involved in the dimer linkage, by which two viral genomes are incorporated into a virion is suggested to be present near the 5' end of the genome (Weiss et al. 1982).

Although the mechanisms of replication of the viral genome has been extensively studied, the mechanism of packaging of the viral genome into virions is not clearly under-

stood. Structural analyses of proviruses of a certain class of mutant viruses were useful in elucidating the possible mechanism of specific packaging of viral genome (Linial et al. 1978; Shank and Linial 1980; Watanabe and Temin 1982; Mann et al. 1983; Sorge et al. 1983; Kawai and Koyama 1984; Koyama et al. 1984). In RSV, as well as in murine leukemia virus (MLV) and reticuloendotheliosis virus, the restriction endonuclease map analyses of the proviruses suggested that the recognition site for packaging may be located near the 5' end of the genomic RNA. In RSV, a second packaging locus was reported to be present in the 115-nucleotide direct repeat that flanks the transforming gene, src (Sorge et al. 1983).

The selective packaging of genomic RNA against the subgenomic mRNAs may be explained by the mechanism of the specific recognition of the signal sequence. In spleen necrosis virus and MLV, the sequence near the 5'-end of the genomes that are necessary for selective packaging are removed from subgenomic mRNAs by splicing; thus, mRNAs of these viruses are not packaged into virions. In contrast, the 5' donor splice site is located within the gag-coding region in RSV (Swanstrom et al. 1982). The evidence supporting this notion was presented recently, in which the precursors for gag and env gene structural proteins were shown to contain a common amino-terminal sequence (Ficht et al.

1984). Therefore, it seems likely that some sequence in the gag-coding region beyond the donor splice site is also involved in selective packaging of the genomic RNA.

Scope of This Work

In the last decade, many avian retrovirus, as well as a small number of murine virus mutants have been isolated. In the early years, the research effort was a mere characterization of the mutants based on a physiological description. As our knowledge of the molecular biology of RNA tumor virus infection progressed, the mutants became more and more useful as tools to determine whether proteins were virus coded, as well as to demonstrate the biological consequence of biochemical changes.

One type of mutant, the conditional lethal mutant, has been particularly useful in biochemical studies, since one can easily manipulate its phenotype by controlling some environmental factor such as temperature.

In general, temperature-sensitive (ts) mutants of retroviruses have permissive temperatures of 31-32°C (mammalian viruses) and 35-37°C (avian viruses) and nonpermissive temperatures of 37-40°C and 40-42°C, respectively.

Conditional and nonconditional mutants of RSV are available for several viral functions. The mutants could

be grouped into three classes: (I) transformation defective mutant or T class, (II) replication defective mutant or R class and (III) coordinate mutant or C class (Vogt, 1977).

The T class mutants contain a lesion in the src gene. The conditional ts mutants belonging to this class can replicate at 41°C but can not transform cells at 41°C. The non-conditional T class td mutnats (trnasformation defective), however, can not transform cells because of partial or complete deletion of src gene. Non-transforming viruses, such as, leukosis or Rous associated viruses (RAV), also have the src gene deleted.

The R class mutants have a lesion in either gag or env gene. The conditional ts mutants can not replicate at the nonpermissive temperature but can transform cells at 41°C. The non-conditional mutants are generally deletion mutants.

The conditional ts, C class mutants can not replicate or transform cells at the nonpermissive temperature and contain lesions in the pol gene. C class mutants can also be generated by mutations in both R funcitons (gag or env gene) and T function (src gene).

The majority of the ts replication mutants of RSV belong to the C class with lesions in the pol gene and

these mutants ;have made important contributions to our understanding of RNA-dependent DNA-polymerase (reverse-transcriptase) structure and function.

The first ts polymerase mutants identified were LA335 and LA337 (Linial and Mason, 1973; Wyke, 1973). These are coordinate mutants which have lesions in the reverse transcriptase gene. Both the mutants have defects in early functions. The viruses lose both transforming and replicating abilities after 8 hours if infection has been initiated at 41°C, and if shifted down to 35°C and maintained at 35°C for 10-15 hours the virus no longer shows the temperature sensitivity with respect to transformation and replication. Both the virions and the reverse transcriptase in LA335 and LA337 are heat labile (Verma 1975). All three enzymatic activities, RNA-dependent and DNA-dependent polymerase and RNase H, are heat labile (Verma 1975). The rate of thermal inactivation suggests that the polymerase and the RNase H activities are located at different sites (Verma 1975). Both of these enzymatic activities are located in the α subunit which is also thermolabile in both LA335 and LA337. Mason et al. (1979) have isolated six additional pol mutants, four of which are like LA337 (PH543, PH553, PH568, and PH620).

Studies to distinguish the defective functions in the various polymerase mutants are in their infancy, but some interesting results have been obtained. The Mn^{++}-dependent DNA endonuclease activity associated with αβ polymerase (Golomb and Grandgenett, 1979) was examined in four ts pol mutants with similar biological phenotypes: LA335, LA337, PH553, and PH568 (Golomb et al., 1981). It was found that the endonuclease activities of LA335 and LA337 were more thermolabile than that of wild type, whereas the activities in PH553 and PH568 were as stable as that of wild type. This correlates well with the fact that LA335 and LA337 appear to map at the same site, whereas PH553 and PH568 contain a different mutation. These results also show that the Mn^{++}-dependent endonuclease activity is a virus coded function, but they do not identify a function for the endonuclease in the virus life cycle. It should be pointed out that all ts pol mutants that have been tested for RNase H activity are ts for both RNase H activity and polymerase activity and that no mutants have been isolated that are temperature-sensitive for RNase H activity alone. In addition, no ts pol mutants in DNA endonuclease activity alone have been isolated. Recently, virus mutants possessing deletions in the pp32 region have demonstrated that this region encodes function(s) essential

for replication of the virus (Hippenmeyer and Grandgenett, 1984). Two separate point mutations generated near the amino-terminus of pp32 resulted in decreased viral replication and cell transformation due to reduced synthesis of the viral RNA from the integrated provirus (Hippenmeyer and Grandgenett, 1985). It appears that transcription is affected by aberrant integration. Similar results were obtained previously by Donehower and Varmus (1984) that a single base change in the 3' region of Moloney murine leukemia virus pol decreased the level of viral DNA integration 10-fold and that most of the proviruses had integrated aberrantly. These results indicate that a viral coded endonuclease activity is required for proper integration of the DNA.

Analysis of two mammalian retrovirus mutants, Rauscher-MLV ts24 (Stephenson and Aaronson, 1973) and Mo-MLV ts3 (Wong et al. 1973), indicates that the reverse-transcriptase is enzymatically inactive in its $Pr180^{gag-pol}$ precursor form and is activated only after cleavage to p80. At the nonpermissive temperature, these mutants fail to cleave $Pr180^{gag-pol}$. Cleavage apparently occurs after virion release, as polymerase activity is found only in virions released after shifting to the permissive temperature cells infected by these mutants (Witte and Baltimore,

1978). These experiments lend credence to the idea that $Pr180^{gag-pol}$ is the actual precursor that is cleaved to mature polymerase. A ts mutant that does not cleave polymerase precursors has not been found in the avian retrovirus system.

Previous studies carried out in this laboratory (Ghosh, 1984) with RSV-conditional lethal mutant LA83 showed that it was defective in both replication and transformation functions. At the nonpermissive temperature LA83 produces non-infectious particles lacking reverse-transcriptase activity. It is apparent that the synthesis of the reverse transcriptase at the nonpermissive temperature is defective in LA83.

The mechanism of the synthesis and processing of active reverse transcriptase has not been studied. The mutant LA83 offers an excellent opportunity to study these two important aspects of reverse transcriptase metabolism. In addition one can also study the interrelationship between precursor polyproteins cleavage and virion assembly.

This thesis describes investigations into reverse-transcriptase biogenesis by two distinct approaches. Broadly stated, these approaches are first by biochemical characterization of a conditional mutant of RSV; second, by using the recombinant DNA methods to express the pol

gene in _E. coli_ to attempt to identify the functional domains of the pol gene and functions involve in the virus life cycle. The specific questions or objectives which these approaches have attempted to answer are as follows:

1) The mechanism of biosynthesis of reverse transcriptase.
2) The interrelationship between the precursor polyprotein cleavage and virion maturation.
3) Expression of the reverse transcriptase gene in _E. coli_.
4) Role of the various domains of reverse transcriptase in the virus life cycle.

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and Reagents

Polybrene and dimethylsulfate (DMS) were obtained from Aldrich Chemical Co. Methyl mercury hydroxide was obtained from Alfa Products, Thiokol/Ventron Division. Ammonium persulfate, dimethyl sulfoxide (DMSO), formaldehyde, formamide, glycerol, NP-40, sodium dodecyl sulphate (SDS), and Na-ethylenediaminetetraacetic acid (EDTA) were purchased from BDH Chemicals Ltd. Acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), ampholines, glycine, Triton X-100 and urea were purchased from Bio-Rad Laboratories. Agarose, isopropylthio-β-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) were obtained from Bethesda Research Laboratories. Poly (dT) and oligo (dT):poly (rA) were purchased from Collaborative Research, Inc. Sodium deoxycholate and dithiothreitol (DTT) were obtained from Calbiochem. Bacto-tryptone, Bactoagar, yeast extract, and tryptose phosphate broth (TPB) were from Difco Laboratories. Hydrazine (HZ) and X-ray films were purchased from Eastman Kodak. Piperidine was purchased fromFisher Scientific. Dulbecco's modified MEM, α-MEM, F-11 MEM, 199 medium, high glucose-Dulbecco medium, Joklik modified MEM, fetal bovine serum

(FBS), new born calf serum (NBCS), calf serum (CS), dialyzed fetal bovine serum (DFBS), chick serum (ChS), penicillin/streptomycin, trypsin, and fungizone were purchased from Gibco. Trasylol (Aprotinin) was purchased from Boehringer Mannheim (Canada) Ltd. Polygram silica N-HR thin layer plates were purchased from Brinkman Instruments Canada, Ltd. Gene Screen and Enhancer were from New England Nuclear Corp. Protein A-Sepharose was purchased from Pharmacia Fine chemicals. dATP, dCTP, dGTP, TTP, and ATP were purchased from P.L. Biochemicals. Nitrocellulose membrane was obtained from Schleicher and Schuell. Bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), ethylene glycol bis (β-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA), ampicillin, tetracycline, chloramphenicol, ethidium bromide, 3-β-indole acrylic acid were purchased from Sigma Chemicals. Soybean trypsin inhibitor was from Worthington.

2.2.1 Enzymes

The restriction enzymes, T4 DNA ligase, T4 DNA-polymerase, T4 polynucleotide kinase, and *E. coli* DNA-polymerase I (Klenow fragment) were obtained from Bethesda-Research Laboratories or Boehringer Mannheim Ltd. Proteinase K was from Boehringer Mannheim Ltd. Micrococcal nuclease was purchased from P.L. Biochemicals. Ribonuclease A was

from Sigma Chemicals or Boehringer Mannheim Ltd. TPCK-treated trypsin, Lysozyme, and deoxyribonuclease were obtained from Worthington. Phospholipase A_2 was obtained from Dr. G.E. Gerber, Department of Biochemistry, McMaster University.

2.1.3 Biochemicals

[^{3}H] Glucosamine, [^{3}H] mannose, [^{3}H] palmitic acid, [^{3}H] TTP, [^{3}H] uridine, and [^{32}P] orthophosphate were obtained from New England Nuclear Corporation. [^{35}S]-Methionine was purchased from New England Nuclear Corp. or Amersham corporation. [α-^{32}P] ATP, [α-^{32}P] CTP, [γ-^{32}P] ATP, and poly [8-^{3}H] adenylic acid were purchased from Amersham Corp. [^{3}H] diazirinophenoxy (DAP) undecanoate (160 mCi/mmole) was provided by Dr. G.E. Gerber and P. Leblanc, Department of Biochemistry, McMaster University.

2.1.4 Antisera

Anti-RSV serum was prepared by injecting rabbits with detergent-disrupted proteins from gradient-purified PR-B. Anti-reverse transcriptase antiserum (anti-pol) was a gift from Dr. H. Oppermann and Dr. T. Pappas. Anti-p27, anti-p19, anti-p15, anti-p12, and anti-gp85 antisera were generously provided by Dr. D. Bolognesi, Department of Surgery, Surgical Virology Laboratory, Duke University

Medical Center, Durham, North Carolina. Anti-pol, anti-pp32, and anti-synthetic pp32 peptide antisera were gifts from Dr. D.P. Grandgenett, Institute for Molecular Virology, St. Louis University Medical Center, St. Louis, Missouri. Tumor Bearing Rabbit (TBR) serum was obtained from Dr. P. Branton, Department of Pathology, McMaster University. Anti-src (carboxy peptide) antiserum was a gift from Dr. B.M. Sefton, Tumor Virology Laboratory, The Salk Institute, San Diego, California. Anti-β-galactosidase antiserum was a gift from Dr. R.C. Huebner, Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Mass.

2.1.5 Cells

Primary chick embryo fibroblasts (CEF) were prepapred from 9- to 11-day-old embryos (Hy-Line) of the C/O, $chf^{-}gs^{-}$ phenotype following the procedure of Vogt (1967). Primary cells were grown at 35°C in 199 medium or high-glucose-Dulbecco (HGD) medium supplemented with 10 % TPB, 5 % CS, and 2 % heat-inactivated chicken serum (HICS). Secondary cells were grown at 35°C in growth medium (GM) containing 199 medium or HGD medium supplemented with 10 % TPB and 5 % CS. All culture media were supplemented with 1 % penicillin/streptomycin, 1 % fungizone, 2 % L-glutamin, and 2 % of a 7.5 % $NaHCO_3$ solution. All monolayer cultures were grown in the presence of 5 % CO_2.

E. coli LE392 [F^-, hsd R514 (r_k^-, m_k^-), supE44, supS58. LacYI, or Δ(lacIZY)6, galK2, galT22, metB1, trpR55, X^-] was obtained from Dr. F.L. Graham, Department of Biology, McMaster University. E. coli HB101 [F^-, hasS20 (r_B^-, m_B^-), recA13, ara-14, proA2, lacY1, galK2, rpsL20 (Sm^r), xyl-5, mtl-1, supE44, X^-] was obtained from Dr. J. Smiley, Department of Pathology, McMaster University. E. coli JM103 [Δ(lac pro), thi, strA, supE, endA, sbcB, hsdR$^-$, F'traD36, proAB, lacI^q, z M15] was obtained from Dr. C.B. Harley, Department of Biochemistry, McMaster University.

2.1.6 Viruses

Wild-type viruses were the Prague strain of Rous-sarcoma virus subgroups A (PR-A), B (PR-B) and C (PR-C), and Schmidt-Ruppin strain of RSV subgroup D (SR-D). The temperature-sensitive mutant LA83 was isolated from PR-B that was grown in the presence of 5-azacytidine (Toyoshima and Vogt, 1969b). The permissive temperature for LA83 is 35^oC, while the nonpermissive temperature is 41^oC. All viruses, both wild type and mutant were obtained from Dr. P.K. Vogt, University of Southern California School of Medicine, Los Angeles, California and purified by cloning before growing the stock virus.

Plaque purified vesicular stomatitis virus (VSV) was of the Indiana serotype, strain HR-LT, originally obtained from Dr. L. Prevec, Department of Biology,

McMaster University.

All virus stock clultures were stored at -90°C.

2.1.7 Plasmids

pBR322, a cloning vector was provided by Dr. W.C. Leung, Department of Pathology, McMaster University. pUC8 and pUC9, pBR322 derivatives containing Lac Z gene with the multicloning sites were provided by Dr. Y. Gluzman, Cold Spring Harbor Laboratories, Cold Spring Harbor, New York. pATV08, pBR322 derivative containing complete proviral DNA of PR-C was provided by Dr. R.V. Guntaka, Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York. pSAL-102, pBR322 derivative containing complete proviral DNA of PR-A was provided by Dr. J.T. Parsons, Department of Microbiology, University of Virginia School of Medicine, Charlottesville, Virginia. pSRA-2, pBR322 derivative containing complete proviral DNA of SR-A was provided by Dr. H.E. Varmus, Department of Microbiology and Immunology, University of California, San Francisco, California. pB5^{gag}, pB5^{pol}, and pB5^{env} are pBR322 derivatives containing gag-specific-, pol-specific-, or env-specific-proviral DNA of PR-A, respectively. These plasmids were provided by Dr. E. Stavnezer, Genetic and Molecular Biology Unit, Graduate School, Sloan-Kettering Institute for Cancer Research, New York. pER103, a prokaryotic expression vector

containing a promoter-operator sequence derived from the trp operon of Serratia marcescens with a potent artificial ribosome binding site (RBS) sequence was provided by Dr. M.B. Dworkin, Department of Biological Sciences, Sherman Fairchild Center, columbia University, New York.

2.2 Methods

2.2.1 Virus Infection

When cells were to be infected with RSV, polybrene was added at a final concentration of 2 μg/ml to facilitate viral adsorption (Toyoshima and Vogt, 1969a). Virus was added to secondary cultures within 3 hours of replating. For the preparation of virus 1 % DMSO was added to the medium 1 day postinfection to increase virus yield and to aid cell monolayer stability. When the infected cells were confluently transformed they were subcultured and incubated either at 35°C or 41°C.

2.2.2 Virus Infectivity

Viral infectivity was determined as focus forming units per ml (FFU/ml) by the focus assay procedure. 60 mm culture plates were seeded with 5×10^5 cells from primary CEF in regular growth medium containing polybrene at a

final concentration of 2 μg/ml. The monolayers were infected with 0.1 ml of an appropriate dilution of RSV serially diluted with PBS (138 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.46 mM KH_2PO_4; Dulbecco and Vogt, 1954). After overnight (12-18 hrs) incubation at 35°C, the monolayers were overlaid with 5 ml of 199 medium containing 10 % TPB, 2 % CS, 1 % Beef embryo extract, 0.25 % DMSO, and 0.9 % agar. After the agar solidified, the plates were incubated at 35°C or 41°C for 5 days in a 5 % CO_2, 95 % air atmosphere. After the incubation period, the cells were overlaid again with 2 to 3 ml of the overlay medium. Foci were checked after 3 days or the plates were further incubated for 2 to 3 days after addition of 1 to 2 ml of the overlay medium.

2.2.3 Virus Purification

When the infected cells were confluently transformed, the medium was collected and clarified by centrifugation at 3600 rpm for 30 min at 4°C. The virus was pelleted from the supernatant by centrifugation in Beckman 50.2 Ti rotor at 48,000 rpm for 1 hour at 4°C and suspended in standard buffer (STE - 0.01 M Tris-HCl, pH 7.4, 0.1 M NaCl, 0.001 M EDTA). The virus suspension was sonicated for 3 to 5 seconds before being pelleted through 25 % (w/v) sucrose onto a 40 % (w/v) potassium tartrate cushion, in

a Beckman SW41 rotor at 36,000 rpm for 2 hours at 4°C. The virus at the interface was collected, diluted with STE, and pelleted by centrifugation in a type 65 rotor (Beckman) at 48,000 rpm for 1 hour at 4°C. In some cases, the labeled viral particles were purified directly from the clarified harvested media by pelleting through 25 % sucrose in a Beckman SW41 rotor at 40,000 rpm for 2 hours at 4°C.

2.2.4 Radioisotopic Labeling of Viral Particles

2.2.4.1 Labeling of Virus with Radioactive Amino-Acids, Sugars, or Orthophosphate.

Viral proteins were labeled by incubating confluently transformed cells (PR-B-infected cells at 35 or at 41°C and LA83-infected cells at 35°C) or LA83-infected nontransformed cells at 41°C with radioactive amino acids, sugars, or orthophosphate. After removal of growth medium, 2 ml of respective labeling medium containing 5 % DFBS and the corresponding radioactive amino acids, sugars, or orthophosphate were added to each plate. The plates were incubated either at 35 or 41°C for 5 hours, then an additional 3 ml of the labeling medium containing 5 % DFBS was added to each plate. After 4 hours incubation, 5 ml of growth medium was added. Finally, the medium was harvested after

14 hours and the viruses were purified as described above. An aliquot of labeled virus was spotted on a Whatman 3 MM filter paper and the sample was precipitated with 10 % TCA. The filter paper was boiled in 5 % TCA for 5 min and then washed with two changes of ice cold 5 % TCA over a period of 20 min. The filter paper was rinsed with 95 % ethanol: ether (1:1. v/v) followed by washing with ether. After drying in a fume hood, the filter paper was placed in a precounted scintillation vial containing 10 ml of a PPO/ toluene based scintillation fluid (4 gm of diphenyloxyzole in 1 liter of toluene). The radioactivity was determined in a scintillation counter.

2.2.4.2 Phosphate Labeling.

Viral proteins were labeled with [^{32}P] phosphate as previously described (Shaikh et al. 1979). Briefly, infected cells were starved for phosphate by 12 hours incubation in phosphate-deficient 199 medium with 5 % DFBS. Cells were then labeled with 250 μCi/ml of [^{32}P] orthophosphate in the above medium for 12 hours, then supplemented with 5 ml of growth medium and the medium was harvested after 12 hours. Supernant medium was clarified and the virus purified. Virus was disrupted in 0.02 M Tris-HCl, pH7.5, 0.05 M NaCl, and 0.1 % NP-40. RNA was hydrolyzed by incubation at 37°C for 2 hours with 20-30 μg of RNase A.

Phospholipids were extracted with 5 vol of ether. The solution was diluted to 1 ml with the lysis buffer and the viral proteins were immunoprecipitated using anti-gag antiserum and protein A-Sepharose.

2.2.4.3 Labeling of Virus with [^{3}H] Uridine.

Viral genomic RNAs were labeled by incubating confluently transformed or nontransformed cells with [^{3}H] uridine (250 µCi/plate) in 2 ml of F-11 MEM supplemented with 2 % CS and 1 % DMSO. After 1 hour incubation at respective temperature, an additional 3 ml of the labeling medium was added and incubated further 11 hours at the respective temperature. The medium was removed and 5 ml of fresh GM was added and further incubated for 12 hours. The combined media were clarified and the labeled viruses purified as described above. The virus pellets were suspended in a buffer containing 1 mM EDTA, 0.5 % SDS, 2.0 A_{260} units of wheat germ tRNA, and 100 µg/ml of Proteinase K in 10 mM Tris-HCl, pH 7.5. The suspensions were incubated at room temperature for 20 min, then extracted with phenol:chloroform (1:1, v/v). The aqueous layers were precipitated with ethanol and the RNAs were suspended in sterile water.

2.2.5 Radioisotopic Labeling of Virus-Infected CEF Cells

Viral proteins were labeled with either [^{35}S] methionine (100 µCi/ml) or [^{32}P] orthophosphate (1 mCi/ml) by incubating confluently transformed or nontransformed cells in 199 medium lacking either methionine or phosphate. In chase experiments, the labeled medium was removed and replaced with normal growth medium. In some experiments, cultures were shifted to the permissive temperature in growth medium containing 50 µg of cycloheximide per ml (Witte and Baltimore, 1978). After incubation, the cells were washed three times with cold STE buffer and the cells were lysed with 1 ml of cold RIPA buffer (50 mM Tris-HCl, pH7.2, 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS, 1 % sodium deoxycholate) containing 100 Kallikrein inactivator units of Trasylol. Lysed cells were scraped with a rubber policeman and collected into an Eppendorf tube and incubated for 30 min in an ice bath with occasional mixing. Cell lysates were centrifuged in an Eppendorf centrifuge for 15 min at 4°C to yield a solubilized fraction.

2.2.6 Radioisotopic Labeling of *E. coli*

The inocula were grown in LB (Luria-Bertani) medium (Per liter: 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g NaCl) containing the appropriate antibiotic, then diluted into 1:100 and grown for about 2.5 hours at 37°C with vigorous shaking until the OD_{600} of the culture was approximately 0.4. Chloramphenicol was added to the culture to a final concentration of 200 µg/ml. The cultures were incubated at 37°C with vigorous shaking for a further 12-16 hours. The *E. coli* cells (5 ml) were pelleted and washed with prewarmed M9 medium (Per liter: 6 g Na_2HPO_4-H_2O, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 1 ml of 1 M $MgSO_4$, and 10 ml of 0.01 M $CaCl_2$). The cells were resuspended in 1 ml of M9 medium supplemented with 0.2 % glucose, 1 µg/ml thiamine, 20 µg/ml standard amino acids (except methionine, which was 2 µg/ml), 20 µg/ml ampicillin, and 20 µCi/ml [^{35}S] methionine. The cells were labeled for 30 min at 37°C and pelleted and resuspended in 10 mM Tris-HCl, pH 7.2, 1 mM EDTA and transferred into Eppendorf tubes and centrifuged for 30 seconds. Total cell extracts were prepared by boiling in 0.5 ml of a buffer containing

150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 100 KIU/ml Trasylol, and 1 mM PMSF in 10 mM Tris-HCl, pH 7.2 and lysozyme to a final concentration of 1 mg/ml. The soluble fractions were collected and immunoprecipitation carried out as described below.

In the case of E. coli containing recombinant plasmids with trp promoter, the overnight E. coli cultures were diluted into 1:100 in M9 medium supplemented with 0.2% glucose, 1 mM nonessential amino acids, 100 μg/ml ampicillin and grown until $A_{660} = 0.1$, then 3β-indole acrylic acid (20 μg/ml) was added to derepress the trp promoter (Hallewell and Emtage, 1980), and then grown a further 1.5 hours. The cells were labeled with [^{35}S] methionine (8 μCi/ml) for 30 min. Total cell extracts were prepared and the soluble cell lysates were immunoprecipitated as described above.

2.2.7 Growth of Staphylococcus aureus and Preparation for Use in Immunoprecipitation

Cultures of Staphylococcus aureus, strain Cowan I were grown in Penassay broth supplemented with 5 mg/ml casitone, 2.5 mg/ml yeast extract, 2.5 mg/ml β-glycerophosphate, 4 μg/ml niacin, and 2 μg/ml thiamine-HCl. Seven hundred milliliters of broth in 2-liter standard Erlenmeyer flasks received inocula from

5 ml broth cultures growth over the previous night at 37°C. The large cultures were then incubated for 16 hours at 37°C in shaker.

Bacteria were collected and washed twice by centrifugation at 3000 rpm for 20 min in PBS containing 0.05 % (w/v) sodium azide. After resuspension to approximately a 10 % (w/v) concentration in PBS-azide, the cells were stirred at 23°C for 1.5 hours in the presence of 1.5 % formalin, washed, and again resuspended to the same concentration in buffer without formalin. They were then added to a large Erlenmeyer flask to a depth of less than 1.5 cm and killed by rapid swirling in an 80°C water bath for 5 min, followed by rapid cooling in an ice-water bath. After two more washes in PBS-azide, the concentration was then set at 10 % (w/v). The bacteria were stored at 4°C. A day before use the bacteria were pelleted by centrifugation at 2000 g for 20 min and incubated in 0.5 % NP-40 in NET (150 mM NaCl, 5 mM EDTA, 50 mM Tris, and 0.02 % sodium azide) buffer, pH 7.4, for 15 min at 23°C. They were then washed once in 0.05 % NP-40 NET buffer and finally resuspended to the volume of the original 10 % suspension in the latter buffer containing 1 mg/ml ovalbumin (Kessler, 1975).

2.2.8 Immunoprecipitation

To an aliquot of the solubilized fraction 5 to 10 µl of the antiserum against RSV or monospecific antisera

were added, and the reaction was incubated for 30 min at 4°C with continuous mixing. The antigen-antibody complex was specifically adsorbed by the addition of either 0.2 ml of a 10 % (v/v) suspension of protein A covalently coupled to Sepharose beads (protein A-Sepharose) or 0.2 ml of a 10 % (w/v) suspension of inactivated Staphylococcus aureus and incubation was continued further for 2.5 hours at 4°C. The antigen-antibody-protein A complex was washed three times with RIPA buffer and twice with TBS buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl) containing 100 KIU of Trasylol, and the precipitated proteins were released into solution by addition of either lysis buffer A [9.5 M urea, 2 % (w/v) NP-40, 5 % 2-mercaptoethanol and 2 % ampholine (comprised of 1.6 % pH range 5 to 7 and 0.4 % pH range 3 to 10, O'Farrell, 1975] for twoOdimensional gel electrophoresis or electrophoresis sample buffer [0.0625 M Tris-HCl (pH 6.8), 3 % SDS, 10 % glycerol, 5 % 2-mercaptoethanol] and subsequent heating in a boiling water bath for 3 min for SDS-polyacrylamide gel electrophoresis.

2.2.9 Assay for Protein Kinase

Immunoprecipitates were used in phosphotransfer reactions. An immunoprecipitation with absorbed TBR serum was performed as described above. The immune-complex was suspended in 50 µl of kinase buffer (20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$) after the washing procedure. The mixture was

preincubated at 35°C or 41°C for 2 min, then [γ-^{32}P] ATP (10 µCi, 3000 Ci/mmole) was added, and the reaction mixture was incubated for 2 min at the respective temperatures. The assay was terminated by addition of 1 vol of cold stop buffer (20 mM EDTA, 4 mM ATP) and 10 vol of TS buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 % Trasylol). The immune-complex was spun down and washed once with buffer containing 25 mM Tris-HCl (pH 6.8) and 1 % Trasylol, then suspended in gel electrophoresis sample buffer. The mixture was boiled (100°C, 3 min), the protein A-Sepharose beads were removed by centrifugation, and the sample was applied to a 10 % polyacrylamide slab gel.

2.2.10 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Discontinuous polyacrylamide slab gels were prepared by the method of Laemmli (1970). Separating gels of the required acrylamide concentration were cast from a filtered stock solution of acrylamide (30:0.8, acrylamide: bis acrylamide, w/w). In addition, the separating gel contained 0.375 M Tris-HCl (pH 8.8), 0.1 % glycerol and 0.025 % TEMED. The solution was degassed prior to the addition of freshly prepared ammonium persulphate to 0.075 % and SDS to 0.1 %.

The stacking gel consisted of 5 % acrylamide, 0.125 M Tris-HCl (pH 6.8), 0.1 % SDS, 0.05 % glycerol, 0.075 % TEMED

and 0.064 % ammonium persulphate. The electrophoresis running buffer was Tris-glycine (pH 8.3) buffer (Per liter: 3.02 gm Tris base, 14.4 gm glycine, and 1 gm SDS). After electrophoresis, the slab gels were soaked in destaining solution (7 % acetic acid, 40 % methanol) for 30 min, then treated for fluorography (Laskey and Mills, 1975). Gels were treated in 100 % DMSO by two 30 min incubations in room temperature. Gels were then treated for 3 hours with a solution of 22.2 % PPO in DMSO (w/v). The PPO was precipitated by washing the gel in water for 1 hour and dried on a sheet of filter paper in vacuo before exposure to Kodak X-Omatic, XAR-5 or XRP-1 X-ray film at -70°C for the appropriate length of time. Quantitation of specific protein bands was obtained by scanning autoradiographs in a Joyce-Loebl microdensitometer.

2.2.11 Two-Dimensional Gel Electrophoresis

2.2.11.1 First Dimension.

Isoelectric focusing gels were made in glass tubing (130 X 2.5 mm inside diameter) sealed at the bottom with Parafilm. The gel mixture (total 10 ml) contained 5.5 gm of urea, 1.33 ml of acrylamide stock solution [30 % acrylamide: 28.38 % (w/v) acrylamide and 1.62 % bis acrylamide], 2 ml of 10 % (w/v) NP-40, 2 % ampholines (1.97 ml of H_2O + 0.4 ml of ampholines, pH range 5 to 7 + 0.1 ml of pH 3 to 10). When the urea was completely dissolved, 10 µl of 10 % ammonium

persulfate was added and the solution was degassed under vacuum for about 1 min. Immediately after addition of 7 µl of TEMED, the solution was loaded into the gel tubes. The gel was overlaid with 8 M urea and after 1 to 2 hours replaced with 20 µl of lysis buffer A overlaid with a small amount of water. The gels were allowed to set for 1 to 2 hours. The gels were then placed in a standard tube gel electrophoresis chamber. The lysis buffer A and water were removed from the surface of the gel and 20 µl of fresh lysis buffer A were added. The tubes were then filled with 0.02 M NaOH. The lower reservoir was filled with 0.01 M H_3PO_4 and the upper reservoir was filled with 0.02 M NaOH which was extensively degassed to remove CO_2. The gels were then prerun according to the following schedule: (a) 200 volts for 15 min; (b) 300 volts for 30 min; (c) 400 volts for 30 min. The upper reservoir was emptied, lysis buffer and NaOH were removed from the surface of the gels, and the samples were loaded and overlayed with 10 µl of sample overlay solution [9M urea, 1 % ampholines (comprised of 0.8 % pH range 5 to 7 and 0.2 % pH range 3 to 10)] then 0.02 M NaOH, and the chamber was refilled. The gels were run at 400 volts for 12 hours and then at 600 to 700 volts for 1.5 hours.

To remove the gels from the tubes, a 5 ml syringe was connected to the tube via a short piece of Tygon tubing, and the gel was slowly forced out by pressure on

the syringe.

The gels were equilibrated in SDS sample buffer [10% (w/v) glycerol, 5% (w/v) 2-mercaptoethanol, 2.3% (w/v) SDS, and 0.0625 M Tris-HCl, pH 6.8] by shaking at room temperature for 2 hours.

2.2.11.2 Second Dimension.

The cylindrical isoelectric focusing gel was loaded onto a discontinuous polyacrylamide slab gel by using a 1% agarose gel solution [1% (w/v) in SDS sample buffer] to keep the gel in place and to avoid mixing as the protein zones migrate out of the cylinder into the slab. The cylindrical gel was placed on a piece of Parafilm, straightened out, and placed close and parallel to one edge of the Parafilm. One milliliter of the melted agarose solution (held at about 80°C) was put in the notch, then the Parafilm was used to transfer quickly the cylindrical gel into this solution.

Appropriate amount of radioactive molecular weight standard samples were mixed with the 1% agarose solution and cylindrical gels were formed with the glass tubing. The gels were removed as described above and cut into about 1 cm length and loaded onto the slab gel at the end of the cylindrical gel. About 5 min were allowed for the agarose to set, then ran at 20 mA constant current until the dye front reaches the bottom of the gel. After electrophoresis, the gels were treated as described above.

2.2.12 Agarose Gel Electrophoretic Fractionation of Genomic RNAs

2.2.12.1 Electrophoresis of RNA through Gels Containing Formaldehyde (Lehrach et al., 1977; Goldberg, 1980).

The gel was prepared by melting agarose in water, cooling to 60°C, and adding 5X gel buffer [0.2 M morpholinopropane-sulfonic acid (MOPS) (pH 7.0), 50 mM sodium acetate, 5 mM EDTA (pH 8.0)] and formaldehyde to give 1 X and 2.2 M final concentrations, respectively. The agarose concentration was usually 1 %. The solution was poured onto a 16 x 20 cm glass plate contained in a horizontal gel apparatus. A sample well comb was placed at one end of the molten gel (close to the -ve electrode), and the gel was allowed to solidify for 30-45 min. After the gel had solidified, it was overlaid with the gel buffer and the comb was removed and samples loaded into the preformed wells. The buffer chambers were filled with the gel buffer until the buffer contacted the edges of the gel. The gels were run at 1.5 volts/cm for 12-16 hours. The buffer was recirculated to avoid generating a pH gradient in the gel.

Samples were prepared by mixing 5 µl of RNA and 20 µl of sample buffer (50 % formamide and 25 % formaldehyde in the gel buffer) and heated at 60°C for 5 min, then added 2.5 µl of 10X dye Ficoll mixture (1 % Ficoll, 0.005 %

bromophenol blue, 0.1 % SDS).

2.2.12.2 Electrophoresis of RNA through Gels Containing Methylmercuric Hydroxide (Bailey and Davidson, 1976).

The gel was prepared by melting agarose (1 %) in gel-running buffer (50 mM boric acid, 5 mM sodium borate, 10 mM sodium sulfate) as described above.

Samples were prepared by mixing equal volumes of the RNA solution and 2X loading buffer (25 µl of methylmercuric hydroxide, 500 µl of 4X running buffer, 200 µl of 100 % glycerol, 275 µl of H_2O, and 0.2 % w/v of bromophenol blue). Samples were loaded and the gel run at 1.5 volts/cm for 12-16 hours with recirculation of the buffer. After electrophoresis, teh gel was stained by incubating for 30 min in 0.5 M ammonium acetate and 0.5 µg/ml of ethidium bromide. The ammonium salt complexes methylmercury and enhances binding of the dye to RNA.

2.2.12.3 Fluorography of Agarose Gel

After electrophoresis, the gels were equilibrated with ethanol by soaking them in approximately 20 volumes of ethanol for about 30 min followed by a second (and if necessary a third change) 30 min immersion in fresh ethanol to remove water from the gels. The gels were then soaked in a 3 % (w/v) solution of PPO in ethanol for about 3 hours. Finally, the gels were immersed in water for about 1 hour to remove ethanol and to precipitate PPO *in situ* and then

they were dried by overlaying several layers of Whatman 3 MM filter papers and a stack of paper towels, with a suitable weight on top to hold it down.

2.2.13 Tryptic Peptide Analysis

2.2.13.1 Ion Exchange Chromatography.

Gel slices containing the protein to be analyzed were incubated in 0.2 M NH_4HCO_3 (pH 8.5) containing 100 µg TPCK-trypsin per ml at 37°C for 16 hours. The solution was replaced with fresh ammonium bicarbonate and fresh trypsin and incubation was continued for an additional 4 hours. The solutions were combined, fresh trypsin was added, and incubation for a further 4 hours was carried out. At the end of the incubation, the solution was filtered and lyophilized. The tryptic peptides were resuspended in water and re-lyophilized. This was repeated three more times after which the tryptic peptides were suspended in 500 µl of 0.05 M pyridine-acetate (pH 3.1) (8 ml pyridine, 139.2 ml glacial acetic acid, 1954 ml water). Tryptic peptides were separated on a water jacketed 0.9 x 22 cm column containing Aminex A5 cation exchange resin and maintained at 52°C (Toneguzzo, 1977). Samples were loaded directly into the precolumn eluent with the aid of an Altex 4 way injection valve containing a 0.5 ml sample loop. Peptides were eluted at a flow rate of 1 ml/min with a Milton Roy mini-pump using a linear gradient composed of

150 ml of 0.05 M pyridine-acetate (pH 3.1) and 150 ml of 1 M pyridine-acetate (pH 5.1)(40.3 ml pyridine, 37.6 ml glacial acetic acid, and water to 500 ml). This was followed by elution with 50 ml of 2 M pyridine-acetate (pH 5.1)(32.5 ml pyridine, 27.8 ml glacial acetic acid, and water to 200 ml). Fractions of 2 ml were collected directly into scintillation vials, dried, suspended in 200 ul of water and counted in 4 ml of Aquasol scintillation fluid.

2.2.13.2 Two Dimensional Peptide Mapping.

Two-dimensional tryptic peptide analysis was carried out basically as in the previously published method (Rettenmier et al., 1979b). Briefly, gel slices containing the protein to be analyzed were incubated with freshly prepared performic acid (formic acid/hydrogen peroxide 95:5, v/v) for two hours on ice. The gel pieces were removed, dried under vacuum and treated with TPCK-trypsin as above. Tryptic peptides were solubilized in a small volume of the electrophoresis buffer (acetic acid-pyridine-water, 10/1/100, v/v/v) and spotted on a 20 X 20 cm Polygram silica N-HR thin layer plate. Phenol-red was spotted in an opposite corner to serve as a tracking dye. Electrophoresis was carried out in the first dimension for about 12 hours at 300 volts. After the plate had dried, ascending chromatography in the second dimension was carried out in 1-butanol-pyridine-acetic acid-water (5/4/1/5, v/v/v/v). After chromatography, the plate was

dried in a chromatography oven at 90°C, sprayed with Enhancer and exposed to Kodak X-Omat XAR film at -70°C for appropriate lengths of time.

2.2.14 Enzyme Assays

2.2.14.1 Preparation of *E. coli* Cell Lysates.

E. coli cultures grown overnight were pelleted by centrifugation at 5000 rpm for 5 min at 4°C. The cells were suspended in TE buffer (10 mM Tris-HCl (pH 8.5), 1 mM EDTA) and transferred into Eppendorf tubes and centrifuged for 30 seconds at 4°C. The pelleted cells were resuspended in a buffer containing 15 % sucrose in 50 mM Tris-HCl (pH 8.5), and lysozyme was added to a final concentration of 1 mg/ml. After 10 min on ice, a second buffer containing 0.1 % Triton-X-100, 0.1 mM EDTA, and 50 mM Tris-HCl (pH 8.5) was added. After 10 min on ice, the mixture was centrifuged at 12,000 g for 15 min at 4°C and the supernatants were collected for enzyme assays.

2.2.14.2 Assay for DNA Polymerase.

Reaction mixture (0.1 ml) containing 50 µl of *E. coli* cell lysate in the buffer containing 0.1 M Tris-HCl (pH 8.1), 0.01 M $MgCl_2$, 0.1 mM of each of dATP, dCTP and dGTP, 2 % 2-mercapto-ethanol, 0.1 % Triton X-100, and 10^6 cpm of [^{3}H] TTP (25×10^3 cpm/pmole) was incubated at 37°C for various length of time. After incubation, the samples were precipitated by adding 0.1 ml of cold 0.1 M Na-pyrophosphate, 0.02 ml of 0.25 % BSA,

and 4 ml of cold 5 % trichloroacetic acid (TCA). The precipitates were collected on glass fiber filter paper and washed with 5 % TCA, ethanol:ether (1:1, v/v), followed by washing with ether. After drying in a fume hood, the filter paper was placed in a precounted scintillation vial containing 10 ml of a PPO/toluene based scintillation fluid. The radioactivity was determined in a scintillation counter.

2.2.14.3 Assay for RNase H.

Reaction mixture (0.05 ml) containing 20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 6 mM DTT, 2 µg BSA, 18.8 pmole of poly(dT), 16.9 pmole of [^{3}H] poly(A), and 10 µl of *E. coli* cell lysate was incubated for 30 min at 37°C. Reaction was stopped by the addition of 0.1 ml of cold 0.1 M sodium pyrophosphate, 0.4 mg of BSA, and 0.25 ml of 10 % TCA. The reaction mixture was centrifuged at 12,000 g for 15 min, and the supernatant was collected and counted in 10 ml of Aquasol scintillation fluid.

2.2.14.4 Assay for Reverse Transcriptase.

Assay using purified virus and poly(rA):oligo(dT) as template was performed as described (Mason et al., 1974). The reaction mixture contained in a final volume of 100 µl, 50 mM Tris-HCl (pH 8.2), 50 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 10 µM each of dATP, dCTP and dGTP, 0.1 % Triton X-100, 100 µCi/ml of [^{3}H] TTP (5 Ci/mmole), 10 µg/ml poly(rA):oligo(dT) and purified particles and was incubated at 35°C for 90 min.

Cold 10% TCA insoluble radioactivity was determined on filter paper (Toneguzzo and Ghosh, 1976).

2.2.15 Transformation of Escherichia coli by Plasmid DNA

Ten milliliters of LB medium were inoculated with 0.1 ml of an overnight culture of *E. coli*. The culture was incubated with vigorous shaking at 37°C to a density of about 5 x 10^7 cells/ml (OD_{550} = 0.5). This usually takes 2 to 4 hours. For each transformation assay, 3 ml of cells were used. The cell cultures were chilled on ice for 10 min, then collected by centrifugation at 4000 g for 5 min at 4°C. The pelleted cells were suspended in half the original culture volume of an ice-cold, sterile solution of 50 mM $CaCl_2$ and 10 mM Tris-HCl (pH 8.0). After incubation in an ice bath for 15 min, the cells were collected by centrifugation as above. The pelleted cells were resuspended in 1/15 of the original volume of an ice-cold, sterile solution of 50 mM $CaCl_2$ and 10 mM Tris-HCl (pH 8.0). The cells were stored at 4°C for 12 to 24 hours. For transformation, 100 µl of sterile TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] containing plasmid DNA was added to 200 µl of competent cells, prepared as above, and stored on ice for 30 min. The suspension was heat shocked at 42°C for 2 min, then 1.0 ml of prewarmed LB medium was added and incubated at 37°C for 1 hour without shaking. Aliquots of an appro-

priate dilution, in LB medium, of the cells were spread on agar plates (LB medium containing 1.85% of Bacto agar) containing the selective antibiotic (tetracycline at 10 μg/ml or ampicillin at 20 μg/ml) and incubated at 37°C. Colonies were detected 16 to 20 hours later.

In the case of transformation of _E. coli_ JM103 by pUC plasmid, the cells were spread on agar plates (containing ampicillin at 50 μg/ml) with 0.01 ml of 100 mM IPTG and 0.05 ml of 2% X-gal per plate. Transformants were visualized either as blue (Lac^+) or colorless (Lac^-) colonies.

2.2.16 Purification of Plasmid DNA

A colony isolate of _E. coli_, harboring the plasmid to be purified, was grown overnight in 5 ml of LB medium at 37°C with vigorous shaking. The next morning, the culture was diluted to 500 ml with LB medium containing the selective antibiotic (usually 20 μg/ml ampicillin) and incubated at 37°C with shaking until the culture reached late log phase (OD_{600} = 0.6). Chloramphenicol was added to 200 μg/ml and incubation continued for a further 16 to 20 hours. Cells were harvested by centrifugation at 5000 g for 10 min at 4°C, washed with TE buffer, and re-pelleted. The pellet was resuspended in 20 ml of a buffer containing 15% sucrose and 50 mM EDTA in 50 mM Tris-HCl (pH 8.5). The mixture was then cooled on ice and 5 ml of a freshly prepared lysozyme solution

(5 mg/ml) was added. The mixture was inverted occasionally during a 10 min incubation on ice and 15 ml of a buffer containing 0.1% Triton X-100 and 50 mM EDTA in 50 mM Tris-HCl (pH 8.5) was added. The mixture was inverted gently and occasionally for 10 min on ice. The partially lysed cells were centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was collected and 0.5 ml of 20% SDS was added. The mixture was heated at 65°C for 10 min and cooled at least 30 min on ice in presence of 5 ml of 5 M potassium acetate. The precipitate was pelleted by centrifugation at 10,000 rpm for 15 min, and the supernatant was collected. The supernatant was extracted with phenol:chloroform (1:1, v/v) and the aqueous layer was precipitated with 2 vol of ethanol in room-temperature. The precipitate was sedimented by centrifugation at 10,000 rpm for 10 min. The pellet was washed with 80% ethanol, dried, and dissolved in a buffer containing 1 mM EDTA and 10 µg/ml RNase A in 10 mM Tris-HCl (pH 7.5). The solution was incubated at room temperature for 15 min, precipitated with 2 vol of ethanol containing 0.2 M potassium-acetate, and left on ice for 20 min. The precipitate was pelleted, washed with 80% cold ethanol and dried. The final pellet was dissolved in a buffer containing 0.1 mM EDTA in 10 mM Tris-HCl (pH 8.0).

2.2.17 Restriction Enzyme Digestion

Restriction enzyme digestions were carried out in

20 µl reaction volume (except in preparative reactions) containing appropriate amount of plasmid DNA, 100 µg/ml of nuclease free BSA, and an appropriate amount of restriction enzyme as recommended by the supplier in a buffer containing 10 mM $MgCl_2$ and 50 mM NaCl in 50 mM Tris-HCl (pH 8.0). For restriction enzyme Kpn I, the reaction buffer contained 10 mM $MgCl_2$, 10 mM NaCl, and 6 mM 2-mercaptoethanol in 10 mM Tris-HCl (pH 7.5). The reaction mixtures were incubated at 37°C for 1 hour (except for the digestion with Taq I was at 65°C) and terminated by the addition of Termination buffer [5X stock: 4 M urea, 50% sucrose (w/v), 50 mM EDTA (pH 8.0) and 0.1% bromophenol blue (w/v)]. In the case of double enzyme digestion, digestion was first carried out with the enzyme requiring lower salt concentration (e.g., Kpn I). In the case of double digestion with same salt concentration, both enzymes were incubated together.

2.2.18 Separation of DNA Fragments by Gel Electrophoresis

2.2.18.1 Agarose Gel Electrophoresis.

Agarose was added to TBE electrophoresis-buffer [89 mM Tris, 89 mM boric acid (pH 8.3), 1 mM EDTA, and 0.5 µg/ml ethidium bromide] to the desired concentration (usually 0.8%) and dissolved as described above. The solution was cooled to

$50^{\circ}C$ and poured onto a preassembled horizontal gel apparatus. After the gel had solidified, the gel was overlaid with TBE buffer and the comb was removed and samples were loaded into the preformed wells. Electrophoresis was carried out at 40-100 volts for appropriate time. The gel was removed and examined under short wave UV light.

2.2.18.2 Photography.

Agarose gels were photographed under short wave UV illumination from a Chromo-Vue model 0-63 transilluminator (Ultra-Violet Products Inc., San Gabriel, Calif.). Photographs were taken with a Polaroid MP-3 Land camera containing Type 107 land film and equipped with a 39 mm SR60-2 filter.

2.2.19 Purification of DNA Fragments from Agarose Gels

DNA bands, separated by agarose gel electrophoresis, were visualized by long wavelength UV illumination of the ethidium bromide stained gels. A slit about 5 mm below the band was made and a strip of DEAE-cellulose paper inserted. Strips of Whatman DE81 DEAE-cellulose paper were cut to a height equal to the thickness of the gel and a width equal to, or slightly greater than, the slot width, and were soaked for several hours in 2.5 M NaCl. The strips were then washed several times in water and stored in 1 mM EDTA at $4^{\circ}C$. After

insertion of the strip, electrophoresis was resumed at 150 volts until the band was transferred into the strip (usually 5-10 min). The DEAE-cellulose paper, into which the DNA had been electrophoretically transferred, was removed from the gel and washed with cold water. The paper was drained and blotted dry with filter paper. This was then placed in a microfuge tube and 300-700 µl of 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1.5 M NaCl was added per 50 mm^2 of paper. The paper was shredded by vortexing and incubated at 37°C for 2 hours, with occasional agitation. The mixture was transferred into a microfuge tube, containing a plug of siliconized glass wool which served to trap the paper fibers. A hole was made in the bottom of the tube and it was placed within the opening of a 12 x 75 mm polypropylene tube (the lip of the microfuge tube rested securely on the edge of the polypropylene tube). After centrifugation for 5 min at 10,000 rpm, the eluate was extracted with 3 vol of n-butanol saturated with water, and the DNA was precipitated after addition of 2 vol of ethanol at -20°C for overnight. The DNA was recovered by centrifugation at 12,000 g for 15 min at 4°C. The DNA pellet was washed with 80 % ethanol, dried, and suspended in a small volume of appropriate buffer.

2.2.20 Nucleotide Sequence Analysis

2.2.20.1 <u>Dephosphorylation of DNA</u>

The DNA fragment was generated by restriction enzyme digestion. The digested DNA was extracted once with phenol/chloroform and precipitated with ethanol. The pelleted DNA was dissolved in a minimal volume of 10 mM Tris-HCl (pH 8.0), then dephosphorylated in a 50 µl reaction mixture containing 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine and appropriate amount of calf intestine alkaline phsphatase (CIP) in 50 mM Tris-HCl (pH 9.0). To dephosphorylate protruding 5' termini, the reaction mixture was incubated at 37°C for 30 min, then a second aliquot of CIP was added and the incubation was continued for a further 30 min. To dephosphorylate DNA with blunt ends or recessed 5' termini, incubation was at 37°C for 15 min and at 56°C for 15 min. Then, a second aliquot of CIP was added and the incubations repeated at both temperatures. The reaction was stopped by adding 40 µl of water, 10 µl of 10X STE [100 mM Tris-HCl (pH 8.0), 1 M NaCl, 10 mM EDTA], and 5 µl of 10% SDS, followed by heating at 68°C for 15 min. The mixture was extracted twice with phenol/chloroform and twice with chloroform. The aqueous layer was precipitated with 2 vol of ethanol. The DNA pellet was dissolved in a small volume of a buffer containing 0.1 mM EDTA in 10 mM Tris-HCl (pH 8.0), then analyzed on agarose gel. The DNA fragment of interest was purified from the gel as described above.

2.2.20.2 <u>Labeling the 5' ends of DNA with T4 Polynucleotide Kinase.</u>

The DNA fragment purified as described above was labeled at 5' protruding termini in a 50 µl reaction mixture containing 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA, 50 pmoles [γ-^{32}P] ATP (sp. act. = 3000 Ci/mmole), and 10-20 units of T4 polynucleotide kinase in 50 mM Tris-HCl (pH 7.6) by incubating at 37°C for 30 min. The reaction was stopped by adding 2 µl of 0.5 M EDTA and extracting once with phenol/chloroform, then precipitated DNA with ethanol. The DNA pellet was redissolved in a small volume of sterile water and digested with a second restriciton enzyme to generate one-end labeled DNA fragments. The labeled DNA fragments were purified from the agarose gel as described above.

DNA molecules with blunt ends or recessed 5' ends label less efficiently with polynucleotide kinase than molecules with protruding 5' ends. The efficiency of labeling may be improved as follow: The dephosphorylated DNA was heated to 70°C in a 40 µl solution containing 20 mM Tris-HCl (pH 9.5), 1 mM spermidine, and 0.1 mM EDTA and chilled quickly on ice. The mixture was supplemented with 5 µl of 10X blunt-end kinase buffer [0.5 M Tris-HCl (pH 9.5), 0.1 M $MgCl_2$, 50 mM DTT, 50 % glycerol], 50 pmoles of [γ-^{32}P] ATP in a volume of 5 µl, and 20 units of T4 polynucleotide kinase. The mixture was incubated at 37°C for 30 min. The reaction was stopped by adding 2 µl of 0.5 M EDTA and extracting once with phenol/chloroform. The aqueous phase was coll-

ected and the DNA precipitated with ethanol in the presence of 5 µl of 3 M sodium acetate (pH 5.2). The DNA pellet was dissolved in a small volume of sterile water and digested with a second restriction enzyme to generate one-end labeled DNA fragments. The labeled DNA fragments were purified from the agarose gel as described above.

2.2.20.3 Sequence Analysis of DNA by Chemical Modification.

This method is essentially the chemical base-modification procedure as described by Maxam and Gilbert (1980). The changes incorporated into the protocol include: (i) Longer reaction times for the chemical modification reactions. (ii) Addition of large amounts of carrier tRNAs to ensure efficient ethanol precipitations.

2.2.20.4 Sequencing Gels.

The gel was made up as follows: To make a 20 % stock acrylamide, the following components were mixed and the volume was brought to 500 ml with sterile distilled water. 96.5 gm acrylamide, 3.35 gm methylene-bis-acrylamide, 233.5 gm ultra-pure urea, 100 ml of 5X TBE to 500 ml with water. To make a 20 % sequencing gel, 50 ml of the 20 % stock acrylamide was mixed with 0.4 ml of freshly prepared 10 % ammonium persulfate. The solution was then degassed under vacuum. The solution was mixed with 50 µl of TEMED and poured into a preformed sequencing gel mold. The

polymerized gel was pre-electrophoresed for 1.5 hours at about 2000 volts. Two microliters of each sample was loaded by means of drawn out capillaries and samples were run for an appropriate length of time at about 2000 volts with constant power setting at 40 watts. The wells were rinsed prior to loading to remove urea that had diffused out of the gel. The gel was transferred to a piece of used X-ray film, covered with plastic wrap, placed on film with an intensifying screen, and autoradiography was performed at -70°C for an appropriate length of time.

2.2.21 Subcloning DNA Fragments into Plasmid Vector

Ligation of vector DNA with the desired DNA fragments was carried out in a 20 µl reaction volume containing an appropriate amount of vector DNA and more than double amounts of DNA fragment to be cloned, 2 µl of 10X ligation buffer [0.1 M $MgCl_2$, 0.2 M DTT, 0.5 M Tris-HCl (pH 7.7), 0.01 M ATP, 500 µg/ml nuclease free BSA] and an appropriate amount of T4 DNA ligase. The amount of T4 DNA ligase was doubled for blunt-end ligation. The mixture was incubated at 4°C for 16 hours and the reaction was terminated by heating at 65°C for 10 min. The reaction mixture was made to 100 µl with TE buffer and used to transform competent *E. coli* cells as described earlier.

2.2.22 Identification of Recombinant Plasmids

Recombinant plasmid with a desired DNA fragment was identified by insertional inactivation of an antibiotic resistant gene where the DNA was inserted. Transformed bacteria with the recombinant plasmids were first selected from untransformed cells by growing in presence of an antibiotic whose resistant gene was not affected. The resultant bacterial colonies were then screened for those cells containing the recombinant plasmids with the desired DNA inserted in the other drug resistant gene. The colonies were transferred into two duplicated plates containing each antibiotic drug. The colonies were identified for those not growing in presence of the antibiotic drug whose resistant gene was affected. These colonies were picked from the other duplicated plates and the plasmids were purified from the cultures.

The recombinant plasmids were also identified by insertional inactivation of an enzyme activity. For example, the recombinant plasmids with the desired DNA fragment inserted at the site of lac Z gene present in pUC plasmid were identified by spreading the transformed cells on ampicillin containing plates in presence of X-gal, the chromogenic substrate for β-galactosidase. The recombinant plasmids do not produce β-galactosidase due to insertional inactiva-

tion of the lac Z gene and are unable to utilize X-gal resulting in formation of colorless colonies. In contrast, intact puC plasmid transformed colonies produce β-galactosidase and form blue colonies. The colorless colonies were picked and inoculated in 5 ml LB medium containing 20 μg/ml ampicillin and allowed to grow to saturation overnight at 37°C. The plasmids were then isolated from these cells and analyzed by restriction enzyme digestion and agarose gel electrophoresis as described earlier.

2.2.23 DNA Mediated Gene Transfer

DNA mediated gene transfer was carried out by the calcium-phosphate transfection procedure (Graham and van der Eb, 1973; Stow and Wilkie, 1976). Primary CEF cells were trypsinized and plated on 60 mm culture dishes at a concentration 5×10^5 cells per plate and transfected with the DNA when the plates were 50 % confluent. An appropriate amount of DNA along with 10 μg of salmon sperm carrier DNA was added to 0.5 ml of HEBS (10X HEBS: 50 gm of HEPES, 80 gm of NaCl, 3.7 gm of KCl, 1.25 gm of $Na_2HPO_4 2H_2O$, 10 gm of dextrose in 1 liter. It was diluted just before use and the pH was adjusted to 7.05). Added 35 μl of 2 M $CaCl_2$ to each tube and incubated on ice for 40 min for precipitation. Medium from dishes was aspirated off and the calcium phosphate

precipitated DNA mixture was poured onto the dish. The culture was incubated at 37°C for 30 min to allow the precipitate to adhere to the cells. The culture was fed with HGD medium supplemented with 2 % chick serum and 1 % FBS (Low serum was used because sera may contain DNase). After 12 hours, the medium was changed to HGD supplemented with 5 % FBS, 2 % chick serum, and 1 % DMSO. When the cells were confluent, they were subcultured into 10 cm culture dishes. Cells were maintained until cell became transformed.

At 5.5 days posttransfection, cells were labeled with [^{35}S] methionine (180 μCi/10 cm plate) for 16 hours and viruses were purified from the medium and analyzed by SDS-PAGE as described earlier. Intracellular viral proteins were labeled for 1 hour with [^{35}S] methionine (225 μCi/10 cm plate) and cells were lysed and immunoprecipitated as described above.

2.2.24 Preparation of Total RNAs from E. coli.

Twenty-five ml of overnight culture was poured onto 10 ml of crushed, frozen media and collected by centrifugation at 10,000 x g for 10 min at 4°C. The cell pellets were resuspended in 4 ml of RNA buffer (0.01 M NaCl, 0.01 M Tris-HCl (pH 7.5), 0.005 M $MgCl_2$, and 300 μg/ml lysozyme) and frozen in an acetone-dry ice bath. After thawing, the

suspension was made to 1 % in SDS and incubated at 37°C until clearing of the solution occurred. Sodium acetate buffer (pH 5.2) was then added to a final concentration of 0.1 M. The samples were heated at 65°C for 2 min in the presence of an equal volume of water-saturated phenol. The aqueous layer was re-extracted four times at 37°C with equal volume of water-saturated phenol. The aqueous layer was made to 2 M in NaCl, and the RNA was precipitated by the addition of 1.5 volume of 95 % ethanol and keeping at -20°C. The precipitates were pelleted at 4°C and reprecipitated from 0.15 M NaCl with 1.5 vol of 95 % ethanol. This was repeated three additional times and the final RNA was washed with ether, dried with N_2 and resuspended in 0.2 ml of sterile water.

2.2.25 Nick Translation

The nick-translation reaction was carried out in a 50 µl reaction volume containing 10 mM $MgSO_4$, 0.1 mM DTT, 50 µg/ml BSA, 1 nmole of each dATP, dGTP and dTTP, 100 pmoles [α-^{32}P] CTP in 50 mM Tris-HCl (pH 7.2). This mixture was chilled to 0°C and 0.5 µl of diluted DNase I (0.1 µg/ml) was added to the reaction mixture, then 5 units of *E. coli* DNA polymerase I was added. The mixture was incubated at 16°C for 60 min and the reaction was stopped

by adding 2 µl of 0.5 M EDTA. The nick-translated DNA was separated from unincorporated [α-^{32}P] CTP by centrifugation through a small column of Sephadex G-50.

2.2.26 <u>The Dot Blot Assay for RNA</u>

Nitrocellulose was prepared by first wetting with distilled water and then equilibrating the filter with 3 M NaCl, 0.3 M Na Citrate (20X SSC). Once filters were dried with a heat lamp they were ready for application of the samples. After positioning the dry, treated nitrocellulose sheets on the Hybrid-Dot (Bethesda Research Laboratories) manifold and applying a vacuum, each dilution (1-5 µl) was dispensed onto the filter and washed once with an equal volume of the tRNA diluent. With the vacuum still applied, the manifold was disassembled and nitrocellulose removed, placed on Whatman 3 MM filter paper, and dried with a heat lamp. The filter was placed at 80°C for 1-2 hours to immobilize the RNA on the filter.

Hybridization with specific radiolabeled sequences was accomplished using conditions identical to those employed in the Southern (1975) technique. A prehybridization step involving a 6-12 hours incubation of the filter in a minimal amount of hybridization buffer (3X SSC, 0.05 M HEPES (pH 7.0), 200 µg/ml tRNA, 50 µg/ml heat denatured salmon sperm DNA, and

Denhardt's (1966) buffer, 0.02 % each BSA, Ficoll, and polyvinylpyrrolidone) minimizes nonspecific binding by the radiolabeled probe. After pre-annealing, the excess buffer was blotted on 3 MM filter paper and hybridization buffer (2-3 mls), which included 1-2 x 10^5 cpm/ml ^{32}P labeled probe, was added and incubated for 48 hours. All incubations were performed at 42°C using this buffer. To remove excess probe after hybridization, filters were washed as follow: (1) 2X SSC for 1 hour at room temperature; (2) 0.1X SSC and 0.1 % SDS for 45 min at 55°C, (3) three rinses with 0.1X SSC at room temperature. Filters were placed on 3 MM filter paper to air dry and exposed to Kodak XAR-5 X-ray film at -70°C using intensifying screen.

2.2.27 S1 Nuclease Mapping of Transcripts

Hybridization of 250 µg of total *E. coli* and 5' end-labeled DNA probe (35,000 cpm) was carried out in 40 mM PIPES (pH 6.4), 1 mM EDTA, 400 mM NaCl and 80 % formamide in a total volume of 10 µl. The reactions were heated for 10 min at 60°C and transferred to 45°C and hybridization was carried for 12 hours. After hybridization, 110 µl of S1 nuclease buffer containing 250 mM NaCl, 30 mM sodium acetate (pH 4.5), 1 mM $ZnSO_4$, 20 µg/ml of single stranded salmon sperm DNA, and 200 U/ml of S1 nuclease was added and incubated

for 40 min at 25°C. The reaction was terminated by extraction with an equal volume of phenol/chloroform (1:1, v/v), followed by ethanol precipitation. The precipitates were dissolved in loading buffer containing 80 % (v/v) formamide, 50 mM Tris-borate (pH 8.3), 1 mM EDTA, 0.1 % (w/v) xylene-cyanol, 0.1 % (w/v) bromophenol blue and analyzed on a 4 % polyacrylamide gel.

RESULTS

SECTION 3.1: BIOCHEMICAL CHARACTERIZATION OF A TEMPERATURE-SENSITIVE MUTANT OF ROUS SARCOMA VIRUS DEFECTIVE IN PROCESSING OF THE REVERSE TRANSCRIPTASE PRECURSOR

Four primary polypeptide translation products of the avian retrovirus genome have been defined (see Fig. 1.1). These are: (i) $gPr92^{env}$, a 92,000 molecular weight glycosylated precursor (England et al., 1977; Shealy and Rueckert, 1978; Klemenz and Diggelmann, 1978) that upon cleavage yields the virion envelope protein gp85 and gp37; (ii) $Pr76^{gag}$, a 76,000 molecular weight precursor, that is cleaved in a series of steps to yield the major internal virion proteins p19, p12, p10, p27 and p15 (Vogt et al., 1975; Hunter et al., 1983b); (iii) $Pr180^{gag-pol}$, a polyprotein of estimated molecular weight 180,000, which is an apparent "read through" product of the combined gag and pol genes that is cleaved to yield the enzyme reverse transcriptase (α and β subunit complex; Oppermann et al., 1977); and (iv) $pp60^{src}$, a protein encoded in src, has a molecular weight of 60,000 (Brugge et al., 1978; Levinson et al., 1978), which is phosphorylated (Brugge et al., 1978; Levinson et al.,

1978) and displays the enzymatic properties of a cyclic nucleotide-independent protein kinase (Levinson et al., 1978; Collett and Erikson, 1978).

The precise mechanisms by which these proteins or their cleavage products aggregate at the cell membrane to form the viral budding structure are unknown. It is well known that proteolytic cleavages play an important role in the maturation of avian as well as murine retroviruses (Eisenman and Vogt, 1978; Jamjoom et al., 1977; Vogt et al., 1979; von der Helm, 1977; Yoshinaka and Luftig, 1977a,b). The interrelationship of macromolecular assembly and specific proteolytic cleavage is difficult to assess in cells infected with wild-type virus because all stages of assembly and processing are occurring simultaneously. In murine viruses, the cleavages of gag precursor appears to be associated with a condensation of the viral core, after budding from the cell membrane has occurred (Yoshinaka and Luftig, 1977a). In avian viruses it is likely that cleavage occurs concomitantly with, and plays an essential role in, viral assembly. This hypothesis is supported by the observation that mammalian cells transformed by avian sarcoma virus do not produce virus particles and also do not cleave $Pr76^{gag}$ (Eisenman et al. 1975). Studies with ts mutants of MLV (Stephenson et al., 1975; Yeger et al., 1978) have shown that a defect in the cleavage of gag gene polyprotein precursor results in defective maturation and assembly of the virus particle (Stephenson et al., 1975; Yeger et al.,

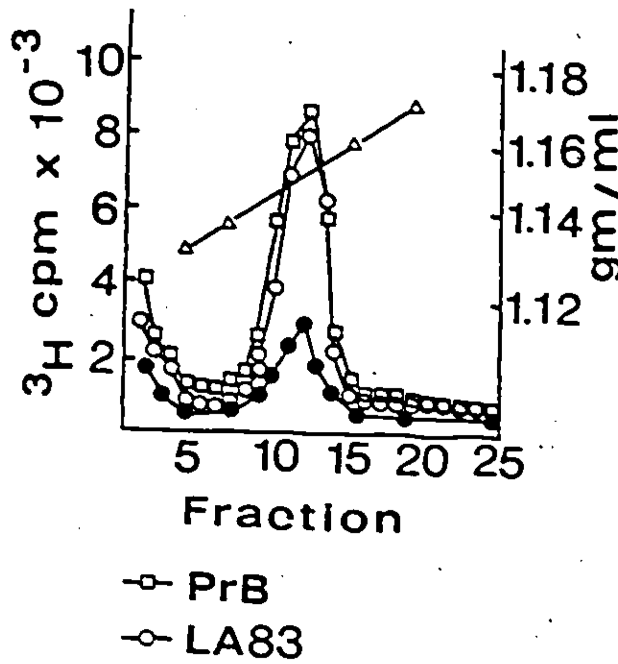


Figure 3.1.1 Sucrose density gradient of [^{3}H] uridine-labeled particles of PR-B and LA83 grown at 35°C and 41°C.

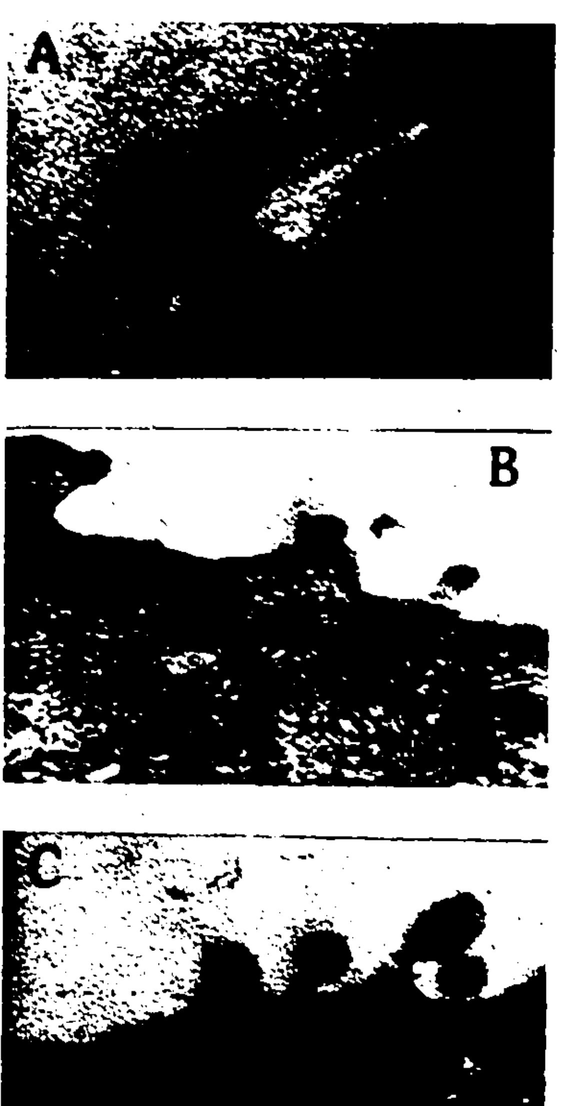
LA83 infected C/O cells were labeled with [^{3}H] uridine for 16 hrs, and the clarified virus was centrifuged in 25% to 50% (w/w) sucrose density gradient for 4 hrs at 45,000 rpm in a Beckman SW 41 rotor. The density was determined with a refractometer. This figure is directly from Ghosh (1984).

1978; van de Ven et al., 1978). It has also been reported that in the case of two MLV mutants ts3 (Moloney) and ts24 (Rauscher) the late budding structures observed at the non-permissive temperature were due to a defect in the cleavage of both gag and gag-pol precursor polyproteins (Witte and Baltimore, 1978). Lu et al., using Mo-MLV ts3 particles, showed that the cleavage of the precursor $Pr65^{gag}$ was accompanied by a change in the virion from an immature to a mature morphology (Lu et al., 1979). In the case of the avian system the virion assembly mutant tsLA3342 was suggested to contain a cleavage defective gag precursor polyprotein $Pr76^{gag}$ (Hunter et al., 1976; Rohrschneider et al., 1976).

I was interested in studying the molecular basis by which RNA tumor viruses are assembled, and the relationship of processing of the reverse transcriptase precursor to virus maturation using avian sarcoma virus (RSV) as model. Previous studies carried out in this laboratory (Ghosh, 1984) with a RSV-conditional lethal mutant RSV-PR-B-tsLA83 (LA83) showed that LA83 was defective in both replication and transformation functions. At the nonpermissive temperature, the yield of particles (NI-LA83) was 30 % of that obtained at 35°C (Fig. 3.1.1). The buoyant density of both NI-LA83 and LA83 particles were the same. Electron microscopic analysis showed that LA83-infected cells grown at 35°C (Fig. 3.1.2A) contained released virus particles

Figure 3.1.2 Electron microscopy of LA83-infected chick-embryo fibroblasts at the permissive and nonpermissive temperatures.

Confluently transformed cells maintained at 35°C were shifted to 41°C and maintained at 41°C for 48 hrs when the cells were no longer transformed. For temperature-shiftdown the cells maintained at 41°C were quickly cooled to 35°C and maintained at 35°C for 2 hrs before fixing *in situ* with glutaraldehyde. (A) LA83-infected cells at 35°C; (B) LA83-infected cells at 41°C; (C) LA83-infected cells after shiftdown from 41°C to 35°C. This figure is directly from Ro and Ghosh (1984).


A
B
C

with typical C-type particle morphology. But infected cells grown at 41°C (Fig. 3.1.2B) showed the accumulation of an increased number of budding structures but fewer released particles. A shiftdown from 41 to 35°C resulted in an increased release of virus particles with a concomitant decrease in budding structures (Fig. 3.1.2C). The infectivity of NI-LA83 produced at 41°C was 0.7 % of the virus particles produced at 35°C. Complementation analyses showed that LA83 synthesized functional gag and env gene products at the nonpermissive temperature (Ghosh 1984). Analysis of reverse transcriptase activity of NI-LA83 showed that it contained only 3 % of the activity of LA83 particles produced at 35°C. Reverse transcriptase activity including RNA-directed DNA polymerase, DNA-directed DNA polymerase, and RNase H present in LA83 were, however, not thermolabile (Noble and Ghosh, unpublished).

Analyses of NI-LA83 Particles

Since the noninfectious NI-LA83 particles produced at 41°C contained only 3 % of the reverse transcriptase activity of LA83 particles, it was decided to examine in detail the polypeptide composition of NI-LA83. The primary technique used was SDS-polyacrylamide gel electrophoresis (PAGE) in which the polypeptide composition of NI-LA83 was fractionated and compared to that of LA83 virions obtained at 35°C and PR-B virions obtained at 35 and 41°C.

One of the first questions asked was whether the loss of infectivity and reverse transcriptase activity in NI-LA83 was due to the absence of the reverse transcriptase or the glycoproteins, or both.

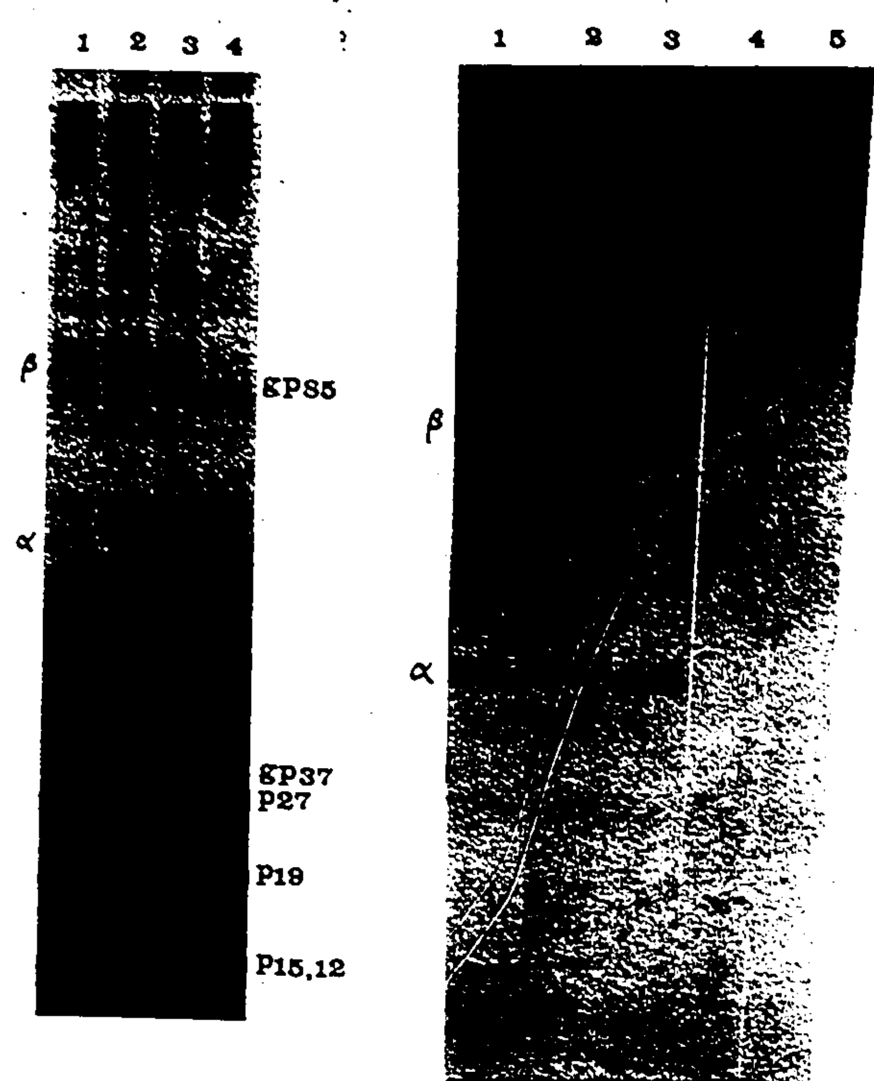
Viruses were labeled with [^{35}S] methionine and purified as described in Materials and Methods. When [^{35}S]-methionine labeled NI-LA83 was analyzed on SDS-PAGE, no significant difference in polypeptide composition of the structural proteins was observed. Results presented in Fig. 3.1.3 showed that the autoradiograph of [^{35}S] methionine labeled NI-LA83 (lane 4) contained all the expected internal structural (gag) proteins (p27, p19, p12/p15) and the envelope glycoproteins (gp85 and gp37) in amounts similar to those present in LA83 and PR-B (lanes 1 to 3). The α and β subunits of reverse transcriptase were absent in NI-LA83 although they were present in LA83 and PR-B virions. The purified particles were further analyzed by immunoprecipitation with antiserum specific for reverse-transcriptase and the results are shown in Figure 3.1.4. PR-B virus grown at 35 or 41°C as well as LA83 virus grown at 35°C contained both subunits of reverse transcriptase (lanes 1 to 3). In contrast, NI-LA83 (lane 4) did not contain any polypeptides corresponding to α and β subunits. A polypeptide of about 200,000 Da was present in all the viruses and it may represent nonspecific precipitation of a host protein as it was also precipitated with preimmune

Figure 3.1.3 Left: Autoradiogram of SDS-polyacrylamide-gel containing electrophoretically separated proteins of purified viruses.

Viruses were labeled for overnight with [^{35}S] methionine and purified as described in Materials and Methods. Lane 1, PR-B grown at 35°C; lane 2, LA83 grown at 35°C; lane 3, PR-B grown at 41°C; lane 4, NI-LA83.

Figure 3.1.4 Right: Autoradiogram of SDS-polyacrylamide-gel containing electrophoretically separated reverse transcriptase subunits.

Viruses were labeled for overnight with [^{35}S] methionine, purified and immunoprecipitated with anti-pol antiserum. Lane 1, PR-B grown at 35°C; lane 2, LA83 grown at 35°C; lane 3, PR-B grown at 41°C; lane 4, NI-LA83; lane 5, NI-LA83 precipitated with normal rabbit serum. This figure is directly from Ro and Ghosh (1984).


1 2 3 4
β
gP85
α
gP37
P27
P19
P15,12
1 2 3 4 5
β
α

serum (lane 5).

The presence of a number of bands at the 200K-Da region was variable, and depended on the labeling conditions and was observed in viruses harvested at early times (see below). The polypeptides may represent host contaminants or they may be the precursor for reverse transcriptase. As mentioned in Section I, the precursor for reverse transcriptase has been shown to be present in the virions of two temperature sensitive mutants of MLV, and functional reverse transcriptase was produced when these cell-free viruses were incubated at the permissive temperature (Witte and Baltimore, 1978). It was, therefore, decided to characterize these virion associated high molecular weight proteins in more detail. The first experiment was to test whether the reverse transcriptase can be produced after incubation of the NI-LA83 particles at the permissive temperature. NI-LA83 particles were harvested every 30 min from LA83-infected cells at 41°C. The harvested media were clarified and incubated at 35°C for various lengths of time, then the particles were purified as described in Materials and Methods. Reverse transcriptase was assayed with the exogenous template and primer (poly rA:oligo dT). The results shown in Fig. 3.1.5 indicated that no apparent increase of the background activity was observed after incubation of NI-LA83 particles at the permissive temperature. These

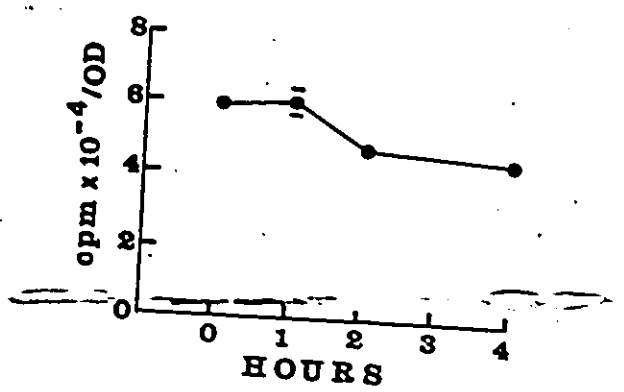


Figure 3.1.5 Reverse transcriptase activity associated with purified NI-LA83 particles.

The media harvested from LA83-infected cells at 41°C were clarified and incubated at 35°C for various lengths of time, then the particles were purified as described in Materials and Methods. The purified NI-LA83 particles were used in standard reverse transcriptase assay using exogenous poly-(rA):oligo (dT) template. The reaction mixture contained in a final volume of 100 µl, 50 mM Tris-HCl (pH 8.2), 50 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 10 µM each of dATP, dCTP and dGTP, 0.1% Triton X-100, 100 µCi/ml of [^{3}H] TTP (5 Ci/mmole), 10 µg/ml poly (rA):oligo (dT) and purified particles and was incubated at 35°C for 90 min. Cold 10% TCA insoluble radioactivity was determined on filter paper (Toneguzzo and Ghosh, 1976).

results support the earlier results (Figs. 3.1.3, 3.1.4) that there are no $Pr180^{gag-pol}$ molecules present in NI-LA83 that can be processed to mature reverse transcriptase.

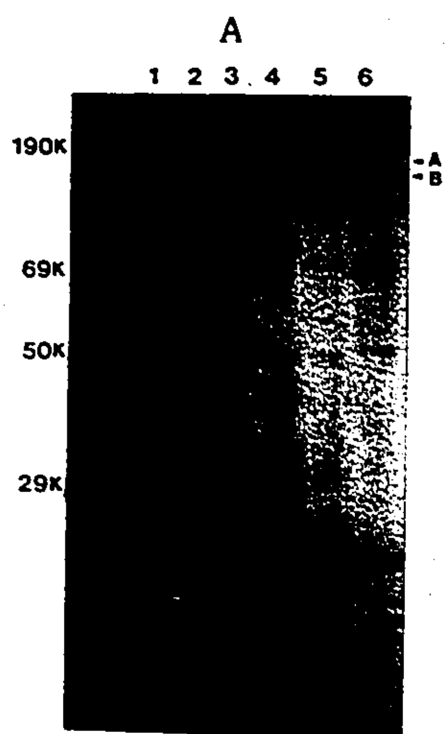
To determine whether the high molecular weight virion associated proteins are accessible to the protease (p15), that is specific for gag related proteins (see Section I), cells infected with LA83 at 41°C were labeled with [^{35}S] methionine for 2 hours and the virus particles released during the labeling period were purified as described in Materials and Methods. The particles were disrupted and immunoprecipitated with anti-p27 antiserum. The immune-complex was suspended in the <u>in vitro</u> protease reaction buffer (0.05 M Tris-HCl, pH 7.2, 0.15 M NaCl, and 0.1 % Triton X-100) and incubated at 37°C for 1 hour in the presence of various amounts of the protease (disrupted PR-B viruses). Results presented in Figure 3.1.6 showed a number of bands around 190,000 molecular weight region which were immunoprecipitated with anti-p27 antiserum (lane 1), however, these same proteins were also precipitated with preimmune serum (lane 6). More significantly, these proteins were apparently unaffected by the treatment with the protease (lanes 2 to 5). Even though the amounts of the proteins were reduced slightly with increased amount of the protease, no cleavage products were detected. Similarly, the <u>in vitro</u> cleavage analysis was carried out with the intracellular proteins

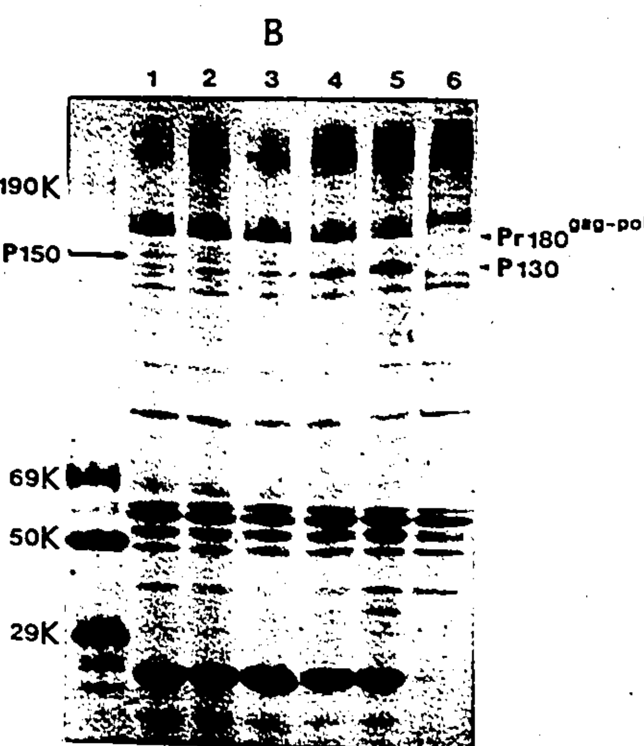
Figure 3.1.6 Left: *In vitro* cleavage analyses of the high Mr proteins from NI-LA83.

LA83-infected cells at 41°C were labeled with [^{35}S] methionine (100 μCi/plate) for 2 hrs. The labeled media were collected, clarified and the viral particles were purified. The particles were immunoprecipitated with anti-p27 antiserum. The immune-complex was suspended in the *in vitro* protease reaction buffer and incubated at 37°C for 1 hr in presence of various amounts of disrupted purified PR-B. Lane 1, no PR-B added; lane 2 to 5, addition of 5, 10, 20, and 40 μg of PR-B, respectively; lane 6, immunoprecipitates with normal rabbit serum. Bands marked with A and B are virion-associated high molecular weight proteins (see Fig. 3.1.8).

Figure 3.1.8 Right: *In vitro* cleavage analyses of the high Mr proteins from LA83-infected cells.

LA83-infected cells at 41°C were labeled as in Fig. 3.1.6. The labeled cells were lysed and immunoprecipitated with anti-p27 antiserum, and *in vitro* processing of $Pr180^{gag-pol}$ was tested. Lane 1, no PR-B added; lanes 2 to 5, addition of 5, 10, 20, and 40 μg of PR-B, respectively; lane 6, precipitates with normal rabbit serum.


A
1 2 3 4 5 6
190K
69K
50K
29K
A
B


B
1 2 3 4 5 6
190K
P150
Pr180gag-pol
P130
69K
50K
29K

from LA83-infected cells. The LA83-infected cells were labeled with [^{35}S] methionine and the cell lysates were immunoprecipitated with anti-p27 antiserum. Results presented in Fig. 3.1.7 showed that the apparent precursor for reverse transcriptase ($Pr180^{gag-pol}$) was precipitated with the antiserum (lane 1). When the immune-complex was treated with the protease the amount of $Pr180^{gag-pol}$ was reduced significantly with concomitant increase in the amount of a protein with molecular weight of 130,000 (P130). This protein may represent an intermediate of reverse transcriptase (see Section 3.2). It is also evident that a relatively small amount of a polypeptide with molecular weight of 150,000 (P150) was precipitated by anti-p27 antiserum and P150 also disappeared with increasing amounts of the protease. This polypeptide was further characterized by immunoprecipitation and tryptic peptide mapping (see Section 3.2).

Above data indicate that the virion associated high molecular weight proteins are not reverse transcriptase-related proteins and they may represent host proteins. More direct evidence was obtained by tryptic-peptide mapping. The tryptic-peptide profiles of these virion associated high molecular weight proteins (designated A & B in Fig. 3.1.6) with apparent molecular weight of $Pr180^{gag-pol}$ were compared to those obtained from $Pr76^{gag}$ and $Pr150^{gag-pol}$ (see Section 3.2 for the characterization of these pre-

cursor proteins). $Pr150^{gag-pol}$ has antigenic determinants for all the gag sequences and pol sequence (Fig. 3.1.17). Consistent with the immunoprecipitation analyses, $Pr150^{gag-pol}$ apparently contained all the tryptic peptides present in $Pr76^{gag}$ (Fig. 3.1.8). In contrast, the tryptic peptide profiles of proteins A and B were quite different from those obtained from $Pr150^{gag-pol}$ or $Pr76^{gag}$, but A and B are related such that all the tryptic peptides of B were also present in A. These results clearly indicated that no apparent $Pr180^{gag-pol}$ polypeptides were present in NI-LA83 particles.

Although the amount of gag proteins present in NI-LA83 was similar to that in LA83 or in PR-B, possible alterations in posttranslational modification were tested. ^{32}P-labeled viral proteins were prepared as described under Materials and Methods, and analyzed by SDS-PAGE. Virus labeled with [^{35}S] methionine was also electrophoresed on the same gel so that the ^{32}P-labeled proteins could be identified. Figure 3.1.9 showed that three internal structural proteins, p23, p19 and p12, were labeled with ^{32}P. The relative amounts of each protein in NI-LA83 (lane 5) were similar to those present in LA83 or in PR-B (lanes 2 to 4). In addition, a polypeptide which comigrated with p15 was phosphorylated. It may represent residual phospholipid which have been also observed by others (Shaikh et al., 1979).

Figure 3.1.8 Tryptic-peptide analyses of the virion-associated high molecular weight proteins.

The [^{35}S] methionine-labeled virion-associated high molecular weight proteins (A and B as in Fig. 3.1.6), Pr150 and Pr76 were eluted from the gel slices in the presence of TPCK-Trypsin, oxidized, and subjected to two-dimensional tryptic-peptide analyses as described in Materials and Methods. Pr150 was immunoprecipitated from LA83-infected cells at 41°C, Pr76 was immunoprecipitated from PR-B-infected cells, and A and B were isolated from NI-LA83. In the schematic diagrams, peptides common to both A and B were indicated in filled outlines.

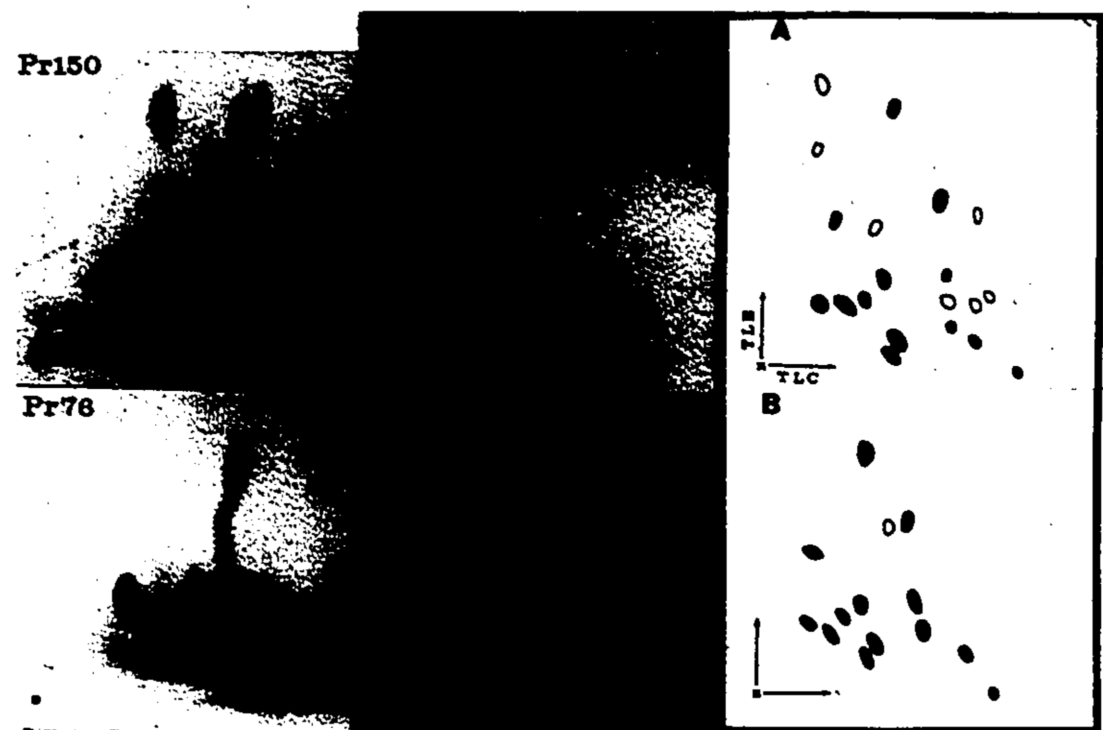
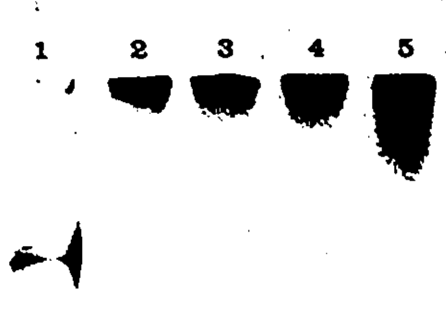
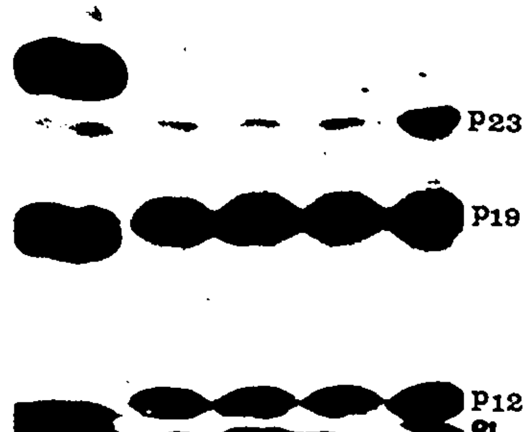

Pr150
Pr76
A
B
TLE
TLC

Figure 3.1.9 Phosphoproteins of PR-B, LA83, and NI-LA83.

Autoradiograph of a 12.5 % polyacrylamide slab gel containing electrophoretically separated polypeptides from PR-B, LA83, and NI-LA83. The cells were labeled for 12 hrs with [^{32}P] orthophosphate (250 µCi/ml) and the viruses were harvested after a 12 hrs chase with regular medium and purified as described under Methods. Lane 1 contains purified PR-B virus labeled for 16 hrs with [^{35}S] methionine; lane 2, [^{32}P] phosphate-labeled PR-B at 35°C; lane 3, [^{32}P] phosphate-labeled LA83 at 35°C; lane 4, [^{32}P] phosphate-labeled PR-B at 41°C; lane 5, [^{32}P] phosphate-labeled NI-LA83. Band marked PL is phospholipid. This figure is directly from Ro and Ghosh (1984).


1
2
3
4
5


P23
P19
P12
PL

To examine whether there are any defects in glycosylation of the envelope glycoproteins in NI-LA83, the virus was labeled with radioactive sugars. Figure 3.1.10 showed that, both gp85 and gp37 were labeled with [^{3}H] mannose (lanes 2 to 5) and [^{3}H] glucosamine (lanes 6 to 9) in NI-LA83. The relative amounts of the glycoproteins present in NI-LA83 and in LA83 or PR-B were also similar.

Finally, the RNA present in NI-LA83 particles was analyzed by labeling the virus with [^{3}H] uridine and fractionating the RNA on an agarose gel. Results presented in Figure 3.1.11 showed that the profile of RNA contents in Ni-LA83 (lane 4) was similar to LA83 or PR-B (lanes 1 to 3), and showed the presence of genomic RNA and small molecular weight host RNAs (see introduction).

Analyses of Intracellular Viral Polypeptides

To determine whether the synthesis of reverse transcriptase was defective or the reverse transcriptase molecules were synthesized but were not incorporated into mature virions, the intracellular viral polypeptides were examined. Cells infected with PR-B or LA83 at 35 and 41°C were labeled for 1 hour with [^{35}S] methionine and the labeled proteins were immunoprecipitated with antiserum specific for RSV proteins. The immunoprecipitates were then analyzed by

Figure 3.1.10 Glycoproteins of PR-B, LA83, and NI-LA83.

Autoradiograph of a 12.5 % polyacrylamide slab gel containing electrophoretically separated glycoproteins from PR-B, LA83, and NI-LA83. The cells were labeled with [^{3}H] mannose (800 μCi) or [^{3}H] glucosamie (125 μCi) for 12 hrs and the viruses were harvested after a 12 hrs chase with regular medium and purified as described under Materials and Methods. Lane 1, molecular weight marker proteins (190K, 69K, 50K, and 29K); lanes 2 to 5, [^{3}H] mannose-labeled PR-B produced at 35°C, LA83 produced at 35°C, PR-B produced at 41°C and NI-LA83, respectively; lanes 6 to 9, [^{3}H] glucosamine-labeled PR-B formed at 35°C, LA83 formed at 35°C, PR-B formed at 41°C and NI-LA83, respectively; lane 10 contains purified PR-B virus labeled for 16 hrs with [^{35}S] methionine. This figure is directly from Ro and Ghosh (1984).

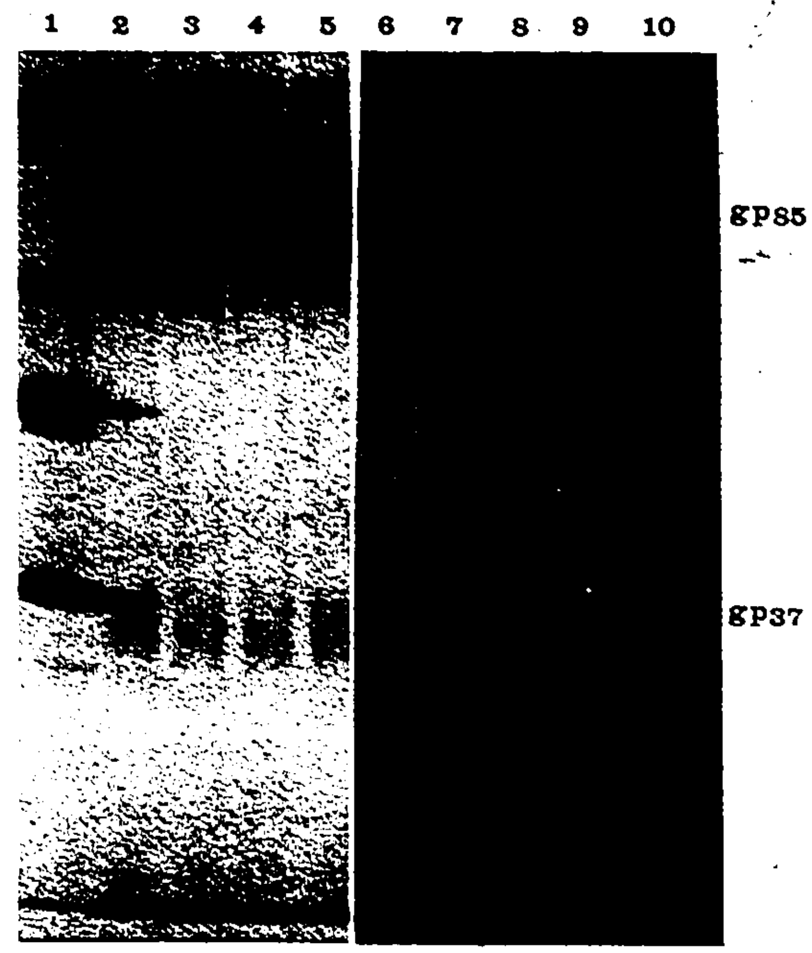
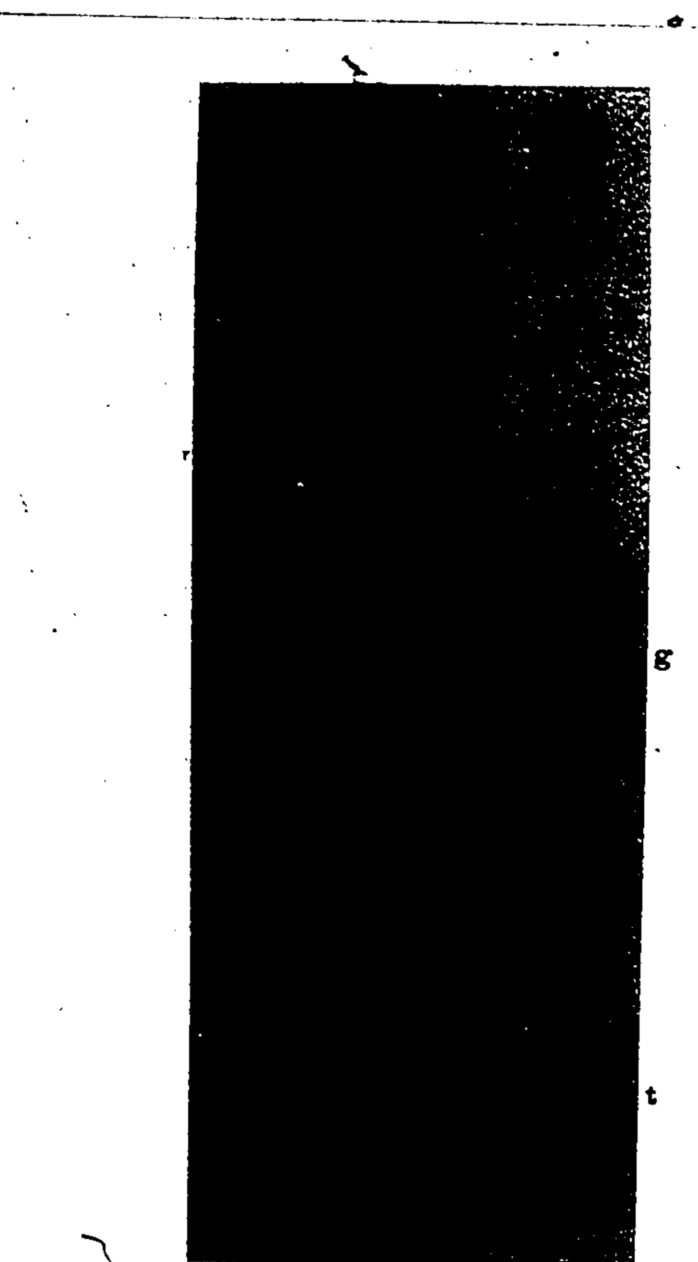

1
2
3
4
5
6
7
8
9
10
gP85
gP37

Figure 3.1.11 Agarose gel electrophoretic analysis of RNAs of NI-LA83, LA83 and PR-B.

Viral genomic RNAs were labeled with [^{3}H] uridine and the labeled RNAs were isolated from the purified viruses as described under Materials and Methods. The isolated RNAs were analyzed on denaturing agarose gels as described under Materials and Methods. Lane 1, RNAs from PR-B grown at 35°C; lane 2, RNAs from LA83 grown at 35°C; lane 3, RNAs from PR-B grown at 41°C; lane 4, RNAs from NI-LA83. g corresponds to the genomic RNA and t corresponds to the host tRNAs and lower molecular weight RNAs.



SDS-PAGE and autoradiography (Fig. 3.1.12). From LA83-infected cells at 41°C, the anti-RSV antiserum precipitated a polypeptide with 180,000 molecular weight (lane d) which comigrated with $Pr180^{gag-pol}$ from PR-B infected-cells at either temperature (lanes a and c) and LA83-infected cells at 35°C (lane b). The anti-RSV antiserum also precipitated the other two major precursors, $gPR92^{env}$ and $Pr76^{gag}$, and the intermediates $Pr66^{gag}$, $Pr60^{gag}$, and $Pr16^{gag}$. The cleavage products of $Pr76^{gag}$, p27, p19 and p12/p15 were also produced during the 1 hour labeling period.

In order to confirm the synthesis of the three primary precursor proteins, monospecific antisera were used for the immunoprecipitation analyses. Results presented in Figure 3.1.13 showed the presence of a single band representing the precursor polypeptide $gPr92^{env}$ which was precipitated with the anti-glycoprotein antiserum. No such band was detected in cell lysates treated with preimmune serum. The synthesis of $Pr76^{gag}$ in LA83-infected cells at the nonpermissive temperature was examined by immunoprecipitation with anti-p27 antiserum (Fig. 3.1.14). It was evident that $Pr76^{gag}$ and a relatively lower amount of p27, as well as $Pr180^{gag-pol}$, were precipitated with anti-p27 antiserum. Since $Pr180^{gag-pol}$ molecules contain both gag and pol sequences, they were precipitated with both gag- and pol-monospecific antisera. As can be seen in Figure

Figure 3.1.12 Polyacrylamide gel analysis of [^{35}S] methionine-labeled intracellular viral proteins immunoprecipitated with anti-RSV antiserum.

Infected cells were labeled with [^{35}S] methionine for 1 hr and the cells were lysed and immunoprecipitated with anti-RSV antiserum. The precipitates were then analyzed on a 12.5 % acrylamide gel. Lane a, PR-B-infected cells at 35°C; lane b, LA83-infected cells at 35°C; lane c, PR-B-infected cells at 41°C; lane d, LA83-infected cells at 41°C.

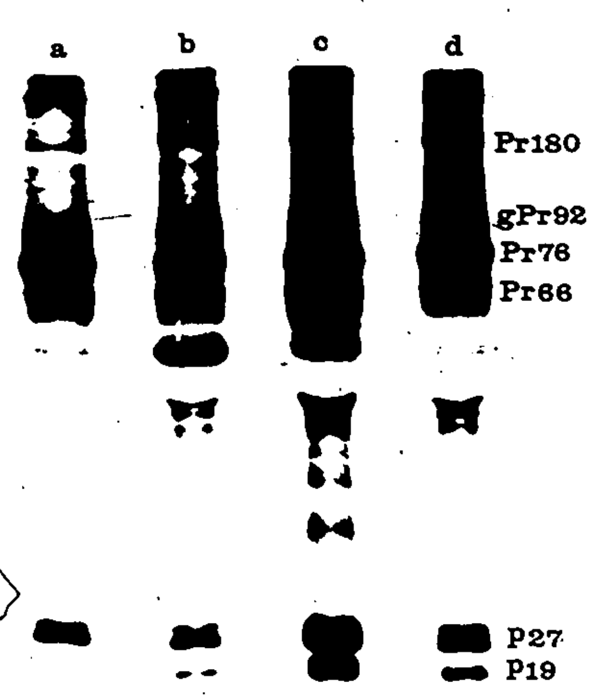

a
b
c
d
Pr180
gPr92
Pr76
Pr66
P27
P19
Pr12
P15,12

Figure 3.1.13 Polyacrylamide gel analysis of [^{35}S] methionine-labeled intracellular viral proteins immunoprecipitated with anti-gp antiserum.

Infected cells were labeled with [^{35}S] methionine for 1 hr and the cells were lysed and immunoprecipitated with anti-glycoprotein antiserum. The precipitates were then analyzed on a 10 % polyacrylamide gel. Lanes 1 and 10, molecular-weight marker proteins; lanes 2 and 3, PR-B-infected cells at 35°C; lanes 4 and 5, LA83-infected cells at 35°C; lanes 6 and 7, PR-B-infected cells at 41°C; lanes 8 and 9, LA83-infected cells at 41°C. Lanes 2,4,6 and 8 correspond to the immunoprecipitates with normal rabbit serum, and lanes 3,5,7, and 9 correspond to the immunoprecipitates with the anti-gp antiserum. The arrow indicates the precursor polypeptide gPr92^{env}.

1 2 3 4 5 6 7 8 9 10

190

69

50

29

Figure 3.1.14 Polyacrylamide gel analysis of [^{35}S]-methionine labeled intracellular viral proteins immunoprecipitated with anti-p27 antiserum.

Infected cells were labeled with [^{35}S] methionine for 1 hr and the cells were lysed and immunoprecipitated with anti-p27 antiserum. The precipitates were then analyzed on a 10% polyacrylamide gel. Lanes 1 and 10, molecular-weight marker proteins; lanes 2 and 3, PR-B-infected cells at 35°C; lanes 4 and 5, LA83-infected cells at 35°C; lanes 6 and 7, PR-B-infected cells at 41°C; lanes 8 and 9, LA83-infected cells at 41°C. Lanes 2, 4, 6, and 8 correspond to the immunoprecipitates with normal rabbit serum, and lanes 3, 5, 7, and 9 correspond to the immunoprecipitates with the anti-p27 antiserum.

1 2 3 4 5 6 7 8 9 10

190K

Pr180

Pr76

69K

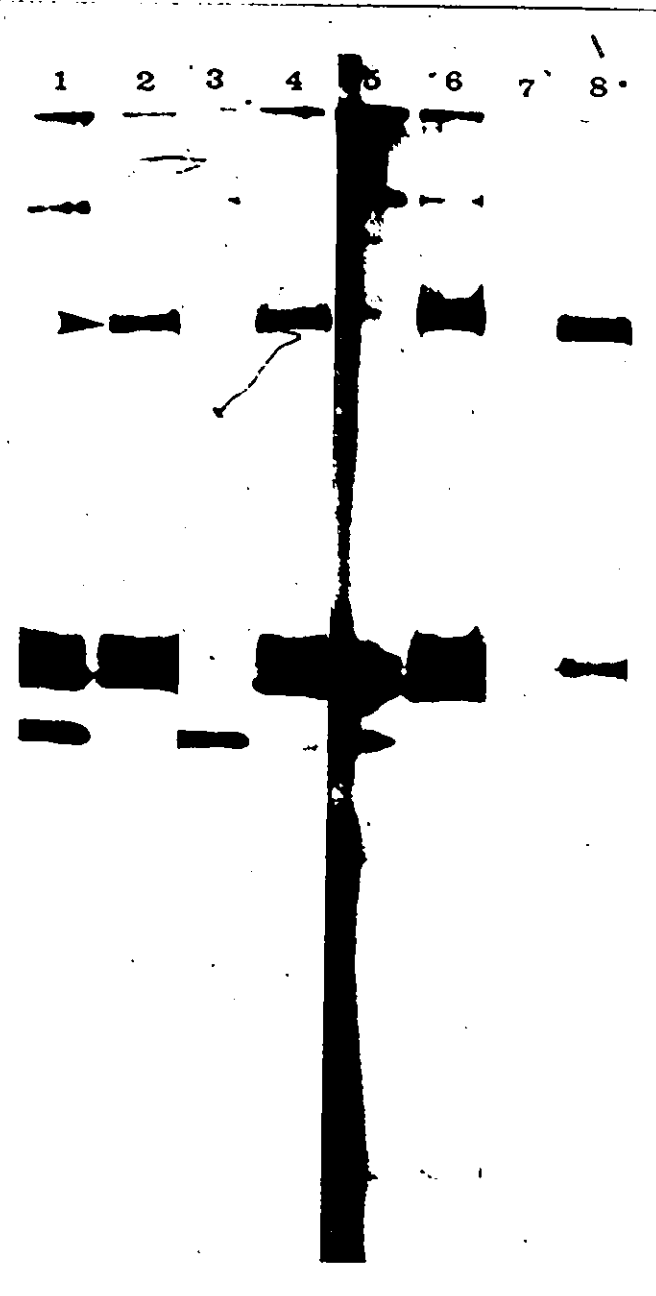
50K

29K

P27

Figure 3.1.15 Polyacrylamide gel analysis of [^{35}S]-methionine labeled intracellular viral-proteins immunoprecipitated with anti-pol-antiserum.

Infected cells were labeled with [^{35}S] methionine for 1 hr and the cells were lysed and immunoprecipitated with anti-pol antiserum. The precipitates were then analyzed on a 10% polyacrylamide gel. Lanes 1 and 2, PR-B-infected cells at 35°C; lanes 3 and 4, LA83-infected cells at 35°C; lanes 5 and 6, PR-B-infected cells at 41°C; lanes 7 and 8, LA83-infected cells at 41°C. Lanes 1, 3, 5, and 7 correspond to the immunoprecipitates with normal rabbit serum, and lanes 2, 4, 6, and 8 correspond to the immunoprecipitates with anti-pol antiserum. The arrow indicates the precursor polypeptide Pr180.


1
2
3
4
5
6
7
8

3.1.15, only Pr180$^{gag-pol}$ molecules were evident after immunoprecipitation with anti-reverse transcriptase antiserum. For the further confirmation of Pr76^{gag} synthesis, the tryptic peptide profile of [^{35}S] methionine-labeled Pr76^{gag} was compared to those obtained from the individual gag proteins. As can be seen in Fig. 3.1.16, Pr76^{gag} contained all the tryptic-peptides (panel D) present in P19 (panel A), p27 (panel B), and p12/p15 (panel C). These results suggest that the synthesis of three precursor proteins was not defective in LA83.

In order to establish that the apparent Pr180$^{gag-pol}$ synthesized in LA83-infected cells at 41°C contains all the gag-specific domains, the labeled cell extracts were immunoprecipitated with each individual anti-gag antiserum. Results presented in Fig. 3.1.17 showed that 180,000 Da polypeptide present in cells infected with LA83 at 41°C was precipitable with anti-RSV (lane a), anti-p19 (lane b), anti-p27 (lanes c,d), anti-p12 (lane e), anti-p15 (lane f), and anti-reverse transcriptase)lanes g,h) antisera, but not with normal rabbit serum (lane i) and anti-glycoprotein antiserum (Fig. 3.1.13). Furthermore, the tryptic-peptide profile of [^{35}S] methionine-labeled Pr180 was compared to those obtained from Pr76^{gag} and reverse transcriptase. Consistent with the immunoprecipitation analyses, Pr180 contained all the tryptic peptides (Fig. 3.1.18A) present in reverse-

Figure 3.1.16 Two-dimensional tryptic-peptide analyses of $Pr76^{gag}$ and the mature gag proteins.

The [^{35}S] methionine-labeled polypeptides were isolated by gel electrophoresis and excised from the gel and treated as described under Materials and Methods. Autoradiographs of the dried plates are shown in the figure; electrophoresis (TLE) was from bottom to top and chromatography (TLC) was from left to right. p19 (A), p27 (B), and p12/p15 (C) were isolated from purified PR-B virus. Pr76 (D) was obtaiined from LA83-infected cells at 41°C.

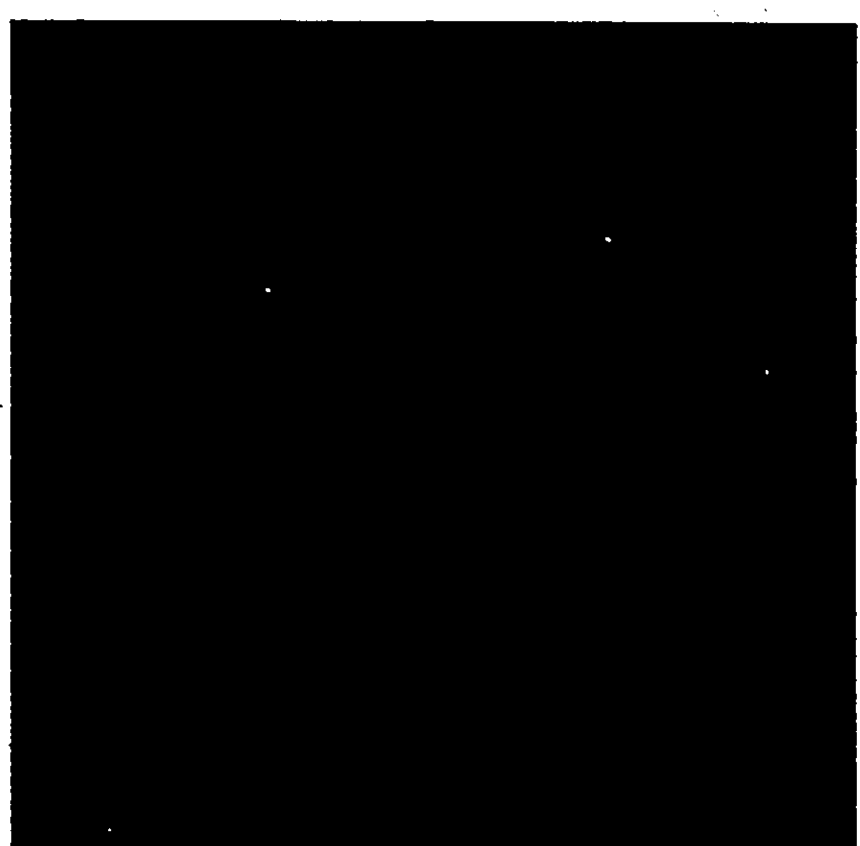


Figure 3.1.17 Immunological characterization of Pr180. Autoradiograph of a 7.5 to 15% gradient polyacrylamide slab gel containing electrophoretically separated polypeptides from LA83-infected cells after immunoprecipitation with monospecific antisera. The cells were labeled 1 hr at 41°C with [^{35}S] methionine. Lane a, anti-RSV serum; lane b, anti-p19 serum; lanes c and d, anti-p27 serum; lane e, anti-p12 serum; lane f, anti-p15 serum; lanes g and h, anti-pol serum; lane i, preimmune serum; lane j, molecular weight marker proteins (190K, 69K, 50K, and 29K). This figure is directly from Ro and Ghosh (1984).

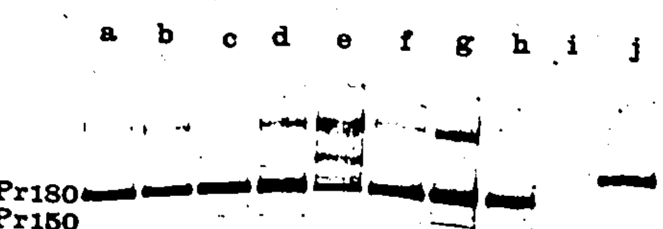
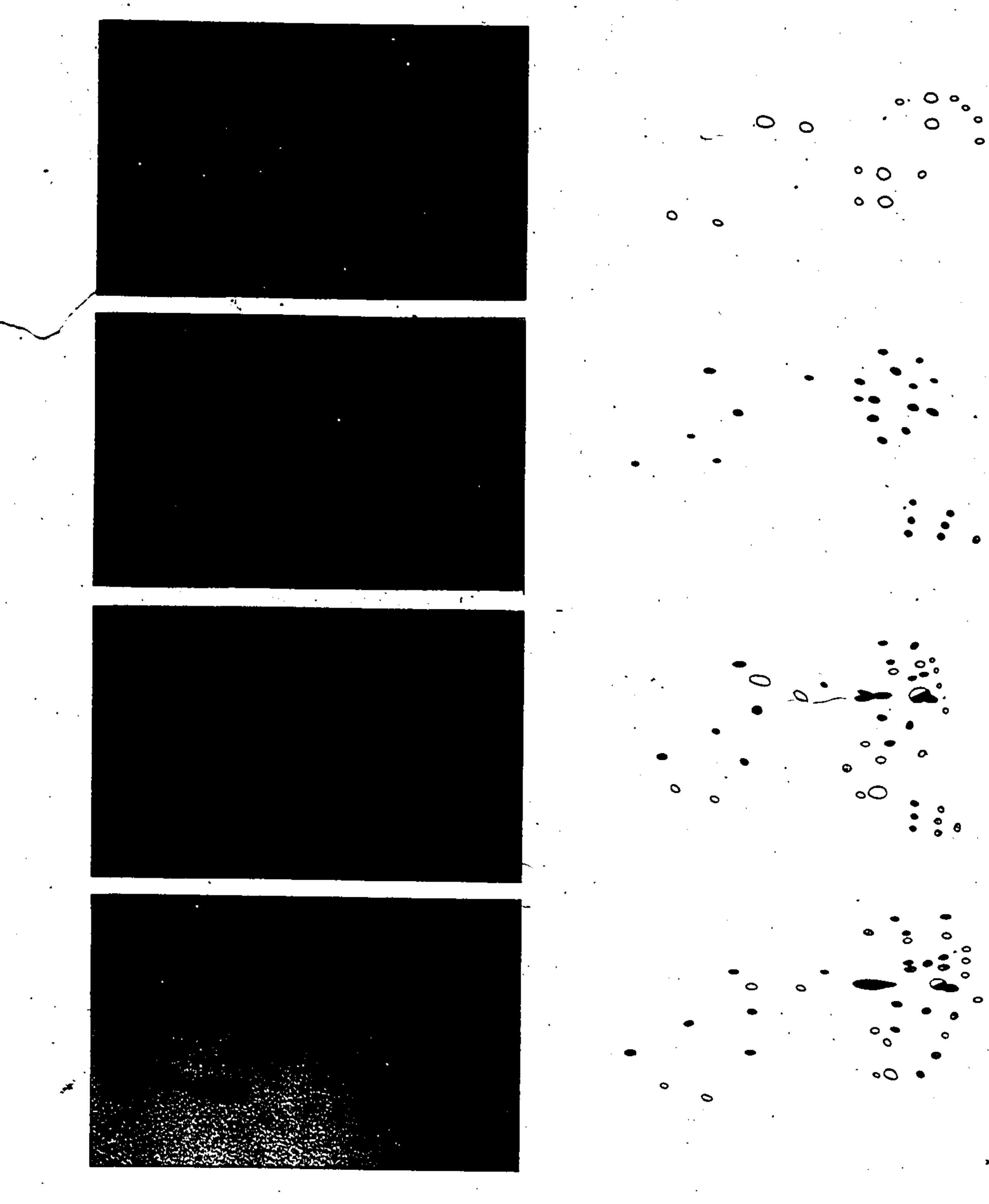

a b c d e f g h i j
Pr180
Pr150

Figure 3.1.18 Two-dimensional tryptic-peptide fingerprinting analyses of Pr180 with $Pr76^{gag}$ and reverse transcriptase subunits.

[^{35}S] Methionine-labeled polypeptides were isolated by gel electrophoresis and excised from the gel and treated as described under Materials and Methods. Autoradiographs of the dried plates are shown in the figures; a schematic diagram of each autoradiograph is shown immediately below it. The origin of each fingerprint, indicated by x in the diagrams, is at the lower left corner, and electrophoresis is from bottom to top. Pr180 (A) was obtainied from LA83-infected cells at 41°C. $Pr76^{gag}$ (D) and reverse-transcriptase (C) were obtained from PR-B-infected cells and purified PR-B virus, respectively. The reverse transcriptase was immunoprecipitated from disrupted virus with anti-pol antiserum and finally isolated by gel electrophoresis. The α and β subunits were combined for digestion with trypsin. $Pr76^{gag}$ was immunoprecipitated from PR-B-infected cells with anti-p27 serum and isolated by gel electrophoresis. In the schematic diagrams, peptides characteristic of reverse transcriptase are indicated in filled (C) and $Pr76^{gag}$ in open outlines (D). The autoradiograph (B) is the mixture of peptides from $Pr76^{gag}$ and reverse transcriptase. The spots indicated by crossed outlines in Pr180 (A) are not seen either in reverse-transcriptase or in $Pr76^{gag}$, while the peptides indicated by crossed outlines in reverse transcriptase (B, C) are not observed in Pr180. This figure is directly from Ro and Ghosh (1984).



transcriptase (Fig. 3.1.18C) and in Pr76^{gag} (Fig. 3.1.18D). In addition to the peptides of Pr76^{gag} and reverse transcriptase Pr180 contained six more tryptic peptides indicated by crossed circles in the schematic diagram (Fig. 3.1.18A) which were not present either in reverse transcriptase or in Pr76^{gag}. Also, there are six tryptic peptides indicated by crossed circles in the schematic diagrams of Figs. 3.1.18B and C which were not present in Pr180. These peptides may represent products of incomplete digestion. The tryptic-peptide profiles of Pr180$^{gag-pol}$ from LA83 infected cells at 41°C were also compared to those from PR-B infected cells. As can be seen in Fig. 3.1.19, no apparent difference was noted. Taken together these data suggested that the Pr180 protein observed in cells infected with LA83 is Pr180$^{gag-pol}$. Although the precursor Pr180$^{gag-pol}$ was synthesized at 41°C without any apparent defect, the NI-LA83 particles did not contain any reverse transcriptase (Fig. 3.1.4, lane 4).

Pr180$^{gag-pol}$ has been previously shown to be phosphorylated (Eisenman et al., 1980). To determine whether there were defects in phosphorylation of Pr180$^{gag-pol}$, LA83-infected cells were labeled for 2 hrs with [^{32}P] phosphate and the cell lysate was precipitated with anti-gag or anti-reverse transcriptase antisera. As shown in Fig. 3.1.20, both Pr76^{gag} and Pr180$^{gag-pol}$ were phosphorylated in LA83-infected cells at the nonpermissive temperature (lanes e and

Figure 3.1.19 Two-dimensional tryptic-peptide finger-printing analyses of $Pr180^{gag-pol}$ from LA83- and PR-B-infected cells

[^{35}S] Methionine-labeled proteins were excised from poly-acrylamide gels, digested with trypsin, and the tryptic-peptides were analyzed as described under Materials and Methods. For the direct comparison of tryptic-peptides, samples were spotted on the same plate on opposite sides of the center line of the plate. Following electrophoresis (TLE), the plate was cut along the center line and the two halves were chromatographed (TLC) at right angles to the direction of electrophoresis.

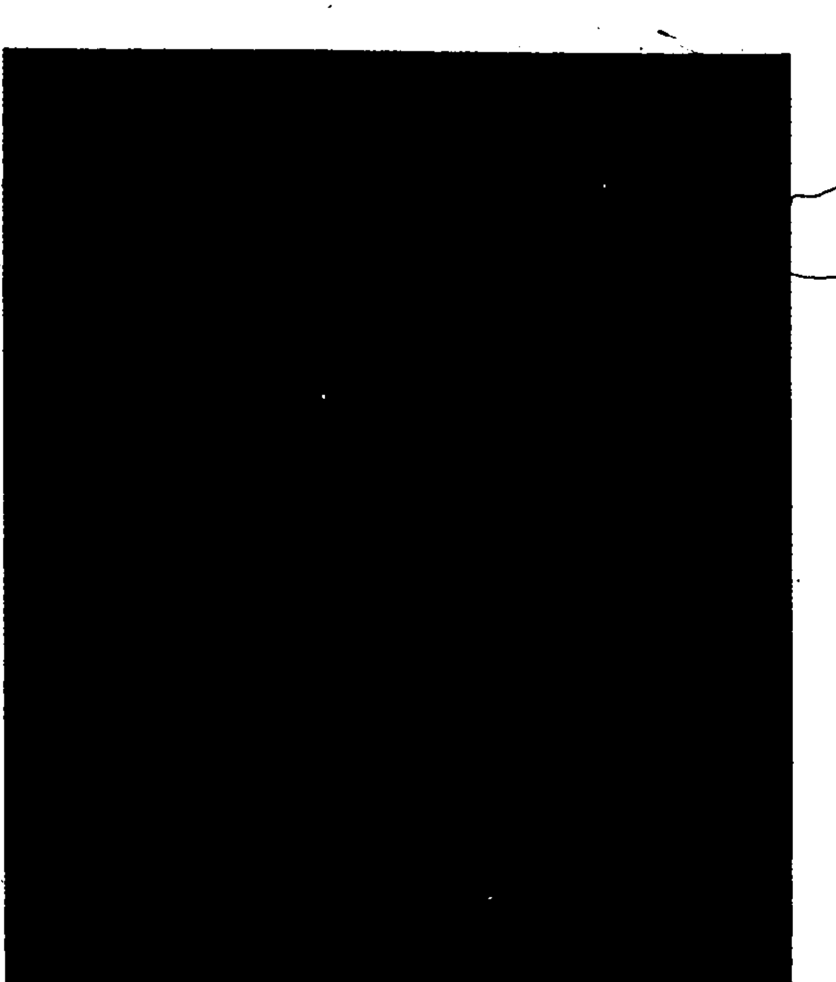
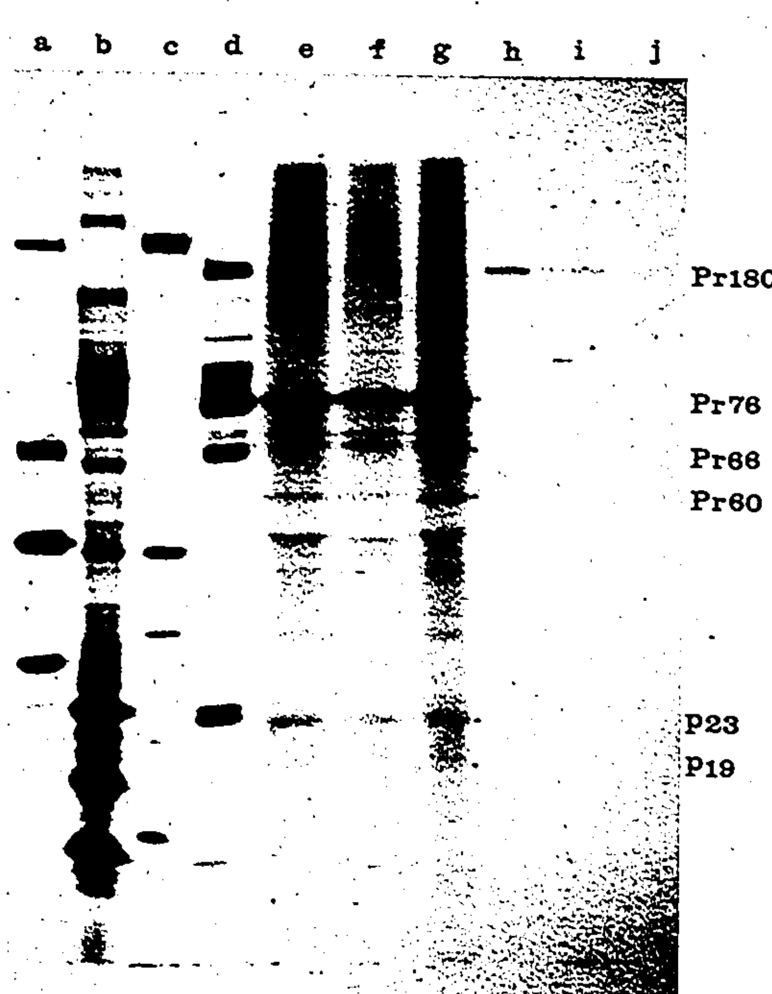


Figure 3.1.20 Autoradiogram of intracellular phosphoproteins.

The infected cells were labeled with [^{32}P] phosphate and the cell lysates were precipitated with anti-gag (lanes d to g) or anti-pol (lanes h to j) antisera and analyzed on a 12.5% polyacrylamide gel. Lane a, molecular weight marker proteins; lane b, [^{35}S] methionine-labeled purified PR-B; lanes c and d, [^{35}S] methionine-labeled PR-B-infected cells immunoprecipitated with normal rabbit serum and anti-gag antiserum, respectively; lanes e and h, LA83-infected cells at 41°C; lanes f and i, PR-B-infected cells at 41°C; lanes g and j, LA83-infected cells at 35°C.



a
b
c
d
e
f
g
h
i
j
Pr180
Pr76
Pr66
Pr60
P23
P19

h). Furthermore, the gag proteins p23 and p19 were also detected in the infected cells (lanes e to g), but no p12 was detected in this particular experiment. These results indicated that $PR180^{gag-pol}$ was normally synthesized and phosphorylated in LA83-infected cells at 41°C but the extent of phosphorylation of $Pr180^{gag-pol}$ has not been determined.

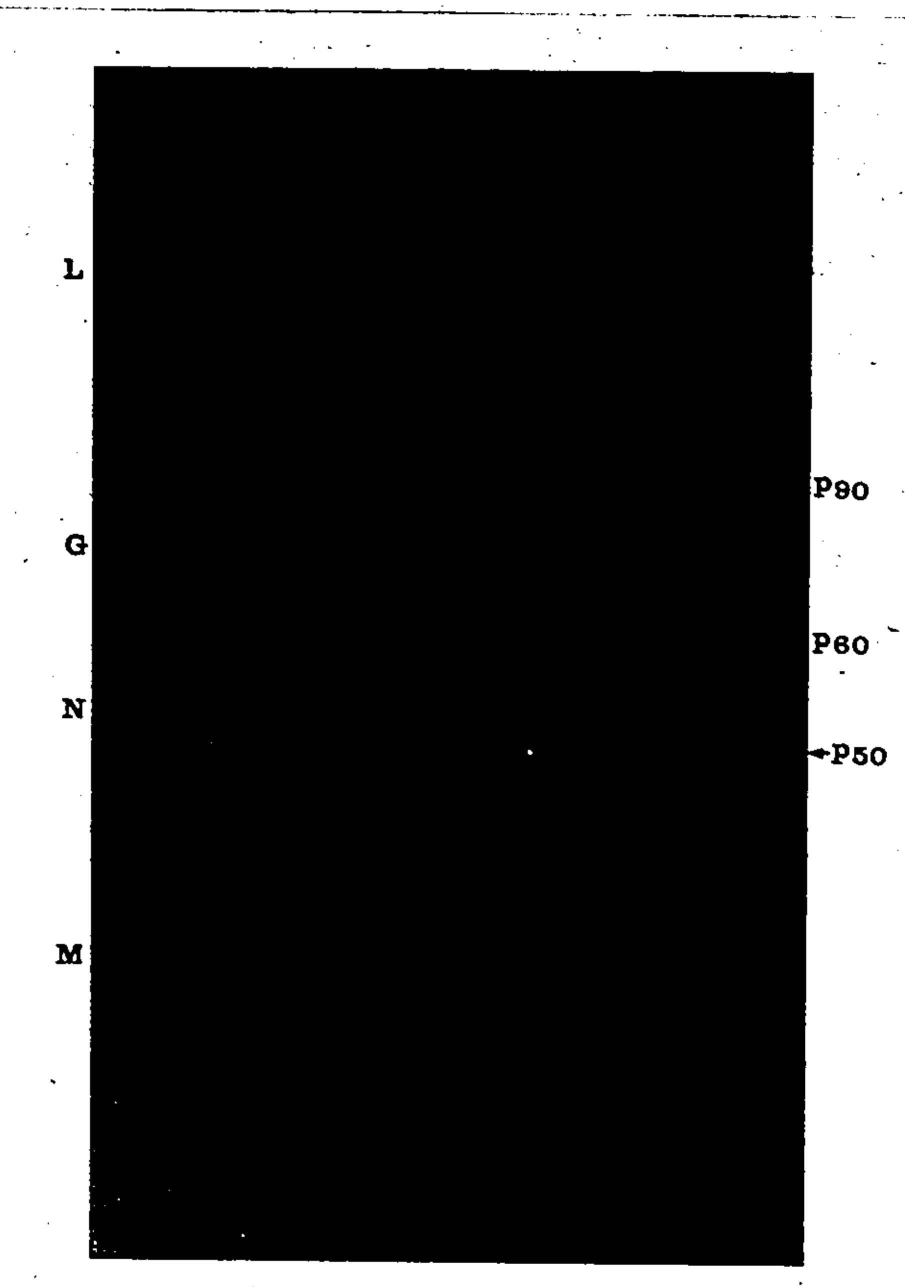
We reported recently a RSV-conditional lethal mutant RSV-PR-B-tsLA83 (LA83) which is defective in both replication and transformation functions (Ghosh, 1984) and the biochemical evidence suggests that LA83 is defective in processing the reverse transcriptase precursor at the nonpermissive temperature (Ro and Ghosh, 1984). Thus it was suspected that LA83 contains a lesion in the pol gene or LA83 could have multiple mutations in the replication and transformation functions. Complementation experiments with avian leukosis viruses defective in transforming function showed that LA83 could complement only replication defects (Ghosh, 1984). These results suggested that the transformation defect in LA83 is due to mutation in the src gene. It was thus decided to examine whether the src gene products are synthesized at the nonpermissive temperature. Virus was added to secondary cultures of chick embryo fibroblast cells within 3 hours of replating. When the infected cells were confluently transformed they were subcultured and incubated at either 35 or 41°C. The infected cells were labeled with $[^{35}S]$ methio-

nine (Amersham) for 2 hours at either 35 or 41°C. The cell extracts were immunoprecipitated with the serum from rabbits bearing Rous sarcoma virus-induced tumors (TBR antiserum) which has been preadsorbed with the detergent-treated unlabeled virus. The immunoprecipitates were solubilized in electrophoresis sample buffer and analyzed on a 10 % SDS-polyacrylamide gel (Ro and Ghosh, 1984). As shown in Figure 3.1.21, $pp60^{src}$ synthesized in LA83-infected cells was comparable to that synthesized in the wild-type PR-B-infected cells at either 35 or 41°C. Therefore, the normal production of $pp60^{src}$ at the nonpermissive temperature indicates that the defect in transformation was due to the functional defects of $pp60^{src}$ rather than its lability.

The protein $pp60^{src}$ has protein kinase activity specific for tyrosine residues. The identification of the proteins phosphorylated by $pp60^{src}$ is important in understanding the events involved in RSV-mediated transformation. One potential substrate is a 50,000 molecular-weight phosphotyrosine containing protein (pp50) which is associated in a complex with $pp60^{src}$ and a cellular 90,000 molecular weight protein (pp90; Brugge et al., 1981) and thus is immunoprecipitated by antibody to $pp60^{src}$ (Hunter and Sefton, 1980; Oppermann et al., 1981). As can be seen in Figure 3.1.21, both pp90 and pp50 were detected in immunoprecipitates from lysates of cells infected with LA83

Figure 3.1.21 Polyacrylamide gel analyses of [^{35}S]-methionine labeled pp60^{src}.

RSV-infected cells wer labeled with [^{35}S] methionine for 2 hrs either at 35 or 41°C. The cell extracts were immunoprecipitated with TBR serum which has been preadsorbed with the detergent treated unlabeled virus. The precipitates were then analyzed on a 10% polyacrylamide gel. Lane 1, molecular weight marker proteins; lanes 2 and 3, PR-B-infected cells at 35 and 41°C, respectively; lanes 4 and 5, LA83-infected cells at 35 and 41°C, respectively; lanes 6 and 7, SR-D-infected cells at 35 and 41°C, respectively; lane 8, the immunoprecipitates with normal rabbit serum.


L
G
N
M
P90
P60
P50

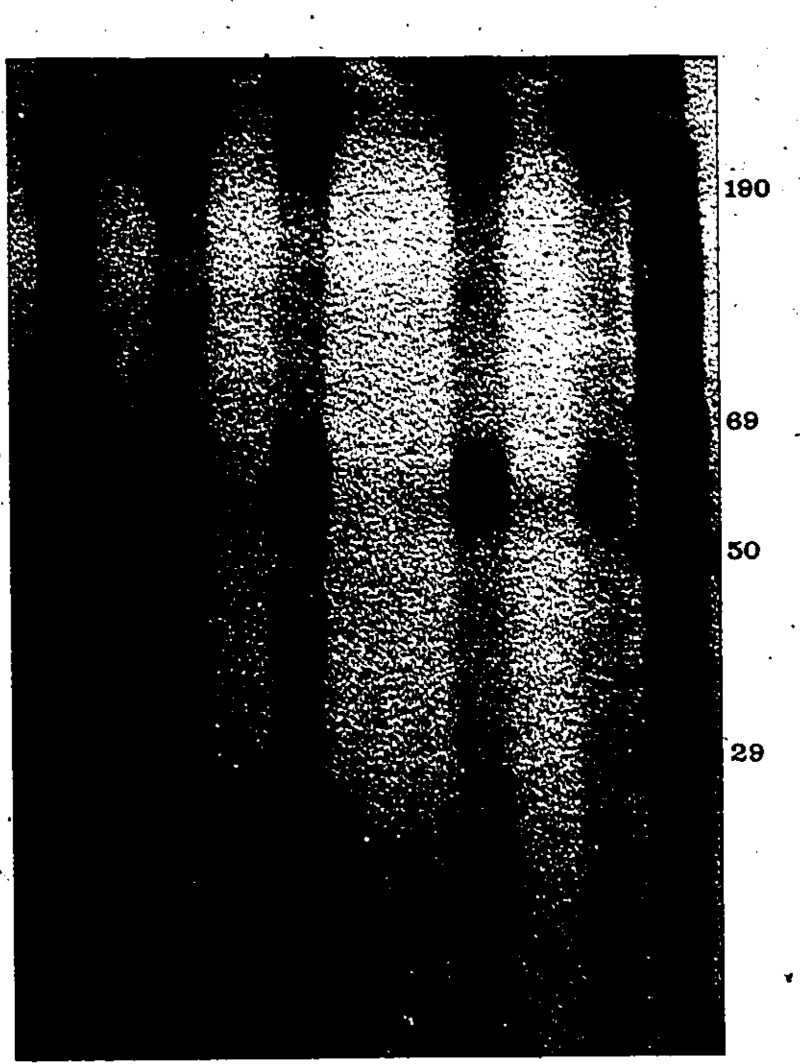
(lanes 4 and 5) as well as those from wild-types (lanes 1,2,6 and 7) at both temperatures. However, the ratio of $pp60^{src}$ to pp90 and pp50 were varied for each virus strain as demonstrated previously (Brugge et al., 1981). One interesting observation was that the relative amount of pp50 associated with $pp60^{src}$ in LA83-infected cells at the nonpermissive temperature (lane 5) was less than that present in LA83-infected cells at 35°C (lane 4). It has been preciously demonstrated that a cellular protein pp50 was phosphorylated at tyrosine with concomitant transformation of the infected cells by RSV (Brugge and Darrow, 1982). This phosphorylation of pp50 at tyrosine was not temperature sensitive in cells infected with mutant viruses containing a temperature sensitive defect in the src gene (Brugge and Darrow, 1982). Thus, it seems that the temperature sensitive defect of src in LA83 may be due to diminished association of pp50 with $pp60^{src}$. However, the possibility of other functional defects can not be ruled out. It was thus decided to examine the possible defect in the kinase activity present in $pp60^{src}$ by assaying the immune-complex phosphotransferase activity.

The immunoprecipitates from the infected cells were assayed for kinase activity. The protein A-sepharose beads containing bound immune-complexes were resuspended in 50 µl of the kinase buffer (20 mM Tris-HCl, pH 7.2 -

5 mM $MgCl_2$) and incubated at 30°C for 10 min in the presence of [γ-^{32}P] ATP (3000 Ci/mmol, Amersham). The reactions were terminated by addition of 10 vol of a buffer containing 300 mM NaCl, 5 mM EDTA, 10 % glycerol, 1 % Triton X-100, 1 % sodium deoxycholate, 100 KIU/ml Trasylol, 1 mM PMSF and 0.1 % SDS in 10 mM Tris-HCl (pH 7.2). The immune-complex was washed once with the above buffer except that the NaCl concentration was 10 mM. Finally, the proteins were solubilized in electrophoresis sample buffer and analyzed on a 10 % SDS-polyacrylamide gel. It is evident in Figure 3.1.22 that the phosphotransferase reaction carried out on $pp60^{src}$ from LA83-infected cells at either temperature resulted in labeling of the heavy chain of immunoglobulin (lanes 7 and 9) as in the reactions from the wild-type infected cells (lanes 3 and 5). However, cells infected with the mutant contain at the nonpermissive temperature (lane 9) less immunoprecipitable protein kinase activity than do cells at the permissive temperature (lane 7). The protein kinase activity of $pp60^{src}$ encoded by the mutant seems more labile than that encoded by wild-type virus (see below). Similar results were obtained previously with other temperature-sensitive mutants (Collet and Erikson, 1978; Sefton et al., 1979, 1980).

The kinase activity present in $pp60^{src}$ synthesized in LA83-infected cells at 41°C was tested for temperature-sensitivity. The immune-complex obtained from LA83-infected

Figure 3.1.22 The protein kinase activity induced by LA83. Lanes 1 and 10, molecular weight marker proteins; lanes 2 and 3, PR-B-infected cells grown at 35°C and immunoprecipitated with normal rabbit serum and TBR serum, respectively; lanes 4 and 5, PR-B-infected cells grown at 41°C and immunoprecipitated with normal rabbit serum and TBR-serum, respectively; lanes 6 and 7, LA83-infected cells grown at 35°C and immunoprecipitated with normal rabbit-serum and TBR serum, respectively; lanes 8 and 9, LA83-infected cells grown at 41°C and immunoprecipitated with normal rabbit serum and TBR serum, respectively.

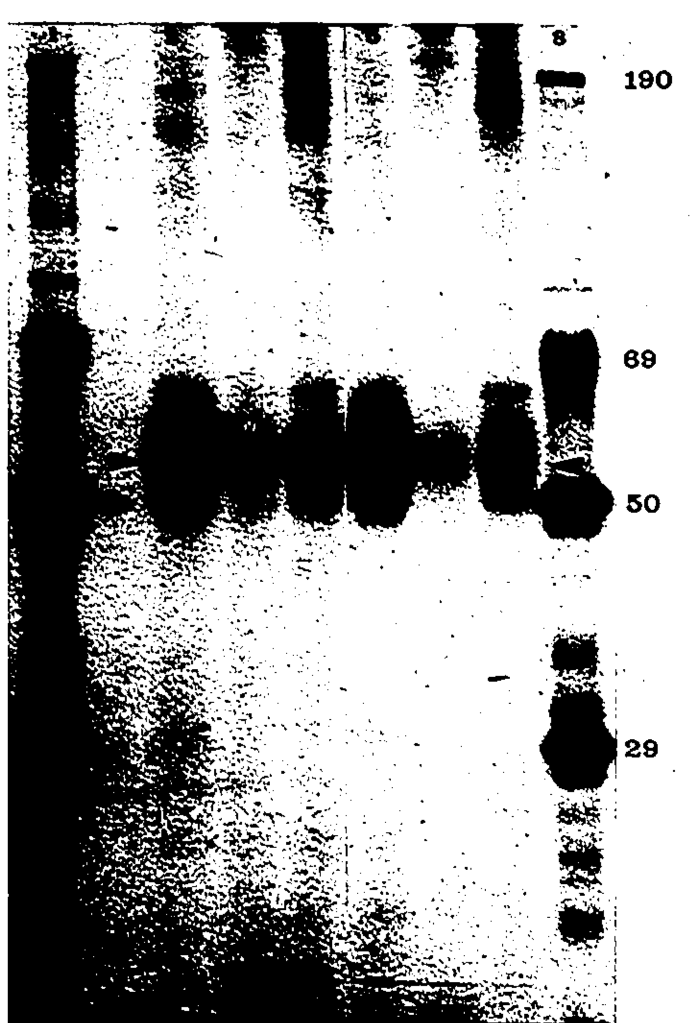

190
69
50
29

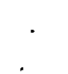
cells grown at 41°C was assayed for the kinase activity at both 35 and 41°C. First, the immune-complexes were preincubated at either 35 or 41°C for 2 min, the [γ-^{32}P] ATP was added and the mixtures were further incubated 5 min at the respective temperatures. The reactions were terminated as above and analyzed on a 10% SDS-polyacrylamide gel. As shown in Figure 3.1.23, the kinase activity was temperature-sensitive. The labeling in the immune-complex from LA83-infected cells at 41°C and assayed at 41°C (lane 6) was relatively less than the one assayed at 35°C (lane 3). No significant difference was evident in the mutant infected cells grown at 35°C and assayed at either temperature (lanes 4 and 7). This result was unexpected and not clear why the activity from mutant extracts grown at 35°C is not ts in the <u>in vitro</u> experiment. One possibility is that the LA83 pp60^{src} is functionally stabilized when synthesized at 35°C. In contrast, the pp60^{src} produced at 41°C was able to recover the activity slightly when assayed at 35°C (lane 3). However, the activity is still much lower than the activity from mutant extracts grown at 35°C (lanes 4 and 7).

The lability of the protein kinase activity of the mutant suggests that this activity is essential for transformation which is temperature-sensitive in LA83-infected cells. The observation of protein kinase activity in cells infected with the mutant at the nonpermissive temperature suggests a possibility that the transformed phenotype of a

Figure 3.1.23 Temperature-sensitivity of the Protein-Kinase Activity Induced by LA83.

The $pp60^{src}$ protein containing protein kinase activity was immunoprecipitated from PR-B-infected cells grown at 41°C, and cells infected with LA83 grown at 35 or 41°C. The cells were lysed and equal volumes of the cell lysates were immunoprecipitated with TBR serum and assayed for the protein-kinase activity at 35 and 41°C, respectively. Lanes 1 and 8, molecular weight marker proteins; lanes 2 and 5, PR-B-infected cells grown at 41°C and assayed at 35 and 41°C, respectively; lanes 3 and 6, LA83-infected cells grown at 41°C and assayed at 35 and 41°C, respectively; lanes 4 and 7, LA83-infected cells grown at 35°C and assayed at 35 and 41°C, respectively.


8
190
69
50
29



chick cell is dependent critically on the actual concentration of src-encoded protein kinase activity, however, consideration of other factors, such as the amount available and/or the level of association of the substrates (eg. pp50) with $pp60^{src}$, in the transformation of a chick cell can not be ruled out.

Stability and Kinetics of Processing of Intracellular Precursor Polypeptides

The intracellular processing of viral precursor polypeptides was analyzed by pulse-chase experiments. Infected cells were pulsed for 30 min with [^{35}S] methionine, then chased for varying amounts of time, and virus-specific proteins were analyzed by immunoprecipitation with anti-RSV, anti-p27, anti-glycoprotein, or anti-reverse transcriptase antisera and electrophoresed on SDS-polyacrylamide gels. Figure 3.1.24 shows the results obtained with anti-RSV antiserum. Both $Pr76^{gag}$ and $gPr92^{env}$ could be chased and hence processed at both 35 and 41°C. A significant amount of $Pr180^{gag-pol}$ was, however, present even after a 4 hrs chase at 41°C in cells infected with LA83. In contrast, $Pr180^{gag-pol}$ present in cells infected with LA83 or PR-B at 35°C as well as in cells infected with PR-B at 41°C could be chased. The processing of $gPr92^{env}$ was presented in Fig. 3.1.25, in which the pulse-chased cell extracts were analyzed by immunopreci-

Figure 3.1.24 Polyacrylamide gel analyses of [^{35}S] methionine labeled viral proteins immunoprecipitated with anti-RSV antiserum.

RSV-infected cells were pulse-labeled for 30 min at 35 or 41°C, then chased for 2 and 4 hrs. The labeled cells were lysed and immunoprecipitated with anti-RSV antiserum. The precipitates were then analyzed on a 7.5 to 15 % gradient polyacrylamide slab gel.

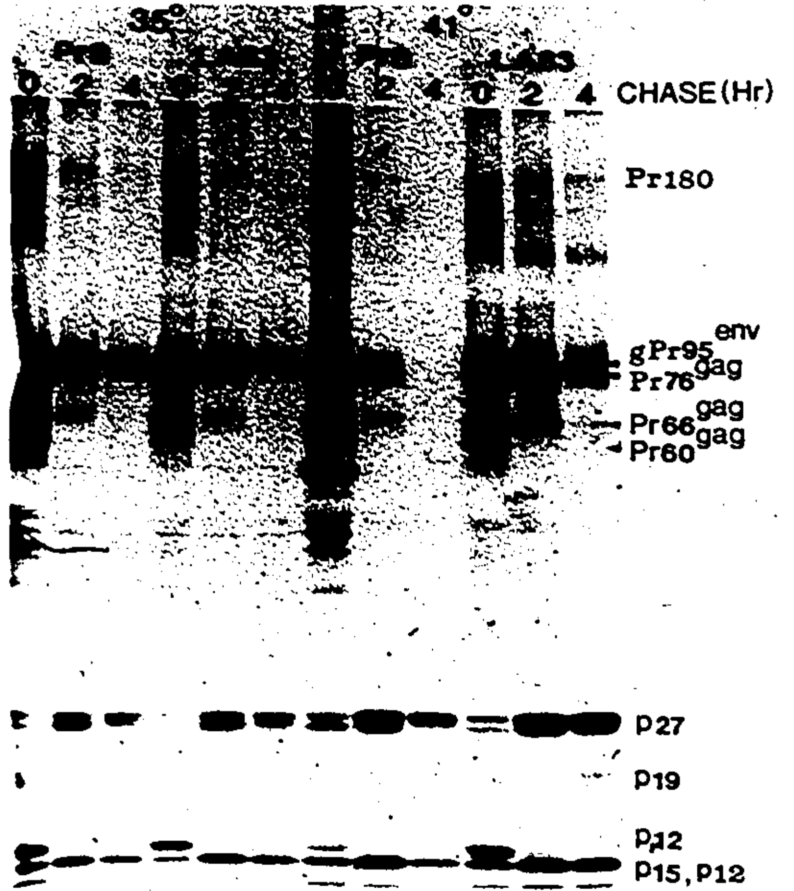

35°
41°
0 2 4
0 2 4 CHASE (Hr)
Pr180
gPr95 env
Pr76 gag
Pr66 gag
Pr60 gag
p27
p19
p12
p15, p12

Figure 3.1.25 Polyacrylamide gel analyses of [^{35}S] methionine labeled viral proteins immunoprecipitated with anti-gp antiserum.

RSV-infected cells were pulse-labeled for 30 min at 35 or 41°C, then chased for 2 and 4 hrs. The labeled cells were lysed and immunoprecipitated with anti-gp antiserum. The precipitates were then analyzed on a 10 % polyacrylamide gel. Lanes 1 to 3, PR-B-infected cells at 35°C were pulsed and chased, respectively; lanes 4 to 6, LA83-infected cells at 35°C were pulsed and chased, respectively; lanes 7 to 9, PR-B-infected cells at 41°C were pulsed and chased, respectively; lanes 10 to 12, LA83-infected cells at 41°C were pulsed and chased, respectively. The arrow indicates the precursor protein $gPr92^{env}$. No sample is present in lane 5.

1 2 3 4 5 6 7 8 9 10 11 12

Figure 3.1.26 Accumulation of Pr180$^{gag-pol}$ in LA83-infected cells at the nonpermissive temperature.

Autoradiograms of polyacrylamide slab gels containing electrophoretically separated polypeptides from LA83-infected cells after immunoprecipitation with different antisera. LA83-infected cells at 35°C were pulse labeled with [^{35}S]-methionine for 30 min (lane a) and chased for 2 (lane b) and 4 hrs (lane c) at 35°C and immunoprecipitated with anti-RSV-antiserum. LA83-infected cells at 41°C were pulse labeled with [^{35}S] methionine for 30 min (lanes d,g, j) and chased for 2 (lanes e, h, k) and 4 hrs (lanes f, i, l) at 41°C, and immunoprecipitated with anti-pol (lanes d to f), anti-p27 (lanes g to i), or anti-gp (lanes j to l) antisera, respectively. This figure is directly from Ro and Ghosh (1984).

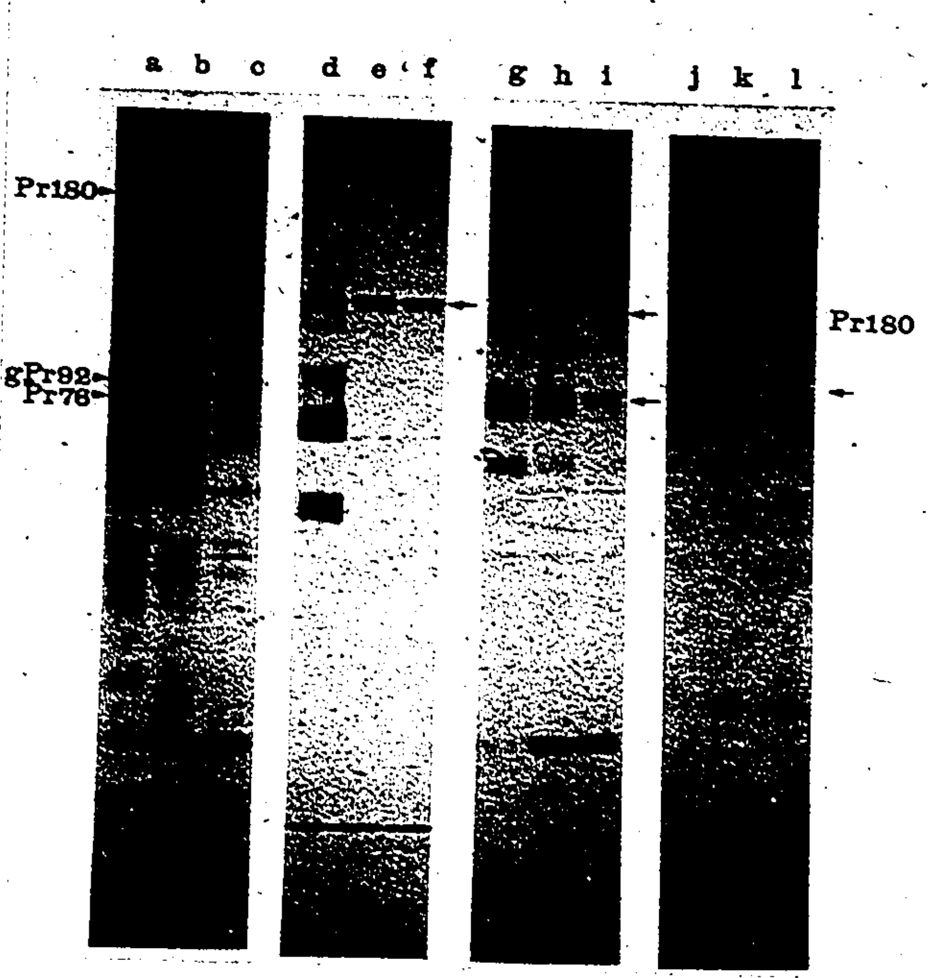
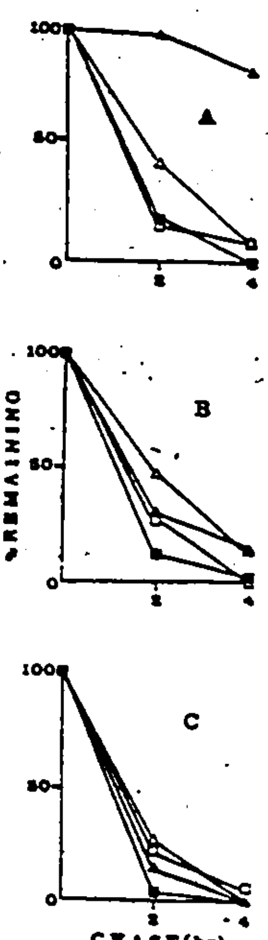

a b c d e f g h i j k l
Pr180
gPr92
Pr78
Pr180

Figure 3.1.27 Disappearance of precursor molecules in RSV-infected cells.

Quantitation of specific protein bands was obtained by scanning autoradiographs in a transmission densitometer. □, PR-B-infected cells pulsed and chased at 35°C; ■, PR-B-infected cells pulsed and chased at 41°C; Δ, LA83-infected cells pulsed and chased at 35°C; ▲, LA83-infected cells pulsed and chased at 41°C. (A) $Pr180^{gag-pol}$, (B) $Pr76^{gag}$, (C) $gPr92^{env}$. The results were obtained from autoradiographs of immunoprecipitations with anti-pol, anti-p27, and anti-glycoprotein antisera, respectively. This figure is directly from Ro and Ghosh (1984).


A
B
C
100
50
0
2
4
%REMAINING
CHASE(hr)

pitation with anti-glycoprotein antiserum. More convincing evidence for accumulation of $Pr180^{gag-pol}$ in LA83 infected cells at 41°C was obtained by immunoprecipitation with anti-reverse transcriptase antiserum. As presented in Fig. 3.1.26, a significant amount of $Pr180^{gag-pol}$ was present even after a 4 hrs chase at 41°C in cells infected with LA83 (lane f). In contrast the amounts of $Pr180^{gag-pol}$ present in cells infected with LA83 at 35°C decreased significantly during this chase (lanes a to c). Therefore, it appeared that in the case of LA83 there was a reduction in the rate of processing of $Pr180^{gag-pol}$ into reverse transcriptase at the nonpermissive temperature. Both $Pr76^{gag}$ and $gPr92^{env}$ could be chased and hence processed at both 35 and 41°C in LA83-infected cells (lanes a to c, g to l). The relative amounts of the three precursor polypeptides, $Pr180^{gag-pol}$, $Pr76^{gag}$, and $gPr92^{env}$ after different periods of chase were determined by microdensitometric analyses of the corresponding bands present in the autoradiograms obtained by immunoprecipitation with anti-reverse transcriptase, anti-p27, or anti-glycoprotein antisera. A plot of the percentage of the precursor remaining after chase against the duration of the chase is presented in Fig. 3.1.27. The results show that only the precursor $Pr180^{gag-pol}$ in the case of LA83 infected cells at 41°C was stable and about 80 % of the precursor was not chased even after 4 hrs. Over 90 % of $Pr180^{gag-pol}$ was, however, chased in 4 hrs in the case of LA83-infected cells

at 35°C or PR-B-infected cells at 35 or 41°C. The $t_{\frac{1}{2}}$ of $Pr180^{gag-pol}$ in LA83-infected cells at 41°C was greater than 4 hrs and at 35°C it was about 1.75 hrs (see Table II). Results of previous experiments (Hayman, 1978a) showed that $t_{\frac{1}{2}}$ for $Pr180^{gag-pol}$ in PR-B-infected cells was about 1.15 hrs at 35°C and 1.25 hrs at 41°C. Also, the $t_{\frac{1}{2}}$ values of 0.75 to 1 hr and 1 to 1.5 hrs were reported earlier for $Pr76^{gag}$ and $gPr92^{env}$, respectively, in wild-type virus-infected cells (Hayman, 1978a; Oppermann et al., 1977; Vogt et al., 1975).

Effects of Temperature Shiftdown on the Processing of $Pr180^{gag-pol}$

It was observed that although LA83-infected cells at the restrictive temperature produced noninfectious particles, a shiftdown to the permissive temperature resulted in synthesis of infectious virions within 2 hrs after shiftdown (Ghosh, 1984). To investigate whether the intracellular precursor $Pr180^{gag-pol}$ was processed and the reverse-transcriptase produced was incorporated in the released virus particles, LA83-infected cells were labeled for 30 min at 41°C with [^{35}S] methionine and the temperature was shifted down to 35°C in the presence of cycloheximide (50 µg/ml) so that only previously synthesized polypeptides were utilized for virus production. The intracellular proteins were analyzed at various intervals after shiftdown by immunoprecipita-

Figure 3.1.28 Processing of $Pr180^{gag-pol}$ in LA83-infected cells after shiftdown to the permissive temperature.

[^{35}S] Methionine-labeled polypeptides from LA83-infected cells were immunoprecipitated with anti-pol antiserum and electrophoresed on a 10% polyacrylamide slab gel. Lane a, molecular weight marker proteins; lane b, LA83-infected cells at 41°C pulse labeled 2 hr with [^{35}S] methionine; lanes c to g, LA83-infected cells at 41°C pulse labeled 2 hr, then shifted down to 35°C and chased for 0.5, 1, 2, 4, 6 hr, respectively. The graph (right) shows the rate of disappearance of $Pr180^{gag-pol}$. Quantitation of $Pr180^{gag-pol}$ was obtained as described in legend to Fig. 3.1.27. This figure is directly from Ro and Ghosh (1984). The arrow indicates the precursor polypeptide $Pr180^{gag-pol}$.

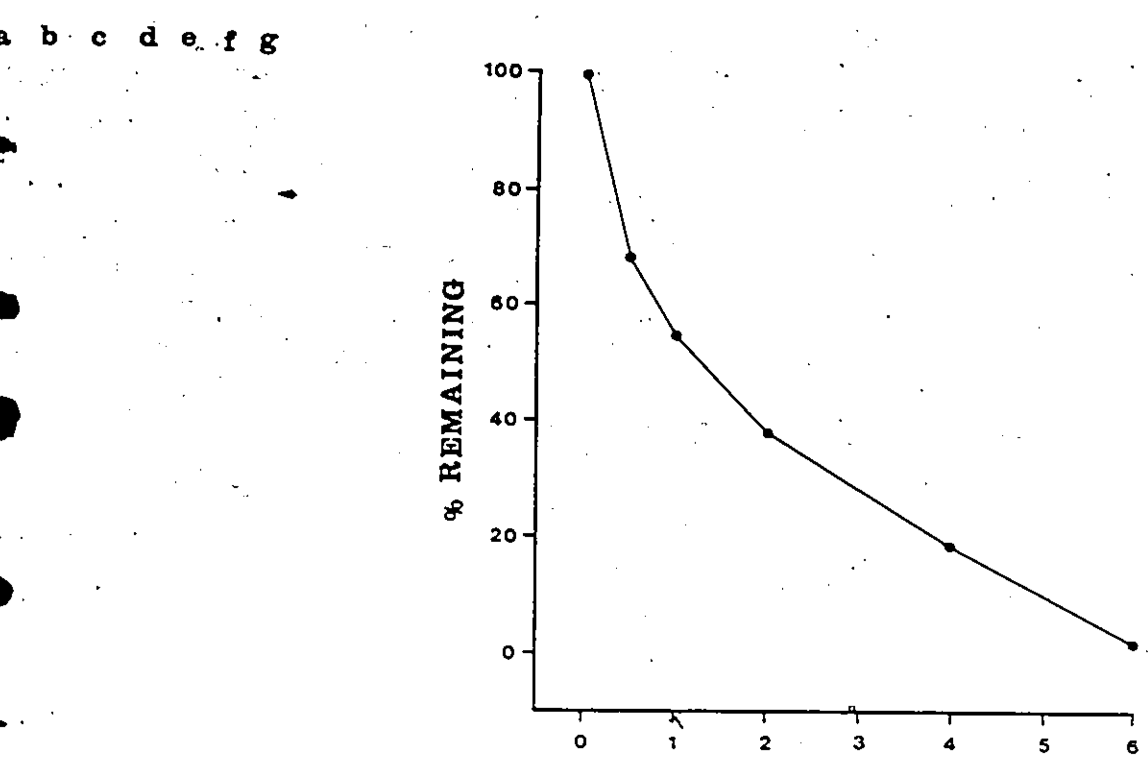

a b c d e f g
100
80
60
40
20
0
% REMAINING
0 1 2 3 4 5 6
HOURS

Table II. Approximate half-life of RSV precursor polypeptides.

Precursors	Cell Cultures			
	PR-B/35°C	PR-B/41°C	LA83/35°C	LA83/41°C
$Pr180^{gag-pol}$	1.15	1.25	1.75	>4
$Pr76^{gag}$	1.35	1.0	1.9	1.45
$gPr92^{env}$	1.3	1.1	1.4	1.2

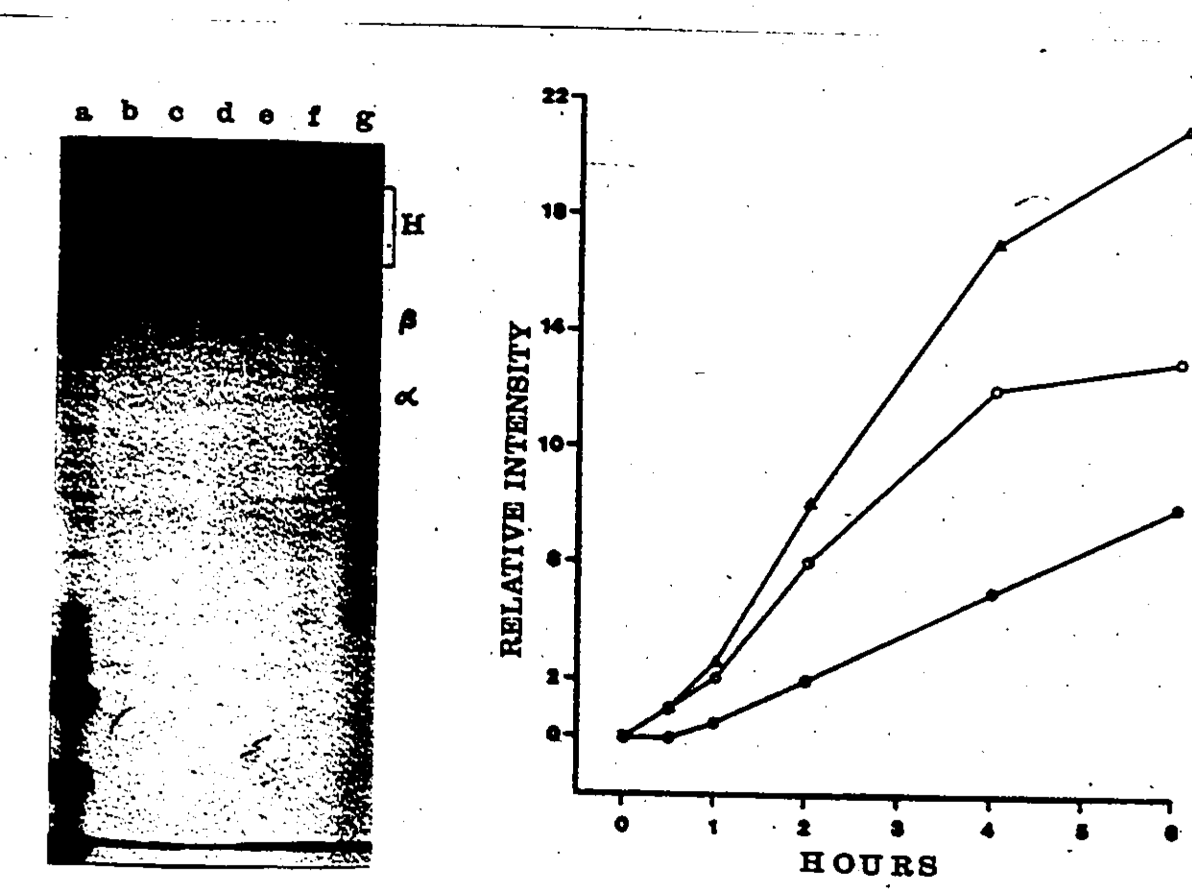
The half-life ($t_{\frac{1}{2}}$) of the three precursors were determined from the results of kinetics of turnover of the precursors from the autoradiograms of immunoprecipitations with anti-polymerase, anti-p27, and anti-glycoprotein sera, respectively.

tion with anti-reverse transcriptase antiserum and SDS-PAGE (Fig. 3.1.28). It was evident that in the infected cells $Pr180^{gag-pol}$ could now be chased after shiftdown. The graph in Fig. 3.1.28 shows the quantitation of the amounts of $Pr180^{gag-pol}$ remaining after temperature shiftdown for various intervals of time. It was observed that $t_{\frac{1}{2}}$ of $Pr180^{gag-pol}$ showed a marked decrease to about 1.25 hrs on shiftdown in the presence of cycloheximide. This value was comparable to the $t_{\frac{1}{2}}$ of wild-type virus-infected cells (Table II).

Since the appearance of the products of processing of $Pr180^{gag-pol}$, namely α and β subunits were not evident in the cells (Figs. 3.1.12, 3.1.15, 3.1.17, 3.1.20, 3.1.24, 3.1.26, 3.1.28), the virus released during the chase after shiftdown was purified and analyzed by immunoprecipitation with anti-reverse transcriptase antiserum and electrophoresis on SDS-polyacrylamide gel (Fig. 3.1.29). It was evident that both α and β subunits appeared and increased with time of chase. The presence of a number of bands at the 200K-Da region was variable and was observed in viruses harvested at early times. The polypeptides may represent host contaminants which were nonspecifically precipitated with pre-immune serum. Tryptic-peptide analyses of two polypeptides at the region of 200K-Da showed no homologies to $Pr180^{gag-pol}$ (Fig. 3.1.8). These data are in agreement with the data presented by Eisenman *et al*. (1980a) and suggested that

Figure 3.1.29 Appearance of reverse-transcriptase subunits in virus particles.

LA83-infected cells at 41°C were pulse labeled with [^{35}S]-methionine then chased after shiftdown to 35°C for varying lengths of time. Virus was purified from the supernatant medium, immunoprecipitated with anti-pol antiserum, then analyzed by SDS-polyacrylamide gel electrophoresis on a 12.5% gel. Lane a contains purified PR-B virus labeled for 16 hr with [^{35}S] methionine; lane b, virus released from cells infected with LA83 after 2 hr labeling at 41°C; lanes c to g, virus released from cells infected with LA83 after 2 hr labeling at 41°C followed by 0.5, 1, 2, 4, and 6 hr chase, respectively at 35°C. The graph (right) shows the relative amounts of reverse-transcriptase subunits in virus released from LA83-infected cells after 2 hr labeling at 41°C (0 hr) followed by 0.5, 1, 2, 4, and 6 hr chase, respectively at 35°C. The relative amounts of radioactivity in each band were determined by scanning densitometry as described in legend to Fig. 3.1.27. Symbols: •, α subunit; o, β subunit; Δ, combined α and β subunits. This figure is directly from Ro and Ghosh (1984).


a b c d e f g
H
β
α
RELATIVE INTENSITY
22
18
14
10
6
2
0
0
1
2
3
4
5
6
HOURS

$Pr180^{gag-pol}$ was not incorporated into virions as a precursor to α and β subunits. Thus, polymerase precursors are probably cleaved concomitant with virus budding, and only the products of these cleavages are actually incorporated into virus particles.

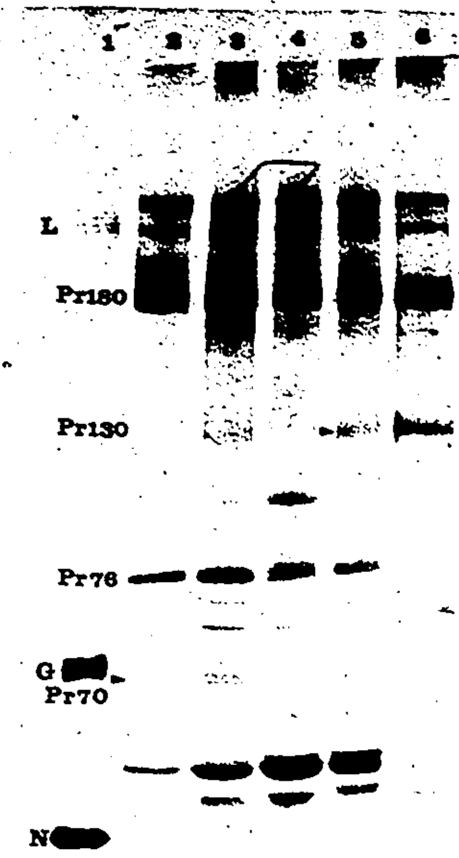
The kinetics of appearance of reverse transcriptase subunits in virions is shown in Fig. 3.1.29. In agreement with the previously published data (Eisenman et al., 1980a), the presence of the β subunit was detected first, whereas the α subunit appeared after a lag period. These results are also in agreement with reports that α is derived by cleavage from β (Gibson and Verma, 1974; Moelling, 1975). The disappearance of $Pr180^{gag-pol}$ corresponded with the appearance of α and β subunits in virus particles (compare Figs. 3.1.28 and 3.1.29).

SECTION 3.2: PROCESSING OF REVERSE TRANSCRIPTASE PRECURSOR MOLECULES

A study of the processing of $Pr180^{gag-pol}$ requires the identification of the possible intermediate(s) involved in the synthesis of α and β subunits of the reverse transcriptase. It has been shown that the immuno-complexed $Pr180^{gag-pol}$ molecules could be cleaved in an *in vitro* experiment, resulting in the formation of a new polypeptide with molecular weight of 130,000 (Fig. 3.1.7). Analyses of intracellular proteins resulted in further identification of two putative intermediate polypeptides with molecular weights of 130,000 (Pr130) and 70,000 (Pr70), respectively. The detection of these polypeptides was variable depending on the labeling conditions. These polypeptides have been further characterized by immunoprecipitation with the monospecific antisera. Results presented in Figure 3.2.1 showed that Pr130 contains antigenic determinant for p15 and reverse-transcriptase sequences, and Pr70 contains antigenic determinants for gag sequences. The relative amount of Pr130 and Pr70 were significantly less than $Pr180^{gag-pol}$. Shortly after the identification of these putative intermediates, Eisenman and coworkers (Eisenman et al., 1980a) presented similar results, however, they were not able to demonstrate unequivocally whether $Pr130^{gag-pol}$ is a cleavage product of $Pr180^{gag-pol}$ or a primary translation product.

Figure 3.2.1 Immunological characterization of Pr130 and Pr70.

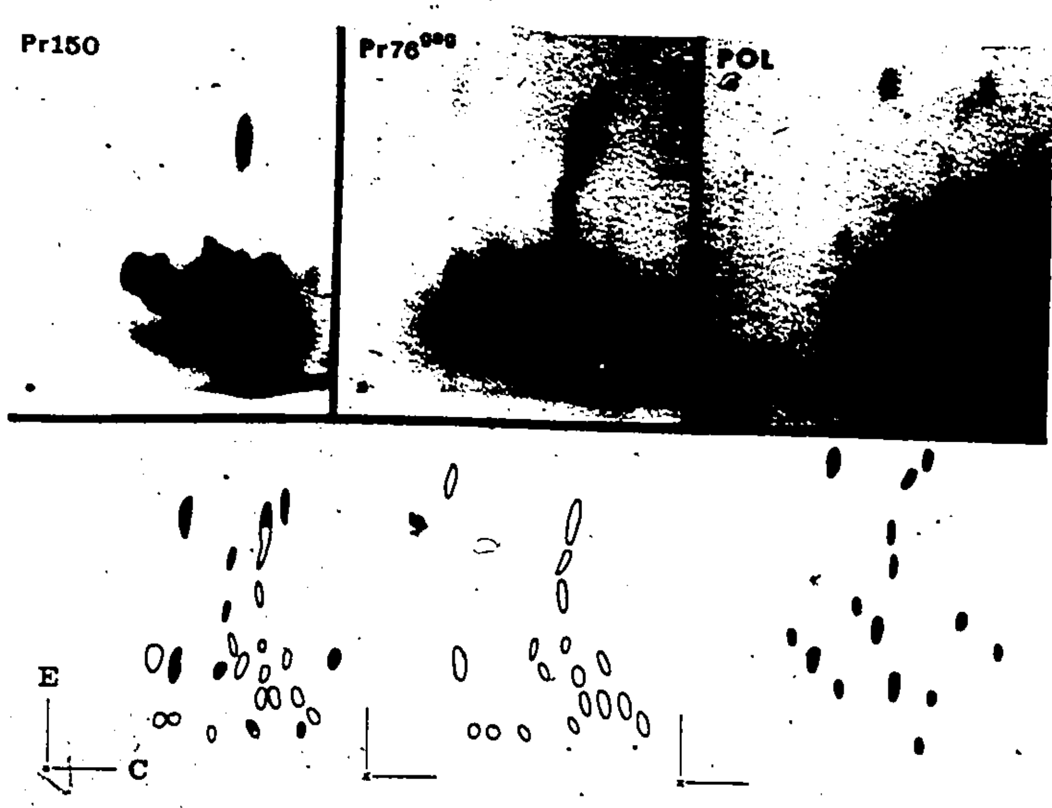
LA83-infected cells at 41°C were labeled with [^{35}S]-methionine for 2 hrs, then labeled cells were lysed and immunoprecipitated with the monospecific antisera. The precipitates were analyzed on a 12.5% polyacrylamide gel. Lane 1, molecular weight marker proteins; lanes 2 to 6, immunoprecipitates with anti-p19, anti-p27, anti-p12, anti-p15, and anti-pol antisera, respectively.


1
2
3
4
5
6
L
Pr180
Pr130
Pr76
G
Pr70
N
M

Further analysis of the intracellular proteins labeled for short time from the infected cells resulted in the identification of an additional putative intermediate polypeptide with apparent molecular weight of 150,000 (Pr150). This protein was shown to contain antigenic determinants for both gag and reverse transcriptase sequences (Fig. 3.1.17). The relative amount of Pr150 was less than $Pr180^{gag-pol}$. These data suggest that Pr150 may represent a minor intermediate product. The peptide make up of Pr150 was established by tryptic peptide mapping. The [^{35}S] methionine-labeled protein was eluted from a gel in the presence of TPCK-trypsin, oxidized, and subjected to two-dimensional tryptic peptide analyses as described in Materials and Methods. Figure 3.2.2. shows the profile of [^{35}S] methionine labelled tryptic-peptides from Pr150 (immunoprecipitated from LA83-infected cells at 41°C), $Pr76^{gag}$ (immunoprecipitated from PR-B-infected cells), and reverse transcriptase (immunoprecipitated from disrupted PR-B virions). It is evident that all the [^{35}S] methionine-labeled peptides of $Pr76^{gag}$ were coincident with those of Pr150. Some of the tryptic peptides of reverse-transcriptase were also coincident with those of Pr150, but a number of them were apparently missing. These results demonstrate that Pr150 does not possess complete pol-related sequences, but possesses complete gag-related sequences. Thus, the possible sequence of Pr150 may be represented as NH_2-Pr76-pol (α)-COOH, where the unique carboxy-terminus

Figure 3.2.2 Two-dimensional tryptic-peptide fingerprinting analyses of Pr150.

[^{35}S] Methionine-labeled polypeptides were isolated by gel electrophoresis and excised from the gel and treated as described under Materials and Methods. Autoradiographs of the dried plates are shown in the figure; a schematic diagram of each autoradiograph is shown immediately below it. The origin of each fingerprint, indicated by x in the diagrams, is at the lower left corner, and electrophoresis (E) is from bottom to top. Pr150 was obtained from LA83-infected cells at 41°C. Pr76^{gag} and reverse-transcriptase (POL) were obtained from PR-B-infected cells and purified PR-B virus, respectively. The reverse transcriptase was immunoprecipitated from disrupted virus with anti-pol antiserum and finally isolated by gel electrophoresis. In the shcmetic diagrams, peptides characteristic of reverse transcriptase are indicated in filled and Pr76 is open outlines.

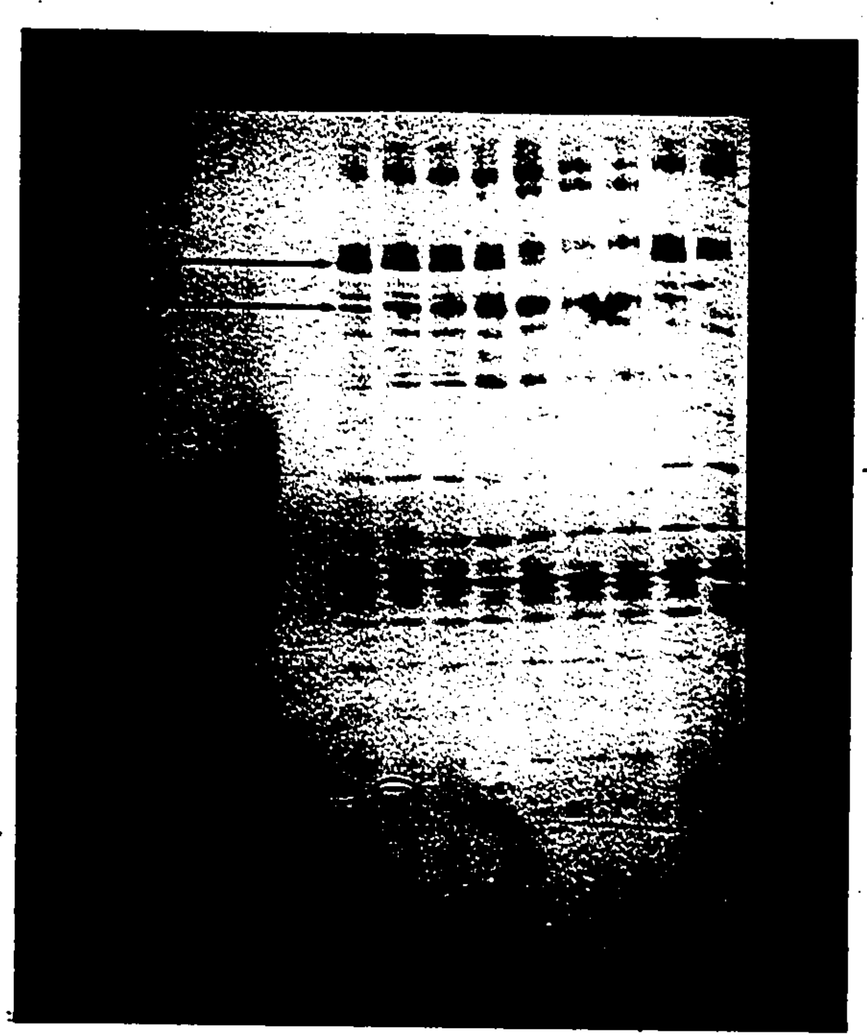

Pr150
POL
E
C

region of β subunit (pp32) is absent. These results further suggest that Pr150 may be cleaved from Pr180$^{gag-pol}$.

In vitro cleavage experiments were, therefore, carried out to determine whether Pr150, Pr130, and Pr70 are intermediates in the processing of Pr180$^{gag-pol}$. Cells infected with LA83 were labeled with [^{35}S] methionine for 2 hours at 41°C and the labeled cells were lyzed and immunoprecipitated with anti-reverse transcriptase antiserum. The immuno-complex bound to protein A-Sepharose was incubated at 35°C for various length of times, in the presence of detergent-disrupted purified PR-B virions, which served as a source of the protease p15. The products were analyzed on a SDS-polyacrylamide gel. Results presented in Figure 3.2.3 showed that Pr180$^{gag-pol}$, Pr150, Pr130, and Pr70 were precipitated from LA83-infected cells at 41°C (lane 3), but they were not precipitated from the cell lysates with preimmune serum (lane 11). These proteins were stable during the 8 hours incubation in the absence of virion protease (lane 10), however, they were processed in the presence of the protease (lanes 4 to 9). The processing of the proteins Pr180$^{gag-pol}$ and Pr150 resulted in increased appearance of Pr130 and Pr70 proteins during the first 2 hours. After 2 hours the amount of these two proteins showed a gradual decrease with increasing time of incubation. Furthermore, a concomitant appearance of a polypeptide, which comigrated with p27, was evident. These data suggest

Figure 3.2.3 *In vitro* processing of reverse transcriptase precursors.

LA83-infected cells at 41°C were labeled with [^{35}S] methionine and labeled cells were lysed and immunoprecipitated with anti-pol antiserum. The immune-complex was incubated at 35°C for various lengths of time in presence of 40 μg of disrupted unlabeled PR-B virus. The products were analyzed on a 12.5% polyacrylamide gel. Lanes 1 and 2, [^{35}S] methionine labeled PR-B virus; lanes 3 to 9, the immune-complexes were incubated in presence of the protease for 0, 0.5, 1, 2, 4, 6, and 8 hrs, respectively; lane 10, the immune-complex was incubated 8 hrs without the protease; lane 11, the precipitates with normal rabbit-serum.



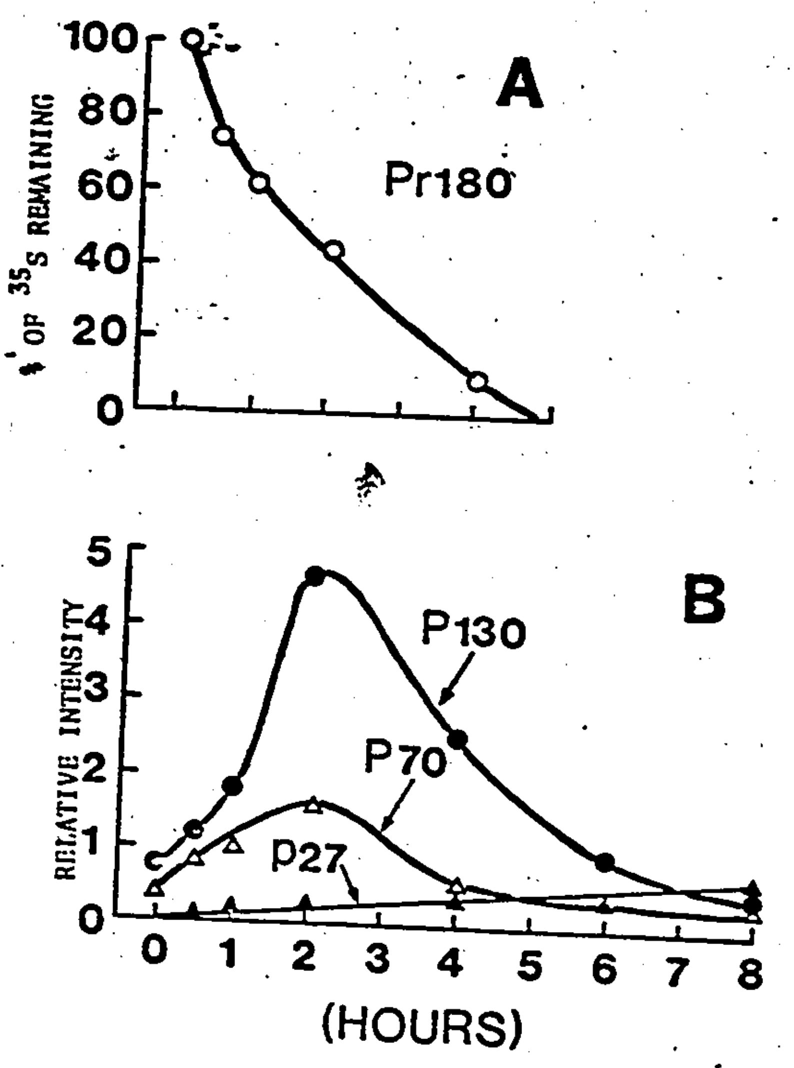


Figure 3.2.4 (A) The rate of disappearance of $Pr180^{gag-pol}$ in the presence of virion protease.

The immune-complex was incubated at 37°C for various lengths of time in presence of the detergent-treated PR-B viruses as in Fig. 3.2.3. The quantitation of $Pr180^{gag-pol}$ was obtained as described in legend to Fig. 3.1.27.

(B) The relative amounts of putative intermediates Pr130 and Pr70 and the product p27 generated during in vitro cleavage of $Pr180^{gag-pol}$.

The relative amounts of radioactivity in each band were determined by scanning densitometry as described in legend to Fig. 3.1.27.

that P130 and Pr70 may represent intermediate molecules cleaved from $Pr180^{gag-pol}$. Immunological analyses of these proteins in infected cells had also shown that they are possible intermediates of the cleavage of $Pr180^{gag-pol}$ (see above). The relative amounts of these polypeptides after different periods of incubation were determined by microdensitometric analyses of the corresponding bands present in the autoradiogram. A plot of the relative amounts of these polypeptides against the time of incubation is presented in Figure 3.2.4. The absence of reverse transcriptase subunits suggested that both subunits may be either unstable during *in vitro* cleavage or the observed intermediate(s) may not be further processed in the *in vitro* system. It should be noted that the production of the reverse transcriptase subunits by *in vitro* cleavage has not been reported. Pr70 may be processed to p27 since Pr70 was shown to be contain the antigenic determinant for p27 sequence. Figure 3.2.5 shows the proposed processing scheme of $Pr180^{gag-pol}$ based on the above data.

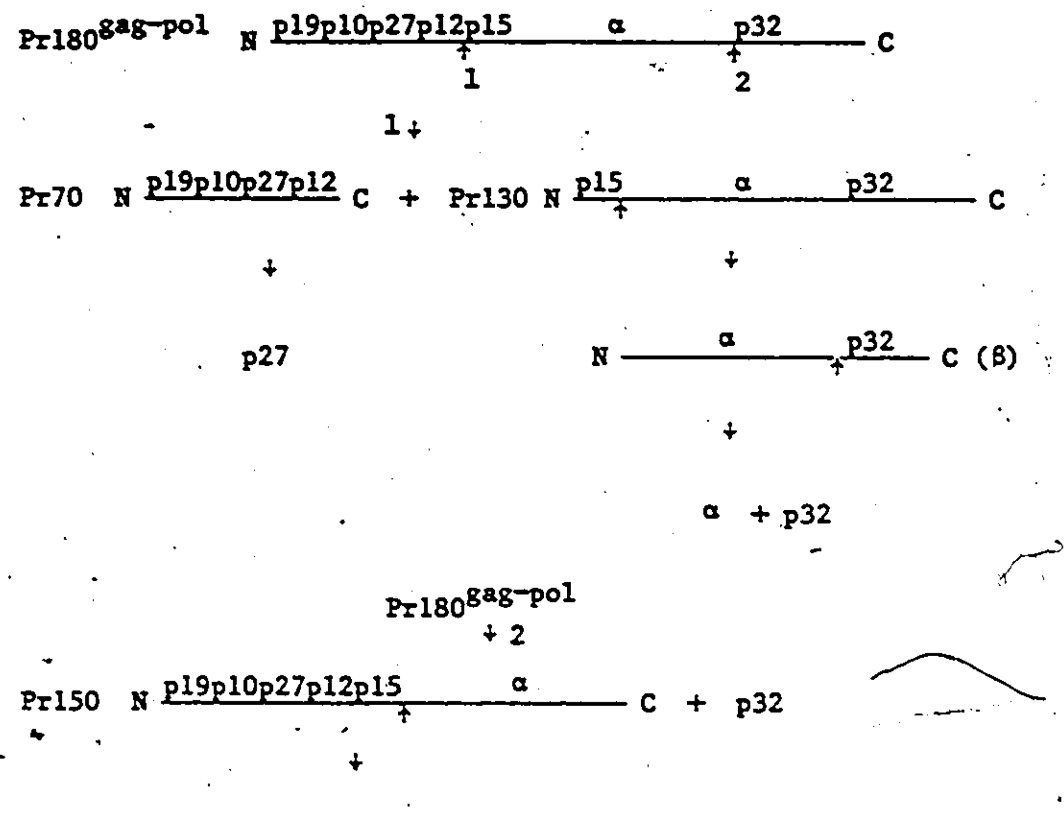


Figure [illegible].2.5 Proposed structures of the precursor and intermediate molecules and proposed scheme for the processing of the precursor molecules to their mature reverse transcriptase.

The initial cleavage at the $Pr180^{gag\text{-}pol}$ molecules are occurring at the gag polypeptide region (1) to liberate Pr70 and Pr130, and the putative cleavage site at the pol polypeptide region (2) between α and p32 to produce Pr150 and p32. Pr130 is further cleaved as in the Figure 1.5 and Pr70 is further cleaved to generate p27. Pr150 is cleaved at the gag-pol junction sequence to produce Pr76 and α.

SECTION 3.3: EXPRESSION OF REVERSE TRANSCRIPTASE GENE IN *E. coli*

The structure and function of the retrovirus reverse transcriptase have been intensively investigated in recent years (Gerard and Grandgenett, 1980; Donehower and Varmus, 1984; Grandgenett et al., 1985; Hippenmeyer and Grandgenett, 1985; Jacks and Varmus, 1985; Roth et al., 1985; Tanese et al., 1985; Varmus, 1985). Reverse transcriptase is required for the conversion of viral genomic RNA into the proviral DNA which is subsequently integrated into the host chromosomal DNA. Purified reverse transcriptase manifests both synthetic and degradative activities. The synthetic activities are characterized by RNA-directed synthesis of cDNA and DNA-directed synthesis of double-stranded DNA. The degradative activities are represented by RNase H activity, which degrades specifically the RNA moiety of DNA:RNA hybrid, and DNA endonuclease activity. Limited information has been obtained about the localization of these various activities on the reverse transcriptase molecule, except for endonuclease activity (Golomb and Grandgenett, 1979; Grandgenett et al., 1978, 1980, 1985; Hippenmeyer and Grandgenett, 1984, 1985). The polymerase β polypeptide is cleaved to the α subunit and the pp32 DNA binding protein *in vitro*. The DNA polymerase and RNase H

activities are located on the NH_2-terminal α polypeptide moiety (Grandgenett et al., 1985). Indirect experiments with various enzymatic inhibitors indicate that DNA polymerase and RNase H are located on different active sites (Gerard and Grandgenett, 1980; Grandgenett et al., 1985). A polypeptide fragment (Mr = 24,000) having only RNase H activity can be isolated by partial chymotryptic digestion of the α moiety (Lai and Verma, 1978). Anti-peptide sera directed against different regions of α subunit have been tested to determine the origin of the p24 protein (Grandgenett et al., 1985). The results suggeted that the RNase H active site is located in the NH_2-terminus of α subunit, consistent with the arrangement found in MuLV pol (Levin et al., 1984). The DNA endonuclease site is apparently located on the carboxyl terminus of the β polymerase subunit (Golomb and Grandgenett, 1979; Samuel et al., 1979; Hizi et al., 1982; Leis et al., 1983; Grandgenett et al., 1985) and a similar endonuclease activity has been demonstrated to be associated with pp32, the phosphoprotein derived from the carboxyl terminus of β(Grandgenett et al., 1978; Eisenman et al., 1980; Copeland et al., 1980; Grandgenett et al., 1985). The endonuclease pp32 is believed to be involved in integration of the synthesized proviral DNA. pp32 was shown to bind to specific sequences on avian retrovirus LTR DNA including the circle junction in *in vitro* studies (Misra et al., 1982; Knaus et al., 1984). The

circular viral molecule containing two LTRs in tandem is the presumed precursor molecule to the integrated provirus (Panganiban and Temin, 1983, 1984). The endonuclease pp32 was shown to nick supercoiled DNA containing LTR circle junction sequences (Grandgenett and Vora, 1985). Recently, virus mutants possessing deletion in the pp32 region have demonstrated that this region encodes functin(s) essential for replication of the virus (Hippenmeyer and Grandgenett, 1984). Two separate point mutations generated near the amino-terminus of pp32 resulted in decreased viral replication and cell transformation due to reduced synthesis of the viral RNA from the integrated provirus (Hippenmeyer and Grandgenett, 1985). It appears that transcription is affected by aberrant integration.

The function of RNase H during the synthesis of proviral DNA is still not very clear. It has been proposed that during the synthesis of cDNA, RNase H degrades the RNA template and provides a primer with a 3'-hydroxyl group to initiate the synthesis of the second strand of DNA (Baltimore and Smoler, 1971; Keller and Crouch, 1972; Leis et al., 1973). Studies involving inhibitors specific for RNase H activity (eg., NaF, $\geq$ 150 mM KCl), however, do not seem to support this notion (Brewer and Wells, 1974; Collett and Faras, 1976). The size of cDNA and the amounts of double-stranded DNA synthesized in their studies are relatively small and thus may not reflect the true picture.

More satisfactory answers can be provided from studies involving mutants that have lesions in only RNase H activity. However, all ts pol mutants that have been tested for RNase H activity are ts for both RNase H activity and polymerase-activity and no mutants have been isolated that are ts for Rnase H activity alone. Another approach would be to use a reconstituted system containing DNA polymerase free of RNase H activity. This approach, however, requires physical separation of the two enzymatic activities.

The recent application of recombinant DNA techniques to the study of retroviruses has made it possible to isolate and to amplify the genetic information of such viruses for detailed biological and molecular analyses, such as sequencing of the viral genome, studies of viral gene expression, and site-specific mutagenesis within the viral genome. By expressing reverse transcriptase gene in _E. coli_ and manipulating the gene by site directed mutagenesis, it may be possible to establish the functional domains of the various activities of this very multifunctional enzyme molecule.

This section describes the expression of reverse-transcriptase gene in _E. coli_ harboring recombinant plasmids which contain either the complete sequence of RSV proviral DNA or cloned reverse transcriptase gene.

The presence of two regions of general homology in prokaryotic transcriptional promoter sequences has been

well documented (McClure, 1985). These are the Pribnow-box, or -10 region, and the -35 region (Pribnow, 1975; Schaller et al., 1975; Gilbert, 1976) centered about 10 and 35 base pairs, respectively, upstream from the transcriptional initiation site. More recently, statistical analyses of promoter sequence composition have resulted in the generation of model promoter sequences containing the most frequently found bases at given positions throughout the RNA polymerase recognized region (Scherer et al., 1978; Rosenberg and Court, 1979; Siebenlist et al., 1980; McClure, 1985).

Previous studies have established that mRNAs are translated with varying efficiencies in prokaryotic cells, and any effort to ensure the efficient expression of cloned eukaryotic genes in bacteria must consider not only the faithful transcription of the inserted gene but also the specificity, efficiency and stability of the mRNA produced. Whereas the initiation of transcription presumably is dictated by the existence of a promoter, the initiation of protein synthesis appears to be governed, at least in part, by the availability of a ribosome binding site (RBS) (Grunberg-Manago and Gross, 1977; Steitz, 1979) upstream to the initiation codon AUG. Nucleotide sequence analysis has revealed a region, about 3-11 bases upstream from the initiation codon, that seems to be conserved among most of the bacterial and phage RNAs (Scherer et al., 1980). This region, referred

to as the Shine-Dalgarno (S/D) sequence, is complementary to the 3' end of the 16S ribosomal RNA (Shine and Dalgarno, 1975) and base pairs with it during the process of initiation of protein synthesis (Steitz and Jakes, 1975). The S/D sequence is generally 4-9 bases long, but the reason for this size variability is unclear. Thermodynamically, one might speculate that the extent of homology is directly proportional to the efficiency of ribosome binding. In addition to sequence specificity, the location of the S/D sequence relative to the translational initiation codon is also important in determining the efficiency of initiation (Dunn et al., 1978; Roberts et al., 1979; Boer and Shepard, 1983).

In many cases, a second sequence, R-R-U-U-U-R-R (R = purine nucleotide), has been found, in part or in full, either in addition to or in the absence of the S/D sequence. Whereas the S/D sequence is recognized by the 16S ribosomal RNA, this R-R-U-U-U-R-R sequence may exist for recognition by the ribosomal protein S1 which has the <u>in vitro</u> property of binding to polyuridylate (Tal et al., 1972) and has been shown to be indispensable for the translation of certain natural mRNAs (Szer et al., 1975). The almost exclusive occurrence of this sequence among the RBSs of phage capsid proteins (ϕX, fd, Qβ, R17, MS2, f2) and ribosomal proteins (L11, L12, S12) suggests a functional role in the initiation of synthesis of those viral and

cellular structural proteins that accumulate to high levels in the cell (Scherer et al., 1980).

Bacterial promoters have now been used extensively to encourage synthesis of large quantities of important eukaryotic proteins. By and large, systems that express unfused eukaryotic proteins (direct expression) have been of somewhat limited use because of the problems of stability of the native eukaryotic proteins in E. coli (Boer and Shepard, 1983). Such systems have been mostly employed in the expression of small soluble proteins such as interferon and human growth hormone (Goeddel et al., 1980a,b; Jay et al., 1984; Tessier et al., 1984; Simmons et al., 1984).

Expression of a fusion protein has been widely employed to express small peptide hormones (Itakura et al., 1977; Goeddel et al., 1979a,b; Shine et al., 1980), as well as more complex proteins such as hepatitis B surface antigen (Charnay et al., 1980; Mackay et al., 1981), foot and mouth disease virus VP3 (Kleid et al., 1981), influenza virus hemagglutinin (Davis et al., 1981; Emtage et al., 1980; Heiland and Gething, 1981), VSV G protein (Rose and Shafferman, 1981), and Polyoma early gene products (Schaffhauson et al., 1985).

The retrovirus reverse transcriptase is a complex protein, not only in its enzymatic capabilities but also in its synthesis and maturation in virus-infected cells (see Section 1). Reverse transcriptase is synthesized in RSV-infected cells as a polyprotein ($Pr180^{gag-pol}$) containing

the polymerase and the precursor of the gag structural proteins ($Pr76^{gag}$). The mechanism of synthesis of $Pr180^{gag-pol}$ was suggested to involve a spliced mRNA species which contains a small deletion near the gag and pol junction sequences. The splicing occurs in such a way that the reading frame for gag coding sequence is continued to the reading frame coding for pol gene products (Schwartz et al., 1983). However, recent studies have suggested that a ribosomal frameshift suppression may be involved in the production of gag-pol fusion protein $Pr180^{gag-pol}$ (Jacks and Varmus, 1985). In order to express the pol gene sequence in E. coli, it is necessary to insert the pol gene sequence next to a bacterial coding sequence as a hybrid gene in the correct reading frame to synthesize a fusion protein, but, there may be a problem in processing to the functional mature products. It is desirable to synthesize the polymerase as an independent product, that is not a fused protein. The nucleotide sequence of the proviral DNA or RSV (PR-C) showed that the codon ATG corresponding to methionine residues located at 295 and 297 position of the polymerase molecule present in RSV have a putative RBS adjacent to its 5' end (Fig. 3.3.1). It was, therefore, decided to examine if any of these apparent initiation sites are used in E. coli to synthesize pol-related proteins and if so whether these pol-derived proteins have any enzymatic activity.

```
AspMetIleGluLeuGlyValIleAsnArgAspGlySerLeuGluArgProLeuLeuLeuPheProAlaValAlaMetValArgGlySer
TGACATGATAGAGTTGGGGGTTATTAACCGAGACGGGTCTTTGGAGCGACCCCTGCTCCTCTTCCCCGCAGTAGCTATGGTTAGAGGGAG    2430
                                              p15                    pl85           pol
IleLeuGlyArgAspCysLeuGlnGlyLeuGlyLeuArgLeuThrAsnLeu***            ThrValAlaLeuHisLeu
TATCCTAGGAAGAGATTGTCTGCAGGGCCTAGGGCTCCGCTTGACAAATTTATAGCGAGGGCCACTGTTCTCACTGTTGCGCTACATCTG    2520
                           Ser    Arg
AlaIleProLeuLysTrpLysProAspHisThrProValTrpIleAspGlnTrpProLeuProGluGlyLysLeuValAlaLeuThrGln
GCTATTCCGCTCAAATGGAAGCCAGACCACACGCCTGTGTGGATTGACCAGTGGCCCCTCCCTGAAGGTAAACTTGTAGCGCTAACGCAA    2610
                          T     G                                        C
LeuValGluLysGluLeuGlnLeuGlyHisIleGluProSerLeuSerCysTrpAsnThrProValPheValIleArgLysAlaSerGly
TTAGTGGAAAAAGAATTACAGTTAGGACATATAGAACCTTCACTTAGTTGTTGGAACACACCTGTCTTCGTGATCCGGAAGGCTTCCGGG    2700
                                     G C
SerTyrArgLeuLeuHisAspLeuArgAlaValAsnAlaLysLeuValProPheGlyAlaValGlnGlnGlyAlaProValLeuSerAla
TCTTACCGCTTACTGCATGATTTGCGCGCTGTTAACGCCAAGCTTGTTCCTTTTGGGGCCGTCCAACAGGGGGCGCCAGTTCTCTCCGCG    2790
              (-35  HpaI   H1 3 (-12)    CAT
LeuProArgGlyTrpProLeuMetValLeuAspLeuLysAspCysPhePheSerIleProLeuAlaGluGlnAspArgGluAlaPheAla
CTCCCCGCGTGGCTGGCCCCTGATGGTCTTAGACCTCAAGGATTGCTTCTTTTTCTATCCCTCTTGCGGAACAAGATCGCGAAGCTTTTGCA    2880
                       C                                                         H1 3
PheThrLeuProSerValAsnAsnGlnAlaProAlaArgArgPheGlnTrpLysValLeuProGlnGlyMetThrCysSerProThrIle
TTTACGCTCCCCTCTGTGAATAACCAGGCCCCCGCTCGAAGATTCCAATGGAAGGTCTTGCCCCAAGGGATGACCTGTTCTCCCACTATC    2970
                                                                                   pl85
                                                 Arg
CysGlnLeuValValGlyGlnValLeuGluProLeuArgLeuLysHisProSerLeuCysMetLeuHisTyrMetAspAspLeuLeuLeu
TGTCAGTTGGTAGTGGGTCAGGTACTTGAGCCCTTGCGACTCAAGCACCCATCTCTGTGCATGTTGCATTATATGGATGATCTTTTGCTA    3060
                                                   C
                                Lys
AlaAlaSerSerHisAspGlyLeuGluAlaAlaGlyGluGluValIleSerThrLeuGluArgAlaGlyPheThrIleSerProAspLys
GCCGCCTCAAGTCACGATGGTTGGAAGCGGCAGGGGAGGAGGTTATCAGTACATTGGAAAGAGCCGGCTTCACTATTTCGCCTGATAAG    3150
                                  A
ValGlnArgGluProGlyValGlnTyrLeuGlyTyrLysLeuGlySerThrTyrValAlaProValGlyLeuValAlaGluProArgIle
GTCCAGAGGGAGCCCGGAGTACAATATCTTGGGTACAAGTTAGGCAGTACGTATGTAGCACCCGTAGGCCTGGTAGCAGAACCCAGGATA    3240
                                                             T
Thr
AlaThrLeuTrpAspValGlnLysLeuValGlySerLeuGlnTrpLeuArgProAlaLeuGlyIleProProArgLeuMetGlyProPhe
GCCACCTTGTGGGATGTTCAAAAGCTGGTGGGGTCACTTCAGTGGCTTCGCCCAGCGTTAGGAATCCCGCCACGACTGATGGGCCCCTTC    3330
A
                                                 295  297                       Gln
TyrGluGlnLeuArgGlySerAspProAsnGluAlaArgGluTrpAsnLeuAspMetLysMetAlaTrpArgGluIleValArgLeuSer
TATGAGCAGTTACGAGGGTCAGATCCTAACGAGGCGAGGGAATGGAATCTAGACATGAAAATGGCCTGGAGAGAGATCGTACGGCTTAGC    3420
                                 RBS ------     XbaI                           A
ThrThrAlaAlaLeuGluArgTrpAspProAlaLeuProLeuGluGlyAlaValAlaArgCysGluGlnGlyAlaIleGlyValLeuGly
ACCACTGCTGCCTTGGAACGATGGGACCCTGCCCTGCCTCTGGAAGGAGCGGTCGCTAGATGTGAACAGGGGGCAATAGGGGTTTTGGGA    3510
GlnGlyLeuSerThrHisProArgProCysLeuTrpLeuPheSerThrGlnProThrLysAlaPheThrAlaTrpLeuGluValLeuThr
CAGGGACTGTCCACACACCCAAGGCCATGCTTGTGGTTATTCTCCACCCAACCCACCAAGGCGTTTACTGCTTGGTTAGAAGTGCTCACC    3600
LeuLeuIleThrLysLeuArgAlaSerAlaValArgThrPheGlyLysGluValAspIleLeuLeuLeuProAlaCysPheArgGluAsp
CTTTTGATTACTAAGCTACGTGCTTCGGCAGTGCGAACCTTTGGCAAGGAGGTCGATATCCTCCTGTTGCCTGCATGCTTTCGGGACGAC    3690
LeuProLeuProGluGlyIleLeuLeuAlaLeuLysGlyPheAlaGlyLysIleArgSerSerAspThrProSerIlePheAspIleAla
CTTCCGCTCCCAGAGGGGATCCTGTTAGCCCTTAAGGGGTTTGCAGGAAAAATCAGGAGTAGTGACACGCCATCTATTTTTGACATTGCG    3780
ArgProLeuHisValSerLeuLysValArgValThrAspHisProValProGlyProThrValPheThrAspAlaSerSerSerThrHis
CGTCCACTGCATGTTTCTCTGAAAGTGAGGGTTACCGACCACCCTGTGCCGGGACCCACTGTCTTTACTGACGCCTCCTCAAGCACCCAT    3870
                                             Val
LysGlyValValValTrpArgGluGlyProArgTrpGluIleLysGluIleAlaAspLeuGlyAlaSerValGlnGlnLeuGluAlaArg
AAGGGGGTGGTAGTCTGGAGGGAGGGCCCAAGGTGGGAGATAAAAGAAATAGCTGATTTGGGGGCAAGTGTACAACAACTGGAAGCACGC    3960
                                              T                              G
AlaValAlaMetAlaLeuLeuLeuTrpProThrThrProThrAsnValValThrAspSerAlaPheValAlaLysMetLeuLeuLysMet
GCTGTGGCCATGGCACTTCTGCTGTGGCCGACAACGCCCACTAATGTAGTGACTGACTCGGCGTTTGTTGCGAAAATGTTACTCAAGATG    4050
                                                      T
                                                    Gly
GlyGlnGluGlyValProSerThrAlaAlaAlaPheIleLeuGluAspAlaLeuSerGlnArgSerAlaMetAlaAlaValLeuHisVal
GGACAGGAGGGAGTCCCGTCTACAGCGGCGGCTTTTATTTTAGAGGATGCGTTAAGCCAAAGGTCAGCCATGGCCGCCGTTCTCCACGTG    4140
                               A                   G
ArgSerHisSerGluValProGlyPhePheThrGluGlyAsnAspValAlaAspSerGlnAlaThrPheGlnAlaTyrProLeuArgGlu
CGGAGTCATTCTGAAGTGCCAGGGTTTTTCACAGAAGGAAATGACGTCGCAGATAGCCAAGCCACCTTCCAAGCGTATCCCTTGAGAGAG    4230
AlaLysAspLeuHisThrAlaLeuHisIleGlyProArgAlaLeuSerLysAlaCysAsnIleSerMetGlnGlnAlaArgGluValVal
GCTAAAGATCTTCATACCGCTCTCCATATTGGACCCCGCGCGCTATCCAAAGCGTGTAATATATCTATGCAGCAGGCTAGGGAGGTTGTT    4320
GlnThrCysProHisCysAsnSerAlaProAlaLeuGluAlaGlyValAsnProArgGlyLeuGlyProLeuGlnIleTrpGlnThrAsp
CAGACCTGCCCGCATTGTAATTCAGCCCCTGCGTTGGAGGCCGGAGTAAACCCTAGGGGTTTGGGACCCCTACAGATATGGCAGACAGAC    4410
PheThrLeuGluProArgMetAlaProArgSerTrpLeuAlaValThrValAspThrAlaSerSerAlaIleValValThrGlnHisGly
TTTACGCTTGAGCCTAGAATGGCCCCCCGTTCCTGGCTCGCTGTTACTGTGGATACCGCCTCATCAGCGATAGTCGTAACTCAGCATGGC    4500
                                                      C
ArgValThrSerValAlaValGlnHisHisTrpAlaThrAlaIleAlaValLeuGlyArgProLysAlaIleLysThrAspAsnGlySer
CGTGTCACATCGGTTGCTGTACAACATCATTGGGCCACGGCTATCGCCGTTTTGGGAAGACCAAAGGCCATAAAAACAGATAATGGATCC    4590
                                                                                        C
```

```
CysPheThrSerLysSerThrArgGluTrpLeuAlaArgTrpGlyIleAlaHisThrThrGlyIleProGlyAsnSerGlnGlyGlnAla
TGCTTCACGTCTAAATCCACGCGAGAGTGGCTCGCGAGATGGGGGATAGCACACACCACCGGGATTCCGGGTAATTCCCAGGGTCAAGCT   4680

MetValGluArgAlaAsnArgLeuLeuLysAspArgIleArgValLeuAlaGluGlyAspGlyPheMetLysArgIleProThrSerLys
ATGGTAGAGCGGGCCAACCGGCTCCTGAAAGATAGGATCCGTGTGCTTGCTGAGGGGGACGGCTTTATGAAAAGAATCCCCACCAGCAAA   4770
                                                                          pRBS
GlnGlyGluLeuLeuAlaLysAlaMetTyrAlaLeuAsnHisPheGluAlaGlyGluAsnThrLysThrProIleGlnLysHisTrpArg
CAGGGGGAACTATTAGCCAAGGCAATGTATGCCCTCAATCACTTTGAGCCTGGTGAAAACACGAAAACACCGATACAAAAACACTGGAGA   4860

ProThrValLeuThrGluGlyProProValLysIleArgIleGluThrGlyGluTrpGluLysGlyTrpAsnValLeuValTrpGlyArg
CCTACCGTTCTTACAGAAGGACCCCCCGTTAAAATACGAATAGAGACAGGGGAGTGGGAAAAAGGATGGAACCTGCTGGTCTGGGGACGA   4950

GlyTyrAlaAlaValLysAsnArgAspThrAspLysValIleTrpValProSerArgLysValLysProAspIleThrGlnLysAspGlu
GGTTATGCCGCTGTGAAAAACAGGGACACTGATAAGGTTATTTGGGTACCCTCTCGAAAAGTTAAACCGGACATCACCCAAAAGGATGAG   5040
                pRBS             splice    KPnI
                                                            Gly   Lys
ValThrLysLysAspGluAlaSerProLeuPheAlaGlyIleSerAspTrpIleProTrpGluAspGluGlnGluGlyLeuGlnGlyGlu
            MetArgArgAlaLeuPheLeuGlnAlaPheLeuThrGlyTyrProGlyLysThrSerLysLysAspSerLysGluLys
GTGACTAAGAAACATGAGGCGAGCCCTCTTTTTGCAGGCATTTCTGACTGGATACCCTGGGAAGACGAGCAAGAAGGACTCCAAGGAGAA   5130
                                                            G    A
ThrAlaSerAsnLysGlnGluArgProGlyGluAspThrLeuAlaAlaAsnGluSer***
 ProLeuAlaThrSerLysLysAspProGluLysThrProLeuLeuProThrArgValAsnTyrIleLeuIleIleGlyValLeuValLeu
ACCGCTAGCAACAAGCAAGAAAGACCCGGAGAAGACACCCTTGCTGCCAACGAGAGTTAATTATATTCTCATTATTGGTGTCCTGGTCTT   5220
                   gp85                                                   CHO
 CysGluValThrGlyValArgAlaAspValHisLeuLeuGluGlnProGlyAsnLeuTrpIleThrTrpAlaAsnArgThrGlyGlnThr
GTGTGAGGTTACGGGGGTAAGAGCTGATGTTCACTTACTCGAGCAGCCAGGGAACCTTTGGATTACATGGGCCAACCGTACAGGCCAAAC   5310
                             C
```

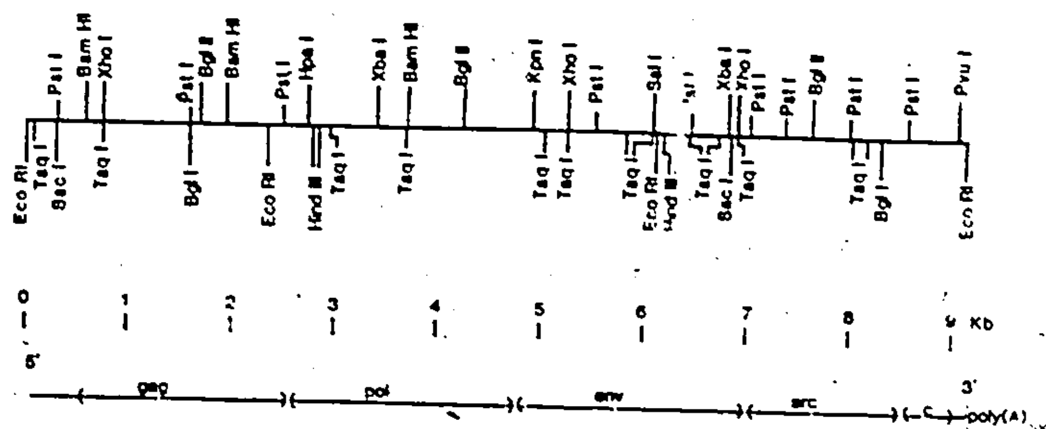
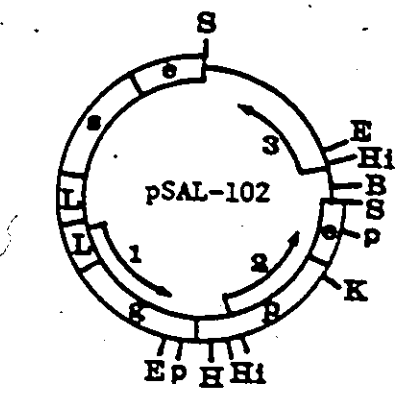


Figure 3.3.1 Nucleotide sequence of the pol gene of PR-C-RSV and restriction enzyme map of PR-A-RSV DNA.

The apparent promoter sequence in the pol gene was indicated as (-35), (-12), and CAT regions. The underlined sequences denote highly conserved sequences. Hpa I corresponds to the restriction enzyme cleavage site recognizing GTTAAC sequence; Hi 3 corresponds to the restriction enzyme Hind III cleavage site recognizing AAGCTT. The putative initiation site in the pol gene was indicated. The sequence representing the hypothetical RBS is located upstream from the Xba I restriction enzyme site (TCTAGA) and two ATG codons are located downstream from the Xba I site, and shown above the codons are the two methionine residues 295 and 297 of reverse transcriptase molecule. Kpn I corresponds to the restriction enzyme cleavage site recognizing GGTACC sequence and located near the 3' end of the pol gene. This figure is directly from Schwartz et al. (1983). The restriction map shown is that of PR-A-RSV DNA. The left hand end of the map (0 kb) corresponds to the 5' end of the RNA genome. The sizes of the individual viral genes are approximate, based on the sizes of known gene products and the structure of deletion mutants. This figure is directly from Highfield et al. (1980).

The work described in this section would not have been possible without the generosity of Dr. H.E. Varmus, Dr. J.T. Parsons, Dr. R.V. Guntaka, Dr. E. Stavnezer and Dr. M.B. Dwokin in providing the recombinant plasmids pSRA-2, pSAL-102, pATV-8, pB5^{pol} and pER103, respectively. The recombinant plasmids pSAL-102 and pSRA-2 contain proviral DNA permuted at the env gene (Highfield et al., 1980; DeLorbe et al., 1980). The possible transcripts for the polymerase like proteins may be promoted from the LTR sequence (Fig. 3.3.2). Recently, it was reported that a sequence in the LTR of both avian and murine tumor virus proviral DNAs were shown to contain a promoter active in E. coli (Guntaka and Mitsialis, 1980; Mitsialis et al., 1981; Prakash et al., 1983). These transcripts may code for gag- and pol- related proteins (Transcript 1; Fig. 3.3.2). The plasmid pATV-8 has RSV proviral DNA permuted at the pol gene (Katz et al., 1982; Fig. 3.3.2). The possible transcripts for the polymerase like proteins can not be synthesized under the control of the LTR, rather they may now be synthesized under the control of one of the two promoters for β-lactamase gene of pBR322 located between Hind III and Bam HI restriction sites (Brosius et al., 1982). These putative pol-transcripts will still contain the putative initiation site as described above. The possible transcripts for the gag-related proteins may be promoted from the LTR sequence (Transcript 1; Fig. 3.3.2).



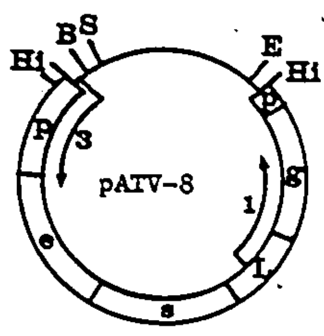


Figure 3.3.2 Schematic representation of RSV proviral DNA recombinant plasmids and proposed transcription strategy.

Symbols: line, pBR322; open bar, RSV proviral DNA (L, LTR: g, gag; p, pol; e, env; s, src; 1 ⟶, viral transcript promoted from the apparent promoter in LTR sequence; 2 ⟶, viral transcript promoted from the apparent promoter in the pol gene; 3 ⟶, viral transcript promoted from one of the two promoters for the β-lactamase gene of pBR322 (Brosius et al., 1982); B, Bam HI; E, Eco RI; H, Hpa I; Hi, Hind III; K, Kpn I; P, Pst I; S, Sal I. pSAL-102, recombinant plasmid containing the proviral DNA of Prague A strain of RSV (Highfield et al., 1980); pATV-8, Prague C strain of RSV (Katz et al., 1982). The sequences are not drawn to scale.

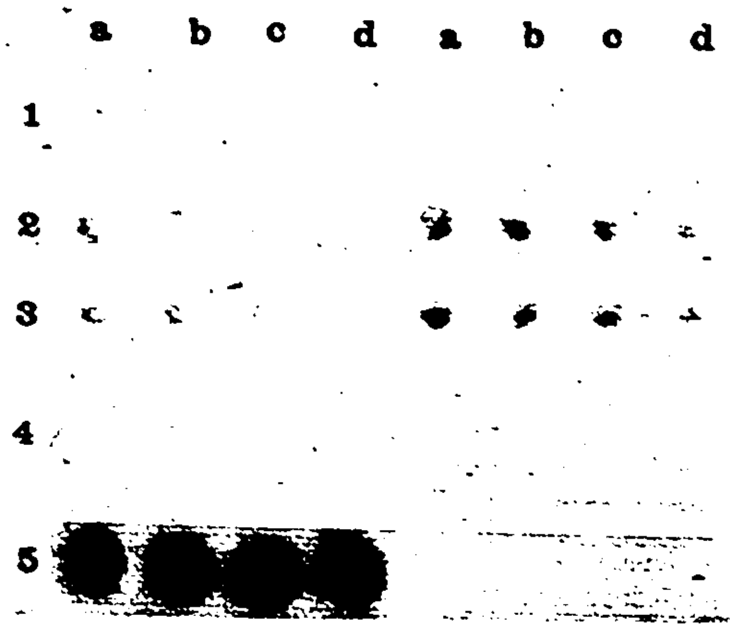


Figure 3.3.3 Autoradiogram of Dot Blot Assay with *E. coli* RNA.

Lane 1, RNAs from *E. coli* harboring pBR322; lane 2, RNAs from *E. coli* harboring pATV-8; lane 3, RNAs from *E. coli* harboring pSAL-102; lane 4, Rabbit tRNAs; lane 5, genomic RNAs from PR-C virus. Rows a to d correspond to serial 1:2 dilutions. Lanes 2 and 3 have duplicated rows.

Dot Blot Assay for in-vivo RNAs from E.coli Harboring the RSV-Recombinant Plasmids

The presence of viral transcripts in *E. coli* harboring the RSV-recombinant plasmids were tested by the dot blot assay (Fig. 3.3.3). Total RNAs were prepared from *E. coli* containing either pBR322 (lane 1) or pATV-8 (lane 2) or pSAL-102 (lane 3). RNA was also prepared from purified PR-C virions for the positive control (lane 5) and rabbit-tRNA was used for the negative control (lane 4). The RNA samples were used at the same concentrations and serial dilutions of samples (3 to 5 μg) were prepared with a rabbit-tRNA diluent (1 mg/ml), and tested. The hybridization probe was prepared by nick-translation of the cloned DNA fragment from pATV-8. pATV-8 was purified and digested with Hind III, the 3.3 kb fragment consisting of pol-env genes sequences (Fig. 3.3.1) was isolated from the agarose-gel. The purified fragment was labeled with ^{32}P by nick-translation as described in Materials and Methods.

As can be seen in Figure 3.3.3, this probe was able to hybridize with RNAs from *E. coli* containing pATV-8 or pSAL-102 and with the genomic RNAs from PR-C virions. In contrast, no hybridization was detected with RNAs from *E. coli* containing pBR322 and with the rabbit tRNAs. These results indicated that *E. coli* harboring the RSV-recombinant plasmids could synthesize the viral transcripts.

Synthesis of gag-Related Polypeptides in E. coli Harboring the RSV-Recombinant Plasmids

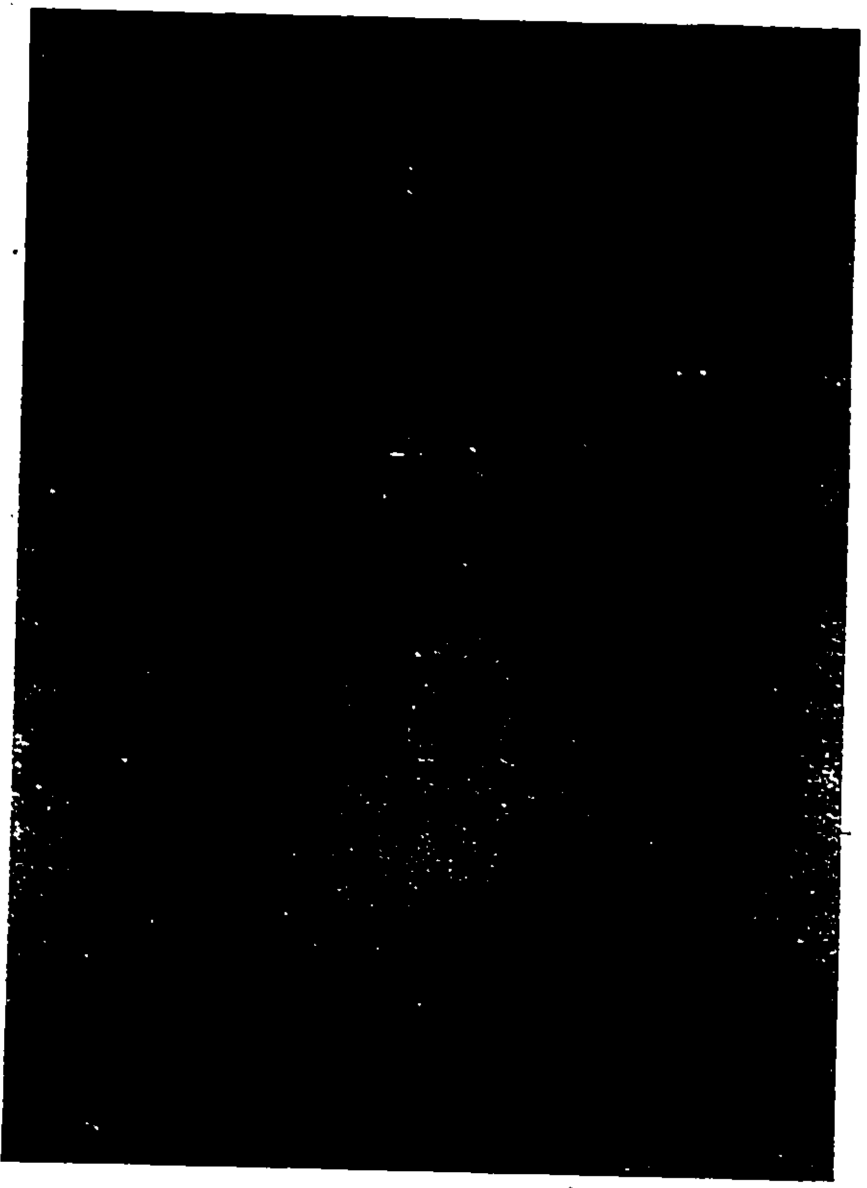
The presence of gag-related proteins in E. coli harboring the recombinant plasmids containing RSV proviral DNAs was determined by immunoprecipitation of the E. coli extracts with anti-gag antiserum. Cells were labeled with [^{35}S] methionine and cell lysates were immunoprecipitated with anti-gag antiserum, then analyzed by SDS-PAGE (Fig. 3.3.4). The predominant stable proteins immunoprecipitable from E. coli containing pATV-8 (lane 5) or pSRA-2 (lane 7) by anti-gag antiserum were very similar in size and antigenicity to the Pr76^{gag} cleavage products p27 and p15 suggesting the same processing of Pr76^{gag} which occurs in eukaryotic cells also occurs in bacteria. Thus, either the cleavage sites for these proteins are accessible to bacterial enzymes, or the p15 protease activity must be active in a precursor form.

Synthesis of pol-Related Polypeptides in E. coli Harboring the RSV-Recombinant Plasmids

The presence of pol-related proteins in E. coli harboring the recombinant plasmids containing RSV proviral DNAs and recombinants containing manipulated pol sequences was determined by immunoprecipitation with anti-pol antiserum. The cells were labeled with [^{35}S] methionine and

Figure 3.3.4 Autoradiogram of SDS-PAGE of the gag-related proteins synthesized in *E. coli*.

E. coli cells were labeled with [^{35}S] methionine and cell lysates were immunoprecipitated with anti-gag antiserum as described under Materials and Methods. Lane 1, molecular weight marker proteins; lanes 2 and 3, *E. coli* harboring pBR322 immunoprecipitated with normal rabbit serum and anti-gag antiserum, respectively; lanes 4 and 5, *E. coli* harboring pATV immunoprecipitated with normal-rabbit serum and anti-gag antiserum, respectively; lanes 6 and 7, *E. coli* harboring pSAL-102 immunoprecipitated with normal rabbit serum and anti-gag antiserum, respectively; lane 8, purified PR-C virus.



cell lysates were immunoprecipitated with anti-pol antiserum, then analyzed by SDS-PAGE (Fig. 3.3.5). A protein with molecular weight of about 65,000 (p65) was detected by the anti-pol antiseum in cells harboring pATV-8 (lane b) and pSAL-102 (lane c). A protein corresponding to p65 was also detected in E. coli containing the deletion mutant pSAL-102DL^{pol}-2 (lane d). However, the amount of p65 present was much less than that present in cells harboring pSAL-102. It should be noted that the plasmid (see Fig. 3.3.6 for the construction) lacks the 126 bp Hind III fragment near the 5'-end of the pol gene (Fig. 3.3.1). In contrast, p65 protein was not detected in E. coli harboring pBR322 (lane a).

The presence of p65 in E. coli harboring the RSV-recombinant plasmids was further analyzed by two-dimensional gel electrophoresis as described in Materials and Methods. Results presented in Fig. 3.3.7 showed the gel analysis of total proteins from cells harboring pBR322 and pSAL-102 (panels A and B). No significant difference could be detected in protein profiles. The labeled cell lysates were, therefore, immunoprecipitated with anti-reverse transcriptase antiserum and analyzed by two-dimensional gel electrophoresis. Results presented in Fig. 3.3.8 showed the presence of p65 in cells harboring pATV-8 and pSAL-102 (panels A and B). p65 was also detected in E. coli containing pSAL-102DL^{pol}-2 (panel C), but the amount was much less than that present in cells harboring

Figure 3.3.5 Autoradiogram of SDS-PAGE of the pol-related proteins synthesized in *E. coli*.

E. coli cells were labeled with [^{35}S] methionine and cell lysates were immunoprecipitated with anti-pol antiserum as described in Materials and Methods. Lane a, *E. coli* harboring pBR322; lane b, *E. coli* harboring pATV-8; lane c *E. coli* harboring pSAL-102; lane d, *E. coli* harboring the deletion mutant pSAL-102DL^{pol}-2.

a b c d

190K–

69K– P65

50K–

29K–

pSAL-102. Again, no p65 protein was detected in E. coli harboring pBR322 (panel D). Thus, it appears that the pol-related proteins may be initiated from the methionine codons which are located downstream from the deletion site in pSAL-102DL^{pol}-2.

Nucleotide Sequence of The Putative Initiation Site in The pol Gene

From the DNA sequence (Fig. 3.3.1), molecular weight of the proteins that initiated from the two ATG codons corresponding to methionine residues 295 and 297 was calculated as 66,000. Therefore, the protein p65 specifically recognized by anti-reverse transcriptase antibody may be initiated from the methionine residues in position 295 or 297 of the reverse transcriptase molecule.

The nucleotide sequence present in Figure 3.3.1 corresponds to the proviral DNA from PR-C-infected cells. It was, thus, decided to determine whether the same putative initiation site is present in pSAL-102 (the PR-A proviral DNA). The DNA fragment used for sequencing analyses was obtained from the recombinant plasmid pBRPOL-62, which contains the complete pol gene with portion of flanking gag and env genes from pSAL-102. The cloned fragment spans from Eco RI site at the 3' end of the gag gene to Sal I site in the middle of env gene (see Fig. 3.3.1). This

Figure 3.3.6 Construction of a RSV proviral DNA recombinant plasmid with specific deletion at the pol gene.

Step 1 - The recombinant plasmid $pB5^{pol}$ (Stavneger et al., 1981), which contains pol gen, was digested with Hind III. The reaction mixture was phenol extracted and DNA was ethanol precipitated. The precipitated DNA was treated with polymerase I (Klenow fragment) for 30 min at 37°C. After phenol extraction and ethanol precipitation, the mixture was treated with T4 DNA ligase at 4°C for 24 hrs. The ligated DNA was introduced into E. coli cells and transformants carrying plasmids were grown in the presence of 20 µg/ml of ampicillin. Colonies containing Hind III resistant plasmids were grown in Luria broth to prepare plasmid DNA. The DNA was further characterized by digestion with restriction endonucleases. One colony ($pB5^{pol}$DL-2) was chosen for further analyses. Lanes 1 to 3, pSAL-102, $pB5^{pol}$DL-2 and $pB5^{pol}$ were digested with Hpa I and Kpn I. Steps 2 and 3 - Plasmids isolated from E. coli harboring $pB5^{pol}$DL-2 or pSAL-102 were digested with Hpa I and Kpn I. The digests were analyzed by electrophoresis in a 0.8% agarose gel, then fragments of 2.1 kb (from $pB5^{pol}$DL-2) and 11.7 kb (from pSAL-102) were isolated from the gel as described in Materials and Methods. Step 4 - The DNAs were ligated with T4 DNA ligase at 4°C for 24 hrs. The ligated DNA was introduced into E. coli cells and transformants (pSAL-$102DL^{pol}$-2) containing plasmids were grown in the presence of 20 µg/ml of ampicillin. The plasmids were further characterized with restriction endonucleases. Lanes 4 and 5, untreated pSAL-102 and pSAL-$102DL^{pol}$-2, respectively. Lanes 6 and 7 correspond to the molecular weight marker DNA digested with Hind III. Lanes 8 and 9, Eco RI digested pSAL-102 and pSAL-$102DL^{pol}$-2, respectively. The resulted fragments are 4.4 kb, 3.3 kb, 3.0 kb, and 2.3 kb from top to bottom. The mobility of 4.4 kb fragment is faster in pSAL-$102DL^{pol}$-2 due to the deletion. Lanes 10 and 11, Kpn I and Eco RI double digested pSAL-102 and pSAL-$102DL^{pol}$-2, respectively. The unique Kpn I site is present in the 4.4 kb fragment and resulted in 2.6 kb and 1.8 kb fragments. In pSAL-$102DL^{pol}$-2, this double digestion resulted in 2.5 kb and 1.8 kb fragment. The 2.6 kb fragment comigrated with the fragment from $pB5^{pol}$ digested with Kpn I and Eco RI (lane 13) and the 2.5 kb fragment comigrated with the fragment generated from $pB5^{pol}$DL-2 (lane 12). Symbols: In $pB5^{pol}$ and $pB5^{pol}$DL-2, line indicates the modified pBR322 sequence (Stavneger et al., 1981), filled bar indicates the 3' end of the gag gene, open bar indicates the pol-gene, and ▲ indicates the specific deletion at the pol gene. The sequences are not drawn to scale.

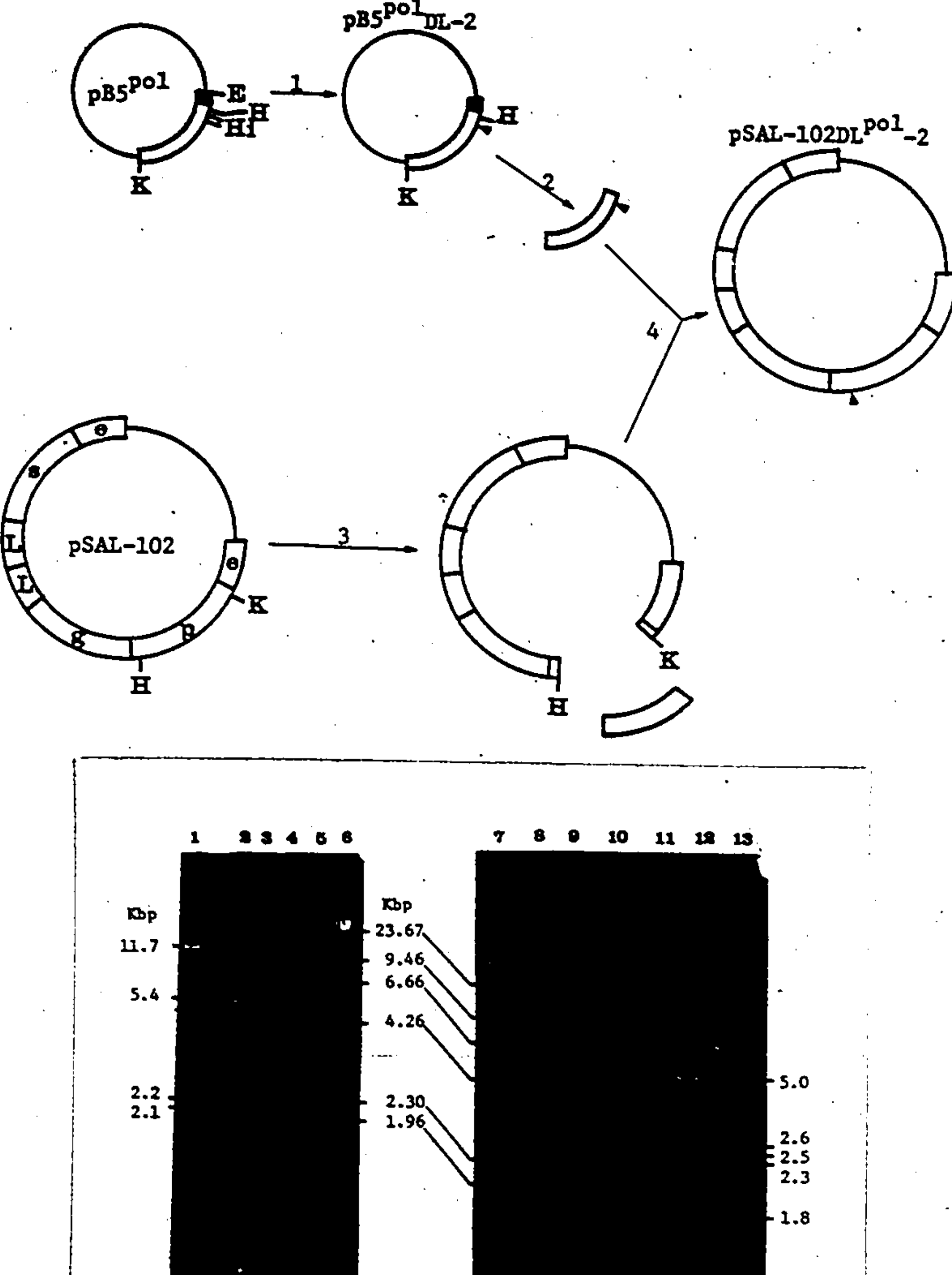
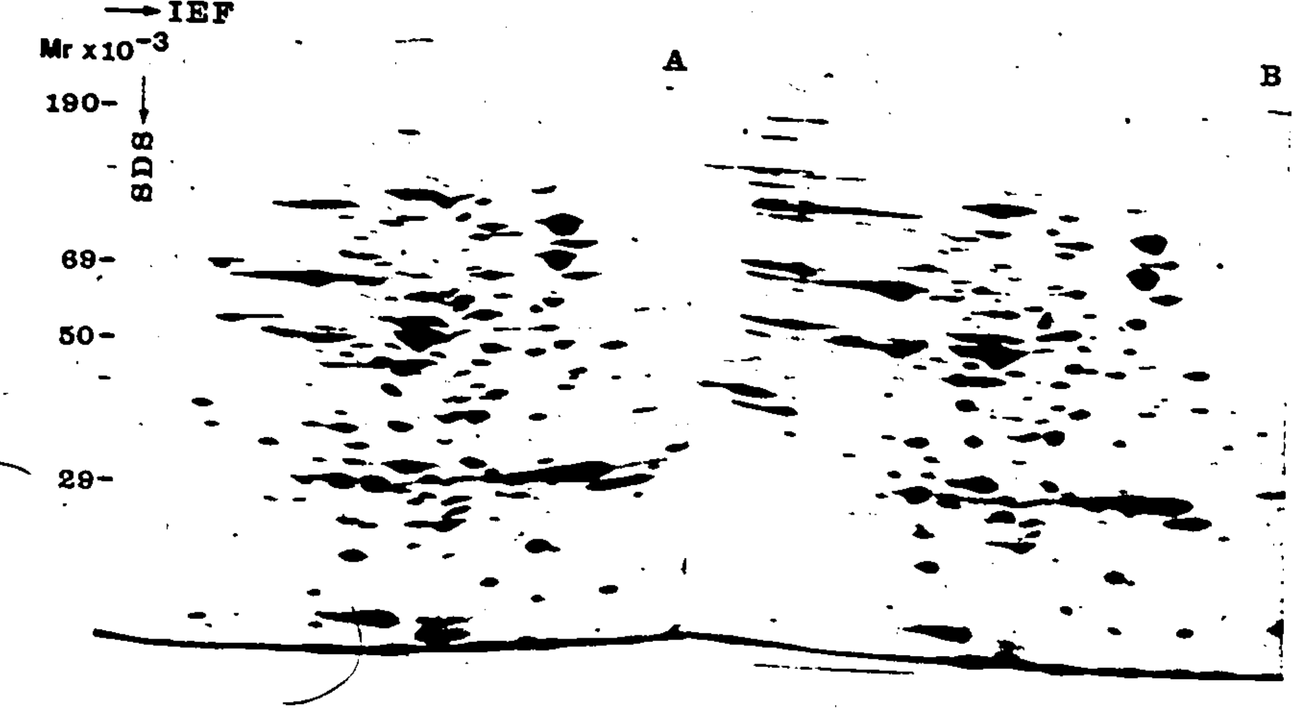

pB5pol
E
H
Hi
K
1
pB5polDL-2
H
K
2
pSAL-102DLpol-2
4
pSAL-102
3
L
L
g
p
K
H
K
H
1
2 3 4 5 6
7 8 9 10 11 12 13
Kbp
11.7
5.4
2.2
2.1
Kbp
23.67
9.46
6.66
4.26
2.30
1.96
5.0
2.6
2.5
2.3
1.8

Figure 3.3.7 Autoradiogram of two-dimensional gel electrophoresis analyses of the total proteins synthesized in *E. coli*.

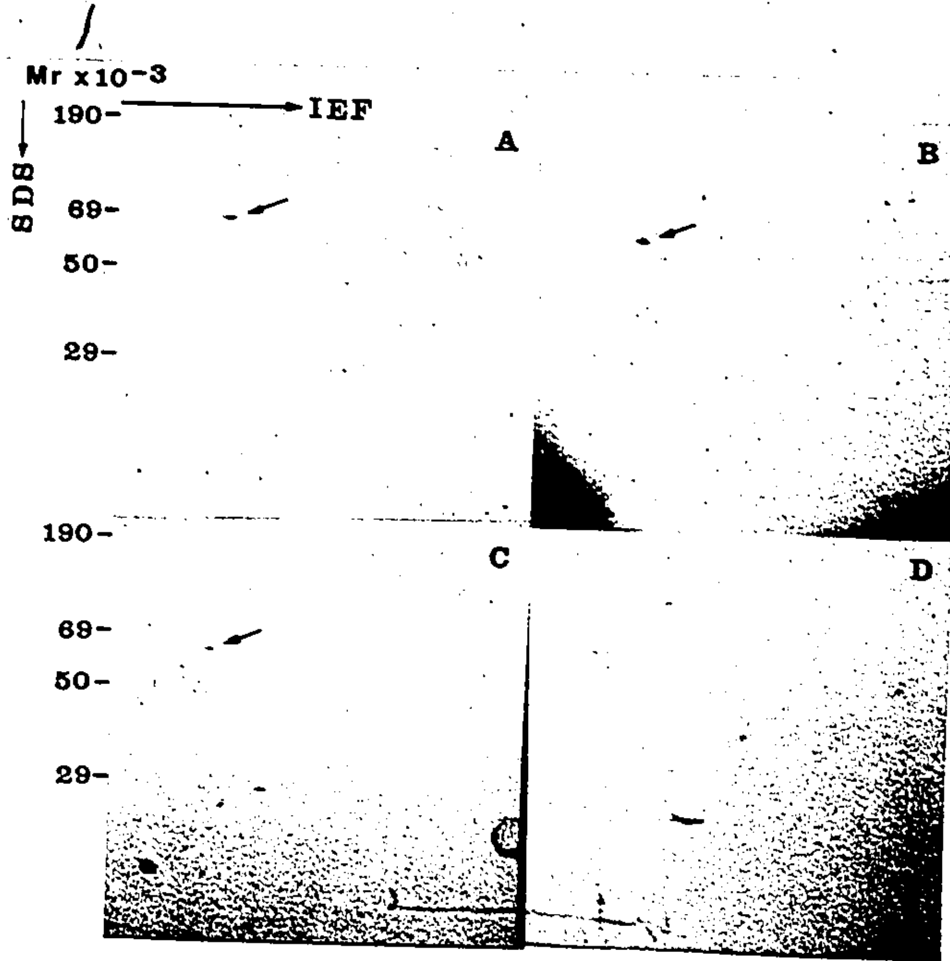
E. coli cells were labeled with [^{35}S] methionine and the labeled proteins were analyzed by two-dimensional gel electrophoresis as described in Materials and Methods. Panel A, *E. coli* harboring pBR322; panel B, *E. coli* harboring pSAL-102.



→ IEF
Mr x10^{-3}
190-
SDS
69-
50-
29-
A
B

Figure 3.3.8 Autoradiogram of two-dimensional gel electrophoresis analyses of the pol-related proteins synthesized in *E. coli*.

E. coli cells were labeled with [^{35}S] methionine and cell lysates were immunoprecipitated with anti-pol antiserum as described in Materials and Methods. Panel A, *E. coli* harboring pATV-8; panel B, *E. coli* harboring pSAL-102; panel C, *E. coli* harboring pSAL-102DL^{pol}-2; panel D, *E. coli* harboring pBR322.



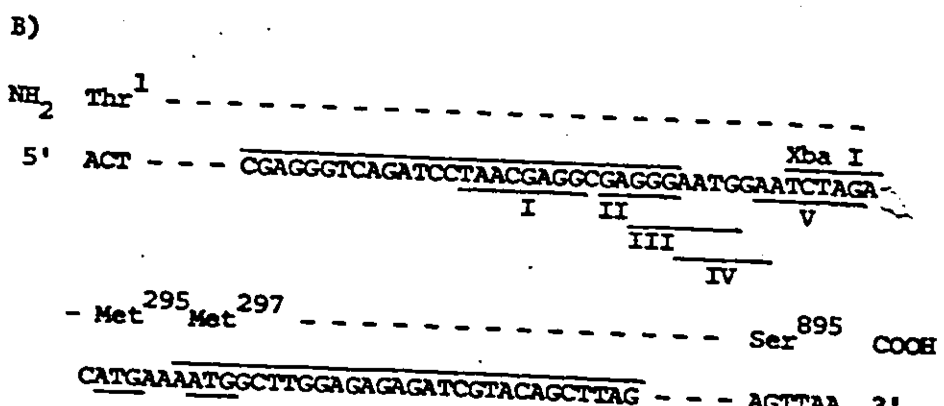
Mr x10-3
190-
IEF
SDS
A
B
69-
50-
29-
190-
C
D
69-
50-
29-

fragment was subcloned into pBR322 which was double digested with the same enzymes (see Fig. 3.3.16 for the construction). The putative RBS is located at the pol gene near the unique Xba I restriciton enzyme site (Fig. 3.3.1). Thus, purified pBRPOL-62 was digested with Xba I to linearize the plasmid, then dephosphorylated at the 5' end with calf intestinal alkaline phosphatase and the DNA was isolated from an agarose gel as described in Methods. The purified DNA was labeled at 5' end with [γ-^{32}P] ATP and polynucleotide kinase and the labeled DNA was digested with Eco RI to generate one end labeled DNA fragments. The labeled DNA fragments (1.1 kb and 6.3 kb) were isolated from the agarose gel, then the purified DNA fragments were analyzed by using the Maxam and Gilbert chemical procedure and the sequences deduced as described in Methods. A representative sequencing gel and the nucleotide sequence at the region of the putative RBS present in the pol gene are shown in Figure 3.3.9. The sequence representing the hypothetical RBS is located upstream from the Xba I site which the two ATG codons are located downstream from the Xba I site. The sequences determined were identical with the PR-C sequences reported previously (Schwartz et al., 1983). Thus, these results indicate that both strains of RSV contain the same hypothetical RBS, and the pol-related proteins p65 may be initiated from the same site in pATV-8, pSAL-102 and pSAL-102DL^{pol}-2.

Figure 3.3.9 Nucleotide sequence of the putative initiation site in the pol gene.

A sequencing gel (A) and the nucleotide sequence (B) over the region of the putative initiation site at the pol gene are displayed. The sequence was derived by 5'-labeling at the Xba I site and sequencing at both direction by the Maxam and Gilbert chemical procedure as described in Materials and Methods. The sequence representing the hypothetical RBS is located upstream from the Xba I site and contains the following features: a variable degree of homology and number of Shine-Dalgarno sequences (I to IV); a heptanucleotide (V), corresponding to the R-R-U-U-U-R-R sequence (R = purine nucleotide) which is present in many bacterial and phage RNAs. The two ATG codons are located downstream from the Xba I site, and shown above the codons are the two methionine residues 295 and 297 of reverse transcriptase molecule. The sequence of Prague A strain determined in this study is identical to the published sequence (Schwartz et al., 1983) of Prague C strain of RSV. The overlined sequences were determined in this study.

B)



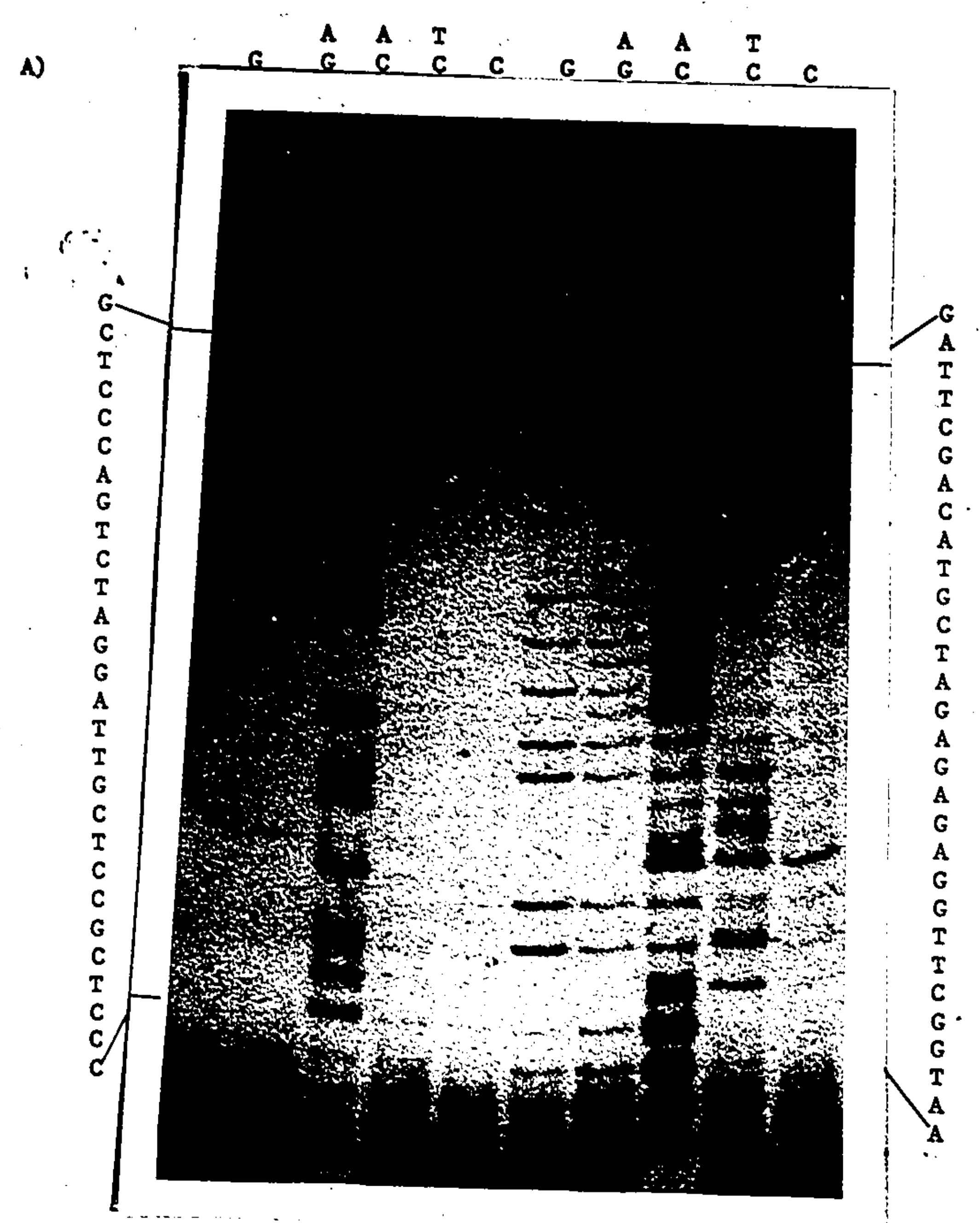

A)
G AG AC TC C G AG AC TC C
GCTCCCAGTCTAGGATTGCTCCGCTCCC
GATTCGACATGCTAGAGAGAGGTTCGGTAA

Table III. DNA polymerase activities of E. coli harboring different recombinant plasmids.

Cell lysates from E. coli harboring	Time of incubation (min)			
	0	15	30	60
	Radioactivity (cpm/A_{280} unit)			
pBR322	10,000	28,000	18,000	13,500
pATV-8	18,000	443,000	753,000	915,000
pSAL-102	11,000	722,000	909,000	983,000
pSAL-102DL^{pol}-2	10,000	64,000	74,000	91,000
RSV*	10,000	207,000	443,000	684,000

E. coli cell lysates (50 µl) prepared as described under Materials and Methods were incubated with 50 µl of the 2X reaction buffer at 37°C for various lengths of time as indicated. The reactions were stopped by addition of cold 100 µl of 0.1 M sodium pyrophosphate, 20 µl of 0.25 % BSA, and 4 ml of cold 5 % TCA. The mixtures were filtered through glass fiber filter paper, washed with 5 % TCA, ethanol:ether (1:1, v/v), and finally with ether. The filters were dried and counted in a Beckman scintillation counter. The values are average of 2 to 5 separate experiments. * Rous sarcoma virus Prague B strain was used for the assay. It was unable to assay with the exogenous template (oligo dT:poly rA) due to high endogenous RNase H activity (see Table VI).

DNA Polymerase Activity in E. coli Harboring Recombinant Plasmids Containing RSV Proviral DNA

Polymerase activity present in lysates of *E. coli* containing the recombinant plasmids was determined by incorporation of [^{3}H] TTP into DNA (Garapin et al., 1970). As shown in Table III, cells harboring recombinant plasmids containing different strains of RSV proviral DNAs (pATV-8 and pSAL-102) showed polymerase activity 30-fold higher than the activity present in cells containing pBR322. The plasmid pATV-8 has RSV proviral DNA permuted at the pol gene (Fig. 3.3.2), however, the polymerase activity present in cells containing this plasmid was comparable to that observed in *E. coli* harboring pSAL-102. The polymerase activity present in *E. coli* harboring pSAL-102DL^{pol}-2 was also tested. This derivative of pSAL-102 lacks the 126 bp Hind III fragment at the 5' end of the pol gene (Figs. 3.3.1 and 3.3.6). The internal ATG codons with putative RBS are located further downstream from these Hind III sites. As shown in Table III the polymerase activity present in *E. coli* harboring pSAL-102DL^{pol}-2 was reduced by about 10-fold, but the residual activity was still about 7-fold higher than that present in cells containing pBR322. The reduced activity could be due to synthesis of an altered pol-related proteins or it could be due to a reduced amount of the translatable pol-transcripts. From the results

Table IV. Effect of ribonuclease on the DNA polymerase activity of *E. coli* cell lysates containing different RSV recombinant plasmids.

Cell lysates from *E. coli* harboring	Conditions	cpm/A_{280}	% inhibition
pATV-8	Preincubated with no addition	819,000	
pATV-8	Preincubated with 100 μg/ml RNase A	63,000	92
pSAL-102	Preincubated with no addition	389,000	
pSAL-102	Preincubated with 100 μg/ml RNase A	69,000	82

Cell lysates were incubated at 22°C for 30 min with or without RNase A, then 2X reaction buffer was added to the chilled reaction mixture and incubated at 37°C for 60 min. After incubation, the samples were precipitated and counted as described in Table III.

described in Figures 3.3.5 and 3.3.8, it seems likely that the reduced activity is due to reduced amount of the pol-related proteins rather than due to synthesis of altered pol-related proteins. The deleted Hind III fragment may, therefore, contain major transcriptional control elements, and the LTR sequence accounts for the residual activity (see below). The protein p65, specifically recognized by anti-reverse transcriptase antiserum, could also possess the observed enzymatic activity.

Reverse Transcriptase Activity in E. coli Harboring RSV Recombinant Plasmids

In order to show that the polymerase activity present in cells harboring RSV recombinant plasmids corresponds to reverse transcriptase activity (Baltimore, 1970; Temin and Mizutani, 1970), the effect of RNase on the template present in the *E. coli* lysates on the polymerase activity was determined. As shown in Table IV, pretreatment of the extracts with RNase A reduced the incorporation of [^{3}H] TTP by 80 to 90%.

The nature of the reaction product was also determined by nuclease digestion (Baltimore, 1970; Temin and Mizutani, 1970). The product was completely hydrolyzed by DNase but was digested only to extent of 25% by RNase A (Table V).

Table V. Characterization of the polymerase products.

Lysates	Treatment	Acid-insoluble radioactivity	% undigested product
pATV-8	Untreated	164,615	(100)
pATV-8	100 μg/ml DNase	1,369	0.8
pATV-8	100 μg/ml RNase A	135,380	82
pSAL-102	Untreated	161,079	(100)
pSAL-102	100 μg/ml DNase	2,320	1.4
pSAL-102	100 μg/ml RNase A	123,097	76
pSAL-102DL ^{pol_2}	Untreated	46,114	(100)
pSAL-102DL ^{pol_2}	100 μg/ml DNase	856	1.9
pSAL-102DL ^{pol_2}	100 μg/ml RNase A	35,207	76
RSV	Untreated	118,903	(100)
RSV	100 μg/ml DNase	31,287	26
RSV	100 μg/ml RNase A	111,401	94

E. coli cell lysates were incubated at 37°C for 1 hour in the buffer containing 0.1 M Tris-HCl (pH 8.1), 0.01 M $MgCl_2$, 10^{-4} M dATP, dCTP, dGTP, 2% 2-mercaptoethanol, 0.1% Triton X-100, and 1 μCi/ml of [^{3}H] TTP. A 200 μl reaction mixture was diluted to 1.6 ml with 10 mM $MgCl_2$ and 0.5 ml aliquots were incubated for 1.5 hours at 37°C with the indicated enzymes. The samples were chilled, precipitated with 5% TCA, washed with 5% TCA, ethanol:ether, and ether, then counted as described in Table III.

Table VI. RNase H activity of *E. coli* harboring different recombinant plasmids.

Cell lysates from *E. coli* harboring	Acid-soluble radioactivity (cpm/A_{280} unit of cell lysate)
pBR322	201,000
pATV-8	218,000
pSAL-102	266,000
pBRPOL-62	203,000
RSV*	116,000
Blank	9,700

Reaction mixture (50 µl) containing 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 6 mM DTT, 2 µg BSA, 18.8 pmol of poly (dT), 16.9 pmol of [^{3}H] poly (rA), and 10 µl of *E. coli* cell lysates were incubated for 30 min at 37°C. Reactions were stopped by the addition of 0.1 ml of cold 0.1 M sodium pyrophosphate, 0.4 mg of BSA, and 0.25 ml of 10% TCA. The reaction mixtures were centrifuged in Eppendorf, and the supernatant were collected and counted in 10 ml of Aquasol scintillation fluid. * Rous sarcoma-virus Prague C strain was used for the assay.

RNase H Activity in E. coli Harboring RSV Recombinant Plasmids

Ribonuclease H activity is also present in the reverse transcriptases found in RNA tumor viruses (Grandgenett et al., 1972). E. coli DNA polymerase I contains a 5' to 3' RNase H activity (Baltimore and Smoler, 1972; Schekman et al., 1972) and E. coli RNase H is an endonuclease (Leis et al., 1973). In order to see if the synthesized pol-related proteins in E. coli have RNase H activity the cell lysates were assayed for the enzymatic activity. The cell lysates were prepared and assayed for RNase H activity as described in Materials and Methods. As shown in Table VI, no significant differences in RNase H activity present in cells harboring RSV recombinant plasmids and in cells harboring pBR322 were observed.

The rates of degradation of $[^3H]$ poly (rA):poly(dT) by RNase H activity present in E. coli harboring pBR322 and in cells harboring pATV-8 are shown in Figure 3.3.10. The rates of degradation were similar in both the cases. Thus, the pol-related proteins lacking NH_2-terminal sequence synthesized in E. coli may not possess any RNase H activity. The activity present in E. coli harboring RSV recombinant plasmids may represent the endogenous activity present in E. coli.

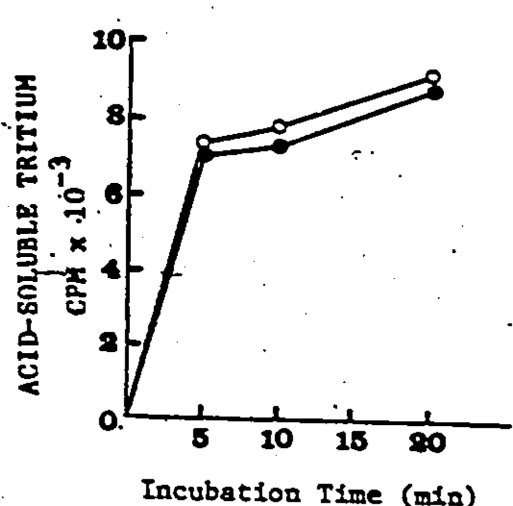


Figure 3.3.10 Rate of degradation of [^{3}H] poly (rA):poly (dT) by RNase H present in E. coli harboring pBR322 or pATV-8.

Cell lysates from E. coli containing pBR322 or pATV-8 were incubated with [^{3}H] poly (rA) (16.0 pmol, 0.022 μCi/pmol) and poly (dT) (18.8 pmol) for various lengths of time at 37°C. Reaction was stopped and the acid-soluble radioactivity was counted as described in Materials and Methods. Symbols: - ● -, pBR322; - o -, pATV-8.

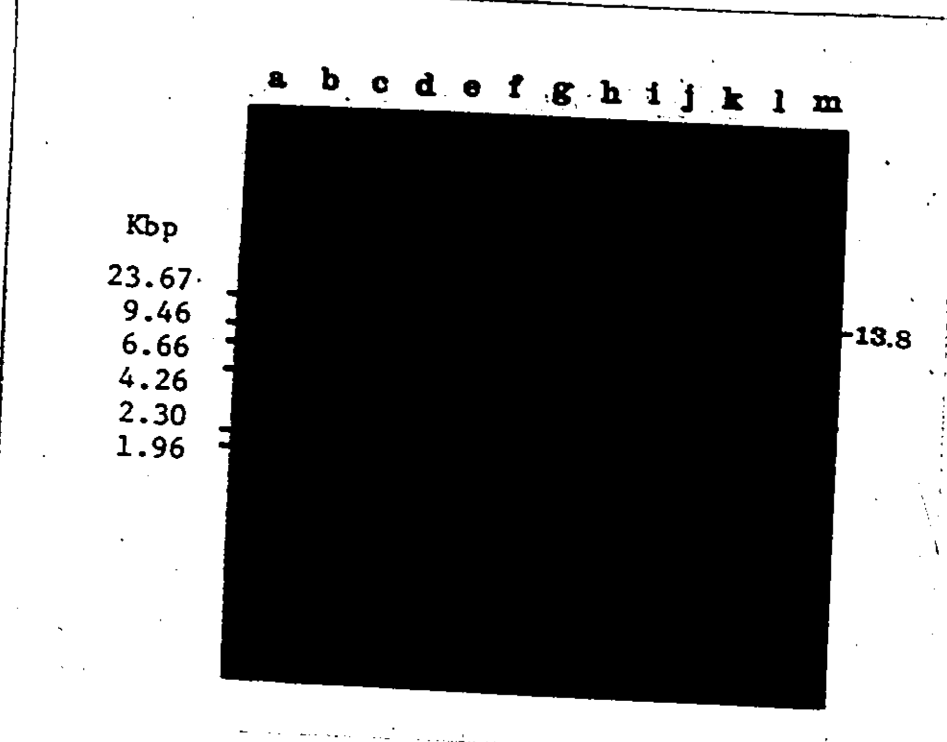


Figure 3.3.11 Agarose gel electrophoresis of the pSAL-102 derivative mutants deleted at the Kpn I site of the pol gene.

The plasmid pSAL-102 was digested with Kpn I at the unique site present at the 3' end of the pol gene. The resulting four base 3' overhang was removed by treatment with E. coli DNA polymerase I (Klenow fragment) and the molecule was religated with T4 DNA ligase. The resulting reaction mixture was transformed into E. coli (HB101) and 12 transformants were analyzed for the plasmids. Lane a, λ DNA fragments molecular weight marker; lanes b to m, the plasmids from the transformants digested with Kpn I. All the plasmids, except in lanes k and m, contain the 13.8 kb linealized DNA corresponding to the intact pSAL-102. The plasmids in lanes k and m were resistant to Kpn I digestion (pSAL-102DL^{pol}-9 and pSAL-102DL^{pol}-11).

Construction of pSAL-102 Derivative mutants Defective at 3' End of The pol Gene

In order to test whether the 3' end region of the pol gene is essential for the polymerase activity, mutants defective at the 3' end of the pol gene were constructed from pSAL-102. The plasmid pSAL-102 was digested with Kpn I at the unique site present at the 3' end of the pol gene. The resulting four base 3' overhang was removed by treatment with E. coli DNA polymerase I (Klenow fragment) and the molecule was religated with T4 ligase. The reaction mixture was used to transform into E. coli and plated on ampicillin containing Luria plates. Colonies produced were screened for plasmids resistant to Kpn I digestion. As shown in Figure 3.3.11, two out of 12 colonies contained the mutated plasmids (lanes k and m). These two pSAL-102 derivatives were resistant to Kpn I digestion (pSAL-102DL^{pol}-9 and -11). They were further analyzed to detect any extensive deletion which might occurred during the manipulation. As shown in Figure 3.3.12, digestion of pSAL-102DL^{pol}-11 with Eco RI resulted in four fragments as with pSAL-102 (lanes e and g). These results indicate that apparently no extensive deletion has occurred in pSAL-102DL^{pol}-11 except possible four base deletion at the Kpn I site. In contrast, pSAL-102DL^{pol}-9 contained apparently large deletion of about 2.6 kb at the LTR and adjacent sequences

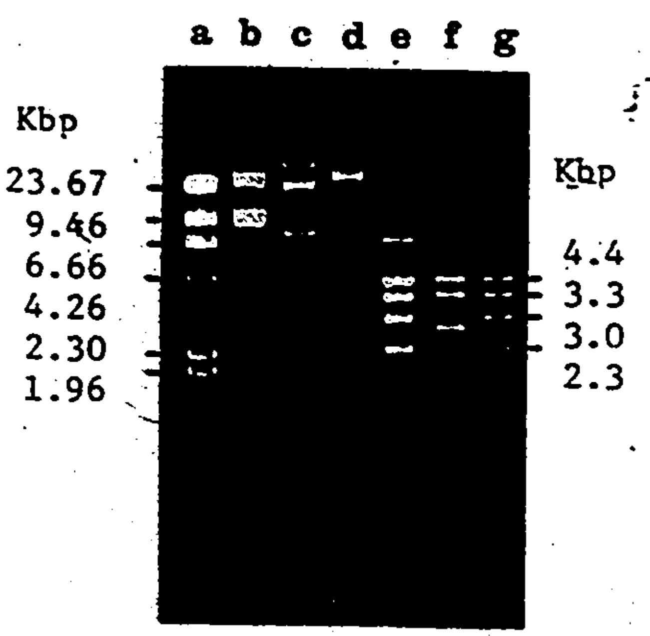


Figure 3.3.12 Restriction enzyme analyses of the pSAL-102 derivative mutants deleted at the Kpn I site of the pol gene.

The transformants containing Kpn I-resistant plasmids were grown, plasmids isolated, analyzed by digestion with Eco RI and the DNA fragments electrophoresed on a 0.8% agarose gel. Lane a, λ DNA molecular weight marker; lanes b to d, the untreated pSAL-102, pSAL-102DL^{pol}-9, and pSAL-102DL^{pol}-11, respectively; lanes e to g, Eco RI digested pSAL-102, pSAL-102DL^{pol}-9, and pSAL-102DL^{pol}-11, respectively.

Table VII. DNA polymerase activities of *E. coli* harboring pSAL-102 derivative mutants.

Cell lysates from *E. coli* harboring	Acid-insoluble radioactivity (cpm/A_{280} unit)
pSAL-102	702,800
pSAL-102DL^{pol}-9	51,000
pSAL-102DL^{pol}-11	48,000
pB5^{pol}	15,000

E. coli cell lysates (50 µl) were incubated with 50 µl of the 2X reaction buffer at 37°C for 60 min and the reaction products were determined as described in Table III.

(gag and src) in addition to the four bases at the Kpn I site. These two colonies were tested for the polymerase activity. As shown in Table VII, the activities were reduced drastically in both cases. The 4 base deletion at the Kpn I site may cause pretermination of the pol coding sequences and may have resulted in the synthesis of a truncated product missing about 60 carboxy-terminal amino acids. Similarly, no polymerase activity was detected in E. coli harboring $pB5^{pol}$ (see Figure 3.3.6), which contains the entire pol gene except 187 base pairs from 3' end of the pol gene (Table VII). These results suggest that the carboxy terminal region of the reverse transcriptase molecule is not dispensable for the polymerase activity.

Cloning The Polymerase Gene Into The Expression Vector pER103

The expression vector pER103 contains a promoter-operator sequence derived from the trp operon of Serratia marcescens (Miozzari and Yanofsky, 1978). Efficient translation of the transcripts was achieved (Dworkin-Rastl et al., 1983) by incorporation into these expression plasmids the potent synthetic RBS described by Jay et al. (1981). The presence of a unique Hind III site following the RBS makes these plasmids suitable as general cloning and expression vectors.

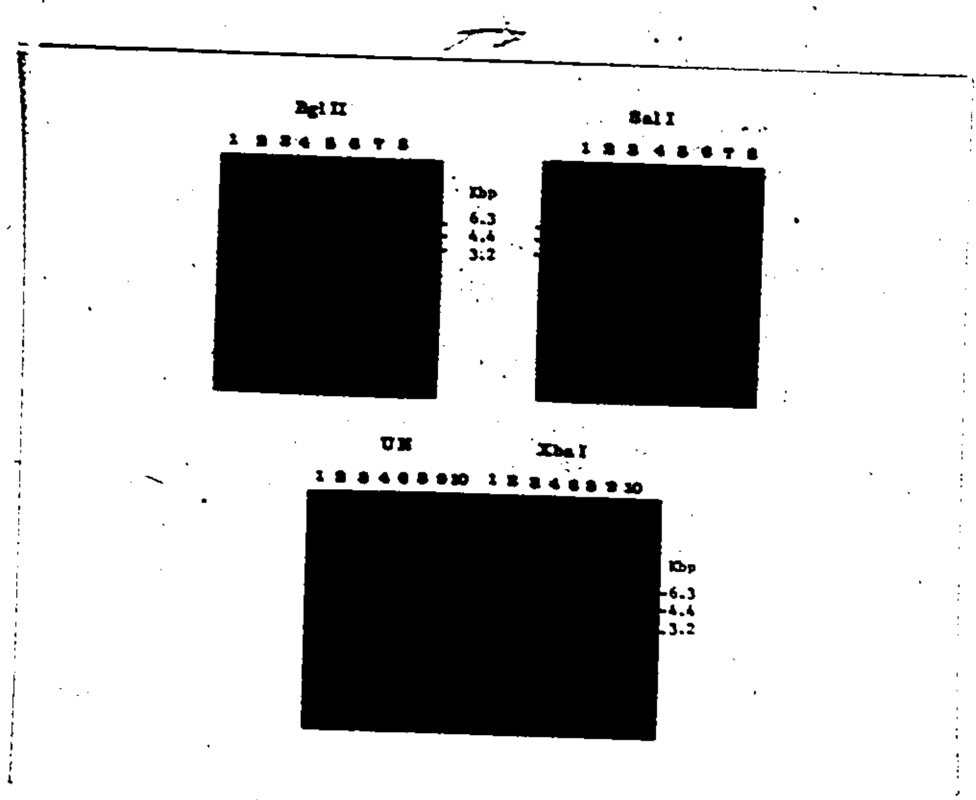


Figure 3.3.13 B Restriction enzyme analyses of pERΔPOL.

The transformants were analyzed for the presence of the recombinant plasmids. Plasmids from each clone were digested with Bgl II, Sal I or Xba I. The numbers in each lane correspond to the clone number. The last lanes of each gel contain the molecular weight marker fragments (Hind III fragments of pATV-8). UN corresponds to the undigested plasmids.

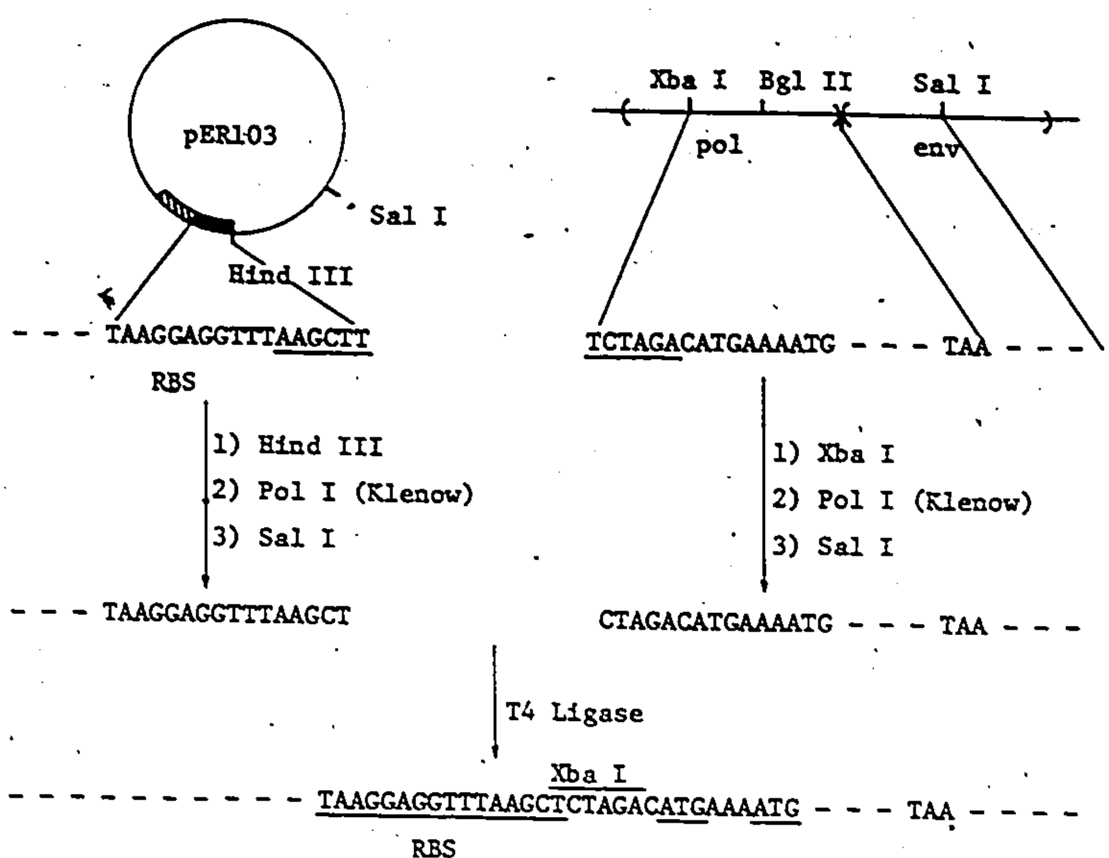
In order to show that the pol-related proteins were synthesized from the putative initiation site, I cloned the putative polymerase coding sequence just downstream from the promoter-RBS site (Fig. 3.3.13A). The ATG initiator codon is now positioned at the optimal distance from the synthetic RBS as reported by Jay et al. (1981) and Dworkin-Rastl et al. (1983). The resulted recombinant plasmids should express the pol gene in inducible manner. The recombinant plasmids were analyzed by restriction enzymes (Fig. 3.3.13B). Out of ten clones analyzed, six clones were shown to contain the recombinant plasmids (pERΔPOL-1, 2, 4, 6, 8, 10). Clones 1, 2, 3, 4, 6 and 8 were shown to contain the recombinant plasmids which could be cleaved with Bgl II (the unique site present in the pol gene) and Sal I. Clones 1, 2, 4, 6, 8 and 10 were shown to contain the recombinant plasmids with intact Xba I site which was regenerated after the blunt-end ligation. The digested DNA gave the expected 7.1 kb linearized plasmid.

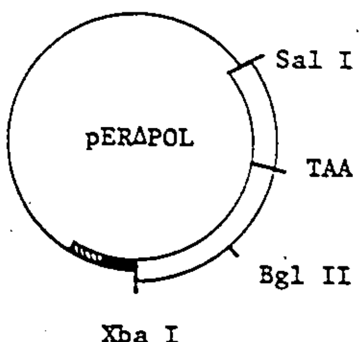
Induction of The DNA Polymerase Activity in E. coli Containing pERΔPOL

Cultures of overnight grown *E. coli* containing pERΔPOL were diluted 1:100 in M9 medium supplemented with 0.2% glucose, 20 μg/ml of 19 amino acids except tryptophan and 100 μg/ml ampicillin and grown until an A_{600} = 0.1

Figure 3.3.13A Cloning of the polymerase gene into the expression vector pER103.

pSAL-102 was first digested with Xba I and treated with polymerase I (Klenow fragment) as described in legend to Figure 3.3.6. After phenol extraction and ethanol precipitation, the DNA was digested with Sal I and the resulted 2.7 kb fragment was isolated from a 0.8% agarose gel. Similarly, pER103 was first digested with Hind III and treated with polymerase I (Klenow fragment). The treated DNA was digested with Sal I and the larger fragment was isolated from the gel. The purified DNA fragments were then ligated and transformed into HB101 as described in Methods. The resulted transformants were analyzed by restriction analysis (Fig. 3.3.13B) and several positive clones (pERAPOL) were obtained. As noted on the figure, pERAPOL retained two unique restriction sites (Xba I and Sal I). The shaded box represents the trp promoter; the closed box represents the RBS; and the open box represents the pol and env genes.


pER103
Sal I
Hind III
- - - TAAGGAGGTTTAAGCTT
RBS
1) Hind III
2) Pol I (Klenow)
3) Sal I
- - - TAAGGAGGTTTAAGCT
Xba I
Bgl II
Sal I
pol
env
TCTAGACATGAAAATG - - - TAA - - -
1) Xba I
2) Pol I (Klenow)
3) Sal I
CTAGACATGAAAATG - - - TAA - - -
T4 Ligase
Xba I
- - - - - - - - - - TAAGGAGGTTTAAGCTCTAGACATGAAAATG - - - TAA - - - -
RBS


pERΔPOL
Sal I
TAA
Bgl II
Xba I

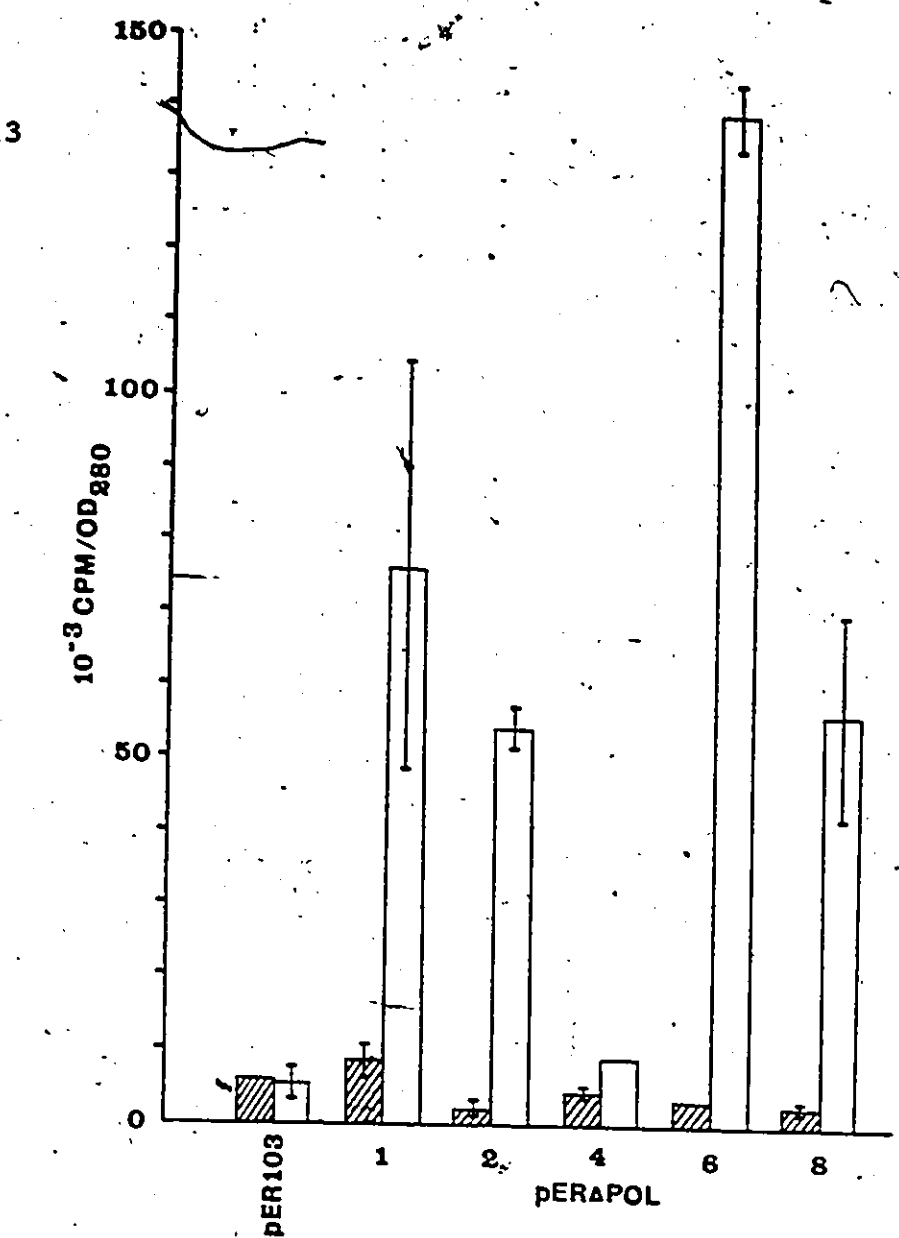


Figure 3.3.14 Histogram of the polymerase activity induced in <u>E. coli</u> harboring pERΔPOL.

<u>E. coli</u> cell lysates were prepared and assayed for the polymerase activity as described in the text. The shaded bars indicate the background activities present before induction and the open bars indicate the activities present after derepression of trp promoter.

was reached. 3β-Indole acrylic acid (20 μg/ml) was then added to derepress trp promoter (Hallewell and Emtage, 1980), and the cultures were grown a further 1.5 hours. Cells were pelleted, lysed and assayed for the polymerase activity as described in Table III. As shown in Figure 3.3.14, the DNA polymerase activity was inducible in *E. coli* harboring pERΔPOL. These results indicate that pol-related proteins possessing enzymatic activity may be initiated from either of the two ATG codons corresponding to methionine residues 295 and 297 of the reverse transcriptase molecule.

Analyses of The pol-Related Proteins From E. coli Harboring pERΔPOL

The presence of pol-related proteins in *E. coli* harboring pERΔPOL was determined by immunoprecipitation and SDS-PAGE. The cells were grown and induced for 1.5 hours as described above, then labeled with [^{35}S] methionine (8 μCi/ml) for 30 min. The cell lysates were immunoprecipitated with anti-synthetic pol-peptide antiserum (Grandgenett et al., 1983), then analyzed on a 10% SDS-polyacrylamide gel (Fig. 3.3.15). A protein with molecular weight of about 65,000 (p65) was detected by the antiserum in cells harboring pERΔPOL (lanes 3 and 4). In contrast, no such protein was detected in cells harboring pER103 (lane 2). Therefore, the protein p65 recognized by anti-synthetic

Figure 3.3.15 Autoradiogram of SDS-PAGE of pol-related proteins synthesized in *E. coli* harboring pERΔPOL.

Cells containing the plasmids were labeled and analyzed by immunoprecipitation as described in Materials and Methods. The precipitated proteins were run on a 10% polyacrylamide gel according to Laemmli. Lane 1 corresponds to molecular weight marker; lane 2, *E. coli* harboring pER103; lanes 3 and 4, *E. coli* harboring pERΔPOL-2 and pERΔPOL-6, respectively.

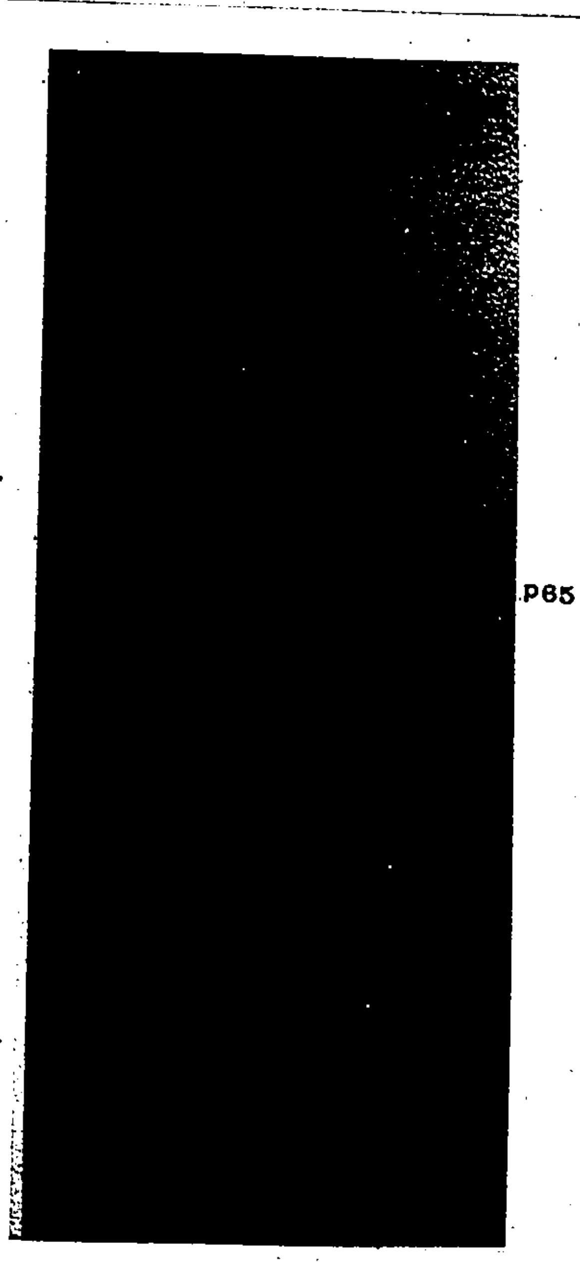

P65

Table VIII. DNA polymerase activities of *E. coli* harboring pol-specific recombinant plasmids.

Cell lysates from *E. coli* harboring	Radioactivity (cpm/A_{280} unit)
None	13,800
pBR322	10,500
pSAL-102	702,800
pBRPOL-62	702,600
pBRPOLDL-2	11,400

E. coli cell lysates (50 µl) were incubated with 50 µl of the 2X reaction buffer at 37°C for 60 min and the reaction products were counted as described in Table III.

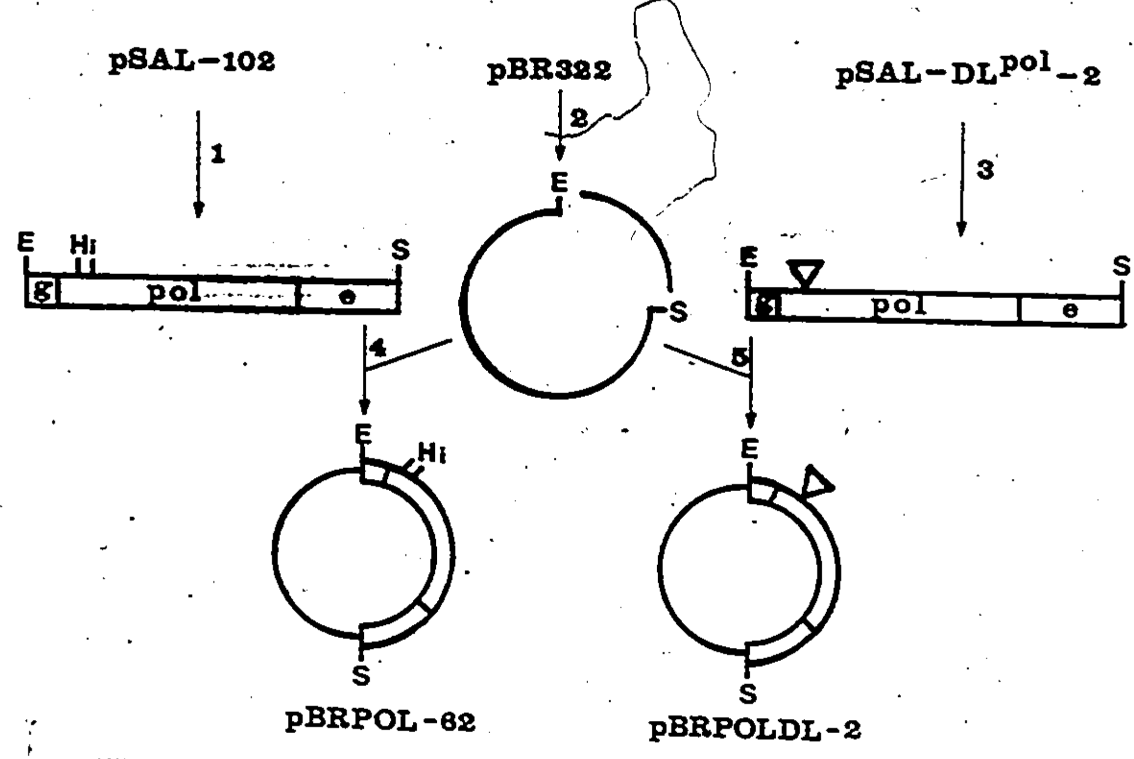
pol-peptide antiserum is initiated from the methionine residues in positions 295 or 297 of the reverse transcriptase molecule and possess the enzymatic activity observed.

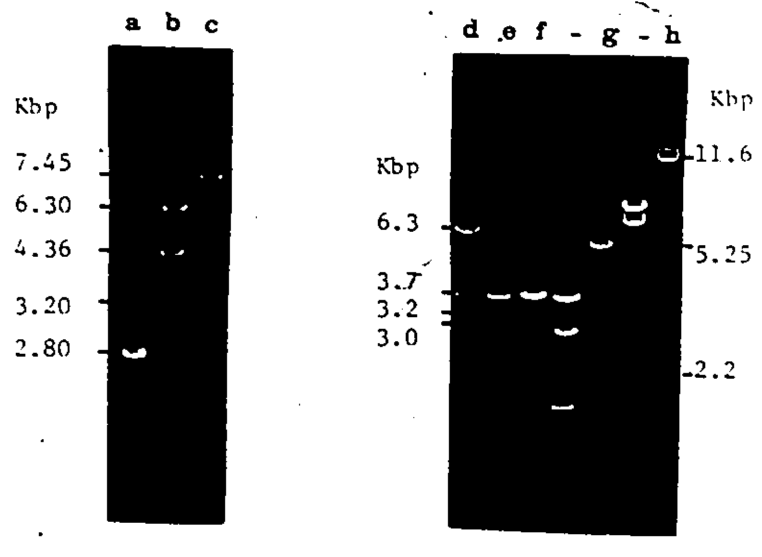
Identification of Putative Promoters For The pol-Transcripts

As described above, the polymerase activity present in *E. coli* harboring pSAL-102DL^{pol}-2 was about 10% of the activity present in cells harboring pSAL-102 (Table III). The reduced activity could be due to a reduced amount of the translatable pol-transcripts. The deleted Hind III fragment may also contain major transcriptional control elements. In order to test this possibility I subcloned the cloned proviral DNA fragment consisting of complete pol gene with portion of flanking gag and env genes into pBR322 (Fig. 3.3.16). The resultant recombinant plasmid pBRPOL-62 contains only the putative promoter located in the small Hind III fragment. I also subcloned the Eco RI and Sal I digested fragment from pSAL-102DL^{pol}-2 as a control (Fig. 3.3.16). The resultant recombinant plasmid pBRPOLDL-2 contains the same sequences present in pBRPOL-62 except that the 126 bp Hind III fragment has been deleted. As shown in Table VIII, the polymerase activity present in cells harboring pBRPOL-62 was comparable to the activity present in cells harboring pSAL-102. In contrast, the extracts from cells

Figure 3.3.16 Cloning of the pol specific fragment into pBR322.

Eco RI and Sal I double digested fragments of pSAL-102 (step 1), pBR322 (step 2), or pSAL-102DL^{pol}-2 (step 3) were isolated from the agarose gel. Appropriate fragments were ligated and transformed into HB101 cells. The resulted colonies on Luria plates containing ampicillin were tested for tetracycline sensitivity on duplicate plates, and the $Amp^{R}Tc^{S}$ phenotype colonies were analyzed for the recombinant plasmids. Lanes a and b correspond to the molecular weight markers, the Hind III digested fragments of pUC9 and pATV-8, respectively. Lane c, Hind III digested pBRPOL-62 which linearize the plasmid to give a 7.45 kb fragment. Lane d, molecular weight marker, the Hind III and Sal I double digested pATV-8; lane e, molecular weight marker, the Eco RI and Sal I double digested pSAL-102; lane f, Eco RI and Sal I double digested pBRPOL-62. Lanes g and h, Kpn I and Hpa I double-digested pBRPOL-62 and pSAL-102, respectively. Symbols: line, pBR322; open bar, RSV proviral DNA; E, Eco RI; Hi, Hind III; S, Sal I; Δ indicates the specific deletion at the pol gene as described in Figure 3.3.6.


pSAL-102
pBR322
pSAL-DL^{pol}-2
1
2
3
4
5
E
Hi
S
g
pol
e
pBRPOL-62
pBRPOLDL-2


a b c
Kbp
7.45
6.30
4.36
3.20
2.80
d e f - g - h
Kbp
6.3
3.7
3.2
3.0
11.6
5.25
2.2

TTG 2-4 TTT 6-9 ATTTGTTATAATG 4-7 CAT Model Promoter[a]

TTG 3 TTT 8 GGCGGTT TAT/ATA A T/A T 5 CAT Model Promoter[b]

TTG 3 TTT 8 ATT T/G GGTATAATG 5 CAT Model Promoter[c]

TTG 3 GCT 8 CTCTGGTAAGGTT 4 AGC Neomycin Resistant Gene[d]

TTG 3 GCT 9 Hind III AAGCTTGTTCCTTTTGGGGCCGT pol Gene[e]

TTGACat TAtAAT cat Consensus Sequence[f]

-35 REGION -10 REGION RNA START

Figure 3.3.17 Comparison of the apparent promoter sequence in the pol gene with model promoter sequences and consensus sequence.

In consensus sequence, the highly conserved bases (>75%; 9S.D.) are underlined. The conserved bases (>50%; 5S.D.) are capital letters. The weakly conserved bases (>40%: 3S.D.) are lower case letters. The superscripts denote literature references as follows: a, Rosenberg and Court (1979); b, Scherer et al. (1978); c, Siebenlist et al. (1980); d, Rothstein and Reznikoff (1981); e, Schwartz et al. (1983); f, McClure (1985).

harboring pBRPOLDL-2 did not show any increase over the background value of extracts from cells containing pBR322. These results suggest that part or all of the major contributing promoter sequence for the pol-transcripts in _E. coli_ is present in the 126 bp Hind III fragment at the 5' region of the pol gene.

Nucleotide Sequence of The Putative Promoter in The pol Gene

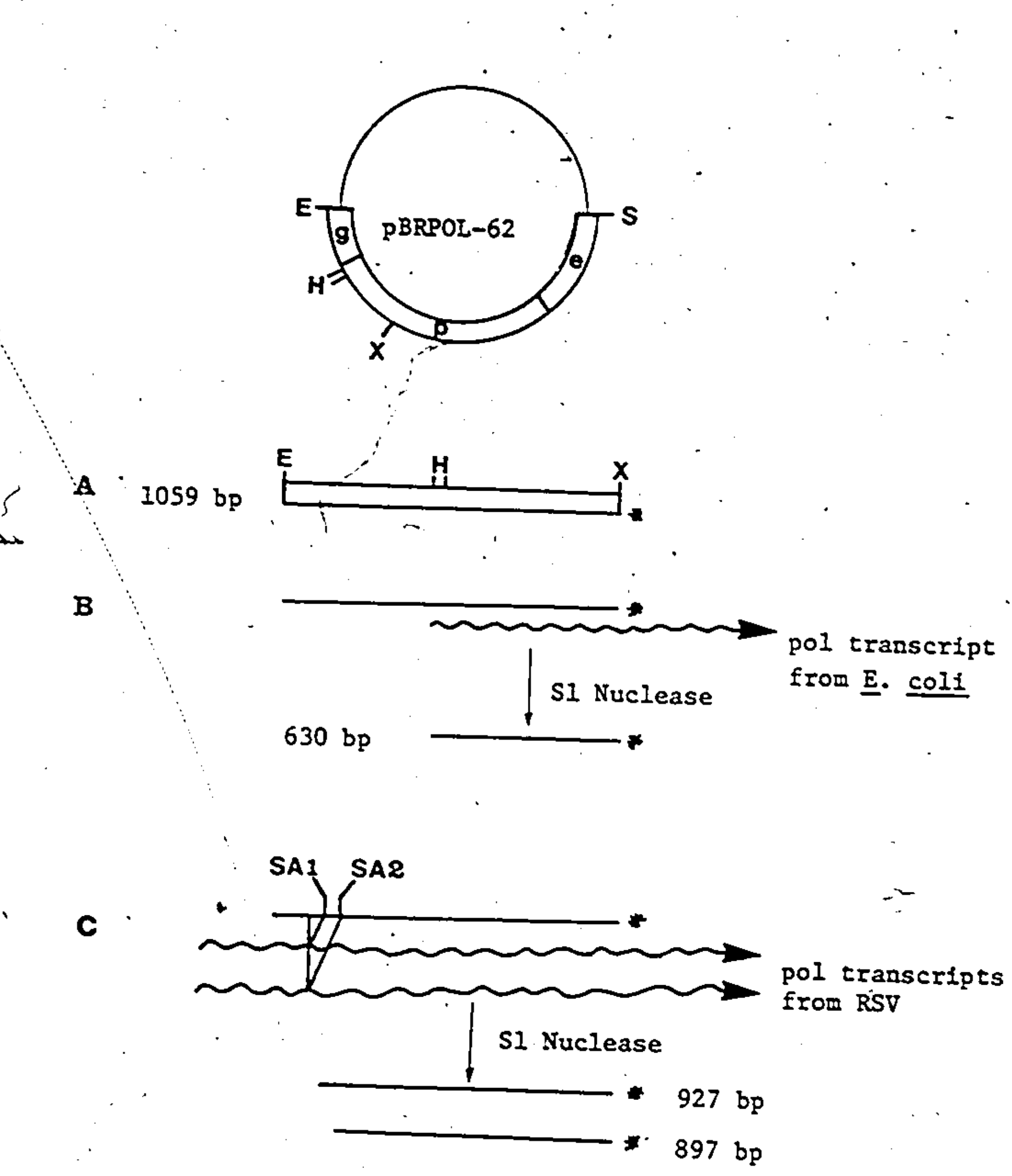
From the DNA sequence (Fig. 3.3.1), it was evident that there is a putative promoter sequence present at the Hind III site near the 5' end of the pol gene. A comparison of the sequences around the Hind III site with known prokaryotic promoter sequences (Scherer et al., 1978; Rosenberg and Court, 1979; Siebenlist et al., 1980; McClure, 1985) showed the presence of conserved sequences similar to a Pribnow box (Fig. 3.3.17). A highly conserved TTG sequences in a region about 35 nucleotides upstream from the mRNA start point was also observed in the LTR sequence of RSV (Mitsialis et al., 1981). This was just upstream from the first Hind III site at the 5' region of the pol gene (Fig. 3.3.17).

Analysis of Viral RNA Transcripts

The above evidence suggests that the initiation of viral RNA transcription may occur near the 5' end of the pol gene. To examine this possibility I used the S1 nuclease protection technique of Berk and Sharp (1978), as modified by Weaver and Weissmann (1979), to map the 5' ends of the viral transcripts synthesized in E. coli harboring pBRPOL-62 or pBRPOLDL-2. Total RNA was isolated and hybridized with a 5' end labeled restriction fragment that overlaps the putative initiaiton site (Fig. 3.3.18). The RNA-DNA hybrids were digested with S1 nuclease, and the protected probe fragments were sized on a 4 % polyacrylamide-gel (Fig. 3.3.19). A new fragment was generated after S1-digestion of the hybrid of RNA from E. coli harboring pBRPOL-62 and the DNA probe (lane a). The size of the protected fragment was estimated to be about 630 bp. This size corresponded well to the expected fragment estimated from the putative promoter sequence in the pol gene of RSV (Fig. 3.3.17). In contrast, no such fragment was generated from the hybrids of RNA from E. coli harboring pBRPOLDL-2 and the DNA probe (lane 2). In both cases, some of the probe remained undigested (lanes 1 and 2). I, therefore, concluded that within the constraints of the S1 nuclease assay, the site of transcription initiation appears to be located at the putative site detected from the nucleotide sequences (Fig. 3.3.17).

Figure 3.3.18 Structures of the ^{32}P-labeled probe and the putative pol-transcripts from E. coli and RSV-infected cells.

(A) The probe labeled at Xba I-site was prepared as described in Methods. (B) The putative pol-transcripts initiate from a site between the two Hind III sites as suggested from the nucleotide sequence of RSV (Schwartz et al., 1983). The protected fragment from S1 nuclease digestion would be about 630 bp in length. (C) Two splicing acceptor sites (SA1 and SA2) for the transcripts for reverse-transcriptase precursor $Pr180^{gag-pol}$ are shown (Schwartz et al., 1983). Hybridization of the putative spliced mRNA and the probe results in formation of single stranded DNA loops at the spliced site. After S1 nuclease digestion, two protected fragments with sizes of 927 bp and 897 bp would be generated.



pBRPOL-62
E
S
g
e
H
X
p
A
1059 bp
E
H
X
B
pol transcript
from E. coli
S1 Nuclease
630 bp
SA1
SA2
C
pol transcripts
from RSV
S1 Nuclease
927 bp
897 bp

Figure 3.3.19 Nuclease-S1 mappling of the pol-transcripts. Lane 1, RNA from E. coli harboring pBRPOL-62; lane 2, RNA from E. coli harboring pBRPOLDL-2; lane 3, RNA from RSV; lane 4, the DNA probe; lane 5, molecular weight markers. pBR322 linearized with Sal I was purified from an agarose-gel and cut with Taq I and labeled with [α-^{32}P] CTP and polymerase I (Klenow fragment).

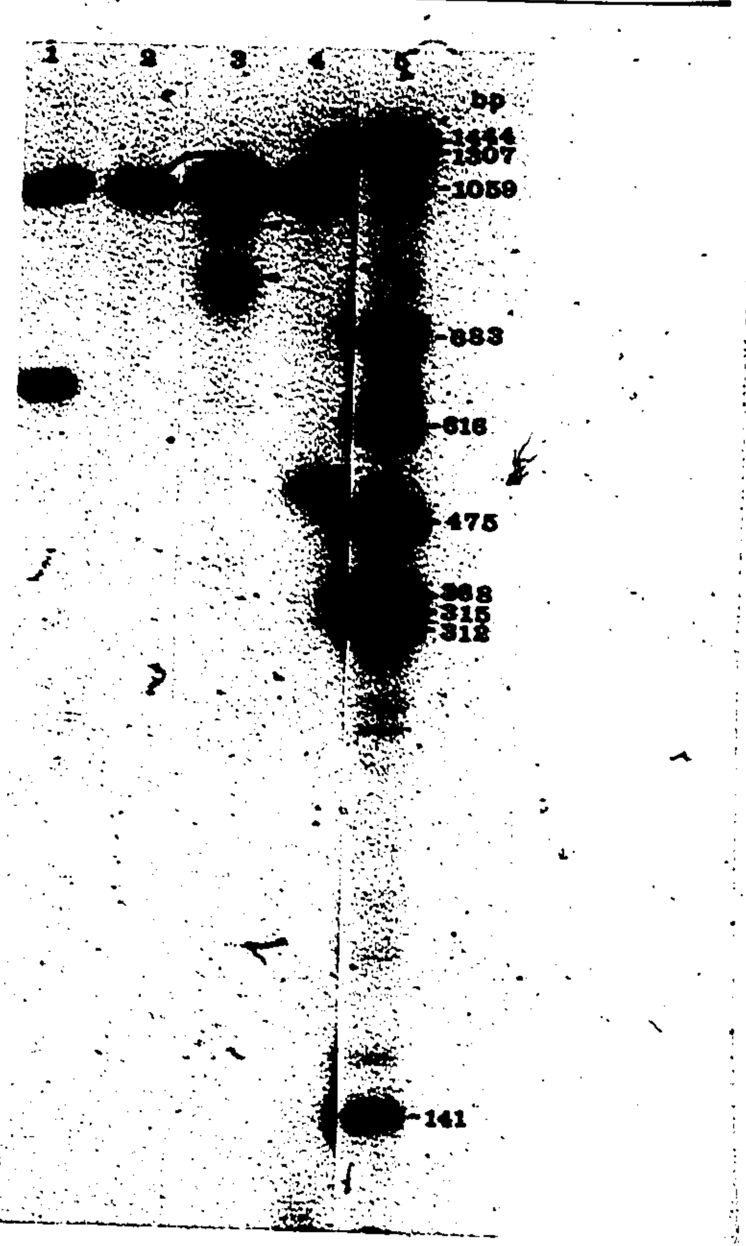

1
2
3
4
5
bp
1444
1307
1059
883
616
475
368
315
312
141

Table IX. Inhibition of DNA polymerase activity of pBRPOL-62 by monospecific antibody to a synthetic peptide of the avian retrovirus pp32 and the β DNA polymerase subunit (anti-synthetic pol-peptide antiserum).

Samples	Treatment	Radioactivity (count/min)
pBRPOL-62	None	17,100
pBRPOL-62	Normal rabbit serum	15,900
pBRPOL-62	Anti-synthetic pol-peptide antiserum	4,600
pBRPOL-62	Anti-p27 (gag) antiserum	15,600
pBRPOLDL-2	None	4,600

E. coli cell lysates (5 μl) were incubated at 22°C for 1 hr with the respective antisera in presence of 1 mM PMSF and 1 mg/ml of Trypsin inhibitor, then assayed for the polymerase activity as described in Table III.

Inhibition of DNA Polymerase Activity by the Monospecific Antiserum

Lysates of E. coli containing pBRPOL-62 were prepared as described in Methods, and the lysates were incubated at 22°C for 1 hour in the presence of different antisera. The antisera treated lysates were then assayed for the polymerase activity. As shown in Table IX, treatment of the cell lysates with anti-synthetic pol-peptide antiserum significantly reduced the polymerase activity to the background activity present in cells containing pBRPOLDL-2. In contrast, treatment of the cell lysates with either normal rabbit serum or anti-p27 (gag protein) antiserum did not reduce the polymerase activity. These results further indicate that the polymerase activity present in E. coli harboring the pol-recombinant plasmids is due to the synthesis of pol-related polypeptides.

Transfection of Wild-Type and Mutant Proviral DNAs into Chick Embryo Fibroblast Cells

E. coli containing pSAL-102DL^{pol}-2 was shown to have DNA polymerase activity (Table III) due to synthesis of the p65 proteins (Figs. 3.3.5 and 3.3.8) that may initiate from the internal methionine (Fig. 3.3.9). pSAL-102DL^{pol}-11 is a deletion mutant at 3'-end of the pol gene (Figs. 3.3.11

and 3.3.12), such that the DNA polymerase activity in E. coli containing pSAL-102DL^{pol}-11 was not significantly higher than background (Table VII). Similarly, the polymerase activity present in E. coli harboring pB5^{pol} was absent (Table VII), which may also be due to a deletion at the 3'-end of the pol gene (Fig. 3.3.6). These results indicate that 5' portion, but not 3', of the pol gene is dispensable for the polymerase activity.

It was, therefore, decided to test this hypothesis in vivo in chicken cells. It was anticipated that there would be synthesis of the fused gag-pol precursor polypeptides in cells transfected by the mutated viral DNAs. In pSAL-102DL^{pol}-2, the deletion in the 5'-end of the pol gene was arranged in such a way that the reading frame was unaltered in the pol gene and synthesis of a 175,000 daltons fused gag-pol polyproteins was expected (42 amino acids deletion at the aminoterminal region of the polymerase molecule). In pSAL-102DL^{pol}-11, the Kpn I site near the 3'-end of the pol gene was mutated so that the synthesis of a truncated polyprotein (Mr = 173,500) was anticipated.

All plasmids were digested with Sal I to remove the pBR322 sequence, and the viral DNAs were purified from the agarose gel. The viral DNAs were treated with T4 DNA ligase just before transfection into 2° CEF cells. Primary CEF cells were trypsinized and plated on 60 mm plates (5 X 10^5 cells per plate) and the ligated DNAs were transfected

into the CEF cells when the plates were about 50% confluent. Calcium phosphate precipitated DNAs (Graham and Van der Eb, 1973) were added to plates and adsorbed for 30 min, then fed with high glucose Dulbecco (HGD) medium supplemented with 2% chick serum and 1% FBS. After 12 hours, the medium was replaced with HGD supplemented with 5% FBS, 2% chick serum, and 1% DMSO. Next day, cells were subcultured into 10 cm plates, then following day the cells were diluted to 1:4.

Transformed cells were detected at 3.5 days posttransfection from the cells transfected with the wild-type DNA. At 4.5 days posttransfection, the cells were completely transformed. No transformation was detected in cells transfected with the mutant DNAs. The lack of transformation in chicken cells is consistent with a requirement for replication in the transfection of chicken cells suggested by Cooper and Okenquist (1978). At 5.5 days posttransfection, cells were diluted to 1:2.

At 6.5 days posttransfection, there was a sign of transformation in cells transfected with the viral DNA from pSAL-102DL^{pol}-2. This indicates that replication of the transfected DNA may have occurred, and the gag-pol fused protein (Pr175$^{gag-pol}$) may have been synthesized and processed to the functional reverse transcriptase, in spite of the deletion at the amino-terminal region of the molecule.

Analyses of The Viral Proteins From Transfected CEF

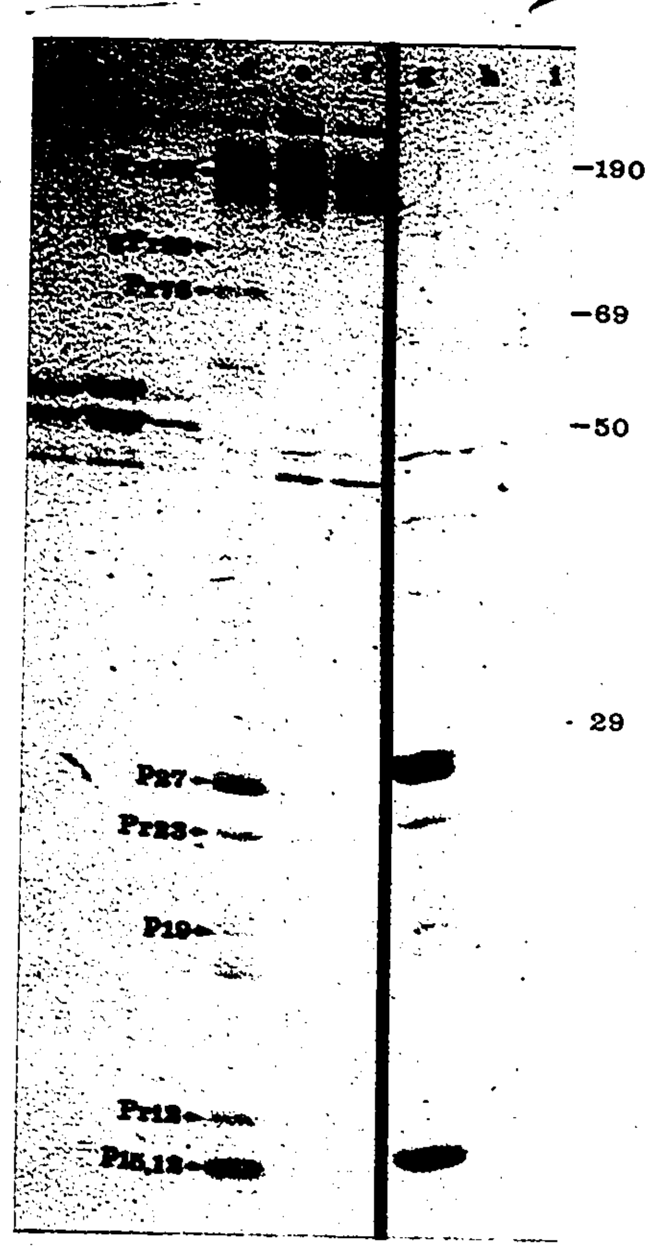
At 5½ days posttransfection, cells were labeled with [^{35}S] methionine (180 µCi/plate) for 16 hours and viruses were purified from the media and analyzed by SDS-PAGE. Intracellular viral proteins were labeled for 1 hour with [^{35}S] methionine (225 µCi/plate). Cells were lysed and immunoprecipitated with anti-RSV antiserum (Fig. 3.3.20).

As expected, the synthesis of $Pr180^{gag-pol}$ was observed in the cells transfected with wild-type DNA (lane d), and slightly lower molecular weight proteins were detected from the cells transfected with the mutant DNAs (lanes e and f). In the labeled wild type virus, all of the gag proteins were present (lane g). No such proteins were detected in the media from the mutant DNA transfected cells (lanes h and i). The intracellular viral proteins present in cells transfected with the mutant DNAs might be synthesized by the transcripts from the unintegrated donor DNAs, since significant amounts of unintegrated viral DNAs may persist for several weeks after infection (Fritsch and Temin, 1977; Guntaka et al., 1976; Varmus and Shank, 1976).

The intracellular viral proteins present in transfected cells were further analyzed by immunoprecipitation with a monospecific antiserum (anti-p27 antiserum) and analyzed on a 7.5% SDS-polyacrylamide gel. As shown in Figure 3.3.21, the synthesis of gag precursors ($Pr76^{gag}$, $Pr66^{gag}$ and $Pr60^{gag}$)

Figure 3.3.20 Polyacrylamide gel analyses of the viral proteins from transfected chick embryo fibroblast cells.

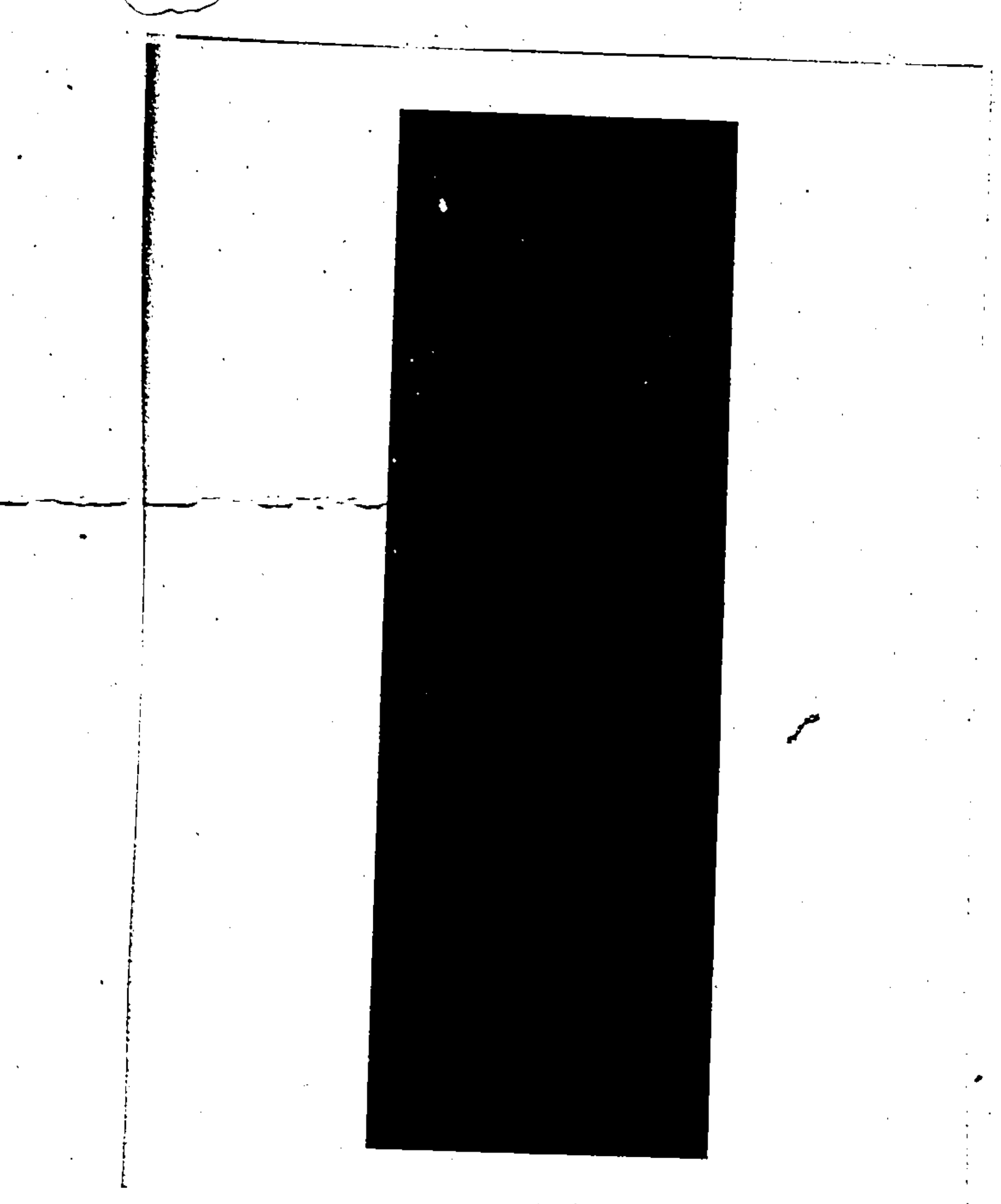
Cells transfected with the viral DNAs were labeled for 1 hour with [^{35}S] methionine and the labeled cells were lysed and immunoprecipitated with anti-RSV antiserum. The immune-precipitates were analyzed on a 12.5% polyacrylamide gel. Lanes a and d, viral DNAs from pSAL-102; lanes b and e, viral DNAs from pSAL-102DL^{pol}-2; lanes c and f, viral DNAs from pSAL-102DL^{pol}-11. Lanes a to c correspond to the precipitates with normal rabbit serum; lanes d to f correspond to the precipitates with anti-RSV antiserum. Lanes g to i, viruses released from cells transfected with the viral DNAs from pSAL-102, pSAL-102DL^{pol}-2, and pSAL-102DL^{pol}-11, respectively.



-190
-69
-50
29
Pr27
Pr23
P19
Pr12
P15,12

Figure 3.3.21 Polyacrylamide gel analyses of the intracellular viral proteins from transfected chick embryo fibroblast cells.

Cells transfected with the viral DNAs were labeled for 1 hr with [^{35}S] methionine and the labeled cells were lysed and immunoprecipitated with anti-p27 antiserum. The immune-precipitates were analyzed on a 7.5% polyacrylamide gel. Lanes a and d, viral DNAs from pSAL-102; lanes b and e, viral DNAs from pSAL-102DL^{pol}-2; lanes c and f, viral DNAs from pSAL-102DL^{pol}-11. Lanes a to c correspond to the precipitates with normal rabbit serum; lanes d to f correspond to the precipitates with anti-p27 antiserum.



was demonstrated with the anti-gag antiserum. Furthermore, as shown above, the synthesis of Pr180$^{gag-pol}$ was observed in the cells transfected with the viral DNA from pSAL-102 (lane d), and slightly lower molecular weight proteins were detected from the cells transfected with the deletion mutant DNAs (lanes e and f).

As mentioned above, cells transfected with the viral DNA from pSAL-102DL^{pol}-2 had exhibited a partial transformed phenotype after about 6 days, but I was unable to detect any viral proteins in the medium. It may mean that the mutant virus could persist by a low level of viral spread and could not be detected by the present method. It is possible that the transformation is a result of the spread of low levels of the virus without integration. Cells infected with a mutant of spleen necrosis virus, that is unable to integrate by virtue of a deletion of the site for integration do in fact support the spread of low levels of the virus (Panganiban and Temin, 1983).

DISCUSSION

Recently, a large amount of information has accumulated on the structure and function of the retrovirus reverse transcriptase. In the present studies, the mechanism of synthesis and processing of reverse transcriptase has been studied. The mutant LA83 described in this work not only offered an excellent method to study these two important aspects of reverse transcriptase biogenesis but also allowed study of the interrelationship between the precursor polyprotein cleavage and virion assembly.

In this discussion, I hope to correlate the observations reported in this thesis with observations of other investigators and discuss how these findings are related to the structure, function and mechanism of synthesis and processing of reverse transcriptase and virion assembly and maturation.

4.1 Impaired Cleavage of the Joint gag-pol Polyprotein Precursor and Virion Assembly in a Temperature-Sensitive Mutant of Rous Sarcoma Virus

The studies reported here provide biochemical

evidence that reverse-transcriptase precursor $Pr180^{gag-pol}$ present in cells infected with the replication-defective conditional lethal mutant LA83 is not cleaved at the nonpermissive temperature. Although temperature-sensitive mutants of MuLV defective in the cleavage of reverse transcriptase precursor have been described (Witte and Baltimore, 1978), in the avian retrovirus system no such mutants have yet been reported. It is believed that in the case of RSV the mature reverse transcriptase is generated by cleavage of the $Pr180^{gag-pol}$, at least in part by the viral protein p15 (Moelling et al., 1980; Vogt et al., 1979; von der Helm, 1977). In the MuLV system the two mutants ts24 and ts3 at the nonpermissive temperature fail to cleave $Pr180^{gag-pol}$ and are also enzymatically inactive. Cleavage of the $Pr180^{gag-pol}$ occurs and reverse-transcriptase activity appears in the released virions after the temperature has been shifted down to the permissive level (Witte and Baltimore, 1978). The experimental data that $Pr180^{gag-pol}$ is accumulated at the nonpermissive temperature in LA83-infected cell and is cleaved into reverse transcriptase in released virions after shiftdown of the temperature (Figs. 3.1.28, 3.1.29) provide strong evidence for the idea that in the RSV system $Pr180^{gag-pol}$ is the precursor to mature reverse transcriptase.

Morphological examination of cells infected with

LA83 at the nonpermissive temperature showed that the maturation of virus particles is blocked at the budding stage and downshift to the permissive temperature overcame the blockage and resulted in the release of viruses (Fig. 3.1.2). Temperature shiftdown experiments also showed that previously synthesized $Pr180^{gag-pol}$ can be cleaved into reverse transcriptase in the released virions (Figs. 3.1.28, 3.1.29). Taken together these data suggest that a relationship may exist between the processing of $Pr180^{gag-pol}$ and the maturation of RNA tumor virus. Previous studies showed that both in the case of mammalian tumor viruses (Jamjoom et al., 1977; Lu et al., 1979; Stephenson et al., 1975; van de Ven et al., 1978; Vogt et al., 1975; Yeger et al., 1978) and avian tumor viruses (Hunter et al., 1976; Rohrschneider et al., 1976) maturation of the virus particles was arrested when the processing of the gag precursors was defective. In the case MuLV mutants, ts3 (Moloney) and ts24 (Rauscher), the maturation defects could be related to the defective processing of both $Pr65^{gag}$ and $Pr180^{gag-pol}$ polypeptides (Witte and Baltimore, 1978). Since the $Pr65^{gag}$ polypeptide had been shown to affect the maturation of virus particles, the primary defect in the case of ts3 and ts24 could be in the processing of the gag precursor. The processing of $Pr65^{gag}$ could also affect the cleavage of $Pr180^{gag-pol}$ in the case of ts3 and ts24. In contrast, my findings showed

that the processing of Pr180$^{gag-pol}$ was independent of the processing of Pr76^{gag}. Furthermore, my results suggested that viral maturation and cleavage of Pr180$^{gag-pol}$ might be interrelated. The precise nature of the relationship between polyprotein processing and virus assembly is yet to be clearly understood.

It has been proposed that the assembly of retroviruses involves processing of polypeptide precursors in association with the membrane at the budding site (Bolognesi et al., 1978). Results of electron microscopic studies with ts mutants of MuLV strongly support this notion (Lu et al., 1979; Yeger et al., 1978). The fact that at the nonpermissive temperature reduced quantities of virion-like particles lacking reverse transcriptase are released suggests that at the restrictive temperature Pr180$^{gag-pol}$ synthesized in LA83-infected cells may not be correctly organized in the budding structure for cleavage consequently affecting virus assembly and maturation. Shiftdown of temperature allows Pr180$^{gag-pol}$ molecules to rearrange correctly on the budding forms resulting in processing of the precursor into the reverse transcriptase subunits and release of virus. The inability to detect any Pr180$^{gag-pol}$ in NI-LA83 suggests that at the restrictive temperature Pr180$^{gag-pol}$ is not localized at the budding site or alternatively uncleaved Pr180$^{gag-pol}$ is not able to be incorporated into the released particles even though it is present at the budding

site.

The viral protein p15 has been implicated in the processing of both $Pr76^{gag}$ and $Pr180^{gag-pol}$ (Moelling, 1975; Vogt et al., 1979; von der Helm, 1977). The proper processing of $Pr76^{gag}$ at 41°C (Figs. 3.1.26, 3.1.27 and Table II) therefore, would seem to rule out any defect in p15 and the p12-p15 junction (the initial cleavage site by a host protease; Vogt et al., 1975) in LA83. It is possible, therefore, that the defect in processing of $Pr180^{gag-pol}$ in LA83 reside in a polypeptide sequence involved in the cleavage of fused gag-pol protein but not in gag protein which may be cleaved by p15 and/or by cellular protease(s). In the case of the ts mutant LA3342 the defect in virus maturation was suggested to be due to a defective core protein (Friis et al., 1976; Hunter et al., 1976; Rohrschneider et al., 1976), indeed new intermediate cleavage products of $Pr76^{gag}$ were noted at the nonpermissive temperature (Rohrschneider et al., 1976).

The cleavage of $Pr76^{gag}$ and $gPr92^{env}$ at the nonpermissive temperature in LA83-infected cells (Figs. 3.1.25, 3.1.26 and Table II) as well as the presence of gag polyprotein cleavage products and the glycoproteins, gp85 and gp37 in the virions, suggest that the gag and env gene may not be affected in LA83. This is corroborated by the biological experiments which show that at the nonpermissive temperature LA83 can complement the gag ts mutant LA3342

and an env deletion mutant - the Brian high-titer strain of RSV [RSV(-)] (Ghosh, 1984). The possibility that post-translational modifications such as, phosphorylation may also be defective in LA83 was ruled out by the proper synthesis of viral phosphoproteins in infected cells at the nonpermissive temperature and by their presence in NI-LA83 (Figs. 3.1.9 and 3.1.20).

To understand the events involved in cleavage of $Pr180^{gag-pol}$ and maturation of virus, the determination of the intracellular localization of $Pr180^{gag-pol}$ molecules in LA83-infected cells at permissive and nonpermissive temperatures would be useful answer. The quantitative analyses of $Pr180^{gag-pol}$ molecules in the cellular fractions would probably show the importance of membrane localization of $Pr180^{gag-pol}$ molecules in the process of virus budding and maturation. It may be possible to demonstrate whether the defect in tsLA83 is due to inefficient transportation of the precursor to the plasma membrane or due to possible conformational change of the molecule.

4.2 The Mechanism of Processing of The Joint gag-pol Polyprotein Precursor

It appears that unlike the cleavage of $Pr76^{gag}$, which is processed just before or during virus budding and produce

gag intermediates and mature gag proteins in the infected cells (Figs. 3.1.12, 3.1.14, 3.1.17, 3.1.20, 3.1.24, 3.1.26), $Pr180^{gag-pol}$ is processed just after or at least during the budding of virus, since mature subunits of reverse transcriptase were not observed in infected cells, but only observed in released virus (Figs. 3.1.3, 3.1.4, 3.1.29).

It is believed that in the case of RSV, mature reverse transcriptase is generated by cleavage of $Pr180^{gag-pol}$, at least in part by the viral coded protease p15. Host protein(s) seems important in processing of the precursor $Pr76^{gag}$. One line of evidence that favors a cellular enzyme arises from a finding that avian tumor viruses, whose replication is unrestricted in avian cells, appear to be blocked prior to precursor cleavage in avian virus transformed hamster cells (Eisenman et al., 1975). The initial cleavage of p15 is thought to occur via a host-mediated process (Eisenman and Vogt, 1978). It is interesting, therefore, to find out whether the crude extracts of uninfected chick cells could cleave p15 from isolated $Pr180^{gag-pol}$ and be processed to its mature products.

Temperature shiftdown experiments showed that previously synthesized $Pr180^{gag-pol}$ can be cleaved into reverse-transcriptase in the released virions (Figs. 3.1.28 and 3.1.29). These results provide strong evidence to the

idea that in RSV system Pr180$^{gag-pol}$ is the actual precursor that is cleaved into mature reverse transcriptase. I, therefore, used Pr180$^{gag-pol}$ molecules accumulated in LA83-infected cells at the nonpermissive temperature as a substrate for <u>in vitro</u> processing. An initial attempt to cleave Pr180 with crude extracts of uninfected chick cells was unsuccessful. Thus the <u>in vitro</u> cleavage of Pr180$^{gag-pol}$ molecules was carried out with detergent-disrupted RSV as a source of p15 protease. As shown in Figure 3.1.7 and indicated in Section 3.2, the Pr180$^{gag-pol}$ molecules were shown to be cleaved with p15 and a number of putative intermediates were identified. It thus, seems that the putative host protease(s) involved in cleavage of Pr76^{gag} may not be involved in the processing of Pr180$^{gag-pol}$ molecules, and the p15 protease generated from Pr76^{gag} may be used for the processing of Pr180$^{gag-pol}$.

Immunoprecipitation experiments have revealed possible intermediates in Pr180$^{gag-pol}$ processing. These are 130,000- and 150,000-daltons intracellular proteins (Pr130$^{gag-pol}$ and Pr150$^{gag-pol}$). Pr130$^{gag-pol}$ contains antigenic determinants of reverse transcriptase and the gag protein p15 (Fig. 3.2.1). Pr150$^{gag-pol}$ contains all of the gag proteins (Figs. 3.1.17 and 3.2.2), but not the complete sequence of reverse transcriptase, in which some of the tryptic-peptides of reverse-transcriptase were missing in Pr150$^{gag-pol}$ (Fig. 3.2.2).

From the in vitro cleavage analyses (Figs. 3.2.3 and 3.2.4), it is apparent that precursor-product relationship could be demonstrated between $Pr180^{gag-pol}$ and $Pr130^{gag-pol}$, although a direct precursor-product relationship could not be demonstrated between $Pr180^{gag-pol}$ and $Pr150^{gag-pol}$. It is tempting to speculate that the cleavage event that releases the α subunit and pp32 from the β subunit may also produce $Pr150^{gag-pol}$ from $Pr180^{gag-pol}$ (Fig. 3.2.5). $Pr150^{gag-pol}$ may only serve for the production of α subunit. This alternative cleavage processing may be important in the regulation of the amounts of reverse transcriptase subunits in the virion. It is also tempting to speculate that $Pr150^{gag-pol}$ is the ultimate precursor for the α subunit and the intermediate $Pr130^{gag-pol}$ may be used for the production of β subunit. Interestingly, the amounts of $Pr150^{gag-pol}$ and $Pr130^{gag-pol}$ present in the cells were approximately equimolar (Fig. 3.2.3, lanes 3 and 10). The fate of the amino-terminal gag segment (Pr70) of $Pr180^{gag-pol}$ is unknown, however, this protein may also serve for the production of the mature gag protein p27 (Figs. 3.2.3 and 3.2.4).

Cleavage of $Pr180^{gag-pol}$ and removal of the gag portion from $Pr150^{gag-pol}$ and $Pr130^{gag-pol}$ apparently occurs within the infected cells, rather than in viral particles, since newly budded virions contain neither of these proteins (Figs. 3.1.6 and 3.1.29). In the virions,

only the mature reverse transcriptase subunits have been detected (Figs. 3.1.3, 3.1.4, 3.1.29), but they were not detected in infected cells (Figs. 3.1.12, 3.1.15, 3.1.17, 3.1.24, 3.1.26, 3.1.28).

The possible biosynthetic schemes for the generation of reverse transcriptase are shown in Figure 3.2.5. Sequence analyses of α and β subunits indicated that the two subunits have common aminoterminal sequences (Copeland et al., 1980). It is not known whether these two subunits are synthesized from common precursor molecules or if two different precursors exist for each subunits. _In vitro_ cleavage analyses showed that the α subunit can be derived from a β subunit by the p15 protein (Moelling et al., 1980). A 32,000 dalton protein pp32 (Schiff and Grandgenett, 1978) found in avian retrovirus cores has the unique carboxyterminal sequence of β subunit (Copeland et al., 1980). Thus, it was suggested that β subunit is the precursor for α subunit and pp32. The identification of $Pr150^{gag-pol}$ suggests an alternative route for the synthesis of α subunit (see Figure 3.2.5). It is not known how the synthesis of the respective subunits is controlled to produce optimum amounts of each subunit. It is tempting to speculate that the ultimate regulatory event in synthesis of reverse transcriptase subunits is at the initial cleavage of $Pr180^{gag-pol}$. The molecules cleaved at the gag polypeptide region (route 1

in Figure 3.2.5) are destined for the synthesis of β subunits through the intermediate Pr130$^{gag-pol}$, and the molecules cleaved at the pol polypeptide region (route 2 in Figure 3.2.5) are destined for the synthesis of α subunits through the intermediate Pr150$^{gag-pol}$. Since pp32 was shown to be present in the virion, it is possible that the pp32 produced in the initial cleavage of Pr180$^{gag-pol}$ in route 2 is incorporated into virus particles, while Pr150$^{gag-pol}$ is quickly processed to α subunits which are incorporated into the virion. The initial cleavage at the pol polypeptide region may render the molecules accessible to the protease and thus allow the cleavage at the gag-pol junction to release α subunits. Intact Pr180$^{gag-pol}$ molecules may not be accessible to the protease at the gag-pol junction sequence due to conformational hindrance but after removal of the carboxy-portion of the polymerase the molecules are now open for the protease to be cleaved at the junction sequence. In route 1, the initial cleavage at the gag-polypeptide region may occur as in the Pr76^{gag} processing (Fig. 1.4). The cleavage intermediates Pr130$^{gag-pol}$ may now be processed to β subunits by removing the aminoterminal gag portion. It has been reported that the half-life of Pr130$^{gag-pol}$ is shorter than the half-life of Pr180$^{gag-pol}$ (Eisenman et al., 1980a). The results of kinetics of synthesis and turnover of these two proteins suggest that only a fraction of Pr180$^{gag-pol}$ may be rapidly converted to

$Pr130^{gag-pol}$. An alternative possibility is that $Pr130^{gag-pol}$ might be a primary translational product. If so, one would predict the existence of a spliced mRNA in analogy with the spliced mRNAs detected for the gag, env, and src gene products (Section 1). Such mRNA has not been identified yet, although its detection would be expected to be difficult because of its low amount and presumed similarity in size to 39S genomic RNA. According to the in vitro cleavage experiments (Figs. 3.2.3 and 3.2.4), one would favour the idea that $Pr130^{gag-pol}$ is the intermediate molecule in the production of β subunit of reverse transcriptase molecule. One can not rule out, however, the possibility that β subunit is released directly from $Pr180^{gag-pol}$ by cleavage at the gag-pol junction sequence.

4.3 Rous Sarcoma Virus Reverse Transcriptase Gene Contains Promoter and Ribosomal Binding Sequencs Needed For Its Expression

The results described in Section 3.3 show that a putative prokaryotic promoter sequence is present in the 5' region of the pol gene which can promote the transcription of reverse transcriptase gene in *E. coli*. The transcripts synthesized contain an internal initiation signal which the prokaryote translation machinery recognizes, and can thus synthesize a pol-related polypeptide with molecular weight of

65,000 that initiates from the methionine residue in position 295 or 297 of the reverse transcriptase molecule. These conclusions were based on the observations that polymerase-activity present in *E. coli* harboring the RSV recombinant plasmids lacking the putative promoter sequence in the 5'-region of the pol gene was significantly lower than the activity present in *E. coli* harboring wild-type RSV recombinant plasmids. The pol-related protein p65 was also synthesized in *E. coli* harboring pATV-8 and polymerase-activity was present in these cells even though the pol gene was interrupted near the 5' end. These results indicate that the synthesis of the pol-related protein containing polymerase-activity was directed by pol gene lacking 5' region upstream from the first Hind III site (permuted site in the pol gene with pBR322 sequence in pATV-8; see Figure 3.3.2). Since the p65 protein synthesized in *E. coli* harboring pATV-8 had the same antigenicity, and electrophoretic mobility to those synthesized by *E. coli* containing the plasmids pSAL-102 and pSAL-102DL^{pol}-2, it is suggested that p65 protein contains the observed polymerase activity.

The synthesis of p65 protein in *E. coli* harboring pERΔPOL strongly support the idea that the protein was initiated at the expected site in the internal methionine residues 295 or 297 of the reverse transcriptase molecules. The inducible activity present in *E. coli* containing pERΔPOL further support the idea that p65 is responsible for the

activities. Thus, the amino-terminal one third of the reverse transcriptase molecule may be dispensable for the polymerase activity.

In contrast to the polymerase activity, no significant differences in RNase H activity present in _E. coli_ harboring RSV recombinant plasmids and in _E. coli_ harboring pBR322 were observed (Table IV). The p65 protein may not have RNase H activity, and the amino-terminal one third of the reverse transcriptase molecule may contain this enzymatic activity. A polypeptide fragment with molecular-weight of 24,000 having only RNase H activity could be isolated by partial chymotryptic digestion of the α moiety (Lai and Verma, 1978). Anti-peptide sera directed against different regions of α subunit have been tested to determine the origin of the p24 protein (Grandgenett et al., 1985). The results suggested that the RNase H activity site is located in the amino-terminus of α subunit, consistent with the arrangement found in MuLV pol (Levin et al., 1984). The results described in Section 3.3 also suggest that the p24 polypeptide may be generated from the amino-terminal of the reverse transcriptase molecules. Direct evidence may be obtained by testing the enzymatic activity of the p65 protein after purification from the _E. coli_ cell lysates. The possibility that p65 is lacking RNase H activity raises a great interest for its usefulness as a tool in molecular biology.

A great number of DNA sequences of *E. coli* promoter has been accumulated (Hawley and McClure, 1983) with a recognition of homologies between promoter regions (Pribnow, 1975; Schaller et al., 1975), and a so-called consensus sequence was later formulated (Rosenberg and Court, 1979; Siebenlist et al., 1980). As discussed by McClure (1985), the surprising finding was that the consensus sequence based only on a compilation of many diverse promoters appears to be maximal in promoter strength. Analyses of many promoter mutations have showed that alterations that decrease homology to the consensus sequence were down mutations and mutations that increase homology to the consensus were up promoter mutations. It has been recognized that the sequences of bona-fide promoters in *E. coli* do not always contain striking homologies to the consensus sequence. The current consensus sequence is shown in Figure 3.3.17. In each region, there are three bases that are highly conserved: the TTG at -35 and the TA---T near -10. It has been demonstrated that the semi-synthetic promoter, in which the -35 and -10 hexamers are precisely homologous to the consensus sequence, was nearly maximal in activity (Amann et al., 1983; DeBoer et al., 1983 Rossi et al., 1983; Brosius et al., 1985). These results support the notion that the consensus promoter is likely to be nearly maximal in activity. As discussed by McClure (1985), the fact that no wild-type *E. coli* promoter has been found that completely matches the consensus promoter even in the

highly conserved hexamer regions suggests that promoter function is ordinarily optimized in vivo and not maximized. In the pol gene, the presence of conserved sequences in -10 hexamers is consistent with there being a Pribnow box (Pribnow, 1975). This highly conserved TTG at -35 is also present just upstream from the first Hind III site at the 5' region of the pol gene (Fig. 3.3.1). The -10 hexamers in the pol gene do not completely match the consensus promoter, however, the pol sequence has two of the three most highly conserved bases in the -10 region. As discussed by McClure, the proposition that "consensus-is-best" for promoter function is supported by the fact that all known E. coli promoters have at least two of the three most highly conserved bases in the -10 region. The results described in Section 3.3 suggested that the promoter like sequences are responsible for the expression of the pol gene, since the deletion of the -10 region resulted in reduction of synthesis of the pol-related proteins and the polymerase-activity was lowered to the back-ground value observed in E. coli containing pBR322.

It is generally assumed that eukaryotic genes cloned into bacterial systems can not be expressed unless they are placed downstream from a bacterial promoter and contain a bacterial ribosome binding site at the 5' end of the initiator ATG codon (Guarente et al., 1980; Villa-Komaroff et al., 1978). Results presented in this thesis, however,

demonstrate that eukaryotic gene can be expressed in *E. coli* by using sequences present within the eukaryotic gene, which can serve as bacterial promoter and ribosomal binding sites adjacent to a methionine codon. It may be possible to maximize the synthesis of p65 and the enzymatic activity, by using a stronger promoter like the semisynthetic promoter as discussed above. It may also be possible to synthesize a more stable enzyme by producing a fusion protein containing an *E. coli* gene product and a large portion of the pol gene product. It has been demonstrated that portions of the pol gene of a mammalian retrovirus can be expressed as a hybrid protein after fusion with the bacterial trp E gene (Tanese et al., 1985). The inserted pol gene sequences include the entire coding region for the mature enzyme and various amounts of additional coding sequences from the trp E gene. Many of these constructs express high levels of reverse transcriptase activity even though the NH_2 and COOH termini of the protein product only approximate the correct termini of the authentic protein. Construction of a fused gene containing a deletion at the 3' terminus resulted in an increase in the level of the reverse transcriptase activity and yielded a high level of production of a stable protein (Roth et al., 1985). It is therefore possible that the 5' region of the pol gene of RSV may also be important in production of the polymerase with high activity. Thus, it would be interesting to construct a fusion gene containing the entire coding region of the pol-

gene. This fused protein may exhibit both polymerase and RNase H activities as in the case of the murine reverse transcriptase expressed in E. coli (Tanese et al., 1985; Roth et al., 1985). It is also interesting to construct a plasmid that expresses only RNase H activity. It has been suggested that NH_2-terminus of the avian reverse transcriptase is responsible for the RNase H activity (Grandgenett et al., 1985) and a polypeptide fragment (Mr = 24,000) having only RNase H activity could be isolated by partial chymotryptic digestion of the α moiety (Lai and Verma, 1978). Furthermore, the p65 protein encoded from 3' two-third of the pol-gene apparently lacks RNase H activity. It is noteworthy that the 5' one-third of the pol gene would express a protein with a molecular weight of 27,000. Therefore, it may be possible to synthesize a fusion protein containing an E. coli gene product and the 5' one-third portion of the pol gene product with RNase H activity only.

I believe that the expression of the avian reverse-transcriptase in bacterial cells will lead to several important projects. Firstly, it may be possible to obtain large quantities of the purified enzyme that will allow extensive characterization of the enzyme. Secondly, mutations can be readily introduced into the cloned gene, as has been done in this study, and large numbers of bacterial cultures can be screened for the presence of rare variants exhibiting desirable changes in the activity. Thirdly, temperature-sensitive

mutations can also be generated, and the effects of the mutations can be analyzed after transfer of the altered DNA back into the viral genome and recovery of virus. Analysis of such mutants may result in a better understanding of the role of the activities of the enzyme in the viral life cycle and the interactions of the protein with the other viral gene products.

Biological Activity of The Viral DNA Inserts

The biological activity of the RSV DNA contained within the recombinant plasmids pSAL-102, pSAL-102DL^{pol}-2, and pSAL-102DL^{pol}-11 was tested in the following manner, Recombinant plasmid DNAs were subjected to Sal I digestion and fractionated by agarose gel electrophoresis, and the genome length (9.5 kb) viral DNAs were recovered. Genome length DNAs obtained from each plasmid were ligated with T4 DNA ligase, and the products were used to transfect secondary cultures of chick embryo fibroblast cells. Viral DNA from pSAL-102 was biologically active. Transfection of chicken cells with viral DNA resulted into transformation of the cells within 4 to 5 days. Virus harvested from the culture media contained all the gag structural proteins. Viral DNA from either pSAL-102DL^{pol}-2 or -11 was unable to induce transformation of chicken cells. The lack of transformation in chick cells is consistent with a requirement for replication in the transfection of chick cells by RSV DNA. Transfection of chicken embryo fibroblast cells by RSV DNA proceeds

by transcription of donor DNA, formation of extracellular progeny virus, and secondary virus infection of sensitive cells (Cooper and Okenquist, 1978). Cells transfected with either pSAL-102DL^{pol}-2 or -11 viral DNA and maintained in culture for 6 days did not make any detectable amount of virus particles. The defectiveness of pSAL-102DL^{pol}-2 and -11 viral DNAs is possibly due to the deletion in the pol gene.

Cells transfected with pSAL-102, pSAL-102DL^{pol}-2 or -11 viral DNA synthesized all three precursor polypeptides at 5.5 days posttransfection (Fig. 3.3.20). In the case of mutant viral DNA transfected cells, these polypeptides may be synthesized by the respective transcripts which were transcribed from donor DNAs. In pSAL-102 viral DNA transfected cells, the transcripts may be synthesized mainly from the stably integrated DNA, since the cells were completely transformed at this stage.

A gag-pol fused polyprotein with molecular weight of 175,000 ($Pr175^{gag-pol}$) was shown to be synthesized in the mutant virus DNA (pSAL-102DL^{pol}-2) transfected cells. The apparent size of the protein indicates that the deletion at the pol gene is such that the pol coding sequence was not interrupted at the deleted site, but continued to the authentic stop codon at the end of the pol gene. Some of the cells transfected with the mutant DNA and maintained in culture for 6.5 days were transformed, indicating that

these cells contain stably integrated viral DNA. It is tempting to speculate that the integration of viral DNA may have occurred as a consequence of the production of functional reverse transcriptase molecules. In spite of the deletion at the amino-terminal region of the molecules, the protein may be still functional though much less efficiently. Alternatively, the precursor protein $Pr175^{gag-pol}$ may be much more stable than $Pr180^{gag-pol}$ and only a very small fraction of the molecules may be processed to functional reverse transcriptase. As expected, the virus production from pSAL-102DL^{pol}-2 viral DNA transfected cells was not high enough to be detected. It is possible that the transformation of the cells is a result of the spread of a low level of the virus without integration. Cells infected with a mutant of spleen necrosis virus that is unable to integrate by virtue of deletion of the site for integration do in fact support the spread of low levels of the virus (Panganiban and Temin, 1983). Further analyses of clonal purified cells from the transformed cells would be helpful to obtain conclusive answers.

The inability to detect any transformation and infectious virions upon transfection by pSAL-102DL^{pol}-11 viral DNA suggest that the 3' region of the pol gene is also required for the production of functional reverse transcriptase. These studies suggest that the pp32 DNA binding protein or the pp32 moiety of the β subunit, or both, are required in the life

cycle of avian retroviruses. The endonuclease pp32 is believed to be involved in integration of the synthesized proviral DNA. pp32 was shown to bind to specific sequences on avian retrovirus LTR DNA including the circle junction in *in vitro* studies (Misra et al., 1982; Knaus et al., 1984). The circular viral molecule containing two LTRs in tandem is the presumed precursor molecule to the integrated provirus (Panganiban and Temin, 1983, 1984). The endonuclease pp32 was shown to nick supercoiled DNA containing LTR circle junction sequences (Grandgenett and Vora, 1985). Recently, virus mutants possessing deletion in the pp32 region have demonstrated that this region encodes function(s) essential for replication of the virus (Hippenmeyer and Grandgenett, 1984). Two separate point mutations generated near the amino-terminus of pp32 resulted in decreased viral replication and cell transformation due to reduced synthesis of the viral RNA from the integrated provirus (Hippenmeyer and Grandgenett, 1985). It appears that transcription is affected by aberrant integration.

SUMMARY AND CONCLUSIONS

The biogenesis of reverse transcriptase was examined by biochemical characterization of a conditional lethal mutant of Rous sarcoma virus. The experimental data that $Pr180^{gag-pol}$ accumulated at the nonpermissive temperature in LA83-infected cell and is cleaved into reverse transcriptase in released virions after shiftdown of the temperature provides strong evidence for the idea that in the RSV system $Pr180^{gag-pol}$ is the precursor to mature reverse transcriptase. Morphological examination of cells infected with LA83 at the nonpermissive temperature showed that the maturation of virus particles is blocked at the budding stage and downshift to the permissive temperature overcame the blockage and resulted in the release of viruses. Temperature shiftdown experiments also showed that previously synthesized $Pr180^{gag-pol}$ can be cleaved into reverse transcriptase in the released virions. Furthermore, the processing of $Pr180^{gag-pol}$ was independent of the processing of $Pr76^{gag}$. Taken together these data suggest that a relationship may exist between the processing of $Pr180^{gag-pol}$ and the maturation of RNA tumor virus. The fact that at the nonpermissive temperature reduced quantities of virion-like particles lacking reverse transcriptase are released suggests that

at the restrictive temperature $Pr180^{gag-pol}$ synthesized in LA83-infected cells may not be correctly organized in the budding structure for cleavage consequently affecting virus assembly and maturation. Shiftdown of temperature allows $Pr180^{gag-pol}$ molecules to rearrange correctly on the budding forms resulting in processing of the precursor into the reverse transcriptase subunits and release of virus. The inability to detect any $Pr180^{gag-pol}$ in NI-LA83 suggests that at the restrictive temperature $Pr180^{gag-pol}$ is not localized at the budding site or uncleaved $Pr180^{gag-pol}$ is not able to be incorporated into the released particles even though it is present at the budding site. The proper processing of $Pr76^{gag}$ at the nonpermissive temperature thus, would seem to rule out any defect in p15 (the viral protease that has been implicated in the processing of gag-related proteins) and the p12-p15 junction (the initial cleavage site by a host protease) in LA83. It is possible, therefore, that the defect in processing of $Pr180^{gag-pol}$ in LA83 reside in a polypeptide sequence involved in the cleavage of fused gag-pol protein but not in gag protein which may be cleaved by p15 and/or by cellular protease(s).

The mechanism of synthesis of reverse transcriptase was investigated. _In vitro_ cleavage of $Pr180^{gag-pol}$ molecules with crude extracts of uninfected chick cells was unsuccessful. Thus, the _in vitro_ cleavage of $Pr180^{gag-pol}$

molecules was carried out with detergent-disrupted RSV as a source of p15 protease. The Pr180$^{gag-pol}$ molecules were shown to be cleaved with p15 and a number of putative intermediates were identified. It thus, seems that the putative host protease(s) involved in cleavage of Pr76^{gag} may not be involved on the Pr180$^{gag-pol}$ molecules, and the p15 protease generated from Pr76^{gag} may be used for the processing of Pr180$^{gag-pol}$. Immunoprecipitation experiments have revealed possible intermediates that may be involved in synthesis of the mature reverse transcriptase complex. These are 130,000- and 150,000-daltons intracellular proteins (Pr130$^{gag-pol}$ and Pr150$^{gag-pol}$). Pr130$^{gag-pol}$ contains antigenic determinants of reverse transcriptase and the gag protein p15. Pr150$^{gag-pol}$ contains all of the gag proteins, but not the complete sequence of reverse transcriptase, since some of the tryptic-peptides of reverse transcriptase were missing in Pr150$^{gag-pol}$. From the in vitro cleavage analyses, it is apparent that precursor-product relationship could be demonstrated between Pr180$^{gag-pol}$ and Pr130$^{gag-pol}$, although a direct precursor-product relationship could not be demonstrated between Pr180$^{gag-pol}$ and Pr150$^{gag-pol}$. The cleavage event that releases the α subunit and pp32 from the β subunit may also produce Pr150$^{gag-pol}$ from Pr180$^{gag-pol}$. Pr150$^{gag-pol}$ may only serve for the production of α subunit. This alternative cleavage mechanism may be important in the regulation of the amounts of reverse transcrip-

tase subunits in the virion. The intermediate $Pr130^{gag-pol}$ may serve for the production of β subunit. The amounts of $Pr150^{gag-pol}$ and $Pr130^{gag-pol}$ present in the cells were approximately equimolar. Cleavage of $Pr180^{gag-pol}$ and removal of the gag portion from $Pr150^{gag-pol}$ and $Pr130^{gag-pol}$ may occur within the infected cells, rather than in viral particles. In the virions, only the mature reverse transcriptase subunits have been detected. Mature reverse transcriptase subunits were not detected in infected cells.

The functional aspects of reverse transcriptase was investigated by recombinant DNA methods. Expression of the reverse transcriptase gene of RSV and the functional role of the enzyme on viral replication were examined. A putative prokaryotic promoter sequence is present in the 5' region of the pol gene which can promote the transcription of reverse transcriptase gene in _E. coli_. The transcripts synthesized contain an internal initiation signal which the prokaryote translation machi[illegible]y recognizes, and can thus synthesize a reverse transcriptase-related polypeptide with molecular weight of 65,000 that initiates from the methionine residues in position 295 or 297 of the reverse transcriptase molecule. The polymerase activity present in _E. coli_ harboring the RSV recombinant plasmids lacking the putative promoter sequence in the 5' region of the pol gene was significantly lower than the activity present in _E. coli_ harboring wild-type RSV recombinant plasmids.

A recombinant plasmid containing a trp promoter and a portion of pol gene was able to express the reverse transcriptase-related protein p65 with the enzyme activity. These results suggested that the amino-terminal one third of the reverse-transcriptase molecule may be dispensable for the polymerase activity. The p65 protein may not have RNase H activity, and the amino-terminal one third of the reverse transcriptase molecule may contain this enzymatic activity. These results demonstrate that an eukaryotic gene could be expressed in E. coli by using sequences present within the eukaryotic gene, which can serve as bacterial promoter and ribosomal-binding sites adjacent to a methionine codon.

Transfection of chicken cells with wild-type viral DNA resulted in the transformation of the cells within 4 to 5 days. Virus harvested from the culture media contained all the gag structural proteins. Cells transfected with viral DNA deleted at either 5' or 3' region of the pol gene did not make any detectable amount of virus particles, however, all three precursor polypeptides were present in the cells. These polypeptides may be synthesized by the respective transcripts which were transcribed from donor DNAs. Some of the cells transfected with the 5'-deleted mutant DNA and maintained in culture for a longer period of time were transformed, indicating that these cells contain stably integrated viral DNA. The integration of viral DNA may have occurred as a consequence of the

production of functional reverse transcriptase molecules. In spite of the deletion at the amino-terminal region of the molecules, the protein may be still functional though much less efficiently. The inability to detect any transformation and infectious virions upon transfection by the 3' deleted mutant DNA suggest that the 3' region of the pol gene is also required for the production of functional reverse transcriptase. These studies suggest that the pp32 DNA binding protein, which is encoded from the 3'-end region of the pol gene, or the carboxy terminal of the β subunit, or both, are required in the life cycle of avian retroviruses.

BIBLIOGRAPHY

Aliperti, G. and Schlesinger, M.J. (1981) Virology 90, 366-369.

Amann, E., Brosius, J., and Ptashne, M. (1983) Gene 25, 167-178.

August, J.T., Bolognesi, D.P., Fleissner, E., Gilden, R.V., and Nowinski, R.S. (1974) Virology 60, 595-601.

Bailey, J.M. and Davidson, N. (1976) Anal. Biochem. 70, 75.

Baltimore, D. (1970) Nature 226, 1209-1211.

Baltimore, D. and Smoler, D. (1971) Proc. Natl. Acad. Sci. 68, 1507-1511.

Baltimore, D. and Smoler, D. (1972) J. Biol. Chem. 247, 7282.

Battula, N. and Temin, H.M. (1978) Cell 13, 387-398.

Berget, S.M., Moore, C., and Sharp, P.A. (1977) Proc. Natl. Acad. Sci. 74, 3171-3175.

Berk, A. and Sharp, P.A. (1977) Cell 12, 721-732.

Bister, K., Hayman, M.J., and Vogt, P.K. (1977) Virology 82, 431.

Blobel, G. and Dobberstein, B. (1975) J. Cell. Biol. 67, 835-851.

Bolognesi, D.P., Bauer, H., Gelderblom, H., and Hoper, G. (1972) Virology 47, 551-566.

Bolognesi, D.P., Luftig, R., and Shaper, J.H. (1973) Virology 56, 549.

Bolognesi, D.P., Montelaro, R.C., Frank, H., and Schafer, W. (1978) Science 199, 183-186.

Bosch, J.V., Schwarz, R.T., Ziemiecki, A., and Friis, R.R. (1982) Virology 119, 122-132.

Brewer, L.C. and Wells, R.D. (1974) J. Virol. 14, 1494-1502.

Brosius, J., cote, R.L., and Perlmutter, A.P. (1982) J. Biol. Chem. 257, 9205-9210.

Brosius, J., Erfle, M., and Storella, J. (1985) J. Biol. Chem. 260, 3539-3541.

Brugge, J.S., Erikson, E., Collett, M.S., and Erikson, R.L. (1978) J. Virol. 26, 773-782.

Brugge, J.S., Erikson, E., and Erikson, R.L. (1981) Cell 25, 363-372.

Brugge, J.S. and Darrow, D. (1982) Nature 295, 250-253.

Buchhagen, D.L. and Hanafusa, H. (1978) J. Virol. 25, 845-851.

Capone, J. (1983) Ph.D. Thesis, McMaster University.

Capone, J., Leblanc, P., Gerber, G.E., and Ghosh, H.P. (1983) J. Biol. Chem. 258, 1395-1398.

Champoux, J.J., Gilboa, E., and Baltimore, D. (1984) J. Virol. 49, 686.

Charnay, P., Gervais, M., Louise, A., Galibert, F., and Tiallais, P. (1980) Nature 286, 893-895.

Chow, L.T., Lewis, J.B., and Bisker, T.R. (1980) Cold Spring Harbor Symp. Quant. Biol. 44, 401-414.

Collett, M.S. and Erikson, R.L. (1978) Proc. Natl. Acad. Sci. 75, 2021.

Collett, M.S. and Faras, A.J. (1976) J. Virol. 17, 291-295.

Cooper, G.M. and Okenquist, S. (1978) J. Virol. 28, 45-52.

Copeland, T.D., Grandgenett, D.P., and Oroszlan, S. (1980) J. Virol. 36, 115-119.

Darlix, J.-L. and Spahr, P.F. (1982) J. Mol. Biol. 160, 147-161.

Davis, A.R., Nayak, D.P., Veda, M., Miti, A.L., Dowbenko, D., and Kleid, D.G. (1981) Proc. Natl. Acad. Sci. 78, 5376-5380.

Davis, N.L. and Rueckert, R.R. (1972) J. Virol. 10, 1010-1020.

DeBoer, H.A., Comstock, L.J., and vasser, M. (1983) Proc. Natl. Acad. Sci. 80, 21-25.

DeLorbe, W.J., Luciw, P.A., Goodman, H.M., Varmus, H.E., and Bishop, J.M. (1980) J. Virol. 36, 50-61.

Denhardt, D. (1966) Biochem. Biophys. Res. Commu. 23, 641-652.

Diggelmann, H. (1979) J. Virol. 30, 799-804.

Dittmar, K.J. and Moelling, K. (1978) J. Virol. 28, 106-118.

Donehower, L.A. and Varmus, H.E. (1984) Proc. Natl. Acad. Sci. 81, 6461-6465.

Dunn, J.J., Birzash-Pollert, E., and Studier, F.W. (1978) Proc. Natl. Acad. Sci. 75, 2741-2745.

Duyk, G., Leis, J., Longiaru, M., and Skalka, A.M. (1983) Proc. Natl. Acad. Sci. 80, 6745-6749.

Dworkin-Rastl, E., Swetly, P., and Dworkin, M.B. (1983) Gene 21, 237-248.

Eisenman, R.N., Vogt, V.M., and Diggelmann, H. (1975) Cold Spring Harbor Symp. Quant. Biol. 39, 1067-1075.

Eisenman, R.N. and Vogt, V.M. (1978) Biochim. Biophys. Acta 473, 187-239.

Eisenman, R.N., Mason, W.S., and Linial, M. (1980a) J. Virol. 36, 62-78.

Eisenman, R.N., Linial, M., Groucline, M., Shaich, R., Brown, S., and Neiman, P.E. (1980b) Cold Spring Harbor Symp. Quant. Biol. 44, 1235-1247.

Eisenman, R.M., Burnette, W.M., Zucco, F., Diggelmanh, H., Heater, P., Tsichlis, P., and Coffin, J. (1980c) In Biosynthesis, Modification and Processing of Cellular and Viral Polyproteins (ed. G. Koch and D. Richter) Academic Press, New York.

Emtage, J.S., Tacon, W.-C.A., Catlin, G.H., Jenkins, B., Porter, A.G., and Carey, N.H. (1980) Nature 283, 171-174.

England, J.M., Bolognisi, D.P., Dietzschol, B., and Halpern, M.S. (1977) J. Virol. 21, 810-814.

Faras, A.J., Taylor, J.M., Levinson, W.E., Goodman, H.M., and Bishop, J.M. (1973) J. Mol. Biol. 79, 163-183.

Ficht, T.A., Chang, L.-J., and Stoltzfus, C.M. (1984) Proc. Natl. Acad. Sci. 81, 362-366.

Friis, R.R., Ogura, H., Gelderblom, H., and Halpern, M.S. (1976) Virology 73, 259-272.

Fritsch, E. and Temin, H.M. (1977) J. Virol. 21, 119-130.

Garapin, A.C., McDonnell, J.P., Levinson, W., Quintrell, N., Fanshier, L., and Bishop, J.M. (1970) J. Virol. 6, 589-598.

Gebhardt, A., Bosch, J.V., Ziemiecki, A., and Friis, R.R. (1984) J. Mol. Biol. 174, 297-317.

Gerard, G.F. and Grandgenett, D.P. (1980) In Molecular Biology of RNA Tumor Viruses (ed. J.R. Stephenson), pp. 345-394, Academic Press, New York.

Ghosh, H.P. (1984) Virology 135, 480-488.

Gibson, W. and Verma, I.M. (1974) Proc. Natl. Acad. Sci. 71, 4991-4994.

Gilbert, W. (1976) In RNA Polymerase, eds. Losick, R. and Chanberlin, M. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) pp. 193-205.

Goeddel, D.V., Heyneker, H.L., Hozumi, T., Arentzen, R., Itakura, K., Yansura, D.G., Ross, M.J., Miozzari, G., Crea, R., and Seeburg, P.H. (1979a) Nature 281, 544-548.

Goeddel, D.V., Kleid, D.G., Bolivar, F., Heyneker, H.L., Yansura, D.G.,

Crea, R., Hirose, T., Kraszewski, A., Itakura, K., and Riggs, A. (1979b) Proc. Natl. Acad. Sci. 76, 106-110.

Goeddel, D.V., Sheppard, H.M., Yelverton, E., Leung, D., and crea, R. (1980a) Nucl. Acids Res. 18, 4057-4073.

Goeddel, D.V., Yelverton, E., Ullrich, A., Heyneker, H.L., Miozzari, G., Holmes, W., Seeburg, P.H., Dull, T., May, L., Stebbing, N., Crea, R., Maeda, S., McCandliss, R., Slona, A., Tabor, J.M., Cross, M., Familletti, P.C., and Pestka, S. (1980b) Nature 287, 411-416.

Goldberg, D.A. (1980) Proc. Natl. Acad. Sci. 77, 5794.

Golomb, M. and Grandgenett, D.P. (1979) J. Biol. Chem. 254, 1606-1613.

Golomb, M., Grandgenett, D.P., and Mason, W.S. (1981) J. Virol. 38, 548-555.

Graham, F.L. and Van der Eb, A.J. (1973) Virology 52, 456.

Grandgenett, D.P., Vora, A.C., and Schiff, R.D. (1978) Virology 89, 119-132.

Grandgenett, D.P., Golomb, M., and Vora, A.C. (1980) J. Virol. 33, 264-271.

Grandgenett, D.P., Knaus, R.J., and Hippenmeyer, P.J. (1983) Virology 130, 257-262.

Grandgenett, D.P. and Vora, A.C. (1985) Nuc. Acids Res. 13, 6205-6221.

Grandgenett, D.P., Quinn, T., Hippenmeyer, P.J., and Oroszlan, S, (1985) J. Biol. Chem. 260, 8243-8249.

Grunberg-Manago, M. and Gross, F. (1977) Prog. Nuc. Acid Res. Mol. Biol. 20, 209-284.

Guarente, L., Laurer, G., Roberts, T.M., and Ptashne, M. (1980) Cell 20, 543-553.

Guntaka, R.V., Richards, O.C., Shank, P.R., Jung, H.-J., Davidson, N., Fritsch, E., Bishop, J.M., and Varmus, H.E., (1976) J. Mol. Biol. 106, 337-357.

Guntaka, R.V. and Mitsialis, S.A. (1980) Gene 12, 113-121.

Hallewell, R.A. and Emtage, S. (1980) Gene 9, 27-47.

Hanafusa, H., Baltimore, D., Smoler, D., Watson, K.F., Yamiu, A., and Spiegelman, S. (1972) Science 177, 1188-1191.

Hawley, D.K. and McClure, W.R. (1983) Nuc. Acids Res. 11, 2237-2255.

Hayman, M.J. (1978) Virology 85, 241-252.

Hayman, M.J., Royer-Polora, B., and Graf, T. (1979) Virology 92, 31-45.

Heiland, I. and Gething, M.J. (1981) Nature 292, 851-852.

Highfield, P.E., Rafield, L.F., Gilmer, T.M., and Parsons, J.T. (1980) J. Virol. 36, 271-279.

Hippenmeyer, P.J. and Grandgenett, D.P. (1984) Virology 137, 358-370.

Hippenmeyer, P.J. and grandgenett, D.P. (1985) J. Biol. Chem. 260, 8250-8256.

Hizi, A., Gazit, A., Guthmann, D., and Yaniv, A. (1982) J. Virol. 41, 974-981.

Hughes, S.H., Shank, P.R., Spector, D.H., Kung, H.-J., Bishop, J.M., Varmus, H.E., Vogt, P.K., and Breitman, M.L. (1978) Cell 15, 1397-1410.

Hughes, S.H., Mutschler, A., Bishop, J.M., and Varmus, H.E. (1981) Proc. Natl. Acad. Sci. 78, 4299-4303.

Hunt, L.A. and Wright, S.E. (1981) J. Virol. 39, 646-650.

Hunter, E., Hayman, M.J., Rongey, R.W., and Vogt, P.K. (1976) Virology 69, 35-49.

Hunter, E., Hill, E., Hardwick, M., Brown, A., Schwartz, D.E., and Tizard, R. (1983a) J. Virol. 46, 920-936.

Hunter, E., Bennett, J.C., Brown, A., Pepinsky, R.B., and Vogt, V.M. (1983b) J. Virol. 45, 885-888.

Hunter, T. and Sefton, B. (1980) Proc. Natl. Acad. Sci. 77, 1311-1315.

Itakura, K., Hirose, T., Crea, R., Riggs, A.D., Heyneker, H.L., Bolivar, F., and Boyer, H.W. (1977) Science 198, 1056-1063.

Jacks, T. and Varmus, H.E. (1985) Science 230, 1237-1242.

Jamjoom, G.A., Naso, R.B., and Arlinghaus, R.B. (1977) Virology 78, 11-34.

Jay, E., Rommens, J., Pomeroy-Cloney, L., Macknight, D., Lutze-Wallace, C., Wishart, P., Harrison, D., Lui, W.-Y., Asundi, V., Dawood, M., and Jay, F. (1984) Proc. Natl. Acad. Sci. 81, 2290-2294.

Jay, F., Khoury, G., Seth, A.K., and Jay, E. (1981) Proc. Natl. Acad. Sci. 78, 5543-5548.

Kacian, D.L., Watson, K.F., Bunny, A., and spiegelman, S. (1971) Biochim. Biophys. Acta 246, 365-383.

Katz, R.A., Omer, C.A., Weis, J.H., Mitsialis, S.A., Faras, A.J., and Guntaka, R.V. (1982) J. Virol. 42, 346-351.

Kawai, S. and Koyama, T. (1984) J. Virol. 51, 147-153.

Keller, W. and Crouch, R. (1972) Proc. Natl. Acad. Sci. 69, 3360-3364.

Kessler, S.W. (1975) J. Immunol. 115, 1617-1624.

Khoury, A.T. and Hanafusa, H. (1976) J. Virol. 18, 383-400.

Kleid, D.G., Yansura, D., Small, B., Dowbenko, D., Moore, D.M., Grubman, M.J., McKercher, P.D., Morgan, D.O., Robertson, B.H., and Bachrach, H.L. (1981) Science 214, 1125-1129.

Klemenz, R. and Diggelmann, H. (1978) Virology 85, 63-74.

Knaus, R.J., Hippenmeyer, P.J., Misra, T.K., Grandgenett, D.P., Muller, V.R., and Fitch, W.M. (1984) Biochemistry 23, 350-359.

Koyama, T., Harada, F., and Kawai, S. (1984) J. Virol. 51, 154-162.

Laemmli, U.K. (1970) Nature 227, 680-685.

Lai, M.-H.T. and Verma, I.M. (1978) J. Virol. 25, 652-663.

Laskey, R.A. and Mills, A.D. (1975) Eur. J. Biochem. 56, 335-341.

Leamnson, R.N. and Halpern, M.S. (1976) J. Virol. 18, 956-968.

Lehrach, H., Diamond, D., Wozney, J.M., and Boedtker, H. (1977) Biochemistry 16, 4743.

Leis, J.P., Berkower, J., and Hurwitz, J. (1973) Proc. Natl. Acad. Sci. 70, 466-470.

Leis, J.P., McGinnis, J., and Green, R.W. (1978) Virology 84, 87-98.

Leis, J.P., Scheible, P., and Smith, R.E. (1980) J. Virol. 35, 722-731.

Leis, J.P., Duyk, G., Johnson, s., Longiaru, M., and Skalka, A. (1983) J. Virol. 45, 727-739.

Leis, J. and Jentoft, J. (1983) J. Virol. 48, 361-369.

Levin, J.D., Grimley, P.M., Ranseur, J.M., and Berezesky, I.K. (1974) J. Virol. 14, 152-161.

Levin, J.D., Hu, S.C., Rein, A., Messer, L.I., and Gerwin, B.I. (1984) J. Virol. 51, 470-478.

Levinson, A.D., Oppermann, H., Levintow, L., Varmus, H.E., and Bishop, J.M. (1978) Cell 15, 561-572.

Linial, M. and Mason, W.S. (1973) Virology 53, 258-273.

Linial, M., Medeiros, E., and Hayward, W.S. (1978) Cell 15, 1371-1381.

Lu, A.M., Soong, M.M., and Wong, P.K.Y. (1979) Virology 93, 269-274.

McClure, W.R. (1985) Ann. Rev. Biochem. 54, 171-204.

MacKay, P., Pasek, M., Magazin, M., Kovacic, R.T., Allet, B., Stahl, S., Gilbert, W., Schaller, H., Bruce, S.A., and Murray, K. (1981) Proc. Natl. Acad. Sci. 78, 4510-4514.

Mangel, W.F., Delius, H., and Duesberg, P.H. (1974) Proc. Natl. Acad. Sci. 71, 4541-4545.

Mann, R., Mulligan, R., and Baltimore, D. (1983) Cell 33, 153-159.

Mason, W.S., Yeater, C., Bosch, J.V., Wyke, J.A., and Friis, R.R. (1979) Virology 99, 226-240.

Mason, W.S., Friis, R.R., Linial, M., and Vogt, P.K. (1974) Virology 61, 559-574.

Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. 65. 499-560.

Meric, c., Darlix, J.-L., and spahr, P.-F. (1984) J. Mol. Biol. 173, 531-538.

Milstein, C., Brownlee, G.G., Harrison, T.M., and Mattews, M.B. (1972) Nature 239, 117-120.

Miozzari, G.F. and Yanofsky, C. (1978) Nature 276, 684-689.

Misra, T.K., Grandgenett, D.P., and Parson, J.T. (1982) J. Virol. 44, 330-343.

Mitsialis, S.A., Young, J.f., Palese, P., and Guntaka, R.V. (1981) Gene 16, 217-225.

Moelling, K. (1975) Cold Spring Harbor Symp. Quant. Biol. 39, 969-973.

Moelling, K. and Hayami, M. (1977) J. Virol. 22, 598-607.

Moelling, K., Scott, A., Dittmar, K.E.J., and Owada, M. (1980) J. Virol. 33, 680-688.

Montelaro, R.C., Sullivan, S.J., and Bolognesi, D.P. (1978) Virology 84, 19-31.

O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007-4021.

Omary, M.B. and Trowbridge, I.S. (1981) J. Biol. Chem. 10, 4715-4718.

Omer, C.A. and Faras, A.J. (1982) Cell 30, 797-805.

Oppermann, H., Bishop, J.M., Varmus, H.E., and Levintow, L. (1977) Cell 12, 993-1005.

Oppermann, H., Levinson, W., and Bishop, J.M. (1981) Proc. Natl. Acad. Sci. 78, 1067-1071.

Panganiban, A.T. and Temin, H.M. (1983) Nature 306, 155-160.

Panganiban, A.T. and Temin, H.M. (1984) Cell 36, 673-679.

Paterson, B.M., Marciani, D.J., and Papas, T.S. (1977) Proc. Natl. Acad. Sci. 74, 4951-4954.

Pauli, G., Rohde, W., and Harms, E. (1978) Arch. Virol. 58, 61-64.

Pawson, T., Martin, G.S., and Smith, A.E. (1976) J. Virol. 19, 950-967.

Pawson, T., Harvey, R., and Smith, A.E. (1977) Nature 268, 416-420.

Pawson, T., Mellon, P., Duesberg, P.H., and Martin, G.S. (1980) J. Virol. 33, 993-1003.

Pelham, H.R.B. (1978) Eur. J. Biochem. 85, 457-462.

Pelham, H.R.B. (1979) Virology 96, 463–477.

Pepinsky, R.B. and Vogt, V.M. (1979) J. Mol. Biol. 131, 819–837.

Pepinsky, R.B., Cappiello, D., Wilkowski, C., and Vogt, V.M. (1980) Virology 102, 205–210.

Pepinsky, R.B. and Vogt, V.M. (1983) J. Virol. 45, 648–658.

Prakash, O., Guntaka, R.V., and Sarkar, N. (1983) Gene 23, 117–130.

Pribnow, D. (1975) Proc. Natl. Acad. Sci. 72, 784–788.

Purchio, A. R., Erikson, E., and Erikson, R.L. (1977) Proc. Natl. Acad. Sci. 74, 4661–4665.

Ramsay, G. and Hayman, M.J. (1980) Virology 106, 71–81.

Rettenmier, C.W., Anderson, S.M., Riemen, M.W., and Hanafusa, H. (1979a) J. Virol. 32, 749–761.

Rettenmier, C.W., Karess, R.E., Anderson, S.M., and Hanafusa, H. (1979b) J. Virol. 32, 102–113.

Rho, H.M., Grandgenett, D.P., and Green, M. (1975) J. Biol. Chem. 250, 5278–5280.

Ro, H.-S.J. and Ghosh, H.P. (1984) Virology 135, 489–502.

Roberts, T.M., Bikel, I., Yocum, R.R., Livingston, D.M., and Ptashne, M. (1979) Proc. Natl. Acad. Sci. 76, 5596–5600.

Rohrschneider, J.M., Diggelmann, H., Ogura, H., Friis, R.R., and Bauer, H. (1976) Virology 75, 177–187.

Rose, J.K. and Schafferman, A. (1981) Proc. Natl. Acad. Sci. 78, 6670–6674.

Rosenberg. M. and Court, D. (1979) Annu. Rev. Genetics 13, 319–353.

Rossi, J.J., Soberson, S., Marumoto, Y., McMahon, J., and Itakura, K. (1983) Proc. Natl. Acad. Sci. 80, 3203–3207.

Roth, M.J., Tanese, N., and Goff, S.P. (1985) J. Biol. Chem. 260, 9326-9335.

Rothstein, S.J. and Reznikoff, W.W. (1981) Cell 23, 191-199.

Samuel, K.P., Papas, T.S., and Chirikjian, J.C. (1979) Proc. Natl. Acad. Sci. 76, 2659-2663.

Schaffhauson, B., Benjamin, T.L., Lodge, J., Kaplan, D., and Roberts, T.M. (1985) Nuc. acids Res. 13, 501-519.

Schaller, H., Gray, C., and Herrman, K. (1975) Proc. Natl. Acad. Sci. 72, 737-741.

Schekamn, R., wickner, W., Westergaard, O., Brutlag, D., Geider, K., Bertsch, L.L., and Kornberg, A. (1972) Proc. Natl. Acad. Sci. 69, 2691-2695.

Scherer, G.E.F., Walkinshaw, M.D., and Arnott, S. (1978) Nuc. Acids. Res. 5, 3759-3773.

Scherer, G.E.F., Walkinshaw, M.D., Arnott, S., and Morre, D.J. (1980) Nuc. Acids Res. 8, 3895-3907.

Schiff, R.D. and Grandgenett, D.P. (1978) J. Virol. 28, 279-291.

Schlesinger, M.J., Magee, a.I., and Schmidt, M.F.G. (1980) J. Biol. Chem. 255, 10021-10024.

Schwartz, D.E., Tizard, R., and Gilbert, W. (1983) Cell 32, 853-869.

Sefton, B.M., Hunter, T., and Beemon, K. (1979) J. Virol. 30, 311-318.

Sefton, B.M., Hunter, T., Beemon, K., and Eskhart, W. (1980) Cell 20, 807-816.

Sen, A. and Todaro, G.J. (1977) Cell 10, 91-99.

Shaikh, R., Linial, M., Brown, S., Sen, A., and Eisenman, R. (1979) Virology 92, 463-481.

Shank, P.R., Hugher, S.H., Kung, H.-J., Majors, J.E., Quintrell, N., Guntaka, R.V., Bishop, J.M., and Varmus, H.E. (1978a) Cell 15, 1383-1395.

Shank, P.R., Cohen, J.C., Varmus, H.E., Yamamoto, K.E., and Ringold, G. (1978b) Proc. Natl. Acad. Sci. 75, 2112-2116.

Shank, P.R. and Linial, M. (1980) J. Virol 36, 450-456.

Shealy, D.J. and Rueckert, R.R. (1978) J. Virol 26, 380-388.

Shimotohno, K., Mizutani, S., and Temin, H.M. (1980) Nature 285, 550-554.

Shine, J. and Dalgarno, L. (1975) Nature 254, 34-38.

Shine, J., Fettes, I., Lan, N.C.Y., Roberts, J.L., and Boxter, J.P. (1980) Nature 285, 456-461.

Shoemaker, C., Goff, S., Gilboa, E., Paskind, M., Mitra, S.W., and Baltimore, D. (1980) Proc. Natl. Acad. Sci. 77, 3932-3936.

Siebenlist, U., Simpson, R., and Gilbert, W. (1980) Cell 20, 269-281.

Simmons, G., Remaut, E., Allet, B., Devos, R., and Fiers, W. (1984) Gene 28, 55-64.

Smith, J.K., Cywinski, A., and Taylor, J.M. (1984) J. Virol 49, 200-204.

Sorge, J., Ricci, W., and Hughers, S.H. (1983) J. Virol. 48, 667-675.

Southern, E.M. (1975) J. Mol. Biol. 98, 503-517.

Stavnezer, E., Magarian, C., Brennan, L., and Alton, T. (1981) J. Virol. 39, 920-934.

Steitz, J.A. and Jakes, K. (1975) Proc. Natl. Acad. Sci. 72, 4734-4738.

Steitz, J.A. (1979) In Biological Regulations and Development, ed. Goldberger, R.F. (Plenum, New York), pp. 349-399.

Stephenson, J.R. and Aaronson, S.A. (1973) Virology 54, 53-59.

Stephenson, J.R., Tronick, R.R., and Aaronson, S.A. (1975) Cell 6, 543-548.

Stohrer, R. and Hunter, E. (1979) J. Virol. 32, 412-419.

Stow, N.D. and Wilkie, N.M. (1976) J. Gen. Virol. 33, 447.

Stromberg, K., Hurley, N.E., Davis, N.L., Rueckert, R.R., and Fleissner, E. (1974) J. Virol. 13, 513-528.

Swanstrom, R., DeLorbe, W.J., Bishop, J.M., and Varmus, H.E. (1981) Proc. Natl. Acad. Sci. 78, 124-128.

Swanstrom, R., Varmus, H.E., and Bishop, J.M. (1982) J. Virol. 41, 535-541.

Szer, W., Hermoso, J.M., and Leffler, S. (1975) Proc. Natl. Acad. Sci. 72, 2325-2329.

Tal, M., Aviram, M., Kanarek, A., and Weiss, A. (1972) Biochim. Biophys. Acta 281, 381-392.

Tanese, N., Roth, M., and Goff, S.P. (1985) Proc. Natl. Acad. Sci. 82, 4944-4948.

Temin, H.M. and Mizutani, S. (1970) Nature 226, 1211-1213.

Tessier, L.-H., Sondermeyer, P., Faure, T., Dreyer, D., Benavente, A., Villeval, D., Courtney, M., and Lecocq, J.-P. (1984) Nuc. Acids Res. 12, 7663-7675.

Tonneguzzo, F. (1977) Ph. D. Thesis, McMaster University.

Tonneguzzo, F. and Ghosh, H.P. (1976) J. Virol. 17, 477-491.

Toyoshima, K. and Vogt, P.K. (1969a) Virology 38, 414-426.

Toyoshima, K. and Vogt, P.K. (1969b) Virology 39, 930-931.

Van de Ven, W.J.M., van Zaane, D., Onnekink, C., and Bloemers, H.P.J. (1978) J. Virol. 25, 553-561.

Varmus, H.E., Heasley, S., Linn, J., and Wheeler, K. (1976) J. Virol. 18, 574-585.

Varmus, H.E. and Levine, A.J. (1983) In Readings in Tumor Virology. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Varmus, H.E. and Shank, P.R. (1976) J. Virol. 18, 567-573.

Varmus, H.E. (1985) Nature 314, 583-584.

Verma, I.M. (1975) J. Virol. 15, 121-126.

Verma, I.M. (1977) Biochim. Biophys. Acta 473, 1-38.

Villa-Komaroff, L., Efstratiadis, A., Broone, S., Lomedico, P., Tizard, R., Naker, S.P., Chick, W.L., and Gilbert, W. (1978) Proc. Natl. Acad. Sci. 75, 3727-3731.

Vogt, P.K. (1967) J. Virol. 1, 729-737.

Vogt, V.M., Eisenman, R., and Diggelmann, H. (1975) J. Mol. Biol. 96, 471-493.

Vogt, V.M., Wright, W., and Eisenman, R. (1979) Virology 98, 154-167.

Von der Helm, K. (1977) Proc. Natl. Acad. Sci. 74, 911-915.

Von der Helm, K. and Duesberg, P.H. (1975) Proc. Natl. Acad. Sci. 72, 614-618.

Watanabe, S. and Temin, H.M. (1982) Proc. Natl. Acad. Sci. 79, 5986-5990.

Watson, K.F., Schendel, P.L., Rosok, M.J., and Ramsey, L.R. (1979) Biochemistry 18, 3210-3219.

Weaver, R.F. and Weissmann (1979) Nuc. Acids Res. 7, 1175-1193.

Weiss, R., Teich, N., Varmus, H.E., and Coffin, J., eds. (1982) Molecular Biology of Tumor Viruses, 2nd edition, RNA Tumor Viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Witte, O.N. and Baltimore, D. (1978) J. Virol. 26, 750-761.

Wong, P.K.Y., Russ, L.J., and McCarter, J.A. (1973) Virology 51, 424-431.

Wyke, J.A. (1973) Virology 52, 587-590.

Yeger, H., Kalnins, V.I., and Stephenson, J.R. (1978) Virology 89, 34-44.

Yoshinaka, Y. and Luftig, R.B. (1977a) Proc. Natl. Acad. Sci. 74, 3446-3450.

Yoshinaka, Y. and Luftig, R.B. (1979b) Cell 12, 709-719.