CHARACTERIZATION OF PICHINDE ARENAVIRUS INFECTION AND

REPLICATION IN CELL CULTURES

By

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ABSTRACT

In order to establish a model of arenavirus infection of monocytes, human peripheral blood monocytes (PBM), human promyelocytic HL60 cells and human THP-1 promonocytic cells were infected with Pichinde virus (PV). PV replication was analysed using a variety of assays which detected viral antigen, RNA and progeny virus. While human PBM were susceptible to PV infection and replication, HL60 cells did not support PV replication, even when cells were induced to differentiate to monocytes with the phorbol ester, PMA. THP-1 cells supported high levels of PV replication only when cells were exposed to PMA. THP-1 cells not treated with PMA supported lower levels of PV replication. Infection of PMA treated THP-1 cells by PV was dependent on protein kinase C (PKC) activation and host cell transcription.

The restriction of PV replication in untreated THP-1 cells was characterized further. Experiments with lysosomotropic compounds demonstrated that equal amounts of PV were bound and internalized by both THP-1 cells and PMA treated THP-1 cells. These studies also indicated that PV enters THP-1 cells by endocytosis into acidic vesicles. The expression of PV specific RNAs in PMA treated and untreated THP-1 cells was also examined. PV S RNA genomes, antigenomes, GPC mRNA, NP mRNA and L RNAs were expressed at higher levels in PMA treated THP-1 cells versus untreated THP-1 cells. Degradation of input viral S RNA could not account for the reduction of PV RNA replication in the untreated THP-1 cells. Increasing the multiplicity of

infection of untreated THP-1 cells with PV was only able to partially overcome

the restriction of virus multiplication. This suggested that the restriction of PV

replication in THP-1 cells occured later than the initial binding and penetration

stages but at, or just prior to, primary transcription of viral mRNAs. These

studies supported a role for host cell factors and a dependence on the

activation or differentiation state of the THP-1 cell in order to support PV

replication.

In order to gain further insight into the mechanisms utilized by PV to

initiate transcription and replication, the 5′ termini of PV S RNA genomes,

antigenomes, GPC mRNA and NP mRNA were characterized. All termini

sequenced had at least one extra nontemplated base. In clones that contained

a single extra nucleotide, this was invariably a G nucleotide. Clones containing

single nontemplated G nucleotides were only derived from PV infected total

cellular RNA. The 5′ termini of NP and GPC mRNAs had on average 4-8

nontemplated bases. In addition, on genomic sense RNAs the base at -1 was

always a G nucleotide, while on antigenomic sense RNAs the base at -1 did not

appear to be conserved. These data have important implications with respect

to the mechanisms of PV transcription and replication initiation and are

discussed in the context of two possible models.

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LIST OF ABBREVIATIONS

PV	Pichinde virus
TAC	Tacaribe virus
LCMV	Lymphocytic Choriomeningitis virus
untreated THP-1 cells	THP-1 cells not treated with PMA
PMA	phorbol myristate acetate
H7	1-(5-Isoquinolinylsulfonyl)-2methylpiperazine Dihydrochloride
Iso-H7	1-(5-Isoquinolinylsulfonyl)-3methylpiperazine Dihydrochloride
PKC	Protein kinase C
MOI	multiplicity of infection
PFU	plaque forming unit
diC8	sn-1,2-diacylglycerol
^{7}MeG	7-methylguanosine
vRNA	viral RNA
vcRNA	viral complementary RNA
RNP	ribonucleoprotein
S RNA	small RNA
L RNA	large RNA
PBM	peripheral blood monocyte
Ci	Curie

dpm	disintegrations per minute
cpm	counts per minute
GPC	glycoprotein precursor
GP1, GP2	glycoproteins 1 and 2
NP	nucleoprotein
L	large protein
Z	zinc finger protein
BHK	baby hamster kidney
Act D	actinomycin D or dactinomycin
RNAse	ribonuclease
RNAs	ribonucleic acids
cDNA	complementary deoxyribonucleic acid
LUS	larger than unit size
rRNA	ribosomal ribonucleic acid
mRNA	messenger ribonucleic acid
CsCl	cesium chloride
MW	molecular weight
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
m	milli, 10^{-3}
μ	micro, 10^{-6}
n	nano, 10^{-9}

l	liter
g	gram(s)
M	molar
°C	degrees celsius
V	volts
rpm	revolutions per minute
(w/v)	weight per volume
(v/v)	volume per volume
U	units
^{35}S	sulfur-35
^{32}P	phosphorus-32
Tris	tris(hydroxymethyl)aminoethane

The laboratory of Dr. D.G. Harnish employs the arenavirus Pichinde (Trapido and Sanmartin, 1971) as a model system to study arenavirus replication and persistence.

The data in this thesis are presented in three chapters. Each chapter represents a manuscript version of a scientific paper that has either been published or has been submitted to a peer reviewed virology journal. The thesis candidate substantially wrote all three papers and performed all the experiments as described. In Chapter 2, Dr. W.E. Rawls was included as an author due to his invaluable discussions during the course of the study. In Chapter 3, Mr. Shilun Zheng was included as an author since he cloned the GPC and L RNAs of PV which were used as hybridization probes. The chapters are preceded by an introductory chapter which details various aspects of arenavirus biology that pertain directly to the studies described in this thesis. The chapters are followed by a chapter which discusess the concepts and issues raised in the papers. This format has been approved by the supervisory committee for Stephen J. Polyak and by the School of Graduate Studies at McMaster University. The experiments described in this thesis were performed to examine two aspects of arenavirus biology. The first investigations concerned the establishment and characterization of a model system to study PV infection of the macrophage. As will be discussed, arenaviruses display a monocytic tropism that may be relevant to arenavirus induced illness. Two chapters of

this thesis deal with this topic. Chapter 2 is a reprint of a publication in the *Journal of Virology* entitled *"Characterization of Arenavirus Pichinde Infection of Cells of the Monocytic Lineage", by S.J. Polyak, W.E. Rawls, and D.G. Harnish*. Chapter 3 entitled *"Analysis of Arenavirus Pichinde Transcription and Replication in Human THP-1 Monocytic Cells", by S.J. Polyak, S. Zheng, and D.G. Harnish,* is a copy of a manuscript that extends the findings presented in Chapter 2. This manuscript has been submitted to *Virology.* Chapter 4 entitled *" The 5' termini of Pichinde Arenavirus S RNAs contain nontemplated nucleotides", by S.J. Polyak and D.G. Harnish,* represents investigations into initiation of PV transcription and replication. This chapter has been submitted to *Virology.* The latter investigation was prompted by a requirement to understand the mechanisms of PV replication with particular reference to the initiation of transcription of viral mRNAs and replication of viral genomic and antigenomic RNAs. The figures in Chapters 3 and 4 are presented as laser photocopies of the original figures. This format has been approved by the School of Graduate Studies at McMaster University.

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CHAPTER 1

1. INTRODUCTION

Arenaviruses are a family of bisegmented, enveloped, negative strand RNA viruses that replicate in the cytoplasm of infected cells. When viewed by electron microscopy, virions appear as pleiomorphic particles 50-300 nm in diameter and have a sandy appearance due to the presence of host cell derived ribosomes (Bishop, 1990). The mean diameter of the infectious unit is approximately 110-130 nm (Bishop, 1990). In addition, the surface of virions contains spikes which are formed by the viral glycoproteins (Bishop, 1990).

Rodents are the natural host for several arenaviruses although in some cases arenaviruses can be transmitted to humans. In these instances, infection of humans can be quite serious and sometimes life threatening. Arenaviruses are the causative agents of Argentine Hemorrhagic Fever (Junin virus; Parodi et al., 1958), Bolivian Hemorrhagic Fever (Machupo virus; Johnson et al., 1966), Venezuelan Hemorrhagic Fever (Guanarito virus; Solas et al., 1991), Lassa Fever (Lassa virus; Buckley and Casals 1970; Frame et al., 1970) and aseptic meningitis (lymphocytic choriomeningitis virus [LCMV]; Armstrong and Lillie, 1934).

Arenaviruses have been useful models to gain insight into the mechanisms involved in virus replication, establishment of persistence and antiviral immunology (Buchmeier et al., 1980). LCMV was used in classical studies to demonstrate the immunological phenomenon known as Major

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Histocompatibility Complex (MHC) Restriction (Zinkernagel and Doherty, 1974).
Thus, the study of arenaviruses has implications ranging from elucidating
mechansims of control of replication to mechanisms of arenavirus induced
illness.

This introduction will review various aspects of arenavirus biology that
pertain to the data presented in the chapters that follow. Specifically, the
introduction will review the current state of the literature on the structure and
function of arenavirus RNAs and proteins. A review of the arenavirus life cycle
including binding and internalization of virus to target cells, transcription,
replication, packaging and release of progeny virions will also be presented.
The introduction will deal with interactions between host cells and arenaviruses
as well as arenavirus cellular tropisms.

1.1 ARENAVIRUS RNAs

The genetic information of arenaviruses is contained in two single
stranded RNA segments termed large (L), and small (S). Analyses of the S
RNAs of several arenaviruses have indicated that S RNA contains two genes
which encode the nucleoprotein (NP) and glycoprotein precursor (GPC) (Auperin
et al., 1982, 1984a, 1984b, 1986; Harnish et al., 1981, 1983; Young et al.,
1981; Dimock et al., 1982; Romanowski et al., 1985; Franze-Fernandez et al.,
1987; Southern et al., 1987; Fuller-Pace and Southern, 1988; Salvato et al.,
1988; Auperin and McCormick, 1989; Clegg et al., 1990; Ghiringhelli et al.,
1991; Wilson and Clegg, 1991). The L RNA also possesses two genes. One

gene encodes a large 200 kD protein (L protein) which is believed to contain the viral RNA dependent RNA polymerase activity (Carter et al., 1974; Leung et al., 1979; Harnish et al., 1981; Singh et al., 1987; Iapalucci et al., 1989a; Salvato et al., 1989) and the other is a smaller 10-14 kD protein, termed Z (Iapalucci et al., 1989b; Salvato and Shimomaye, 1989; Salvato et al., 1992). The genes on arenavirus RNAs are encoded in an ambisense coding strategy. In this strategy, both genomic and antigenomic RNAs encode mRNAs but only from their respective 3′ termini. S and L genomes encode NP and L mRNAs which are of antigenomic sense, while S and L antigenomes encode GPC and Z mRNAs which are of genomic sense.

1.1.1 Genomic RNAs

The sizes of several arenavirus S RNAs have been determined. In general, arenavirus S RNA molecules are approximately 3.4 kb in length, while the L RNA molecules are approximately 7 kb in length. The open reading frames for NP and GPC on S RNA and the open reading frames for L and Z on L RNA are separated by an intergenic region. The exact lengths of these RNAs and the open reading frames are shown in Table 1.

Table 1: The Sizes of Arenavirus Genomic RNAs
and Open Reading Frames

Note: The lengths of S and L genomic RNAs are given in nucleotide bases. The boundaries of the open reading frames (orf) for GPC, NP, Z, and L are presented based on the nucleotide sequences of the respective genomic RNA molecules.

References:

1. Auperin et al., 1984b; 2. Franze-Fernandez et al., 1987; 3. Iapalucci et al., 1989a; 4. Iapalucci et al., 1989b; 5. Ghiringhelli et al., 1991; 6. Clegg et al., 1990; 7. Auperin and McCormick, 1989; 8. Wilson and Clegg, 1991; 9. Salvato et al., 1988; 10. Salvato et al., 1989; 11. Salvato and Shimomaye, 1989; 12. Romanowski et al., 1985.

TABLE 1: Sizes of Arenavirus Genomic RNAs and ORFs

Virus	S RNA	GPC orf	NP orf	L RNA	Z orf	L orf	Reference
Pichinde	3419	52-1560	3335-1653				1
Tacaribe	3421	52-1539	3356-1645				2,3,4
Junin	3400	88-1533	3318-1623	7102	31-316	7071-439	5
Lassa-Nigeria strain	3417	71-1542	3314-1814				6
-Josiah strain	3402	55-1528	3301-1592				7
Mopeia	3419	47-1516	3352-1777				8
LCMV-Arm 53b strain	3376	77-1573	3314-1640	7200	89-360	7167-555	9,10,11
-WE strain	3375	78-1574	3313-1641				12

Arenavirus genomic S and L RNAs possess other structural features. The 5' and 3' termini of all arenavirus S and L RNAs sequenced to date are complementary over a stretch of 19 nucleotides (Auperin et al., 1982). Figure 1 depicts the terminal complementarity. This complementarity may permit the RNAs to form panhandled, circular structures which have been observed by electron microscopy (Palmer et al., 1977; Vezza et al., 1977; Gard et al., 1977; Young and Howard, 1983). It has been suggested that the panhandle represents the promoter region for binding of arenavirus polymerase or recognition signal for association of NP onto RNA to form ribonucleoprotein (RNP) nucleocapsid complexes (Bishop, 1990). PV S genomic RNAs are neither methylated nor capped at their 5' termini, do not contain poly A tracts at the 3' termini, and are not translatable in a cell-free in vitro translation system (Auperin et al., 1982; 1984a; 1984b; Carter et al., 1973; Clegg and Oram, 1985; Franze-Fernandez et al., 1987; Fuller-Pace and Southern, 1988; Leung et al., 1977; 1979; 1981; Lukashevich et al., 1986; Riviere et al., 1985; Southern et al., 1987; Vezza et al., 1977; 1978; 1980). In addition, L genomic RNA does not appear to be polyadenylated (Singh et al., 1987).

Notes for Figure 1: Nucleotides which would not be expected to form base pairs are in bold-face type, while those nucleotides which are different between the S and L RNA 5' terminal sequences of one virus family are underlined. Gaps have been introduced in some cases to optimize the terminal complementarity. G-U base pairing is also permitted.

References:

1. Auperin et al., 1982; 2. Auperin et al., 1984b; 3. Franze-Fernandez et al., 1987; 4. Iapalucci et al., 1989a; 5. Iapalucci et al., 1989b; 6. Ghiringhelli et al., 1991; 7. Clegg et al., 1990; 8. Auperin and McCormick, 1989; 9. Wilson and Clegg, 1991; 10. Salvato et al., 1988; 11. Romanowski et al., 1985; 12. Salvato et al., 1989; 13. Salvato and Shimomaye, 1989.

Figure 1: Terminal Complementarity of Arenavirus Genomic S and L RNAs

Virus	5' and 3' Terminal Sequence	Reference
Pichinde S RNA (and PV Munchique)	5' CGCACCGGGAUCCUAGGC 3' GCGUGUCACUAGGAUCCG	1,2
Pichinde L RNA (and PV Munchique)	3' GUGUG<u>GC</u><u>U</u>CUAGGAUCCG	
Tacaribe S RNA	5' CGCACCGGGGAUCCUAGGC 3' GCGUGUCACCUAGGAUCCG	3
Tacaribe L RNA	5' CGCACCGGGGAUCCUAGGC 3' GCGUG<u>GC</u><u>U</u>CCUAGGAUCCG	4,5
Junin S RNA	5' UGCAGUAAGGGGAUCCUAGGC 3' GCGU-GU-CACCUAGGAUCCG	6
Lassa S RNA -Nigeria strain	5' -GCACCGGGGAUCCUAGGC 3' GCGUGUCACCUAGGAUCCG	7
-Josiah strain	5' CGCACCGGGGAUCCUAGGC 3' GCGUGUCACCUAGGAUCCG	8
Mopeia S RNA	5' GUGGA-----UCCUAGGC 3' CGUGUCACCUAGGAUCCG	9
LCMV S RNA -Arm 53b strain	5' CGCACCGGGGAUCCUAGGC 3' GCGUGUCACCUAGGAUCCG	10,11
-WE strain	5' CGCACCGGGGAUCCUAGGC 3' GCGUGUCACCUAGGAUCCG	
LCMV L RNA -Arm 53b strain	5' CGCACCGGGGAUCCUAGGC 3' GCGUGGCUCCUAGGAUCCG	12,13

1.1.2 Viral mRNAs

Arenavirus mRNAs possess several structural features. PV, TAC and LCMV S RNA derived mRNAs do not contain polyadenylated sequences at their 3' termini (Auperin et al., 1984b; Iapalucci et al., 1991; Meyer and Southern, 1993). In addition, the open reading frames for the mRNAs encoded on arenavirus S and L RNAs are separated by a stretch of nucleotides that have the capacity to form hairpin structures. Of the arenavirus RNAs sequenced to date, most of the intergenic regions contain a single hairpin, while the S RNAs of Tacaribe, Mopeia, and Junin arenaviruses are capable of forming two hairpin structures (Franze-Fernandez et al., 1993; Wilson and Clegg, 1991; Ghiringhelli et al., 1991). The intergenic regions of several arenavirus S and L RNAs are depicted in Figure 2 as hypothetical stem-loop structures. This intergenic region has been shown to be the region where transcription of Tacaribe and LCMV arenavirus mRNAs terminate (Iapalucci et al., 1991; Meyer and Southern, 1993), suggesting that the structure of the hairpin itself, rather than a specific sequence provides the signals for transcription termination.

Notes for Figure 2: Superscript and subscript numbers represent nucleotide boundaries of hairpin structures. The numbering is based on the genomic sequence in a 5' to 3' direction of the RNA in question. Nucleotides printed in a smaller font represent bases which are not involved in base-pairing. Gaps were introduced in the original publications.

References:

1. Auperin et al., 1982; 2. Auperin et al., 1984b; 3. Franze-Fernandez et al., 1993; 4. Iapalucci et al., 1989a; 5. Iapalucci et al., 1989b; 6. Ghiringhelli et al., 1991; 7. Clegg et al., 1990; 8. Auperin and McCormick, 1989; 9. Wilson and Clegg, 1991; 10. Salvato et al., 1988; 11. Romanowski et al., 1985; 12. Salvato and Shimomaye, 1989; 13. Salvato, 1993.

Figure 2: Predicted Hairpin Structures of Arenavirus

S and L RNA Intergenic Sequences

VIRUS	HAIRPIN SEQUENCE	REFERENCES
Pichinde S RNA	^{1595}GGCCUCGACGGCACUCCCC$_{AAUA}$ $_{1636}$CCGGAGCUGCAGUGAGGGG	1,2
Tacaribe S RNA	^{1536}CCACCGGCCCGGGC$_{UUU}$ $_{1566}$UGUGGCCGGGCCCG ^{1568}GCC-CCCCGAUCCGCGGC$_{GUU}$ $_{1605}$CGGAGGGGCUAAGCGCCG	3
Tacaribe L RNA	^{373}GUCGCUGACCCCCGGGGGGCCCCC$_{AA}$ $_{421}$G-CGG--UGGGGGUGCCCCCGGGGG	4,5
Junin S RNA	^{1551}CACCAGCCCGGGC$_{AUU}$ $_{1679}$GUGGUCGGGCCCG ^{1682}GCCCCCCAGUCCGCGGGC$_{CUG}$ $_{1618}$CGGGGGGUCAGGCGCCCG	6
Lassa S RNA -Nigeria strain	^{1555}GCCCCCGUGACCCACCGCCAA $_{1597}$CGGGGGCACUGGGUGGCGGUU	7
-Josiah strain	^{1545}CCCCCGUGACCCACCGCC$_{UAUU}$ $_{1584}$GGGGGCACUGGGUGGCGG	8
Mopeia S RNA	^{1530}CCCCCGAGACCCACCGCC$_{UUC}$ $_{1568}$GGGGGCUCUGGGUGGCGG ^{1593}CCUCCACGACCCACCGCC$_{AGA}$ $_{1631}$GGAGGUGCUGGGUGGCGG	9
LCMV S RNA -Arm 53b and WE strains	^{1583}GCCUCCCUGACUUCUCCACCUC$_{GAAA}$ $_{1628}$CGGAGGGACU---GAGGUGGAG	10,11
LCMV L RNA -Arm 53b strain	pseudoknot at positions 490-540	12,13

The processes involved in transcription of arenavirus mRNAs are not well characterized. Nucleotide sequence data indicate that the open reading frames start approximately 50-120 nucleotides downstream of the respective 3' termini of arenavirus RNAs (see Table 1). This indicates that arenavirus mRNAs possess 5' untranslated regions (UTRs) but it is not known where on the 3' terminus of the viral RNA template, viral transcription initiates. Raju et al. (1990) and Garcin and Kolakofsky (1990, 1992) have demonstrated the presence of extra nucleotides at the 5' termini of Tacaribe virus NP and GPC mRNAs. Immediately 5' to the extra nucleotides was a 7-methylguanosine cap structure. The authors suggested that the 5' structure on the mRNAs represented the end product of an unusual mechanism of initiation of arenavirus transcription. Recently, the 5' termini of LCMV NP and GPC mRNAs have also been shown to possess 1 to 7 nontemplated nucleotides containing a methylated cap structure (Meyer and Southern, 1993). Despite these findings, information concerning arenavirus transcription initiation is limited.

1.2 ARENAVIRUS PROTEINS

There are five known major arenavirus proteins. As discussed above, NP and GPC (cleaved to GP1 and GP2) are encoded on S RNA and L and Z proteins are encoded on L RNA. In purified virions, 70% of total viral protein is contributed by NP (Vezza et al., 1977). Glycoproteins constitute approximately 25% of total virion protein, while the contribution of L protein is approximately 5% (Vezza et al., 1977). This data has permitted an estimation of the number

of viral proteins per virion. For PV, there are approximately 1,500 molecules of NP and 400 molecules each of GP1 and GP2 per virion (Vezza et al., 1977). The same number of NP molecules per virion was calculated for LCMV (Bruns and Lehmann-Gruhe, 1983; Salvato et al., 1992). Approximately 650 molecules each of GP1 and GP2, 25 molecules of L protein, and 400 molecules of Z protein are also found per LCM virion (Salvato et al., 1992).

1.2.1 Nucleoprotein

The molecular weight of NP ranges from 60 kDa for Junin virus (Grau et al., 1981) to 72 kDa for Lassa virus (Kiley et al., 1981). PV NP is approximately 64 kD (Harnish et al., 1981). NP is the predominant protein identified in infected cells and intact virions. Although the functions of NP are not well characterized the protein is likely involved in packaging viral RNA due to its association with viral RNA (Gard et al., 1977; Palmer et al., 1977; Vezza et al., 1977; Young et al., 1983). The relative abundance of basic amino acids lysine and arginine (Ghiringhelli et al., 1991) may provide NP with its RNA binding functions (Zamore et al., 1990). In this context, a conserved arginine rich consensus motif has been detected in NP of LCMV (Lazinski et al., 1989). This motif was initially identified as a required element for bacteriophage antiterminators which are also RNA binding proteins (Lazinski et al., 1989). The putative RNA binding domain in NP of LCMV has the sequence Arg_{52}-Ile-Met-Arg-Lys-Glu-Lys-Arg-Asp-Asp-Lys_{62}. It is interesting that the antiterminators that possess this sequence display specificity toward RNA

molecules that possess hairpin structures (Lazinski et al., 1989). Thus, NP may also regulate arenavirus replication specifically during transcription antitermination at the intergenic hairpin although the specific details of these processes are lacking. Unfortunately, a search of the amino acid sequence of PV NP by the thesis candidate did not detect this sequence. However, other regions of NP containing basic amino acids may also participate in interacting with RNA. In addition, a number of smaller polypeptides related to NP have been documented in the size range 48, 38, 28, 17, 16.5 and 14 kDa (Harnish, 1982; Harnish et al., 1981). Similar NP related polypeptides have been observed in Lassa virus (Clegg and Lloyd, 1983). These proteins may represent cleavage products or premature transcription or translation termination products of NP. The function of these NP related polypeptides is not known but a NP related protein of 28kDa has been documented in nuclei of infected cells (Young et al., 1987). Based on this, it was suggested that NP or NP related proteins may also regulate cellular gene expression. At present, there are insufficient data to support this suggestion.

Interaction between NP and other viral proteins has been documented. When intact virions were treated with crosslinking agents, NP was found associated with LCMV Z protein (Salvato et al., 1992) and LCMV GP2 (Burns and Buchmeier, 1991). It was postulated that the interaction of NP with Z related to a replicative function (Salvato et al., 1992), while the association of NP with GP2 was related to a function required during virus maturation and

budding (Burns and Buchmeier, 1991).

<u>1.2.2 Glycoproteins</u>

The glycoprotein precursor, GPC, is approximately 75kDa in size. PV GPC is 79 kDa in size. This precursor protein has been documented in LCMV (Buchmeier et al., 1987), PV (Harnish et al., 1981), Lassa virus (Clegg and Lloyd, 1983) and Tacaribe virus (Giminez et al., 1983; Franze-Fernandez et al., 1987). The amino terminal half of GPC from LCMV Arm and WE strains has 6 potential N-linked glycosylation sites while PV GPC possesses 11 sites (asn-X-ser/thr) (Parekh and Buchmeier, 1987). The carboxy terminus has fewer sites; 3 for LCMV and 5 for PV. LCMV GPC is rich in mannose and glucosamine but possesses low levels of fucose and galactose (Buchmeier and Oldstone, 1979). However, GP1 contains glucosamine, fucose and galactose while GP2 contains glucosamine and galactose (Buchmeier and Oldstone, 1979). LCMV GPC is cleaved proteolytically at an Arg-Arg site (amino acids 262-263) by a host cell protease in the Golgi complex (Buchmeier et al., 1987) to yield two membrane glycoproteins, GP1 and GP2. PV GPC is cleaved at an Arg-Lys site. The sizes of these proteins range from 44-60 kDa for GP1 and 34-40 for GP2. As GP1 is derived from the amino half of GPC and GP2 from the carboxy half, GP1 is more heavily glycosylated than GP2 (Parekh and Buchmeier, 1987). It has also been shown that the glycosylation of GPC is necessary for intracellular transport and cleavage of the protein (Wright et al., 1990). Further, inhibition of glycosylation also profoundly reduces the amount

of progeny virus that buds from the infected cell (Wright et al., 1990). In addition, the amino acid sequence of GP1 diverges more than GP2, which appears to be highly conserved between different members of the arenaviridae (Parekh and Buchmeier, 1987). GP1 has been shown to be associated with the exterior of the virion envelope, while GP2 is a transmembrane protein (Burns and Buchmeier, 1991). GP1 and GP2 combine in the form of native tetramers and are thought to compose the glycoprotein spike observed on arenavirus virions by electron microscopy (Burns and Buchmeier, 1991). This spike is believed to interact directly with the receptor for arenaviruses present on the surface of cells and allow binding of the virion to the cell. Interestingly, there appears to be no disulfide or covalent linkages between GP1 and GP2 (Burns and Buchmeier, 1991). However, when proteins in virions are crosslinked with a membrane permeable crosslinker, an association of GP2 with NP is found (Burns and Buchmeier, 1991). As discussed above, the interaction of GP2 with NP may be critical in the process of virus assembly and budding.

1.2.3 L Protein

Arenavirus L protein is approximately 200kDa in size and is not glycosylated. Because of the size of its cDNA which hampers in vitro expression studies, low level of expression in infected cells (Harnish et al., 1981) and low level in virions (Harnish et al., 1981; 1983), it has been extremely difficult to determine the functions of this protein. Most of the activities ascribed to L protein are based on inferences from RNA dependent

Compans, 1985; Fuller-Pace and Southern, 1989; Garcin and Kolakofsky, 1992). Only one study clearly demonstrated the production of full length genomic S and L RNAs as well as viral mRNAs in extracts of cells infected with LCMV (Fuller-Pace and Southern, 1989). A correlation between accumulation of L protein at 48-72 hours postinfection and decreased viral replication and transcription was also reported. This suggested that transition from acute to persistent infection may be regulated in part by the amount of L protein. Alternatively, an increase in the amount of L protein towards the end of the replication cycle may serve as a signal to shift from replication to virus assembly and release.

A recent study has investigated the processes involved in Tacaribe virus replication. The generation of full length TAC S molecules was not observed using purified S nucleocapsids although examination of the 5′ termini of the RNAs produced revealed the presence of genomic termini containing a single nontemplated G nucleotide (Garcin and Kolakofsky, 1992). This suggested to the authors that replication rather than transcription activity was being assessed. As will be described, the presence of an extra G nucleotide at the 5′ termini of arenavirus S RNA genomes and antigenomes is thought to reflect a novel mechanism of genome and antigenome replication initiation as proposed by Garcin and Kolakofsky (1990).

Tacaribe virus protein synthesis in a coupled transcription and translation system has also been characterized (Boersma and Compans, 1985). It was

reported that the purified virions had extremely low transcriptase activities in the cell free system. One explanation of incomplete or inefficient replication in vitro may be due to loss or lack of some polymerase components in these systems. This suggests, as alluded to above, that a functional arenavirus polymerase may consist of a multisubunit complex containing, in addition to the L protein, other virus proteins or host cell derived proteins. At present, however, this is only speculative.

As stated above, the main drawback of these studies is the use of purified arenavirus nucleocapsid preparations. By default, any replicative functions observed in the in vitro systems have been ascribed to the L protein. Thus, an understanding of the functions of the L protein and putative arenavirus polymerase complex awaits the generation of an in vitro replication system using recombinant L protein.

1.2.4 Z Protein

The Z protein of arenaviruses is approximately 10-14 kDa in size and is associated with viral nucleocapsids (Salvato et al., 1992). The protein has been demonstrated in LCMV and TAC. Z has amino acid sequences that resemble zinc finger proteins and are capable of binding zinc (Salvato and Shimomaye, 1989). As a result, Z is believed to possess transcription factor-like activities. By extension, Z might be associated with L protein in a polymerase complex. In this regard, immunodepletion of TAC Z protein in in vitro transcription and replication systems inhibits these activities (Garcin et al.,

1993), and LCMV Z protein is associated with nucleocapsids (Salvato et al., 1992).

1.3 ARENAVIRUS LIFE CYCLE

1.3.1 Binding and Internalization

Arenavirus binding to target cells is thought to involve the glycoprotein spike which is composed of tetramers of GP1 and GP2 (Burns and Buchmeier, 1991). The cell receptor for arenaviruses has not been determined. Upon binding to target cells arenaviruses are internalized through acidic endosomes. These data were derived from studies with the lysosomotropic agents ammonium chloride and chloroquine which raise the pH of endosomal vesicles and inhibit the fusion of viral membranes with endosomal membranes. As a result, viral RNP is not released into the cytoplasm. Lysosomotropic agents have been shown to inhibit the internalization of PV, Lassa, Junin and Mopeia arenaviruses (Godlewski, 1990; Glushkova et al., 1990; Castilla et al., 1991). After internalization of the arenavirus particle and fusion of the viral membrane with the endosomal membrane, viral RNA is released into the cytoplasm where RNA transcription and replication occur.

1.3.2 Ambisense Model of Arenavirus Replication

A model of arenavirus replication has been proposed based on the orientation of the open reading frames on viral RNA and on observations of the kinetics of viral RNA and protein accumulation in infected cells in the absense and presence of inhibitors of protein synthesis. The model suggests that upon

infection of cells with genomic copies of S and L RNA, the first events to occur are the transcription of NP and L mRNAs. RNA and protein analyses indicate that NP is the first molecule to be observed in arenavirus infections at 6-8 hours post infection (Bishop, 1990; Franze-Fernandez et al., 1987; Harnish et al., 1981). Transcription is mediated by the viral polymerase complex that is contained within virions. Initiation of transcription is thought to occur at the 3' termini of S and L RNA genomic templates and proceeds in a 5'-3' direction. Transcription of NP and L mRNAs then terminates at the intergenic hairpin. Transcription termination is believed to occur as a result of the secondary structure at the hairpin which does not allow the polymerase to continue transcription. NP and L mRNAs are complementary to genomic RNA and are designated antigenomic sense. These mRNAs are subsequently translated and NP and L proteins start to accumulate. At later times post infection, transcription does not terminate at the hairpin. This is termed antitermination. The reasons for antitermination are not known but by analogy with influenza virus, the accumulation of soluble NP could relax the secondary structure of the hairpin (Beaton and Krug, 1986) and allow the arenavirus polymerase to continue transcription. The net result of antitermination is the generation not of subgenomic mRNAs but full length RNAs which are complementary to S and L RNAs. These complementary RNAs are termed antigenomes and represent an intermediate in the replication of arenaviruses.

According to the ambisense model, full length antigenomic S and L RNAs

then serve as templates for the transcription of GPC and Z mRNAs, respectively. Similar to transcription of NP and L mRNAs, transcription of GPC and Z mRNAs continues to the intergenic hairpin. The GPC and Z mRNAs produced are of genomic sense. These mRNAs are then translated. By processes that are not understood, transcription antitermination occurs at later times post infection to generate full length genomic S and L RNAs. The genomic RNAs are packaged into virions which bud from the plasma membrane.

1.3.3 Packaging and Maturation of Virions

During the replication of arenaviruses, the levels of full length genomic S and L RNAs and virus proteins peak around 24-48 hours post infection in Vero and BHK cells infected at an MOI of 1. Titres of progeny virus in supernatants typically are in the order of $1\text{-}5 \times 10^8$ plaque forming units per millilitre. The slow kinetics of arenavirus replication distinguishes this virus family from other negative stranded virus families which usually reach peak levels of progeny virus production within the first 6-24 hours post infection. The events preceding release of infectious progeny virus into the supernatants are not well characterized. Assembly of virus may commence when newly transcribed genomic RNA adopts a panhandle configuration. The RNP is assembled at the plasma membrane into virions which bud from the plasma membrane. The terminal complementarity of S and L RNAs may provide encapsidation signals. As discussed, GP2 interacts with NP and may also be

involved in assembly. Structures resembling ribosomes are frequently observed in areas where arenaviruses concentrate prior to budding (Bishop, 1990). The presence of ribosomes in virions appears to be fortuitous in that the ribosomes are not required for the replication of arenaviruses. When PV was passaged for two generations in a cell line with a temperature sensitive mutation affecting ribosome function, viral replication still occurred when cells were incubated at the non permissive temperature (Leung and Rawls, 1977). In addition, virions grown in the presence of low concentrations of Actinomycin D do not contain ribosomes and are still infectious (Pedersen, 1971; Carter et al., 1973; Rawls et al., 1976). A peculiar feature of the packaging process is that it appears to be relatively non-specific. At least two copies of S and L genomic RNAs, viral mRNAs, antigenomes and host cell derived ribosomes appear to be packaged into progeny virions (Bishop, 1990). Indiscriminate packaging of arenavirus RNAs obviously complicates the ambisense model of arenavirus replication in that molecules that are usually synthesized later in infection are present at the beginning of the replication cycle. In addition, arenaviruses are diploid with respect to S RNA and L RNA (Romanowski and Bishop, 1983). This phenomenon is most likely a consequence of indiscriminate packaging . It is not known if the packaging of viral RNAs other than genomes or the presence of S and L diploid or polyploid virions has any biological significance. The net result of budding is the release of approximately 100-500 infectious progeny virions from a single cell (Mifune et al., 1971).

1.4 ARENAVIRUS EFFECTS ON HOST CELL FUNCTIONS

In vitro models of infection in tissue culture cells by arenaviruses were established many years ago. A particular feature of some arenavirus infections in vitro is the lack of demonstrable cytopathic effect (CPE), except in Vero cells. Despite the generalized lack of CPE, it has been shown that PV infection of mouse peritoneal macrophages (Friedlander et al., 1984) and Tacaribe virus infection of Vero cells (Lopez and Franze-Fernandez, 1985) inhibits host cell DNA synthesis in a dose dependent manner. LCMV infection of murine neuroblastoma cells inhibits neurotransmitter release (Oldstone et al., 1977). In addition, LCMV infection of mice inhibits growth hormone production and results in stunted growth of the infected animal (Oldstone et al., 1982). Thus, arenavirus infection usually does not kill the host cell but appears to affect the homeostatic functions of the cell. This aspect of arenavirus infections may be relevant to virus-induced disease.

1.5 HOST CELL EFFECTS ON ARENAVIRUS REPLICATION

A requirement for host cell factors for arenavirus replication has been suggested in many studies. A number of studies have examined the role of the nucleus and nucleus-derived gene products in the replication process. Initial studies suggested that the nucleus was required for arenavirus replication based on the pattern of nuclear fluorescence of LCMV-infected cells of virus-carrier mice (Mims, 1966). Similarly, PV-specific nuclear inclusions related to the 28kDa version of NP in Vero cells have also been reported (Young et al.,

1987). The function of these inclusions is not known but may relate to potential effects of NP on nuclear function. In another study, inhibition of arenavirus replication in cells enucleated with cytochalasin B was observed (Banerjee et al., 1976). Other studies have examined the effect of inhibitors of nuclear function on arenavirus replication. Inhibition of PV growth by the nuclear transcription inhibitor Actinomycin D late in the replication cycle has also been reported (Rawls et al., 1976). It was demonstrated that viral protein synthesis and antigen on the cell surface was increased, but virus yield was substantially decreased. This study suggests a host cell nucleus-derived product is involved in virus maturation and budding. Similarly, LCMV replication is inhibited in L cells (Buck and Pfau, 1969) and Machupo virus titres are reduced 2 logs or more in the presence of actinomycin D (Lukashevich et al., 1984). In contrast, Lopez et al. (1986) did not observe any effect of actinomycin D on yields of PV, TAC or Junin virus from virus-infected BHK-21 cells. This may due to the fact that treatment of cells with inhibitor was restricted to the first 6 hours of the infection. In summary, these studies suggest that host cell factors derived from the nucleus are involved in arenavirus replication.

1.6 ARENAVIRUS CELLULAR TROPISMS

Arenaviruses appear to demonstrate tropisms for particular cell types. For example, when mice are injected with LCMV, the virus appears to target the anterior lobe of the pituitary gland (Oldstone et al., 1982). It has also been

suggested that PV is tropic for cells of the reticuloendothelial system (Rawls et al., 1981). One cell that belongs to this system is the monocyte-macrophage. Indeed, reports of monocyte infections by arenaviruses, both in vivo (Ambrosio, et al., 1990; Mims and Tosolini, 1969; Murphy et al., 1977, 1976; Gonzalez et al., 1980) and in vitro (Friedlander et al., 1984; Lewis et al., 1989) have been documented. However, in many of the studies the infected cells were not unequivocally classified as monocytes. For example, only one study has formally demonstrated arenavirus infection of human monocytes. In this case, Junin virus was detected in monocytes of patients with Argentinian hemorrhagic fever (Ambrosio et al., 1990). In another human study, Junin virus replication was described in large reticular cells (Gonzalez et al., 1980). In addition, only one study has demonstrated infection of human monocytic U937 cells by PV in vitro (Lewis et al., 1989). The remaining studies examined infection of rodents with arenavirus. In several cases, the infected cells observed in in vivo infections were only characterized as large reticuloendothelial cells (Mims and Tosolini, 1969; Murphy et al., 1976, 1977). Friedlander et al, (1984) also demonstrated in vitro infection of hamster peritoneal macrophages with PV. This apparent predilection for certain cell types in vivo has been suggested to affect the host antiviral immune response and establishment of persistence but the mechanisms remain obscure.

1.7 RATIONALE AND PURPOSE OF STUDIES

As detailed above, arenaviruses may be tropic for the monocyte-macrophage. However, in several of the studies which indicate arenavirus infection of macrophages, there is some uncertainty as to whether the infected cells are indeed macrophages. Further, as mentioned in the previous section, only one study which was published when these investigations were in progress had carefully demonstrated infection of human monocytes with Junin virus (Ambrosio et al., 1990). Therefore, the purpose of part of this thesis was to develop a model of PV infection and replication in cells of the monocytic lineage and to characterize the infection in detail. These analyses would reveal potential effects of the monocyte on PV replication and vice-versa.

The second portion of this thesis investigated the mechanisms used by PV to initiate viral transcription and replication. This was accomplished by an examination of the 5' termini of PV S RNAs. These studies also pertain to the functions of the PV RNA dependent RNA polymerase and the role of the host cell in the replication of PV. A detailed understanding of the arenavirus life cycle and may eventually provide further insight into the nature of the apparent restriction of PV replication in promonocytic THP-1 cells.

CHAPTER TWO

Characterization of Pichinde Virus Infection of Cells of the Monocytic Lineage

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To establish a model of viral infection of monocytes, we examined infection of human cells and cell lines of the monocytic series with the arenavirus Pichinde virus. We demonstrate for the first time that human peripheral blood monocytes are susceptible to Pichinde virus infection, as shown by immunoprecipitation of virus-specific polypeptides from infected cells, immunofluorescence analyses, and quantitation of virus production from infected cells. The human promyelocytic leukemia cell line HL60 did not support Pichinde virus replication, even if cells were induced with the phorbol ester phorbol myristate acetate (PMA) to differentiate to monocytes. However, the human promonocytic leukemia cell line THP-1 did support Pichinde virus replication. Replication depended on exposure of the cells to PMA. We examined the nature of the effect of PMA in the induction of THP-1 cells to support Pichinde virus replication. We found that 5 min of exposure of THP-1 cells to PMA is sufficient to support virus growth and that PMA-treated THP-1 cells remain susceptible to infection up to 4 days after the initial PMA treatment. We also showed that infection of PMA-treated THP-1 cells is mediated through protein kinase C (PKC). H7, a PKC inhibitor, was able to block both PMA-induced differentiation and Pichinde virus infection of THP-1 cells. The synthetic diacylglycerol and PKC agonist, diC8, was able to stimulate THP-1 cells to support virus growth, albeit to lower levels than PMA. Dactinomycin abrogated the ability of virus to replicate and suggested a requirement for host cell transcription. The PMA effect did not appear to relate to receptor modulation. These results suggest that PMA-induced susceptibility to Pichinde virus infection occurs at a point later than the initial binding and penetration stages and that infection depends on the activation or differentiation state of the cell.

Many viruses induce persistent infections that may interfere with host immunoregulation and result in disease. Many studies have focused on the effects of viral infection on immune effector cells, monocytes and lymphocytes. For example, human immunodeficiency virus infects CD4+ T lymphocytes and monocytes. Monocytes have been suggested to act as a reservoir for human immunodeficiency virus, allowing the virus to escape immune surveillance, while CD4+ T-cell infection by human immunodeficiency virus decreases the number of these cells in peripheral blood, which may lead to immunosuppression and to the clinical manifestations of AIDS (13, 21, 24, 35).

Recent studies indicate that only cells in a particular activation or differentiation state are susceptible to infection. The virus may directly activate the cell, as in the case of cytomegalovirus, which induces a wide spectrum of biochemical changes in infected cells in vitro (1), or prior cell activation may be required, as for vesicular stomatitis virus infection of B cells activated by anti-immunoglobulin or lipopolysaccharide (36).

The arenaviruses are a family of negative-stranded RNA viruses capable of establishing persistent infections and fatal illness in humans and several rodent hosts. One member of this family, Pichinde virus, is capable of inducing persistent infections in newborn hamsters but can be fatal when adult animals are infected (reviewed in references 6 and 29).

It has been suggested that Pichinde virus is tropic for cells

of the reticuloendothelial system, perhaps the monocyte (33). Indeed, two recent studies have demonstrated arenavirus replication in monocytic cells, both in vitro (25) and in vivo (2). To establish a model of monocytic infection by Pichinde virus, we screened human peripheral blood monocytes (PBM) and two human cell lines, the promonocytic leukemia cell line THP-1 (37) and HL60 cells (9, 10), for susceptibility to infection by Pichinde virus. We demonstrate for the first time that human PBM support Pichinde virus replication, while the promyelocytic cell line HL60 does not. The promonocytic cell line THP-1 supported Pichinde virus replication only if cells were induced to differentiate to monocytes by treatment with phorbol myristate acetate (PMA).

MATERIALS AND METHODS

Cells and reagents. Human promyelocytic leukemic cells (HL60) were cultured essentially as described previously (9). Briefly, cells were grown in RPMI medium (pH 7.4) containing 10% fetal calf serum, 1% penicillin, 1% streptomycin, and 1% HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid). THP-1 cells (human promonocytic leukemic cell line) were maintained in RPMI medium containing 10% fetal calf serum, 1% penicillin, 1% streptomycin, 1% HEPES, and 1% L-glutamine. BHK-21 (clone 13) cells were grown in Dulbecco modified Eagle medium containing the above additives in addition to 1% anti-PPLO (GIBCO). African green monkey cells (Vero) were cultured as previously described (30). PBM were isolated with Ficoll-Paque (Pharmacia) from two donors. All cells were maintained at

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† Deceased 11 October 1990.

37°C with 5% CO₂. HL60 and THP-1 cells were induced to differentiate by treatment with PMA (Sigma) (16 nM) for various times, as stated in the Results. H7 (19) and dactinomycin (Sigma) were solubilized in distilled water, while diC8 (a synthetic diacylglycerol) (22) (Sigma) was reconstituted in 100% ethanol. Ammonium chloride was solubilized in distilled water and used at a concentration of 20 mM, which has been shown to be effective in preventing internalization of Pichinde virus into BHK cells (14).

Infection. Unless otherwise stated, all infections were done at a multiplicity of infection (MOI) of 1. High-titer Pichinde virus (>5 × 10⁸ PFU/ml) in 1.0 ml of medium was adsorbed to cells in six-well tissue culture plates (Corning) for 1 h at 37°C. At the end of this period, medium was added to the cells and the infection was allowed to proceed for a minimum of 24 h. Cell supernatants were assessed for Pichinde virus by a standard plaque assay (30).

Immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Radiolabeling, immunoprecipitation, and product analysis were performed essentially as described previously (16). Cells were washed twice in methionine-free medium prior to metabolic labeling for 1.5 h in methionine-free medium containing 50 μCi of [³⁵S] methionine (1,060 Ci/mmol; Dupont, NEN Research Products) per ml. Labeled cells were subsequently washed once in phosphate-buffered saline (PBS) and lysed on ice for 10 min with RIPA buffer (50 mM Tris hydrochloride [pH 7.2], 150 mM NaCl, 0.1% [wt/vol] SDS, 1.0% Na deoxycholate, 1.0% Triton X-100). Cellular and nuclear debris was removed by microcentrifugation in a Fisher centrifuge (model 235B) for 15 min. Cell lysate (1 ml) was mixed with 250 μl of protein A and 5 μl of hamster anti-Pichinde virus polyclonal antiserum or nonimmune hamster serum and allowed to mix at 4°C for 4 h. Immunoprecipitates were washed three times in cold RIPA buffer, 30 μl of SDS sample buffer was added, and tubes were heated at 100°C for 5 min. Samples (20 μl) were loaded onto continuous 8% polyacrylamide gels and electrophoresed at 50 V overnight. Gels were then fixed in water-methanol-acetic acid (50:50:7) for 1 h and impregnated with Enlightening (Dupont) for 30 min. Polypeptides were visualized after exposure to Kodak AR-50 film at −70°C for 1 to 7 days.

Immunofluorescence. Adherent cells were grown on glass coverslips and infected as described above. Nonadherent cells were collected by cytocentrifugation at various times after infection. Cells were air dried and fixed in cold acetone for 10 min. Dried, fixed slides were then rinsed in PBS containing 0.1% bovine serum albumin (BSA). Pichinde virus antiserum (1:25) was added to the slides, and they were incubated for 30 min at 37°C. The slides were washed six times in the PBS-BSA solution and covered with goat anti-hamster fluorescein isothiocyanate-conjugated antibody (1:25) for 30 min at 37°C. Slides were again washed six times and air dried, and coverslips were mounted. Fluorescence was observed with a Leitz inverted microscope.

RESULTS

To determine whether human PBM were permissive for Pichinde virus replication, we infected human PBM with increasing amounts of virus. There was a dose-dependent increase in the virus-specific proteins glycoprotein precursor (GPC) and nucleoprotein (NP) (Fig. 1). At an MOI of 10, viral protein synthesis was lower at 72 h postinfection. As previously demonstrated (15, 16), BHK cells also supported viral protein synthesis. The kinetics of viral protein synthe-
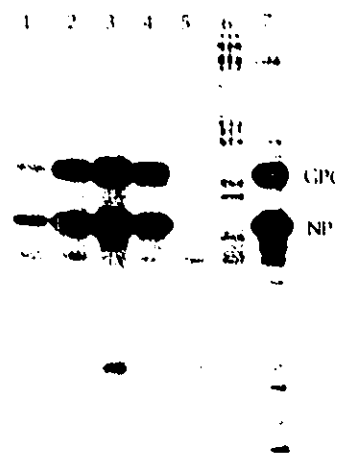
FIG. 1. Effect of MOI on Pichinde virus protein synthesis in PBM. PBM were infected at various MOIs, and proteins were labeled with L-[³⁵S]methionine at 72 h postinfection. Viral polypeptides were visualized following immunoprecipitation with hamster anti-Pichinde virus serum and SDS-PAGE as described in Materials and Methods. Lanes 1, 2, 3, and 4 represent PBM infected at MOIs of 0.01, 0.10, 1.0, and 10.0 PFU per cell, respectively. Lane 5 represents mock-infected PBM, and lane 6 represents mock-infected BHK cells. Lane 7 depicts proteins from BHK cells which were radiolabeled 24 h postinfection with an MOI of 1.0 PFU per cell and represents the positive control. GPC and NP are the previously characterized viral glycoprotein precursor and nucleoprotein, respectively (15, 16).

sis was assessed after infection of cells with an MOI of 1 and immunoprecipitation of L-[³⁵S]methionine-labeled polypeptides at 24-h intervals (Fig. 2). Synthesis was low at 24 h postinfection, and maximum synthesis of viral proteins in
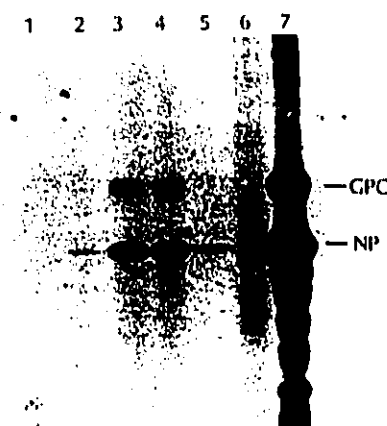
FIG. 2. Kinetics of Pichinde virus protein synthesis in PBM. PBM were infected with Pichinde virus at an MOI of 1.0 PFU per cell as described in Materials and Methods. After incubation at 37°C for various times, cells were metabolically labeled with L-[³⁵S]methionine for 1.5 h. Viral polypeptides were immunoprecipitated and visualized as described in the legend to Fig. 1. Lane 1 represents mock-infected PBM. Lanes 2, 3, 4, and 5 represent Pichinde virus-infected PBM at 24, 48, 72, and 96 h postinfection, respectively. Lane 6 represents mock-infected BHK cells. Lane 7 represents Pichinde virus-infected BHK cells 24 h postinfection with an MOI of 1.0 PFU per cell.

1 2 3 4 5 6 7 8 9 10

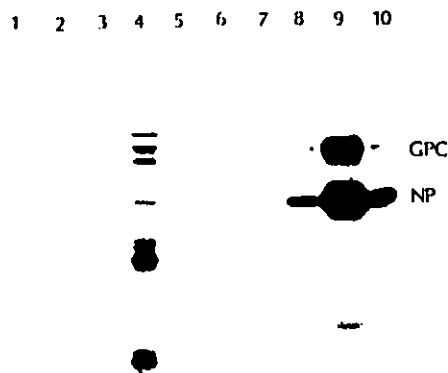FIG. 3. Infection of HL60 cells and THP-1 cells with Pichinde virus. Cells were infected with Pichinde virus at an MOI of 1.0 PFU per cell, incubated at 37°C for various times, radiolabeled with L-[³⁵S]methionine, and prepared as described in Materials and Methods. Lane 1, polypeptides from PMA-treated, mock-infected HL60 cells at 24 h. Lane 2, PMA-treated HL60 cells 24 h postinfection. Lane 3, PMA-treated HL60 cells 48 h postinfection. Lane 4, mock-infected uninduced HL60 cells at 24 h. Lane 5, polypeptides from mock-infected, PMA-treated THP-1 cells at 24 h. Lane 6, uninduced THP-1 cells 24 h postinfection. Lanes 7, 8, and 9, immunoprecipitable proteins from PMA-treated THP-1 cells at 24, 48, and 72 h postinfection, respectively. Lane 10, BHK cells at 24 h postinfection with Pichinde virus. All cultures which received PMA stimulation were treated with 16 nM PMA overnight prior to infection with Pichinde virus.

PBM occurred between 48 and 72 h postinfection. Declines in the amounts of in virus-specific polypeptides were observed by 96 h.

Since PBM cannot be maintained for long periods in vitro, we determined whether human hematopoietic cell lines were permissive to infection by Pichinde virus. As depicted in Fig. 3, HL60 cells did not support Pichinde virus protein synthesis, nor did we observe virus-specific polypeptide synthesis after monocytic differentiation of the cells induced with PMA. Infections of HL60 cells have been attempted up to an MOI of 50 PFU per cell, and these have also been unsuccessful (data not shown). THP-1 cells did support viral replication. Virus replication was observed when the cells were induced to differentiate to monocytes by treatment with PMA (Fig. 3). Viral protein synthesis in infected THP-1 cells peaked at 48 to 72 h, similar to the kinetics of infection of PBM by Pichinde virus. Infected THP-1 cells and PBM produced infectious virions. Both infected PBM and PMA-treated THP-1 cells produced maximal titers of 5.08 × 10⁵ and 2.05 × 10⁶ PFU/ml, respectively, at 72 h postinfection. PBM titers were consistently lower than those in infected THP-1 cells. Unstimulated THP-1 cells did not produce detectable levels of virions, nor did HL60 cells, regardless of the mode of stimulation. Mock-infected cells did not produce infectious virus (data not shown).

To assess the proportion of cells in culture which were virus infected, we undertook immunofluorescence analysis (Fig. 4). As expected, infected THP-1 cells treated with PMA, BHK cells, and PBM synthesized viral antigen which reacted with polyclonal anti-Pichinde virus antiserum. Un-stimulated THP-1 and HL60 cells and PMA-treated HL60 cells did not. Approximately 50, 70, and 60% of PBM, BHK cells, and PMA-stimulated THP-1 cells, respectively, were infected in most cultures during peak viral polypeptide

synthesis. In some experiments, we observed that 1 to 4% of THP-1 cells were infected without pretreatment with PMA. The data indicate that THP-1 cells become susceptible to Pichinde virus infection after PMA treatment and that HL60 cells do not support virus replication whether PMA treated or not.

The kinetics of PMA pretreatment of THP-1 cells required for permissivity to replication of Pichinde virus was examined. Figure 5 illustrates that as little as 5 min of exposure of THP-1 cells to PMA resulted in comparable NP and GPC synthesis 48 h postinfection. Immunofluorescence analysis and Pichinde virus plaque assays demonstrated that infected THP-1 cells treated for various times with PMA exhibited similar proportions of infected cells (approximately 30%) and produced infectious virus to a level of (1.31 ± 0.21) × 10⁶ PFU/ml (mean ± standard deviation) (data not shown). Virus titers were not significantly different between any of the time points of PMA treatment.

We subsequently determined the length of time after PMA treatment that THP-1 cells remained permissive to Pichinde virus infection and replication. THP-1 cells were susceptible to infection for up to 96 h after PMA exposure (Fig. 6). Restimulation with PMA prior to infection at 48 to 96 h after the initial PMA stimulus appeared to decrease the relative levels of viral polypeptides which were synthesized. The decreases were not due to decreases in cell viability (data not shown).

To examine the role of protein kinase C in viral replication, we pretreated THP-1 cells with H7 followed by PMA or with diC8 alone (Fig. 7). H7 at concentrations greater than 600 µM inhibited synthesis of NP and GPC. The inability to support viral replication paralleled the apparent lack of differentiation of the cells (data not shown). Although at higher H7 concentrations cell viability was reduced (50%), it was insufficient to account for the large drop in viral protein synthesis. Figure 7 also reveals that 20 or 40 µM of diC8 was sufficient to induce THP-1 cells to support viral replication. Note that diC8 did not induce any morphological differentiation of THP-1 cells at any concentration tested. Concentrations of diC8 exceeding 40 µM were cytotoxic. The data indicate that Pichinde virus replication in PMA-treated THP-1 cells is mediated through protein kinase C activation.

To determine whether viral replication required PMA-induced de novo transcription, we pretreated THP-1 cells with dactinomycin (Fig. 8). Dactinomycin was able to abrogate replication at concentrations of 40 ng/ml or higher. Morphological differentiation was inhibited at concentrations of 80 to 100 ng/ml.

The requirement for pretreatment of THP-1 cells with protein kinase C-stimulating agents to obtain efficient Pichinde virus replication could relate to upregulation of the receptor for Pichinde virus on THP-1 cells. As illustrated in Fig. 9, this did not appear to be the case. When virus was first preadsorbed to uninduced THP-1 cells at 4 and 37°C, before PMA stimulation, viral replication still occurred. This suggested that the receptor for Pichinde virus was present on unstimulated THP-1 cells and that PMA addition to cells did not influence this aspect of the infection process. We then determined whether virus was internalized more readily in PMA-treated THP-1 cells than in uninduced cells. To this end, we performed studies with the lysosomotropic agent ammonium chloride, which has been shown to inhibit lysosome-mediated entry of Pichinde virus into BHK cells (14). Interestingly, virus was capable of entry into uninduced THP-1 cells, indicating that Pichinde virus enters THP-1 cells through an acidic lysosome (Fig. 10). These findings
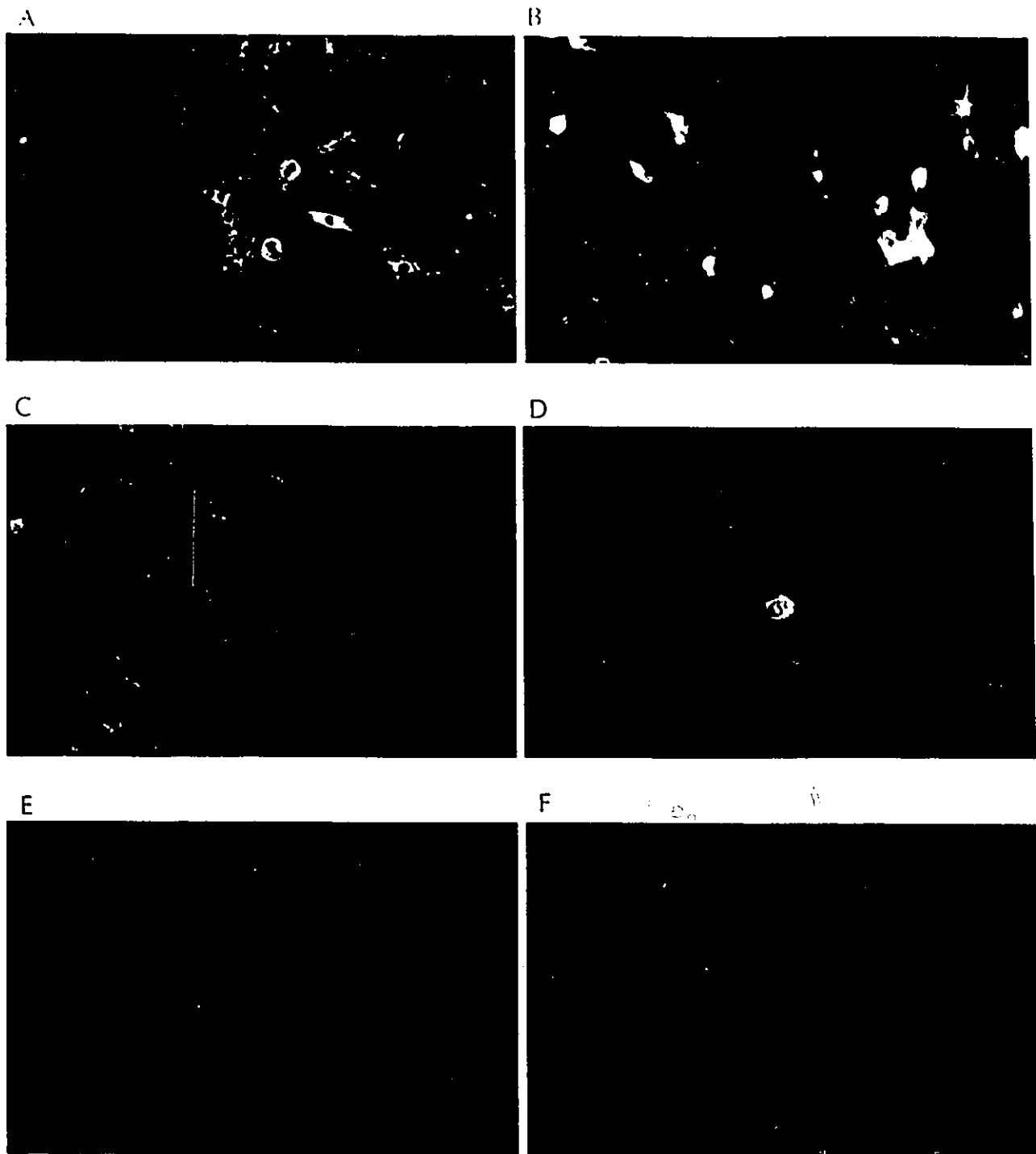
FIG. 4. Immunofluorescence analysis of infected cells. All cells were infected with Pichinde virus at an MOI of 1.0 PFU per cell, incubated for 48 h, fixed in cold acetone, air dried, and incubated with polyclonal hamster anti-Pichinde virus antiserum, which was followed by fluorescein isothiocyanate-conjugated goat anti-hamster antiserum, as described in Materials and Methods. (A) BHK cells; (B) PBM; (C) THP-1 cells treated with PMA (16 nM for 2 h); (D) uninduced THP-1 cells; (E) HL60 cells treated with PMA (16 nM for 2 h); (F) uninduced HL60 cells.
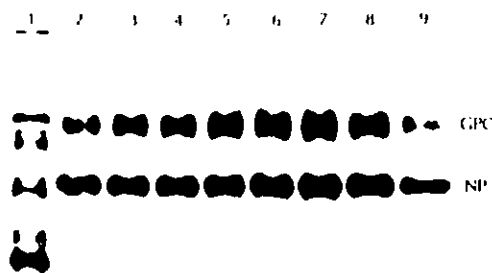
FIG. 5. Kinetics of PMA treatment of THP-1 cells required for Pichinde virus infection. Cells were pretreated with PMA (16 nM) for various times and then were washed thoroughly with fresh medium twice. Pichinde virus (MOI of 1.0 PFU per cell) was adsorbed for 1 h at 37°C, medium was added, and the cells were incubated at 37°C for 48 h. The cells were metabolically radiolabeled with L-[35S]methionine, viral proteins were immunoprecipitated with hamster anti-Pichinde virus antiserum, and polypeptides were visualized by SDS-PAGE as described in Materials and Methods. Lane 1 represents uninduced THP-1 cells 48 h postinfection with Pichinde virus. Lanes 2 to 9 depict polypeptides from Pichinde virus-infected THP-1 cells pretreated with PMA for 5, 15, and 30 min and 1, 2, 4, 8, and 24 h, respectively, at 48 h postinfection.

agree with other studies of Pichinde virus entry into BHK cells, as well as foot-and-mouth disease virus and Semliki Forest virus entry into target cells (5, 14, 18). These data suggest that PMA-induced susceptibility to viral replication

FIG. 6. PMA-treated THP-1 cells remain susceptible to infection at 96 h after PMA treatment. THP-1 cells were induced to differentiate for 2 h with 16 nM PMA, and infection was delayed for 0 to 96 h after PMA stimulation. At the indicated time points, THP-1 cells were infected with Pichinde virus at an MOI of 1.0 PFU per cell as described in the text or restimulated with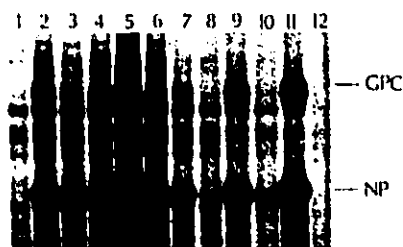 16 nM PMA for an additional 2 h prior to infection with Pichinde virus (MOI of 1.0 PFU per cell). All cultures were incubated at 37°C for an additional 48 h. Cells were subsequently labeled with L-[35S]methionine, and viral polypeptides were visualized by immunoprecipitation and SDS-PAGE analyses as described in Materials and Methods. Lanes 1 and 2 represent proteins from cells mock infected and Pichinde virus infected 2 h after PMA treatment. Twenty-four hours after the initial 2-h PMA treatment, cells were infected with Pichinde virus (lane 3) or restimulated with PMA for 2 h prior to infection with Pichinde virus (lane 4). Forty-eight hours after the initial 2-h PMA treatment, cells were infected with Pichinde virus (lane 5) or restimulated with PMA for 2 h prior to infection with Pichinde virus (lane 6). Seventy-two hours after the initial 2-h PMA treatment, cells were infected with Pichinde virus (lane 7) or restimulated with PMA for 2 h prior to infection with Pichinde virus (lane 8). Ninety-six hours after the initial 2-h PMA treatment, cells were infected with Pichinde virus (lane 9) or restimulated with PMA for 2 h prior to infection with Pichinde virus (lane 10). Lanes 11 and 12 represent Pichinde virus-infected and mock-infected BHK cells, respectively, at 24 h postinfection.

FIG. 7. Effects of H7 and diC8 on Pichinde virus infection of THP-1 cells. Cells were treated with various concentrations of H7 (lanes 1 to 6) for 30 min prior to PMA addition (16 nM) for 2 h. The cells were then washed, infected with Pichinde virus at an MOI of 1.0 PFU per cell, and incubated at 37°C for 48 h. In a separate experiment, cells were incubated with different concentrations of diC8 (lanes 7 to 13) for 30 min prior to infection with Pichinde virus at an MOI of 1.0 PFU per cell and incubation at 37°C for 48 h. All cells were radiolabeled with L-[35S]methionine, and proteins were immunoprecipitated and visualized by SDS-PAGE as described in Materials and Methods. Lanes 1 to 6, THP-1 cells treated with 0, 100, 300, 600, 800, and 1,000 μM H7, respectively, and then infected by Pichinde virus. Lanes 7 to 10, THP-1 cells treated with 20, 40, 80, and 200 μM diC8, respectively, and then infected by Pichinde virus. Lane 11, THP-1 cells treated with ethanol carrier only and then infected by Pichinde virus. Lane 12, PMA-treated (2 h, 16 nM) THP-1 cells, subsequently infected by Pichinde virus. Lane 13, THP-1 cells treated with 80 μM diC8 and then mock infected.

in THP-1 cells is regulated at events following entry into acidic lysosomes.

DISCUSSION

We analyzed infection of monocytes and monocytic cell lines with Pichinde virus. Human PBM and promonocytic THP-1 cells stimulated with PMA were both able to be infected with Pichinde virus, although THP-1 cells supported relatively higher levels of viral growth. This was demonstrated by increased amounts of virus-specific proteins, higher virus titers, and an increased proportion of infected cells in PMA-treated THP-1 cells compared with PBM. Monocyte infection and replication exhibited a dependence on MOI, and at an MOI of 1.0, intracellular viral protein synthesis was maximal between 48 and 72 h postinfection (Fig. 1 and 2). The peak was delayed approximately 24 h
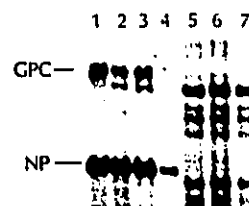
FIG. 8. Infection of PMA-treated THP-1 cells by Pichinde virus is transcriptionally dependent. Cells were treated with various concentrations of dactinomycin for 30 min at 37°C before being treated with 16 nM PMA for 2 h. Subsequently, cells were infected by Pichinde virus at an MOI of 1.0 PFU per cell. Cells were then maintained at 37°C for 48 h. Radiolabeling with L-[35S]methionine and then immunoprecipitation with hamster anti-Pichinde virus antiserum and SDS-PAGE were employed to visualize viral proteins. Lanes 1 to 6 represent polypeptides from THP-1 cells treated with 0, 2, 20, 40, 80, and 100 ng of dactinomycin per ml, respectively, prior to PMA stimulation and Pichinde virus infection. Lane 7 depicts proteins from THP-1 cells treated with 100 ng of dactinomycin per ml, stimulated with PMA, and mock infected.
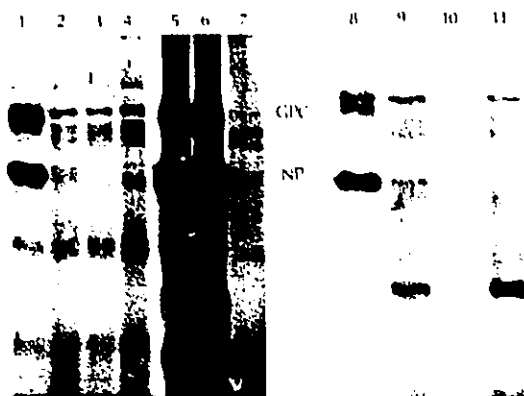
FIG. 9. Infection of PMA-treated THP-1 cells is not due to receptor upregulation. Pichinde virus at an MOI of 1.0 PFU per cell was adsorbed to THP-1 cells for 2 h at 4°C (lanes 1 to 4) or 1 h at 37°C (lanes 8 to 11). The cells were then washed to remove unadsorbed virus, PMA was added at a concentration of 16 nM, and the cells were maintained for 48 h at 37°C. Lanes 1 and 2 represent immunoprecipitable proteins from cells on which Pichinde virus was adsorbed for the indicated time and then which were treated with PMA or not treated, respectively. Lanes 3 and 4 represent proteins from mock-infected cultures treated with PMA or not treated, respectively. Lanes 5 and 6 depict proteins from BHK cells treated with PMA or not treated, respectively, prior to infection with Pichinde virus. Pichinde virus-infected BHK cells were incubated at 37°C for 24 h. Lane 7 represents mock-infected BHK cells. Lanes 8 to 11 are exactly the same conditions as lanes 1 to 4 except that the adsorption was for 1 h at 37°C. Lanes 8 and 9 represent immunoprecipitable proteins from cells on which Pichinde virus was adsorbed for the indicated time and then which were treated with PMA or not treated, respectively. Lanes 10 and 11 represent proteins from mock-infected cultures treated with PMA or not treated, respectively.

relative to that which we have observed previously in BHK-21 cells (16). The subsequent decline in viral protein synthesis by 96 h postinfection is consistent with the viral replication cycle we have previously observed in BHK-21, Vero, and MDCK cells (15, 16). We did not observe a cytopathic effect in either cell type, and the morphology of infected THP-1 cells and PBM appeared normal by indirect immunofluorescence.

Infection of THP-1 cells required treatment of cells with the phorbol ester PMA. We demonstrated that treatment of cells with 16 nM PMA for times as short as 5 min is sufficient to induce cellular changes which permit Pichinde virus replication (Fig. 5). This is consistent with previous demonstrations of PMA-mediated differentiation-inducing effects on leukemic cells with treatment times of 20 min (34), with previous reports of arenavirus replication in U937 cells treated with PMA (25), and with reports of cytomegalovirus replication in monocytic cells treated with PMA (39). The PMA-induced cellular changes were stable for at least 96 h after THP-1 cell treatment as measured by the ability of Pichinde virus to infect cells after removal of PMA (Fig. 6). In this context, we restimulated these same cells at various times after the initial stimulation and did not observe an increased ability to support viral replication. Rather, we consistently observed slight reductions in viral protein synthesis which were independent of viability as measured by trypan blue exclusion. We observed similar reductions in the ability of PBM to support viral replication after PMA treatment (Fig. 6 and unpublished observations). In some exper-
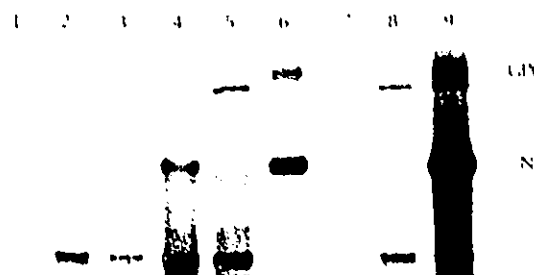

FIG. 10. Pichinde virus entry into uninduced THP-1 cells. Pichinde virus was adsorbed to THP-1 cells in the presence or absence of 20 mM ammonium chloride at 4°C for 2 h. Extra medium with or without 20 mM ammonium chloride was then added, and the cells were incubated for 48 h at 37°C. Radiolabeling with L-[³⁵S]methionine, immunoprecipitation, and SDS-PAGE analyses of viral proteins were performed as described in Materials and Methods. All cells were infected with Pichinde virus at an MOI 1.0 PFU per cell unless otherwise stated. Lanes 1 and 2 represent proteins from THP-1 cells on which virus was adsorbed at 4°C in the presence or absence of NH₄Cl, respectively. Lanes 3 and 4 depict proteins from THP-1 cells which were pretreated with 16 nM PMA for 2 h prior to virus adsorption at 4°C in the presence or absence of NH₄Cl, respectively. Lanes 5 and 6 represent proteins from THP-1 cells on which virus was adsorbed at 4°C in the presence or absence of NH₄Cl, respectively, after which both groups of cells were stimulated with 16 nM PMA for 2 h. Lane 7 depicts proteins from mock-infected THP-1 cells. Lanes 8 and 9 represent proteins from BHK cells on which Pichinde virus was adsorbed at 4°C for 2 h in the presence or absence of NH₄Cl, respectively.

iments, we observed 1 to 4% THP-1 cell infection in the absence of PMA, which coincides with the proportion of unstimulated cells that adhere in culture and appear to undergo spontaneous differentiation to monocytes. The proportion increased to approximately 30% following PMA treatment (Fig. 4). This argues that the differentiation status or activation state of the cell is critical for Pichinde virus replication.

The PMA effect seems to be mediated through protein kinase C. We were able to specifically block NP and GPC synthesis by pretreatment of THP-1 cells with a protein kinase C inhibitor, H7 (19). Moreover, treatment with a specific protein kinase C activator, diC8 (22), was sufficient to support viral protein synthesis in THP-1 cells.

The induced permissivity of THP-1 cells requires new transcription (Fig. 8). Pretreatment of cells with dactinomycin abrogated the PMA-induced effect. In an attempt to examine whether the inability of uninduced THP-1 cells to be infected was related to receptor expression which was inducible by PMA treatment, we examined the binding of radiolabeled virus to various cell types with or without PMA treatment. In preliminary experiments, we could not demonstrate any differences in L-[³⁵S]methionine-labeled virus binding to monocytes, THP-1, BHK-21, and HL60 cells whether PMA treated or not. We therefore conducted experiments in which Pichinde virus was absorbed to cells, residual virus was removed, and cells were subsequently treated with PMA. We observed that virus bound to unstimulated THP-1 cells which then supported NP and GPC synthesis when the cells were stimulated with PMA. Viral infection and replication was abrogated by ammonium chloride treatment as previously documented for arenaviruses (14, 14a). Our data suggest that PMA-induced susceptibility to viral replication in THP-1 cells is regulated at events following entry into acidic lysosomes.

The nature of the PMA-induced, transcriptionally dependent events which are required to support Pichinde virus replication in THP-1 cells is a matter of speculation. The requirement for host cell factors for viral replication has been a matter of controversy. Banerjee and coworkers (4) observed inhibition of arenavirus replication in cells enucleated with cytochalasin B, and more recently, two groups have reported dose-dependent inhibition of host cell DNA synthesis by Pichinde virus infection of mouse peritoneal macrophages (12) and Tacaribe virus infection of Vero cells (26).

Conflicting results have been reported on the effects of dactinomycin on arenavirus growth. Rawls and others (32) have reported inhibition of Pichinde virus late in the replication cycle since viral protein synthesis and antigen on the cell surface were increased, with an apparent decrease in virus yield. Lymphocytic choriomeningitis virus replication is similarly inhibited in L cells (8), and Machupo virus titers are reduced 200-fold or more in the presence of dactinomycin (28). On the other hand, Lopez et al. (27) did not observe any effect of dactinomycin on yields of Pichinde, Tacaribe, or Junin virus from virus-infected BHK-21 cells. The latter study limited dactinomycin treatments to 6 to 10 h during the replication cycle, and differences in the cited studies may relate to the half-life of host cell components required for replication.

A role for nuclear function in arenavirus replication was suggested by Mims (31) on the basis of nuclear fluorescence of lymphocytic choriomeningitis virus-infected cells of virus carrier mice. Young and colleagues (38) have carefully documented Pichinde virus-specific nuclear inclusions in Vero cells. Defined monoclonal antibodies further identified the antigen as an NP-related 28-kDa polypeptide or a conformational variant of NP. The inclusions were observed between 10 and 20 days postinfection, but antigen could be detected as early as 10 to 15 h after infection. To date, the evidence is circumstantial for a role of the nucleus or nuclear transcription products in arenavirus replication.

PMA is known to activate protein kinase C (20), leading to inositol phosphate hydrolysis, increased intracellular pH and calcium, and protein phosphorylation. These events are believed to induce specific changes in cellular transcription. Specific repression or activation of transcription is believed to culminate in further transcriptional changes required to complete the cascade of biochemical events following PMA treatment. For example, complexing of c-fos and c-jun on PMA-responsive elements (AP-1 binding sites) in the promoter regions results in transcriptional activation of a number of genes (3, 7, 11, 23). It is possible that PMA influences Pichinde virus replication by indirectly regulating the expression of a molecule obligatory for Pichinde virus replication through c-fos-c-jun interaction. Alternatively, AP-1-like molecules themselves could be responsible for activation. The nature of the induced sequences is unknown.

It is of interest that we were unable to infect HL60 cells with Pichinde virus even when the cells were treated with PMA. HL60 cells, like THP-1 cells, differentiate to more mature monocytes under the influence of PMA (17). We do not know at present whether the defect relates to receptor or postreceptor binding events, but clearly the monocytes obtained in each case are distinct with respect to the ability to support Pichinde virus replication.

ACKNOWLEDGMENTS

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CHAPTER 3

ANALYSIS OF PICHINDE ARENAVIRUS TRANSCRIPTION

AND REPLICATION IN HUMAN THP-1 MONOCYTIC CELLS

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running title: Pichinde Arenavirus RNA expression in human THP-1 cells

ABSTRACT

Human promonocytic THP-1 cells were previously shown to be nonpermissive for Pichinde virus (PV) replication unless THP-1 cells were induced to differentiate to macrophages by stimulation with phorbol ester (Polyak et al., 1991). The restriction did not involve receptor modulation, virus binding nor internalization of virus but was hypothesized to reflect a requirement for a host cell function in PV replication. In this report we demonstrate that PV S RNA genomes and antigenomes, GPC mRNA, NP mRNA and L RNA are expressed at high levels in PMA treated THP-1 cells but at lower levels in untreated cells. We also show that degradation of input viral S RNA cannot account for decreased PV RNA synthesis in the undifferentiated cells. This suggests that the restriction of PV replication in THP-1 cells may occur at the level of transcription of viral mRNAs and replication of viral genomes and implies a role for differentiation-specific host cell factors in PV replication.

INTRODUCTION

Pichinde virus (PV) belongs to the negative stranded, enveloped virus family, Arenaviridae (Bishop, 1990). The genetic information is contained in two single stranded RNA segments termed large (L), and small (S). The S RNA contains two genes (Auperin et al., 1982, 1984a, 1984b, 1986; Romanowski et al., 1985; Auperin and McCormick, 1989; Clegg et al., 1990; Franze-Fernandez et al., 1987; Fuller-Pace and Southern, 1988; Salvato et al., 1988; Ghiringhelli et al., 1991; ; Southern et al., 1987; Wilson and Clegg, 1991) encoding the nucleoprotein (NP) and glycoprotein precursor (GPC) (Giminez et al., 1983; Dimock et al., 1982; Harnish et al., 1981, 1983; Bishop, 1990). The L RNA also possesses two genes which encode the proteins, L and Z (Iapalucci et al., 1989a; Salvato et al., 1989; Singh et al., 1989). The L protein (Harnish et al., 1981; 1983) is thought to possess the viral RNA dependent RNA polymerase activity observed in in vitro replication systems (Carter et al., 1974; Leung et al., 1979; Fuller-Pace and Southern, 1989). The Z protein is a small zinc-finger binding protein which is hypothesized to contain transcription factor-like activities (Iapalucci et al., 1989b; Salvato and Shimomaye, 1989; Salvato et al., 1992). NP and L mRNAs are encoded on genomic RNA and are of viral complementary sense, while GPC and Z mRNAs are encoded on antigenomic RNA and are of viral sense. This coding strategy has been termed ambisense (Bishop, 1990).

The influence of host cell functions on arenavirus replication has not

been well characterized. It is known that PV replication does not occur in cells enucleated with cytochalasin B (Banerjee et al., 1976), suggesting a nuclear requirement for arenavirus replication. A possible nuclear function may involve host cell transcription as numerous studies indicate that arenavirus replication is inhibited in the presence of actinomycin D (Buck and Pfau, 1969; Martinez-Segovia and Grazioli, 1969; Mifune et al., 1971; Stanwick and Kirk, 1971; Rawls et al., 1976; Harnish, 1982; Lukashevich et al., 1984; Polyak et al., 1991). Studies with other viruses indicate that the activation or differentiation state of the target cell can be a critical determinant of virus tropism. For example, herpes simplex virus type 1 (HSV-1) and Rift Valley fever virus infection of U-937 monocytic cells are both dependent on previous cellular activation or differentiation (Tenney and Morahan, 1987, 1991; Lewis et al., 1989). The restriction of viral infection may be as a result of several or all of the following aspects of the viral infection cycle: 1) lack of binding to target cell that lacks the viral receptor protein, 2) lack of internalization and uncoating of the viral genome, 3) inefficient viral transcription, replication, and translation, 4) defects in virus assembly and release.

Recent studies have suggested that arenavirus infections also exhibit dependency on the differentiation state of the target cell for replication as demonstrated by lymphocytic choriomeningitis virus (LCMV) infection of immature spermatogonia in the testes of mice (Fazakerley et al., 1991). Furthermore, arenaviruses have been shown to be tropic for macrophages as

evidenced by reports of monocyte infection by arenaviruses, both in vivo in humans with Argentine hemorrhagic fever (Gonzalez et al., 1980; Ambrosio, et al., 1990) in rodents infected with LCMV, PV and Tamiami arenaviruses (Mims and Tosolini, 1969; Murphy et al., 1976, 1977); and in in vitro cultures of hamster peritoneal macrophages (Friedlander et al., 1984) and monocytic U937 cells infected with PV (Lewis et al., 1989). This predilection for monocytes may affect the host antiviral immune response and establishment of persistence. We have previously demonstrated that human promonocytic THP-1 cells (Tsuchiya et al., 1980; 1982) support PV replication only if monocytic differentiation of cells is induced with the phorbol ester, PMA (Polyak et al., 1991). We showed that infection was dependent on protein kinase C (PKC) activation and host cell transcription. We further characterized the restricted replication of PV in the untreated THP-1 cells and showed that the PMA effect was not due to changes in receptor modulation, virus binding or internalization however viral protein production and production of infectious virus was substantially reduced in untreated THP-1 cells (Polyak et al, 1991). We suggested from these studies that the restriction of PV replication in THP-1 cells occurs after the binding and internalization phases. We have now utilized molecular probes for PV RNAs to examine the expression of PV specific RNAs in PMA treated and untreated THP-1 cells to further delineate the nature of the restriction in THP-1 cells.

MATERIALS AND METHODS

THP-1 and BHK cells were grown as previously described (Polyak et al., 1991). Induction of monocytic differentiation of THP-1 cells was achieved by treatment with 16 nM PMA (Sigma) for 2 hours prior to addition of virus. Infection of cells with high titre PV ($\geq$ 1.0 X 10^8 PFU/ml) was performed as described (Polyak et al., 1991).

Total cellular RNA was extracted from cells at various times post infection by guanidinium, phenol, chloroform extraction as described by Chomynski and Sachi (1987). Equal amounts of RNA were separated by electrophoresis in 1% agarose gels containing 2.2M formaldehyde at 60V for 5 hours. RNAs were transferred to Immobilon N membranes (Millipore) overnight in 10X SSC (1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0). Membranes were baked for 1 hour at 80°C and prehybridized at 65°C for 2 hours in 5X SSC, 5X Denhardt's (0.5 g Ficoll 400, 0.5 g polyvinylpyrrolidone, 0.5 g bovine serum albumin), 1% SDS (sodium sodecyl sulfate), and 100 μg per ml sonicated salmon sperm DNA. DNA probes were labelled with 50 μCi of α-[^{32}P] dCTP (3000 Ci/mmol)(NEN research products) using a T7 DNA polymerase labelling kit (Pharmacia). Radiolabelled probes with specific activities greater than 5 X 10^8 dpm per μg were then added to the prehybridization mixtures and Northern blots were incubated at 65°C overnight.

Four different PV RNA-specific cDNA probes were used in the Northern blot studies reported here. Nucleotide positions for PV RNA-specific probes

were based on the original genomic sense sequence of PV S RNA reported by
Auperin and coworkers (1984b), and from direct RNA sequencing of the 3' end
of PV L RNA as reported by Auperin et al. (1982). One probe was single
stranded and consisted of a portion of the S RNA of PV encoding the
nucleoprotein (NP) gene in the genomic sense from nucleotides 25-880
(Shivaprakash et al., 1988). This probe detected full length viral
complementary (antigenomic) RNA (vcRNA) as well as NP mRNA. A second
probe was complementary to the probe described above and was capable of
detecting only full length viral RNA (vRNA). The two probes were generated
as described (Shivaprakash et al., 1988). The next probe was double stranded,
encompassed the entire GPC gene (nucleotides 1-1589) and detected vRNA,
vcRNA, and GPC mRNA. The PV L RNA-specific probe was double stranded
and represented nucleotides 17-600 derived from the 3' end of PV L RNA. This
clone was generated by standard cDNA cloning procedures using an
oligonucleotide spanning positions 17-33 of the 3' end of PV L RNA to prime
cDNA synthesis. (Harnish, D.G., Zheng, S., and Polyak, S.J., unpublished).
Membranes were also probed for β-actin mRNA using a commercially available
double stranded β-actin probe (Oncor Sciences). Membranes were washed
according to the following protocol: 2 X 10 minute washes in 2X SSC and 1%
SDS at room temperature, followed by 2 X 10 minute washes in 2X SSC and
1% SDS at 65°C. Two final washes, 15 minutes each, in 0.1X SSC and 0.1%
SDS at 65°C were also performed. Membranes were exposed to Kodak AR-50

film at -70°C.

RESULTS

PV S RNA Expression in THP-1 Cells

We have previously demonstrated that human THP-1 promonocytic cells would not support the replication of PV (Polyak et al., 1991). However, when virus binding assays were performed at 4°C in the presence of the lysosomotropic agent, ammonium chloride, we determined that PV was capable of binding and internalization into undifferentiated THP-1 cells (Polyak et al., 1991). We have now examined transcription and replication in PV infected THP-1 cells in order to determine if the restriction of PV replication involves an early event in the virus life cycle. Figure 1 represents the expression of PV S RNAs in PMA treated and untreated THP-1 cells at various times post infection with PV at a MOI of 1. Untreated THP-1 cells infected with PV synthesized very low levels of S antigenome (viral complementary RNA), NP mRNA, S genome (viral RNA), and GPC mRNA. Neither mock infected untreated nor mock infected PMA treated THP-1 cells expressed any of these virus RNA molecules. In marked contrast, PMA treated THP-1 cells which were infected with PV expressed significantly higher levels of S antigenomes and genomes. The synthesis of NP and GPC mRNAs was also enhanced in PMA treated THP-1 cells which were infected with PV. Densitometric scanning of the Northern blots revealed that the magnitude of enhancement of PV S genome and antigenome replication after PMA treatment as compared with untreated THP-1 cells for figures 1A, B, and C, was approximately 60-90 fold, when assessed

at the time point of maximum PV RNA synthesis. Maximum S RNA synthesis

occurred at 72 hours post infection (lane 8). Thus, the values representing

PMA mediated enhancement of PV S RNA replication cited above represent the

ratio of the densitometry readings from lane 8 compared to the densitometry

readings from lane 4. The observed peak in S RNA expression agrees with our

finding that PV protein synthesis in PMA treated cells peaks between 48-72

hours post infection (Polyak et al., 1991). Figure 1 also depicts β-actin mRNA

expression in untreated and PMA treated THP-1 cells. Clearly, there was higher

expression of β-actin mRNA in untreated versus PMA treated THP-1 cells.

Effect of MOI on PV S RNA Replication in THP-1 Cells

In order to study the effect of increased MOI on PV S RNA replication,

untreated and PMA treated THP-1 cells infected with PV at a MOI of 10. As

shown in Figure 2, PV S RNA genomes, antigenomes, NP mRNA and GPC

mRNA were expressed to higher levels in untreated THP-1 cells when the cells

were infected at a MOI of 10 as compared with a MOI of 1 (Compare Figure 2

with Figure 1). The level of expression of the S RNA molecules was detected

at 24, 48 and 72 hours post infection. PMA treated THP-1 cells also expressed

higher levels of S RNA genomes, antigenomes, NP mRNA and GPC mRNA

when the cells were infected at a MOI of 10 as compared with a MOI of 1.

Peaks in expression of the S RNA molecules were observed at 72 hours post

infection in PMA treated cells. Figure 2 also shows that the amount of S RNA

expression in untreated THP-1 cells is proportionally lower than observed in

PMA treated THP-1 cells when cells are infected at a MOI of 10. This suggests that transcription and replication of PV RNAs is restricted in undifferentiated THP-1 cells.

Stability of Input S RNA Molecules

In order to determine whether the difference in PV S RNA replication in untreated THP-1 cells was the result of differential stability of input virus RNA, we examined the relative concentration of S RNAs in PMA treated or untreated THP-1 cells which were infected with PV at a MOI of 10.0. Cells were harvested for RNA isolation at 6 hours post infection. This strategy was employed in an attempt to limit the analysis to a time preceeding the amplification of virus RNA. The result of this analysis is presented in Figure 3. As can be seen, virus S RNA is intact at 6 hours post infection in both untreated (lane 2) and PMA treated (lane 4) THP-1 cells, and in BHK cells (lane 6) and the relative levels of RNA are approximately the same. Thus, degradation of input virus S RNA in untreated THP-1 cells cannot account for the reduced PV S RNA transcription and replication in these cells.

PV L RNA Expression in THP-1 Cells

We then examined L RNA expression in infected cells. As can be seen in figure 4, the L RNA probe detected an RNA species that was the correct size for L RNA (approximately 7 kb). This RNA species was not evident in mock infected cells. PMA treated THP-1 cells expressed detectable levels of L RNA which peaked at 48-72 hours post infection (lanes 7 and 8). There also

appeared to be L RNAs in untreated THP-1 cells at 48 hours post infection (lane 3). The difference in the levels of L RNA in PMA treated THP-1 cells as compared to uninduced cells appeared to be lower than was observed in the analysis of S RNAs and S-derived mRNAs. When normalized for β-actin mRNA expression, densitometric readings indicated that the enhancement of PV replication in PMA treated THP-1 cells was approximately 4 times higher than in untreated THP-1 cells. Thus, there was an accumulation of L RNAs in PMA treated THP-1 cells that correlated with increases in S RNA molecules. Figure 4 also demonstrated the presence of L specific RNA that was larger than the unit size of L RNA in PV infected BHK cells at 24 hours post infection (lane 10). We have previously documented a PV S RNA-derived larger than unit size RNA (Shivaprakash et al., 1988). At present, the precise nature of LUS RNA is not known. However, LUS detected with PV L RNA probes must be distinct from LUS detected with PV S RNA probes in that the size of LUS RNA detected with a PV L specific probe is much larger than the LUS RNA detected with S RNA specific probes (Shivaprakash et al., 1988).

DISCUSSION

We have shown that PMA treated THP-1 cells support higher levels of PV RNA transcription and replication than untreated THP-1 cells. Replication was assessed by the production of virus specific RNA molecules. Full length genomic and antigenomic S RNA, subgenomic mRNA for NP and GPC, and L RNAs, which peaked at 72 hours post infection, were observed by Northern blot analysis of total cellular RNA from PMA treated THP-1 cells which had been infected with PV. However, in untreated THP-1 cells, lower levels of these virus RNA molecules were observed. Although L RNA expression in undifferentiated THP-1 cells was lower than in PMA stimulated cells, the relative difference between PMA stimulated and unstimulated cells was of lower magnitude than for S RNA. This may indicate the existence of a differential restriction of PV RNA replication in THP-1 cells or that there are different requirements for the replication of L and S RNA.

Our Northern blot data suggest that little or no PV S RNA replication occurs in undifferentiated THP-1 cells. Indeed, a proportion of both L and S RNA expression in untreated THP-1 cells could be due to infection by PV of a small proportion (≤10%) of spontaneously differentiating THP-1 cells as we have suggested previously (Polyak et al, 1991). We have examined whether input PV S RNAs in untreated THP-1 cells are degraded. At high MOI at 6 hours post-infection (prior to any detectable PV RNA synthesis), input viral RNA is intact in both PMA treated and untreated THP-1 cells (figure 3). Thus, active

degradation of input PV RNAs cannot account for the lack of PV RNA synthesis in untreated THP-1 cells.

When PMA treated cells were infected at a MOI of 10, an increase in PV replication was also observed relative to PMA stimulated cells which were infected at a MOI of 1. This supports our previous data that differences in the number of input virion molecules cannot account for the difference in PV replication observed between PMA treated and untreated THP-1 cells (Polyak et al., 1991). Most importantly, the level of PV S RNA expression in undifferentiated versus PMA-differentiated THP-1 cells was still lower when cells were infected with PV at a MOI of 10. This indicates that the restriction of PV replication in untreated THP-1 cells cannot be overcome by increasing the MOI.

Studies in other virus systems have also determined that the activation or differentiation status of the host cell can influence the infection process. For example, HSV replicates efficiently in peripheral blood monocytes (PBM) that have been cultured for several days prior to infection, suggesting that differentiation of freshly isolated PBM to macrophages is a prerequisite for productive infection (Plaeger-Marshall and Smith, 1978; Linnavuori and Hovi, 1981; Daniels et al, 1978). In addition, cytomegalovirus infection of U-937 cells also appears to depend on differentiation of the cells to macrophages, as PMA treatment of the cells changes the infection to a productive one (Weinshenker et al, 1989). Vesicular stomatitis virus infection of B cells

occurs only if the cells are activated by anti-immunoglobulin or liposaccharide (Schmidt and Woodland, 1990), while poliovirus mRNA translation appears to be restricted in U-937 cells (Lopez-Guerro et al. 1989). Similarly, restricted measles virus replication in human peripheral blood mononuclear cells is changed to a productive infection when cells are treated with PMA and/or calcium ionophore (Vainionpaa et al., 1991). Gendelman et al. (1986) examined visna virus replication in mature and immature macrophages. It was shown that a few immature monocytes were infectable and these supported limited RNA replication. However, induction of macrophage differentiation was associated with profound increases in visna virus replication (Gendelman et al., 1986). Tenney and Morahan (1991) have observed lack of HSV-1 immediate early mRNA accumulation in undifferentiated but not PMA differentiated U937 macrophages, suggesting that virus replication occurs preferentially in mature macrophages. It was also suggested that the restriction of HSV-1 replication in undifferentiated U937 cells may have been due to an RNase activity present in the cells.

It was previously demonstrated that both untreated and PMA treated THP-1 cells bound and internalized PV into acidic endosomes (Polyak et al., 1991). Based on the observations reported here we suggest that viral transcription or replication are the earliest points in the replication cycle of PV where we can detect a major defect that contributes to the restriction of PV replication in THP-1 cells. This is supported by the low levels of PV specific

RNAs in THP-1 cells documented in this report and the low levels of PV specific

polypeptides in infected cells and reduced titres of extracellular virus (Polyak et

al., 1991). Possible explanations for this transcriptional block include the lack

of permissive factors or the presence of inhibitory factors in untreated THP-1

cells, both of which prevent transcription initiation or elongation. Stimulation

of the cells with PMA may induce the permissive factor(s) or remove the action

of the inhibitory factor(s), and would permit PV replication to be completed.

Consistent with this notion is the observation that PV replication in PMA

treated THP-1 cells is dependent on host cell transcription (Polyak et al., 1991).

The mechanism by which PV transcription and replication is restricted in

THP-1 cells is not known. The PV polymerase may be defective in initiation of

transcription or extension of RNA transcripts, due to the presence or absence

of a host cell factor. The functions of host cell factors are not known, but

there is some evidence currently available. A candidate stimulatory host cell

function required for arenavirus replication may be that of a kinase. Recently,

a cellular kinase has been shown to phosphorylate VSV phosphoprotein (P

protein) (Barik and Banerjee, 1992). P protein is a component of VSV

transcriptase and the phosphorylation of P protein was shown to be required

for activation of transcription. For arenaviruses, phoshphorylated forms of NP

have been documented for LCMV, PV (Howard and Buchmeier, 1983; Bruns et

al., 1986) and Tacaribe virus (Gimenez et al., 1983), but it is not known if

phosphorylation affects the activity of NP nor is it known which protein(s)

regulate the level of phosphorylation. In addition, the cellular proteins, tubulin and actin, have been shown to be required for both Rhabdovirus (VSV) and Paramyxovirus (Sendai virus and Measles virus) and human parainfluenza virus type 3 (HPIV-3) replication, perhaps during RNP transcription or during assembly and budding (Banerjee, 1992; De et al., 1991). At present, no such role for these proteins in arenavirus replication has been determined.

We previously demonstrated the presence of LUS RNA with PV S RNA-specific probes (Shivaprakash et al., 1988). In this report, we also demonstrated the presence of LUS RNA with PV L RNA-specific probes. At present, the nature of LUS and its role in PV replication is not known. As previously suggested, LUS may be an intermediate in the replication of PV (Shivaprakash et al., 1988). Indeed, further studies must be performed to elucidate the function(s), if any, of LUS RNA.

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FIGURE 1
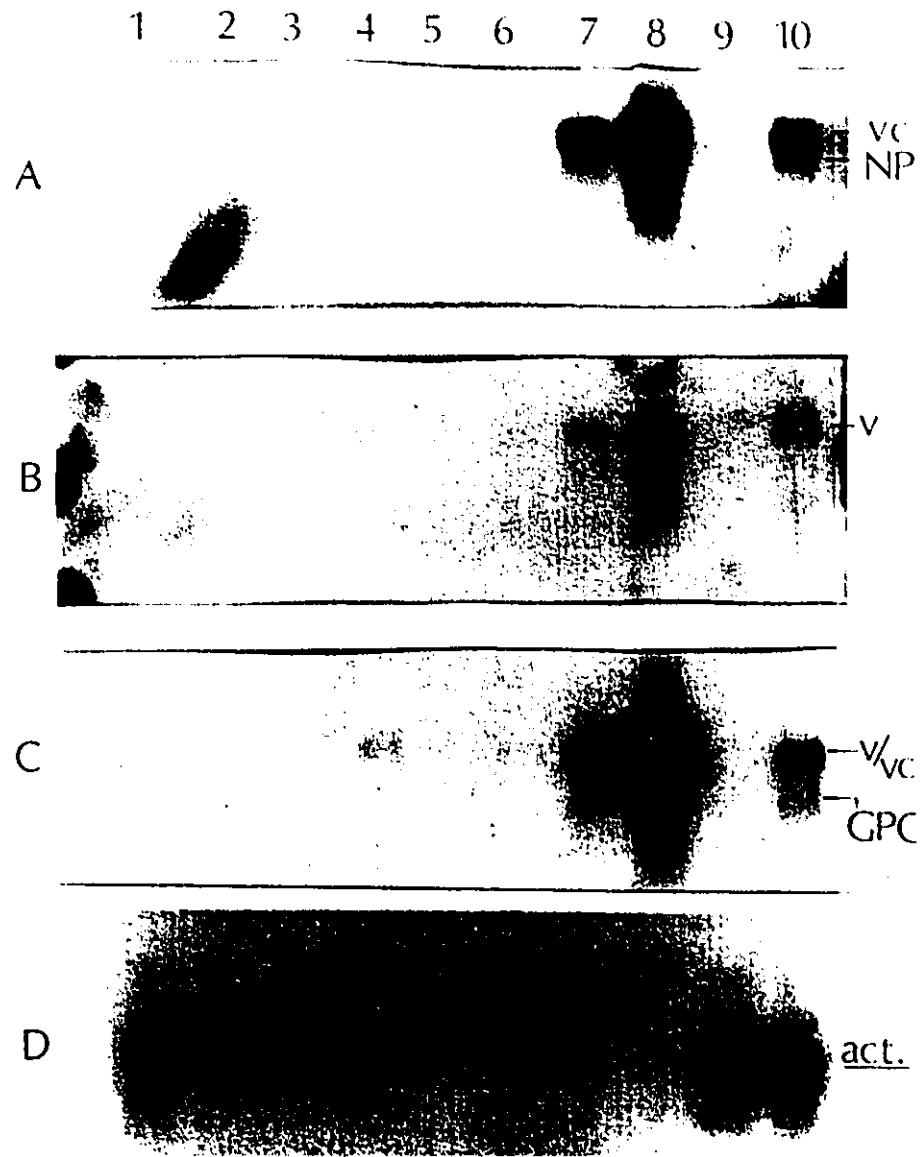
FIGURE 2
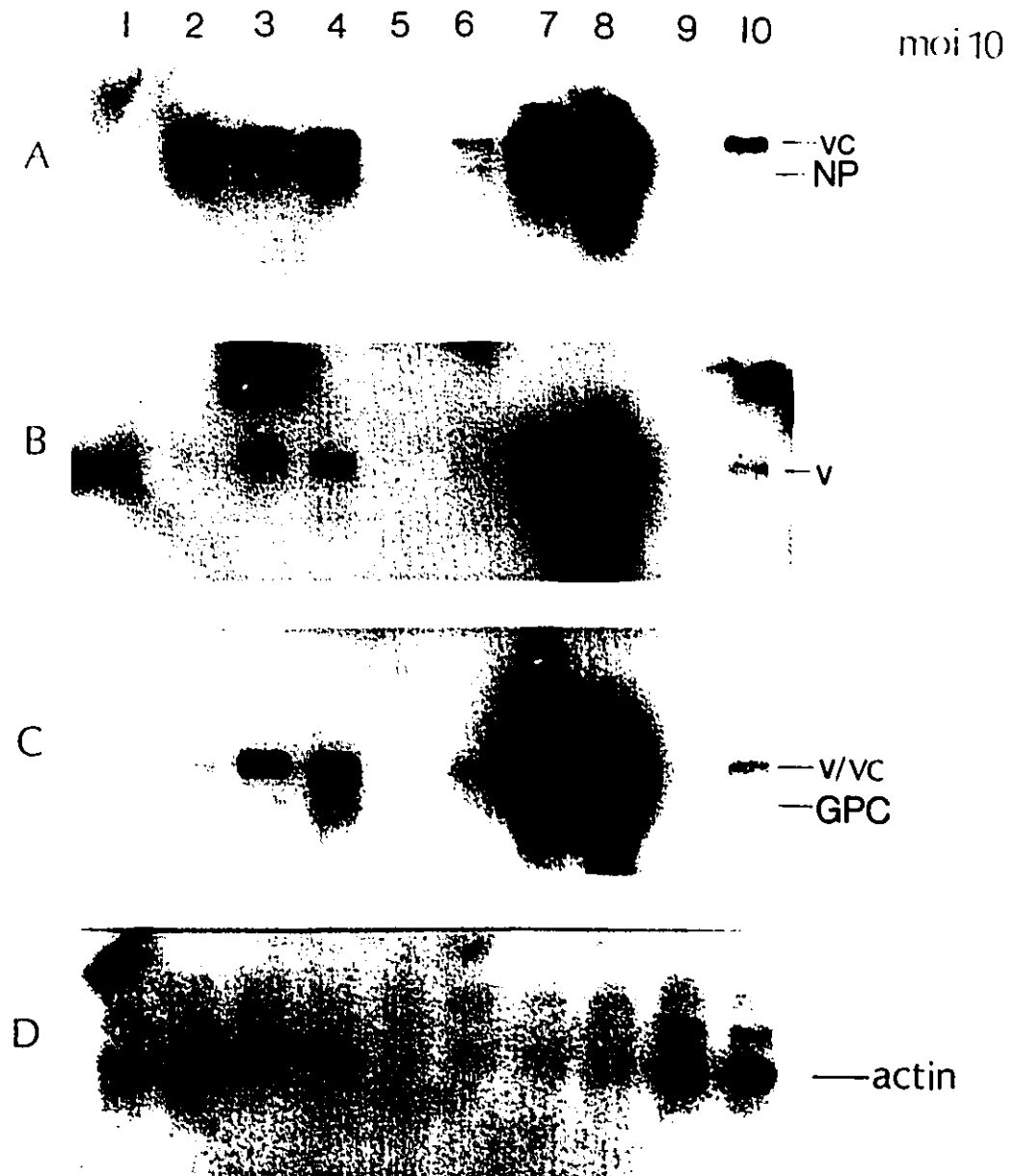
FIGURE 3

FIGURE 4
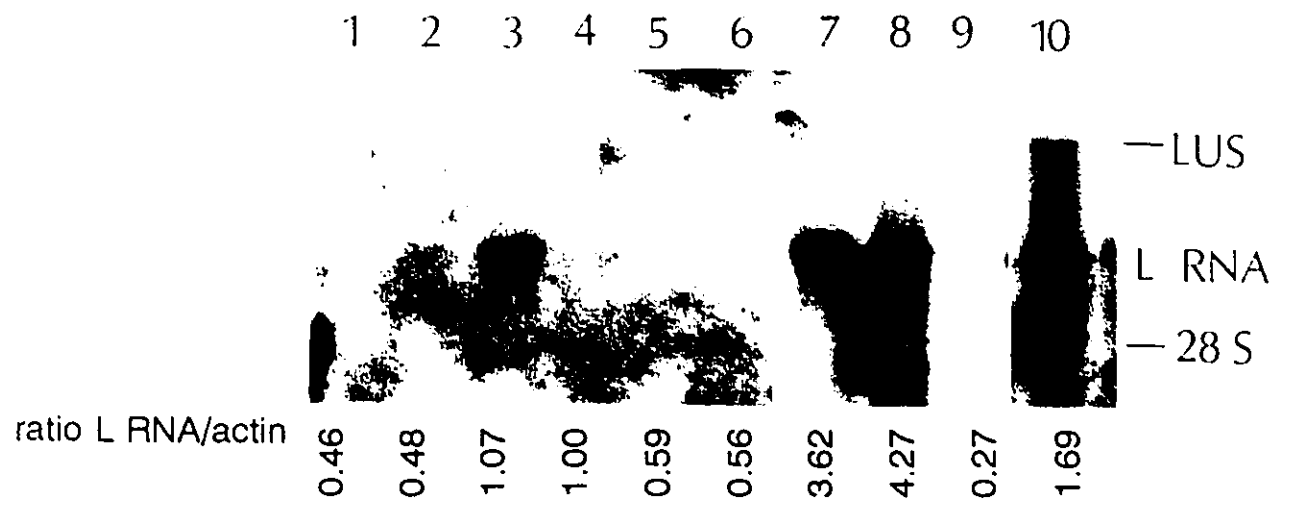
FIGURE LEGENDS

Figure 1: Expression of PV S RNA molecules in PMA treated THP-1 cells and untreated THP-1 cells. RNA isolated from cells at various times post infection was separated by electrophoresis on formaldehyde-containing agarose gels, blotted to nylon membranes and was hybridized with various probes as described in the Materials and Methods. Panel A represents vcRNA (vc) and NP mRNA species, panel B represents vRNA (v), panel C represents v and vcRNA (v/vc) and GPC mRNA, and panel D represents the expression of β-actin mRNA (act.). The positions of the RNAs are indicated. Lane 1 represents RNA harvested from mock infected THP-1 cells at 24 hours. Lanes 2, 3, and 4 represent RNAs from PV infected THP-1 cells at 24, 48, and 72 hours post infection with PV at a MOI of 1, respectively. Lanes 5-8 are the same as lanes 1-5, with the exception that THP-1 cells were pretreated for 2 hours at 37°C with 16 nM PMA prior to infection with PV. Lanes 9 and 10 represent viral RNAs from mock infected and PV infected BHK cells at 24 hours post infection at a MOI of 1, respectively.

Figure 2: Synthesis of PV S RNA molecules in untreated and PMA treated THP-1 cells at high multiplicity of infection. Lane assignments (1-10) and abbreviations are as described for Figure 1 except β-actin mRNA is designated as actin. Cells were infected at a MOI of 10 as described in the Materials and Methods.

Figure 3: Pichinde virus input S RNA is not selectively degraded after infection of THP-1 cells versus PMA stimulated THP-1 cells. THP-1 cells (lane 2), PMA treated THP-1 cells (lane 4) and BHK cells (lane 6) were infected with PV at an MOI of 10.0, and total cellular RNA was extracted at 6 hours post infection. The RNAs were separated by electrophoresis and probed for S viral RNA (vRNA) as described in the Materials and Methods. The position of input vRNA is indicated. Lane 7 represents RNA from PV infected BHK cells at 24 hours post-infection with an MOI of 1. LUS represents the larger than unit size RNA previously described for S RNA (Shivaprakash et al, 1988). Lanes 1, 3, and 5 represent RNAs from mock infected THP-1, PMA stimulated THP-1 and BHK cells, respectively.

Figure 4: Synthesis of Pichinde virus L RNA in PMA treated and untreated THP-1 cells. Lane assignments (1-10) are as described for Figure 1. The position of L RNA is indicated. LUS represents the position of an PV specific RNA that is larger than the unit size of PV L RNA. The position of the 28 S rRNA species (28S) as determined by ethidium bromide staining of the formaldehyde gel is indicated. Numbers below each lane represent the ratio of L RNA to β-actin mRNA as determined by densitometric scans of the autoradiograph. The normalization of L RNA expression to β-actin mRNA expression provides a measure of the difference in L RNA expression between PV infected, PMA treated THP-1 cells and PV infected, untreated THP-1 cells.

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CHAPTER FOUR

THE 5' TERMINI OF PICHINDE ARENAVIRUS S RNAs and mRNAs CONTAIN NONTEMPLATED NUCLEOTIDES

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ABSTRACT

In order to gain insight into the mechanisms of arenavirus transcription and replication, the 5′ termini of Pichinde virus (PV) S RNA genomes, antigenomes, GPC mRNA and NP mRNA were determined using a novel procedure which allowed unambiguous determination of the 5′ sequence of viral RNA. All termini sequenced had at least one extra nontemplated base. In clones that contained a single nontemplated base, the base was invariably a G nucleotide. The 5′ termini of NP mRNAs and GPC mRNAs contained 3-9 and 4 nontemplated bases, respectively. In addition, on genomic sense RNAs the base at -1 was usually a G. However, the base at -1 on antigenomic sense RNAs was not conserved, while on NP mRNAs, a G was found preferentially at position -1. These data are discussed in the context of PV mediated transcription and replication.

Pichinde virus (PV) is a member of the negative stranded, enveloped virus family, Arenaviridae. The genetic information is contained in two single stranded RNA segments termed large (L; approximately 7 kb), and small (S; 3.4 kb). Molecular cloning of several arenavirus genomes has revealed that the S RNA contains two genes encoding the nucleoprotein (NP; approximately 68 kD) and glycoprotein precursor (GPC; approximately 75 kD) (Auperin et al., 1982, 1984a, 1984b, 1986, 1989; Clegg et al., 1990; Franze-Fernandez et al., 1987; Ghiringhelli et al., 1991; Harnish et al., 1981, 1983; Southern et al., 1987; Wilson and Clegg, 1991). The L RNA also possesses two genes encoding the presumptive viral RNA dependent RNA polymerase (L protein; approximately 200 kD) (Harnish et al, 1981; Iapalucci et al., 1989a; Salvato et al., 1989a; Singh et al., 1987) and a smaller protein, termed Z (10 kD) (Iapalucci et al., 1989b; Salvato et al., 1989b, 1992). The genes on both L and S RNAs are arranged at opposite ends of the respective RNA segment and in opposite orientations on their respective RNAs, separated by a stretch of nucleotides that have the potential to form stable secondary structures in the form of a hairpin(s). Viral S mRNA transcription has been shown to terminate in the hairpin (Iapalucci et al., 1991). NP and L mRNAs are transcribed from genomes and are of viral complementary sense, while GPC and Z mRNAs are transcribed from antigenomes and are of viral sense. This coding strategy has been termed ambisense (reviewed in Bishop, 1990).

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Recent studies with Tacaribe (TAC) arenavirus have examined the 5′
ends of S RNA and have revealed an extra G nucleotide at the 5′ ends of TAC
S RNA genomes and antigenomes (Raju et al., 1990). An extra G nucleotide
has also been observed in the sequence of clones of PV S RNA and Lassa Fever
virus S RNA (Auperin et al., 1984b, 1986). Garcin and Kolakofsky (1990) have
demonstrated that the 5′ termini of TAC NP and GPC mRNAs have an
additional 2-5 nontemplated bases and that the nontemplated bases on TAC S
RNAs appear to possess 7 methylguanosine caps, which are proposed to be
derived from host cell mRNAs by a cap snatching mechanism.

This study was performed to further examine mechanisms of arenavirus
mediated transcription and replication initiation. We have cloned the 5′ ends
of PV S RNA genomes, antigenomes, NP mRNA and GPC mRNA using a
method that employs primer extension, ligation of cDNA in a head to tail
fashion with RNA ligase and a polymerase chain reaction to amplify the cDNAs
(Hoffman and Brian, 1991).

MATERIALS AND METHODS

Cells and Virus: PV was prepared as previously described and was used to infect BHK21 cells at an MOI of 1.0 as described (Harnish et al., 1981). A stock of plaque purified PV was passaged 4 times in BHK cells prior to use. Cells were harvested by scraping in PBS and were processed for isolation of total cellular RNA or sedimentation of PV S mRNAs.

Isolation of RNA: Total cellular RNA was extracted from cells at various times post infection by guanidinium, phenol and chloroform extraction as described (Chomczynski and Sacchi, 1987). To separate PV mRNAs from nucleocapsids, cells were lysed in TNE (10 mM Tris.Cl (pH 7.4), 150 mM sodium chloride, 1 mM EDTA) containing 0.2% (v/v) NP-40 and lysates were centrifuged through continuous 10-40% CsCl gradients at 35K rpm for 16 hours in a SW41 rotor (Beckman) (Raju et al, 1990). RNA in the pellet fraction was resuspended in water, ethanol precipitated, and the pellet was dried before resuspension and storage at -70°C in water.

Northern blots: RNAs were separated by electrophoresis in 1% (w/v) agarose gels containing 2.2M formaldehyde at 60V for 5 hours. RNAs were transferred to Immobilon N membranes (Millipore) overnight in 10X SSC (1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0). Membranes were baked for 1 hour at 80°C and prehybridized at 65°C for 2 hours in 5X SSC, 5X Denhardts (0.5 g Ficoll 400, 0.5 g polyvinylpyrrolidone, 0.5 g bovine serum albumin), 1% SDS (sodium sodecyl sulfate), and 100 μg/ml sonicated salmon sperm DNA.

Radiolabelled probes were then added to the prehybridization mixtures and incubated at 65°C overnight. DNA probes were labelled with 50 μCi of α-[^{32}P] dCTP (3000 Ci/mmol)(NEN) using a T7 DNA polymerase labelling kit (Pharmacia). Unincorporated α-[^{32}P] dCTP was removed by chromatography on G50 Sephadex columns (Pharmacia). The specific activities of probes prepared in this way was greater than 5 X 10^8 dpm per μg. One probe consisted of a portion of the S RNA of PV encoding the nucleoprotein (NP) gene in the genomic sense from nucleotides 25-880 cloned into the EcoR1 site of M13mp18 and detects full length viral complementary RNA (vcRNA) as well as NP mRNA (Shivaprakash et al., 1988). The GPC probe is double stranded, represents nucleotides 1-1589 which encompasses the entire GPC gene and detects vRNA, vcRNA and GPC mRNA. The L probe represents a small segment of 60 nucleotides derived from the 3' end of PV L RNA spanning nucleotides 17-76. Membranes were washed initially at low stringency followed by two high stringency washes for 15 minutes in 0.1X SSC/0.1% SDS at 65°C. Membranes were exposed to Kodak AR-50 film for various times at -70°C.

Oligonucleotide Primers: Oligonucleotides were designated by the abbreviations, vc, which refers to viral complementary sense, while vs refers to viral sense. For primer extension to detect genomic S RNA and GPC mRNA 5' termini, the oligonucleotide vc 3319-3328 (5'TTGCAGGACTTCAGGAATGG) was used. To amplify the ligated cDNA molecules, oligonucleotides vs 80-99

(5'CCATTCCTGAAGTCCTGCAA) and vc 3351-3367 (5'AGTCACAATTTGTCCCAT) were used in the PCR. For antigenomic RNA and NP mRNA primer extensions the oligonucleotide vs 3300-3319 (5'ATTGCACCCAGCGGAACGAT) was used. The oligonucleotides used to PCR amplify the ligated antigenomic cDNAs were vc 100-119 (5' ATCGTTCCGCTGGGTGCAAT) and vs 3346-3365 (5' GTTCACACTTCCTCTGAGTC).

Oligonucleotide labelling: 100 ng of oligonucleotide was labelled with 50µCi of [γ^{32}P]ATP in the presence of T4 Polynucleotide Kinase (PNK; Pharmacia) as described (Ausubel et al., 1991). End labelled oligonucleotides were ethanol precipitated dried under vacuum and reconstituted in 10 mM Tris.Cl, 1 mM EDTA (TE; pH 7.9) before use in primer extension reactions.

Primer extensions

20 µg of total cellular RNA from PV infected BHK cells was precipitated with 2.5 volumes of ethanol and 0.3M sodium acetate (pH 5.2) at -186°C for 5 minutes. Pellets were collected by centrifugation (10,000 X g, 15 minutes), washed with 70% ethanol and dried under vacuum for 1 hour. The RNA was then resuspended in 8µl of TE (pH 7.9) with 4X10^5 dpm of labelled oligonucleotide or was mixed with a similar amount of oligonucleotide that had been previously end labelled with cold ATP. 2µl of TKE (10 mM Tris.Cl, pH 7.9, 1mM EDTA, 1.25 M KCl) was added and the mixture was incubated at 55°C for 1 hour. After incubation, 25µl of primer extension buffer (20mM

Tris.Cl, pH 8.3, 10 mM $MgCl_2$, 5mM DTT, 0.4 mM of each dNTP) and 10 units of AMV reverse transcriptase (Pharmacia) was added and the mixture was incubated at 37°C for 1 hour. cDNA reaction products were precipitated with ethanol, pelleted, and dried under vacuum. The cDNA was resuspended in sequencing gel loading buffer and was boiled for 2 minutes prior to electrophoretic separation in a 6% acrylamide sequencing gel which contained 7M urea. Sequencing gels were exposed directly to Kodak AR50 film at -70°C overnight. Bands of interest corresponding to the termini of PV RNAs and extensions beyond the termini were excised from the gel, and eluted by diffusion in elution buffer (0.5M ammonium acetate, 1mM EDTA) overnight at 37°C. Eluted cDNAs were ethanol precipitated and PCR amplified as described (Hoffman and Brian, 1991). The PCR products were then blunt ended with the Klenow fragment of DNA polymerase 1 (Pharmacia), phosphorylated at the 5' end with T4 PNK, ligated into the EcoRV site of Bluescript KS plasmid, and transformed into DH5α cells. Clones were sequenced using the Sequenase Version 2.0 sequencing kit (US Biolabs).

RESULTS

Several negative strand RNA viruses have non-templated bases at the 5′

ends of viral RNA including Tacaribe arenavirus (Raju et al., 1990; Garcin and

Kolakofsky, 1990, 1992), Bunyavirus (Bishop et al., 1983; Bouloy et al., 1990;

Ihara et al, 1985; Vialat and Bouloy, 1992), and Influenza virus (reviewed in

Krug, 1981). For TAC, it has been demonstrated that there are differences in

the number of non-templated nucleotides added to either nucleocapsid or

mRNA molecules. Therefore, we separated viral mRNA from nucleocapsid (NC)

RNA by CsCl gradient centrifugation of RNA from PV infected cells at various

times postinfection. We were unable to identify and isolate PV genome and

antigenome nucleocapsids from the gradient using protocols previously

published for TAC (Raju et al., 1990). However, we were able to separate

mRNA from nucleocapsids. The results are depicted in Figure 1. Purified viral

RNA contained L and S RNAs as well as NP mRNA (lane 1). NP mRNA and S

RNAs were also observed in total cellular RNA from 24 hour infected cells (lane

2). Longer autoradiographic exposure of the northern blot clearly revealed the

presence of L RNA in lane 2 (data not shown). Lanes 3-5 represented the pellet

fractions of the CsCl gradients and indicated that NP mRNA synthesis peaked

at or before 24 hours post infection (lane 3), was reduced at 48 (lane 4) and

was barely detectable 72 (lane 5) hours post infection. Similarly, when the

RNA samples were probed with mixture of GPC and L probes, total cellular RNA

from infected cells was found to contain both L and S RNAs as well as GPC

mRNA (lane 6). GPC mRNA synthesis also peaked at or before 24 hours post infection (lane 7) and had declined at 48 hours post infection (lane 8) and 72 hours post infection (lane 9). We could not detect full length S genomes or antigenomes in any of the pellet fractions derived from CsCl gradients (lanes 3-5, 7-9), nor did we detect virus RNA corresponding to L mRNA. This may be due to low expression of L mRNA in infected cells by inference from the low levels of L protein (Harnish et al., 1981) and L RNA (Southern et al., 1987) detected in infected cells, and low levels of L RNA replication in vitro (Fuller-Pace and Southern, 1989).

Since we could not isolate nucleocapsids, we compared primer extension products obtained from reactions with total virus infected cell RNA with primer extension products obtained with reactions with purified virus mRNA derived from isolations performed at 24 hours post infection. The data are shown in Figure 2. When the NP specific oligonucleotide (vs 3300-3319) was extended on both total cellular RNA from virus infected cells (lane 1) and purified viral mRNA (lane 2), the products extended from position -1. In both cases the primer extension products were heterodisperse and extended to position -4 and longer. Furthermore, the primer extension products generated from NP mRNA template appeared to be longer. Similarly, when primer extension products were generated with a GPC specific oligonucleotide (vc 3319-3328) from total infected cell RNA (lane 4) and GPC mRNA (lane 5), the products were observed to extend to position -1 and longer. GPC mRNA extension products were also

longer than the primer extension products derived from total cellular RNA from infected cells. Primer extension products were not observed when total cellular RNA from mock infected cells was used as a template (lanes 3 and 6). Thus, both NP and GPC mRNAs appeared to have extensions beyond the known 5′ termini and these extensions appeared longer than those detected on virus infected total cellular RNA (which contained genome and antigenome RNAs as well as viral mRNAs).

The primer extension products from each region of the acrylamide gel corresponding to extension products were excised, eluted and cloned as described in the Materials and Methods. Sequencing gels of cDNA clones which contained genomic sense and antigenomic sense termini are shown in Figure 3. As can be seen, from 1 to 5 nontemplated bases were found beyond the known 5′ terminus of PV RNAs. These sequences are representative of clones which contain from 1-9 nontemplated bases beyond the 5′ terminus of PV S RNAs. A summary of all clones sequenced is presented in Table 1.

In total, 19 genomic sense termini (Table 1A) and 20 antigenomic sense termini (Table 1B) were sequenced. Of the 17 genomic sense clones derived from total infected cell RNA, 11 clones had a single nontemplated G. There were 5 clones which possessed a dinucleotide, all of which had a G at position -1. In addition, one clone contained 5 nontemplated bases. The 5′ termini of 2 GPC mRNA clones possessed 4 nontemplated bases. In all genomic sense clones derived from PV infected total cellular RNA a G nucleotide was always

found at position -1, whereas the base at -1 on GPC mRNAs did not appear to be conserved in the limited number of clones examined. Beyond position -1, however, there was no clear preference for a particular base.

Similarly, 5 of 15 antigenomic sense termini isolated from total cellular RNA had a single nontemplated G base (Table 1B). Five clones contained 3 nontemplated bases, while 1 clone contained 4 additional bases. Three clones had 5 nontemplated bases and 1 clone contained 9 extra bases. Other than the antigenomic sense clones obtained from total cellular RNA which contained a single G, the base at position -1 did not appear to be conserved. The 5' termini of 5 NP mRNAs possessed 3-8 nontemplated bases. In addition, the base found at -1 was usually a G (4 of 5 cases). As observed in the case of genomic sense clones, there was no preference for a particular base beyond position -1. There were significantly more genomic sense clones containing a single nontemplated G base (11 of 18) than antigenomic sense clones (5 of 15). In all but two clones the sequences up to the known 5' termini of PV genomes and antigenomes were found. The two genomic sense clones were found to initiate at +5 of the genomic sequence. One clone was isolated from PV infected total cellular RNA while the other clone was derived from purified virus mRNA. It is not known whether these clones represent functional GPC mRNA transcripts.

DISCUSSION

We have demonstrated the presence of extra nucleotides beyond the prototype 5′ terminal sequences of PV S RNA genomes, antigenomes, NP mRNA and GPC mRNA. In all instances a minimum of a single extra G nucleotide was detected. GPC mRNAs possessed 4 nontemplated bases, while NP mRNAs had 3-8 extra bases.

Our data suggest that PV S RNA genomes and antigenomes have an extra G nucleotide on the 5′ end. Although we could not isolate genomes and antigenomes two lines of evidence suggest that clones which contained a single G nucleotide are in fact genomes and antigenomes. One is the observation that clones containing a single nontemplated G nucleotide were obtained only from PV infected total cellular RNA and not purified PV mRNA. Second, PV genomic clones were initially reported to contain an extra G at the 5′ end (Auperin et al., 1984b). Thus, our findings are in agreement with data from Auperin and colleagues (1984b) on PV and with Garcin and Kolakofsky (1990) on TAC genomes and antigenomes which also contain a single G at their 5′ ends. This would support the suggestion that the clones obtained from total PV infected cellular RNA containing two or more nontemplated bases are in fact 5′ termini of PV S mRNAs. With respect to NP mRNA synthesis, we were able to characterize more antigenomic sense termini containing multiple nontemplated nucleotides which corroborates the observation that NP mRNA is the predominant transcript in PV infected cells (Auperin et al., 1984b).

One explanation for the nontemplated bases on PV S mRNAs is that the PV polymerase steals the 5′ 7-methylguanosine caps of cellular mRNAs and several nucleotides of the sequences immediately downstream of the cap. This strategy, termed cap-snatching, has been extensively characterized in the influenza virus system (Krug, 1981) and bunyavirus system (Vialat and Bouloy, 1992). The mechanism can be summarized as follows. The viral RNA dependent RNA polymerase possesses an endonuclease activity which recognizes the cap and cleaves the cellular mRNA at a preferred distance downstream. For influenza virus, the host cell mRNA is cleaved 10-13 nucleotides from the cap (Krug, 1981) while cleavage occurs at 13-18 nucleotides from the cap in bunyavirus (Vialat and Bouloy, 1992). For influenza virus, it has been shown that cap recognition and endonucleolytic cleavage is mediated by the viral PB2 protein (Krug, 1981). The bunyanvirus polymerase has also been shown to possess an endonuclease function (Patterson et al., 1984; Jin and Elliot, 1993). At present, the functions of arenavirus proteins in the putative cap snatching mechanism are not known. However, NP derived proteins have been detected in the nuclei of infected cells (Young et al., 1987) which may suggest a link between arenavirus replication in the cytoplasm and nucleus derived products. Furthermore, our results are also consistent with previously described studies which suggest a nuclear function for PV replication (Banerjee et al., 1976). Specifically, the nuclear function may involve host cell transcription. In this regard, a number of studies have reported that the cellular

transcription inhibitor, Actinomycin D, when added to cultures during or immediately after adsorption, inhibits arenavirus protein synthesis (Harnish, 1982; Polyak et al., 1991) and progeny virus production (Buck and Pfau, 1969; Martinez-Segovia and Grazioli, 1969; Mifune et al., 1971; Stanwick and Kirk, 1971; Rawls et al., 1976; Lukashevich et al., 1984). In contrast, one study reported that the yields of TAC, PV and Junin virus were not affected by actinomycin D (Lopez et al., 1986). In this study, BHK cells were exposed to actinomycin D for the first 11 hours of the replication cycle and virus titres assessed immediately after this time. However, Rawls et al. (1976) clearly demonstrated that when virus titres are examined within the first 12 hours postinfection in the presence of the same concentration of actinomycin D, the titres produced are too low to be significantly different. It was not until 24 hours postinfection that differences in virus titres became apparent (Rawls et al., 1976) and hence the differences observed between the studies may relate to the half-life of the host cell components required for the replication. The apparent sensitivity of arenavirus replication to actinomycin D also parallels the α-amanitin sensitive step in influenza virus replication (Krug, 1981). Therefore, the actinomycin D studies support the hypothesis that PV initiates transcription by a cap snatching mechanism but the intermediate sensitivity relative to influenza virus suggests that cytoplasmic mRNAs are utilized.

TAC arenavirus has also been suggested to initiate transcription using a mechanism that is equivalent to cap snatching (Garcin and Kolakofsky, 1990).

It was demonstrated that a portion of TAC mRNAs contained 7 methylguanosine caps and 2-5 nontemplated bases (Garcin and Kolakofsky, 1990). At present, however, it has not been unequivocally demonstrated that TAC polymerase possesses cap snatching activities. Despite this caveat, our results suggest that PV mediated initiation of transcription may be mechanistically similar to TAC. Further support for the role of cap snatching in arenavirus replication is provided by studies which indicate that Junin virus and TAC replication in Vero cells is inhibited by S-adenosylhomocysteine hydrolase inhibitors (Andrei and De Clercq, 1990). The inhibitors interfere with methylation reactions including the generation of the 7 methylguanosine cap. Viruses which either steal caps (orthomyxoviruses) or synthesize caps (poxviruses, rhabdoviruses and paramyxoviruses) during viral transcription are inhibited in the presence of these inhibitors (reviewed in De Clercq, 1987). This suggests that the inhibitors inhibit viral transcription by interfering with the capping process.

If indeed PV polymerase is involved in stealing caps, then with respect to the limited number of antigenomic sense and NP mRNA clones that contain 8-9 nontemplated bases, the process may not be as stringent as for TAC which steals 2-5 extra bases. In addition, the range in the size of caps stolen appears to be longer for NP mRNA initiation. This finding distinguishes the putative PV cap snatching mechanism from Influenza virus, Bunyavirus, and TAC cap snatching processes.

A model has been proposed in which the TAC polymerase initiates replication of genomes and antigenomes at the penultimate base of the 3' end of the S RNA with pppG and slips backward by two bases (Garcin and Kolakofsky, 1990). The model would explain how TAC polymerase generates the nontemplated G found on genomes and antigenomes and has been supported by in vitro replication of genomes and antigenomes with the dinucleotide primer GpC (Garcin and Kolakofsky, 1992). Thus, PV polymerase may initiate replication in a similar fashion to TAC polymerase. In addition, the mechanism of PV replication initiation may be quite different from PV transcription initiation.

Garcin and Kolakofsky (1990) were unable to determine whether extra C nucleotides are found beyond the known termini because termini were G tailed prior to cloning. We note that with the cDNA ligation protocol employed in this study there was no evidence of single or poly C nontemplated bases and that every clone sequenced had at least one extra nucleotide.

FIGURE 1

FIGURE 2

NP oligo
GPC oligo

FIGURE 3

GPC 5' (vs)

A

NP 5' (vc)

B
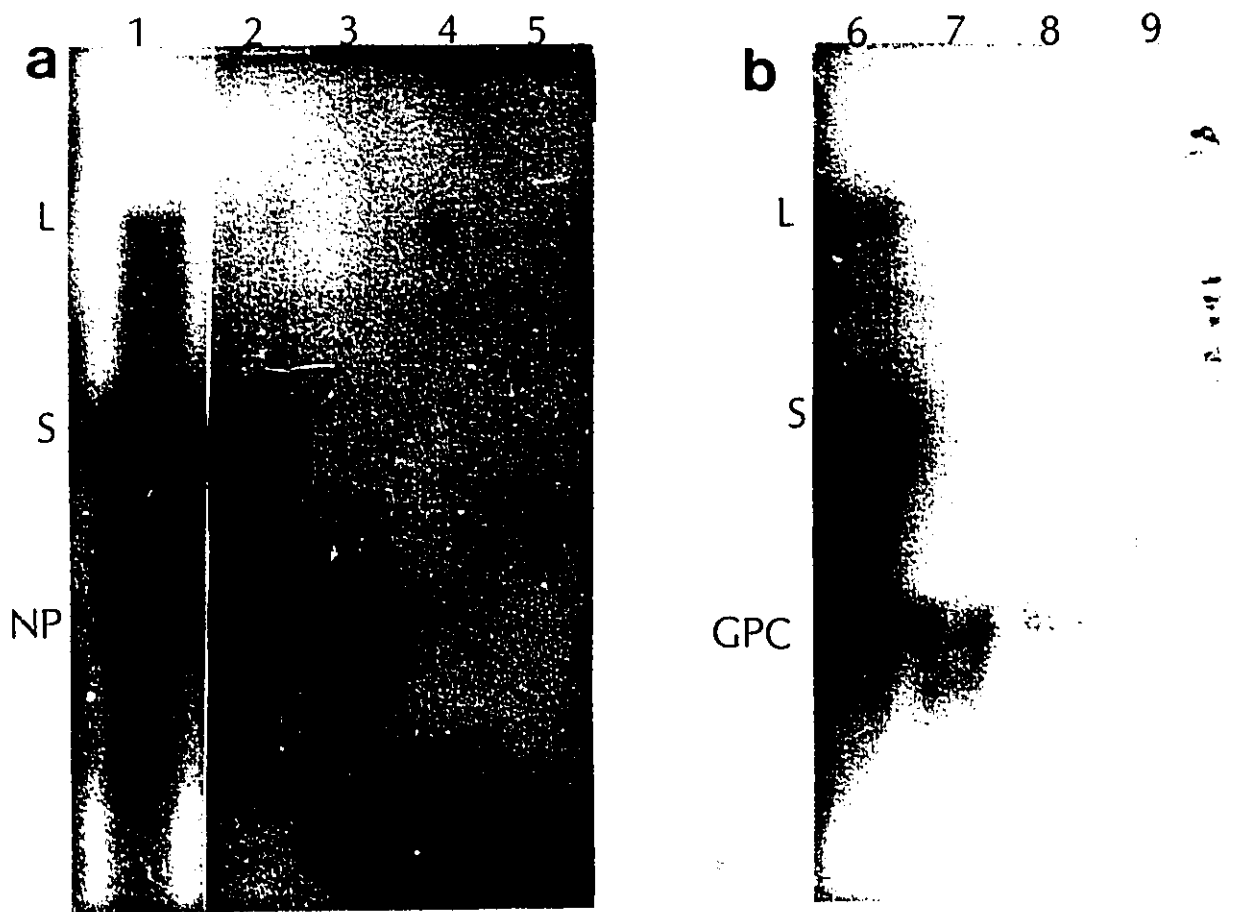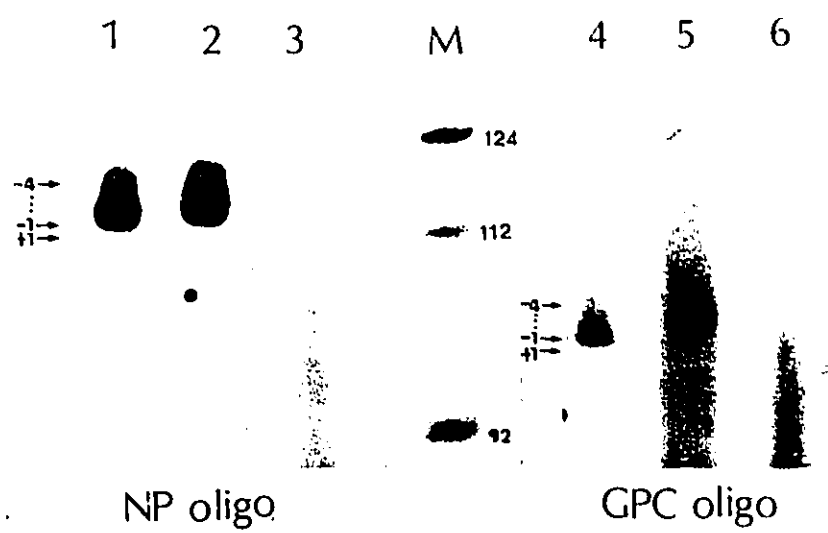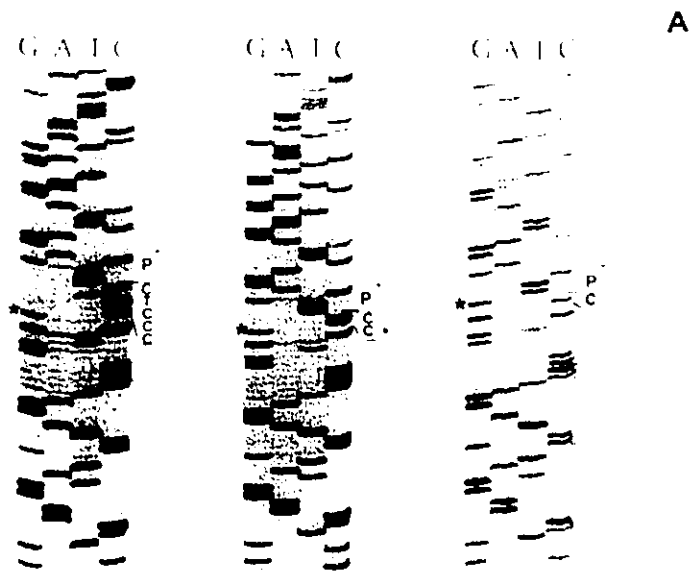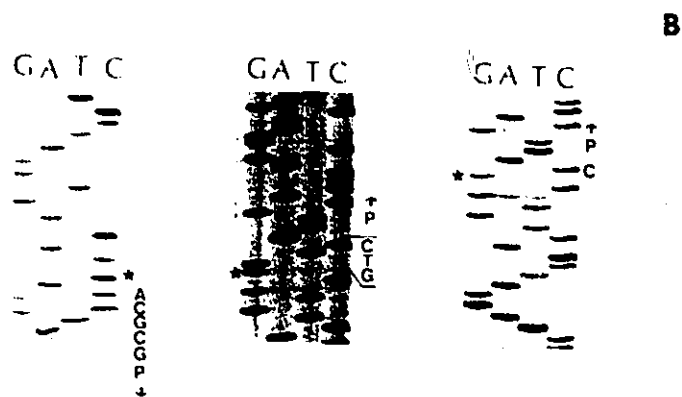
TABLE 1

A:

	GENOMIC 5' SEQUENCE	NUMBER OF CLONES

Cell RNA

```
        -1 +1
         :  :
            CGCACCGGGG...
         G                      11
        UG                       2
        GG                       2
        CG                       1
     CAGGG                       1
```

GPC mRNA

```
      AACU                       1
      GCAC     .                 1
```

B:

ANTIGENOMIC 5' SEQUENCE

Cell RNA

```
        -1 +1
         :  :
            CGCACAGUGG...
         G                       5
       UGU                       1
       GGU                       1
       CCU                       1
       GAC                       1
       AGC                       1
      GACC                       1
     GACCU                       1
     GCGCA                       2
 GGCGUGGCG                       1
```

NP mRNA

```
       UGU                       1
      GCAG                       2
  GCCCGACG                       2
```

FIGURE LEGENDS

<u>Figure 1</u>: Separation of PV S mRNAs from S nucleocapsids. RNA was isolated from PV infected BHK cells (MOI 1.0) at various times postinfection and PV mRNAs were pelleted through CsCl gradients as described in the Materials and Methods. Equal amounts of duplicate samples of total cellular RNA from infected cells (lanes 2 and 6), and purified viral mRNA at 24 (lanes 3 and 7), 48 (lanes 4 and 8), and 72 hours (lanes 5 and 9) post infection, respectively were separated on denaturing formaldehyde gels, transferred to Nylon membranes and hybridized with either a mix of NP and L probes (a) or GPC and L probes (b). Lane 1 represents 5 μg of purified viral RNA. The positions of L RNA (L), S RNA (S), NP mRNA (NP) and GPC mRNA (GPC) are indicated.

<u>Figure 2</u>: Heterogeneous sequences at the 5′ termini of PV S RNA genomes, antigenomes, NP mRNA and GPC mRNA. Total cellular RNA 24 hours post infection (lanes 1 and 4), purified viral mRNA 24 hours post infection (lanes 2 and 5), or mock infected total cellular RNA at 24 hours (lanes 3 and 6) were extended with an NP oligonucleotide (vs 3300-3319; labelled as NP oligo in lanes 1-3) or a GPC oligonucleotide (vc 3319-3328; labelled as GPC oligo in lanes 4-6) with AMV reverse transcriptase. cDNA molecules were separated on 6% acrylamide/7M urea gels. The marker positions of pBR322 DNA digested with Hpa II and labelled with [α^{32}P] dCTP and Klenow fragment are indicated. +1 represents the known 5′ termini of PV S RNAs, obtained by performing sequencing reactions on cloned NP and GPC DNAs with the

respective oligonucleotides as listed above. -1 and -4 represent PV RNAs whose termini are one and four nucleotides longer than the known 5' termini of PV RNAs, respectively.

Figure 3: PV S RNAs contain nontemplated bases at their 5' termini. Representative sequencing gels of cloned PV S RNA termini are depicted. Part A shows the sequences of three genomic sense 5' termini and Part B shows three antigenomic sense 5' termini. The asterisk indicates the position of the terminal base of the prototype sequence of Auperin and coworkers (1984b). Bracketed sequences depict the presence of nontemplated bases, while P followed by an arrow indicates the primer sequences used in the PCR reaction.

Table 1: Nontemplated bases at the 5' termini of PV S RNAs.

The known 5' genomic sense terminus (A) and antigenomic sense terminus (B) as given by Auperin and others (1984b) is depicted in bold. By definition, the termini start at position +1. Nontemplated bases are aligned with the respective termini. -1 represents the first nontemplated base beyond the known terminus. Cell RNA indicates that the clones were derived from PV infected total cellular RNA, while the respective viral mRNAs were derived by sedimentation in CsCl gradients.

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CHAPTER FIVE

5. DISCUSSION

5.1 SUMMARY

Chapters 2 and 3 addressed the issues concerning PV infection and repliction in cells of the monocytic lineage. Human PBMs supported high levels of PV replication as detected by immunoprecipitation SDS-PAGE, immunofluorescence, and virus titre assays. HL60 promyelocytic cells did not support PV replication whether cells were differentiated to macrophages with phorbol ester or not. Human THP-1 cells supported PV replication when the cells were induced to differentiate to macrophages with PMA. The effect of PMA in the induction of THP-1 cells with respect to supporting PV replication was dependent on protein kinase C activation and changes in host cell transcription. Untreated THP-1 cells only supported low levels of PV replication. The restriction of PV replication in THP-1 cells was not related to differences in PV binding and internalization, or degradation of input virus S RNA. Virus protein and RNA synthesis, however, was limited in the untreated THP-1 cells. It was concluded from these studies that the restriction of PV replication in THP-1 cells occurred at, or just prior to the transcription of PV mRNAs.

Chapter 4 presented an analysis of the 5' termini of PV S RNAs. This was performed in order to gain insight into mechanisms of PV transcription and replication initiation. The 5' terminus of every PV S RNA sequenced contained at least 1 nontemplated nucleotide. NP and GPC mRNAs contained from 4-8

nontemplated bases at their 5' termini, while S RNA genomes and antigenomes may have contained a single nontemplated G nucleotide. To account for the apparent difference in the number of nontemplated bases between PV mRNAs and full length S RNA genomes and antigenomes, two different models were proposed. A model of cap snatching was invoked to explain the addition of the extra bases to the 5' termini of PV mRNAs, while one of "polymerase slippage", previously proposed for TAC (Garcin and Kolakofsky, 1990), was proposed to explain the addition of single G nucleotides to the 5' termini of PV genomes and antigenomes.

Each of these components of this thesis will be discussed in turn.

CHAPTERS 2 AND 3

5.2 RESISTANCE OF HL60 CELLS TO PV INFECTION

HL60 human leukemia cells were not susceptible to infection and replication with PV. Resistance to PV infection also occurred when HL60 cells were induced to differentiate to macrophages with PMA. However, human promonocytic THP-1 cells could support high levels of PV replication only if the cells were induced to differentiate to macrophages. Macrophages derived from PMA treated HL60 and THP-1 cells have been shown to possess all the characteristics of human PBMs including monocyte-specific CD14 antigen expression, non-specific esterase staining, macrophage morphology, phagocytic activity, lysozyme production, F_c receptors, and adherence to plastic (Collins, 1987; Tsuchiya et al., 1980, 1982). Thus, both THP-1 and HL60 derived

macrophages may possess similar phenotypes. However, the two cell lines clearly differ in their ability to support PV replication. The reason for this differential susceptibility to PV infection is not known. THP-1 cells appear to be solely restricted to the monocytic lineage whereas HL60 cells are multipotential in that they differentiate to granulocytes, eosinophils and basophils under certain conditions (Collins, 1987; Hutt-Taylor et al., 1988). The difference in differentiation potential of HL60 cells and THP-1 cells may contribute to the differential susceptibility of the cell lines to PV infection by as yet undefined mechanisms. For example, HL60 cells may not express the receptor for PV under any in vitro conditions. In addition, HL60 cells may not express a factor that is required for efficient replication of PV even after PMA induced differentiation to macrophages. As discussed previously, undifferentiated THP-1 cells may not express a factor required for PV replication unless macrophage differentiation the cells occurs. Moreover, the restriction of replication of PV in HL60 cells may be unique to this cell line. In this regard, HL60 cells are also resistant to cytomegalovirus replication, even if the cells are induced to differentiate to macrophages by PMA (Weinshenker et al., 1988).

Alternatively, HL60 cells may mount a more potent cellular antiviral response than PMA treated THP-1 cells when infected with PV. Perhaps the best characterized cellular antiviral responses are those induced by the interferons (IFN-α, β, and γ). The human double-stranded RNA-activated protein kinase is one mediator of the antiviral response induced by interferon.

The protein is 68 kDa in size (p68), 551 amino acids long and has been shown to possess all the conserved consensus domains that are characteristic of serine-threonine protein kinases (Meurs et al., 1990). p68 is activated when it binds double-stranded (ds) RNA formed during the replication of a number of viruses, and in the presence of ATP becomes autophosphorylated on serine and threonine residues. Autophosphorylation of p68 is believed to be required before the kinase can phosphorylate its major target, the α-subunit of eukaryotic initiation factor 2 (eIF-2) (Hovanessian, 1989). Phosphorylation of eIF-2 traps the factor in an eIF-2-GDP-eIF-2B complex, which prevents recycling to eIF-2-GTP. eIF-2 recyling is inhibited because the guanine nucleotide exchange factor, eIF-2B, is trapped in the eIF-2-GDP-eIF-2B complex in its inactive form (Konieczny and Safer, 1983; Safer, 1983; Panniers and Henshaw, 1983). These changes result in reductions of active eIF-2-GTP-Met-tRNA which mediates transfer of the initiator Met-tRNA to the 40S ribosomal subunit, and as a consequence, protein synthesis initiation is profoundly reduced. Because a number of viruses produce dsRNA intermediates during viral RNA replication that can activate p68, some viruses have evolved mechanisms which inhibit the kinase, thus allowing the virus to escape the inhibitory effects on protein synthesis mediated by p68 activation. For example, adenovirus VAI RNA binds to and inactivates p68 (Katze et al., 1987), the tat gene product of HIV downregulates p68 expression (Roy et al., 1990), while influenza virus induces an endogenous cellular inhibitor of p68 (Lee et al., 1990). Poliovirus

also escapes the action of p68 by directly degrading the kinase (Black et al., 1989). Thus, a number of viruses have evolved unique mechanisms to deal with the inhibitory effects of p68 activation on viral protein synthesis. At present, it is not known if arenavirus infections result in the induction and activation of p68, nor if arenaviruses employ any strategies to deal with protein synthesis inhibition in the context of activated p68. However, if HL60 cells expressed p68 upon infection with PV, then the restriction of PV replication in these cells could be due to an inhibition of PV protein synthesis. Another protein induced by interferon is 2,5 Oligoadenylate Synthetase (2,5 OS). 2,5 OS is also activated by dsRNA produced during viral infection and catalyzes the synthesis of oligonucleotides with the general structure $ppp(A2'p5')_nA$, where n is usually greater than 2 (Samuel, 1991). These oligonucleotides are abbreviated 2,5A. The synthesis of 2,5A results in the activation of a cellular endoribonuclease termed RNase L. RNase L cleaves both cellular and viral single stranded RNAs (Samuel, 1991). Thus, PV replication may not have occurred in HL60 cells due to the activation of 2,5A OS which would result in the degradation of PV RNAs. Such a RNAse activity could also contribute to the restriction of PV replication in untreated THP-1 cells. Hence, the presence of a heightenend interferon-induced antiviral response in HL60 cells may provide an explanation for the lack of PV replication in HL60 cells.

5.3 PROTEIN KINASE C AND PV REPLICATION IN THP-1 CELLS

Chapter 2 indicated that THP-1 cells require PKC activation and

differentiation to macrophages in order to support PV protein synthesis and progeny virion production to high levels. This is based on the observation that PV protein synthesis in PMA treated THP-1 cells was inhibited in the presence of H7, an inhibitor of PKC. However, H7 not only inhibits PKC, but it also inhibits cyclic AMP and cyclic GMP- dependent protein kinases, albeit to lower levels (Nixon et al., 1991; Hidaka et al., 1984). Furthermore, data published after the completion of the studies reported in chapter 2 indicated that the structure and biological activities of various commercial sources of H7 differed markedly (Quick et al., 1992). The authors demonstrated that the form of H7 available from Sigma Chemicals was actually an isomer of H7, which placed the methyl group at position 3 of piperazine instead of at position 2 as for the original form of H7 (Figure 3). In the studies reported in this thesis, H7 was obtained from Sigma. Higher concentrations of iso-H7 than H7 were required to inhibit purified rat brain PKC ($\geq$ 100μM) (Quick et al., 1992). This may help to explain the higher concentrations of H7 required to inhibit PV replication in PMA treated THP-1 cells. However, the observations that i) PMA stimulation replaces the activation of PKC by membrane associated diacylglycerol (Kikkawa et al., 1983), ii) PV protein synthesis in PMA treated cells is inhibited in the presence of iso-H7, and iii) PV protein synthesis occurs in THP-1 cells pretreated with synthetic diacylglycerol, suggest that PKC activation is required for the replication of PV in THP-1 cells. In addition, PKC activation in THP-1 cells is required in order for the cells to differentiate to macrophages. In fact,
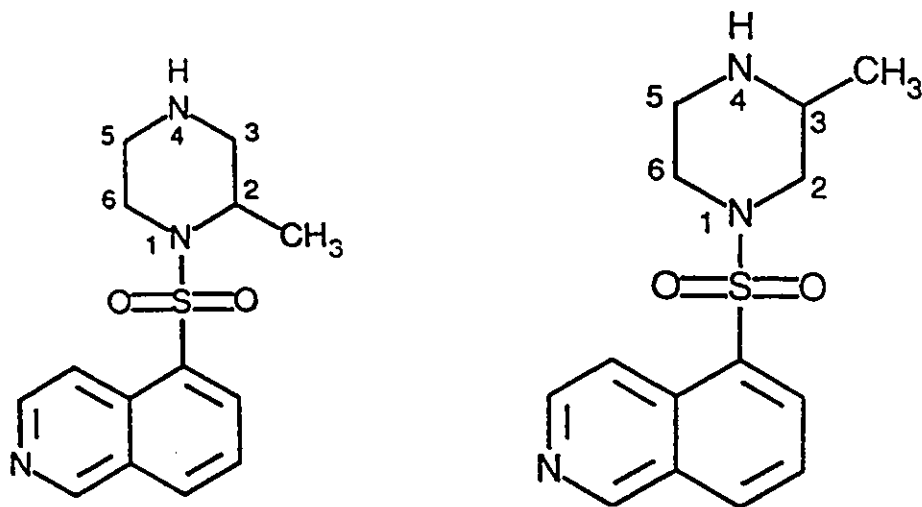
FIGURE 3: The structures of H7 (left) and iso-H7 (right) indicating the location

of the methyl group.

at the higher concentrations of iso-H7 tested, morphological differentiation of

THP-1 cells did not occur. Although PV protein synthesis occurred in diC8

treated THP-1 cells, the level of synthesis was reduced when compared to the

level of PV protein synthesis in PMA treated THP-1 cells, and macrophage

differentiation of diC8 treated THP-1 cells did not occur at any concentration

tested.   This is similar to what was observed during PMA induced

differentiation of HL60 cells. It was demonstrated that PMA stimulation of

HL60 cells induces a sustained activation of protein kinase C that results in

translocation of PKC from the cytosol to membrane portion of the cells (Aihara,

et al., 1991). diC8, however, was shown to be metabolized very quickly by

the cells and did not substantially activate PKC or induce monocytic

differentiation of the cells (Aihara et al., 1991). In these studies, the α-

subspecies of PKC was implicated in the differentiation of HL60 cells to

macrophages because this species was depleted in PMA treated cells to the

highest degree. However, these studies contrast with another study which also

examined PKC isozyme gene expression during PMA induced macrophage

differentiation of HL60 cells. The levels of PKC-β isozyme were shown to be

deficient in cloned variants of HL60 cells that were resistant to PMA induced

macrophage differentiation (Tonetti et al., 1992). The involvement of the β-

subspecies of PKC was therefore implicated in the differentiation of HL60 cells

to macrophages. It is likely that differentiation of HL60 cells (and THP-1 cells)

requires the activation of several PKC subspecies. In fact, there are eight

isozymes of PKC that have been cloned and biochemically characterized. Therefore, it is suggested that PKC activation is required for both efficient PV replication in THP-1 cells and differentiation of THP-1 cells to macrophages. This suggestion also helps to explain why PV protein synthesis was lower in THP-1 cells treated with diC8 as compared to cells that were treated with PMA. Because suboptimal activation of PKC was achieved by the use of diC8, differentiation of THP-1 cells to macrophages did not occur and hence high levels of PV protein synthesis were not observed.

5.4 THE MECHANISM OF PV ENTRY INTO THP-1 CELLS

One conclusion from the studies reported in this thesis is that PV enters cells through endocytosis into acidic vacuoles. In general, viruses which employ this mechanism of entry into cells do so by the following mechanism. Firstly, virus binding to the target cell via specific cell surface receptor interaction with virus coat proteins occurs. Secondly, the bound virus particles are endocytosed when the virus-receptor complexes are aggregated into coated pits. The lumen of the endosome is acidified by an ATP-dependent proton pump. However, the virus has still not reached its destination as it must then be released into the cytoplasm. This is accomplished by the viral coat proteins, which, in the presence of the acidic endosomal environment undergo structural changes which result in fusion of the coat proteins with the inner membrane of the endosome. The virion membrane becomes fused with the endosomal membrane and the contents of the virion are released into the cytoplasm of the

infected cell (reviewed in Kielian and Junerwirth, 1990).

5.4.1 Fusion in Other Virus Systems

Studies with Semliki Forest virus (SFV) and Influenza virus have provided valuable information concerning the virus proteins involved in fusion with endosomal membranes (Kielian and Junerwirth, 1990). For SFV, the fusion protein is contained within the spike protein found on the membrane of SFV virions. The spike is composed of two proteins, E1 and E2. E1 contains a hydrophobic stretch of 20 amino acids near its amino terminus that is highly conserved among all alphaviruses and is believed to be contain the domain for fusion. Numerous studies indicate that in an acidic environment (pH 6.2), E1 and E2 undergo irreversible conformational changes. These changes result in the fusion of E1 with artificial liposome membranes. These data have been supported by mutants of E1 which map to the fusogenic domain and display poor fusogenic activity and reduced neurovirulence in mice. Fusion activity in Influenza virus resides in the hemagglutinin protein (HA) found on the surface of influenza virions. HA is produced as a precursor protein which is cleaved to yield HA1 and HA2. The crystal structure of this molecule has been solved and the following picture of the structure of HA on the surface of virions has emerged. HA1 is found furthest away from the membrane and contains a globular head which permits binding of virions to the cellular receptor for influenza virus, sialic acid. Found under HA1 is HA2, which contains the hydrophobic fusion domain near its amino terminus. HA2 is anchored in the

viral membrane by a transmembrane domain. Therefore, in the native structure of HA the hydrophobic fusion domain on HA2 is hidden beneath HA1. It has been demonstrated that upon binding to cells via the sialic acid-HA1 interaction, Influenza virions are endocytosed in acidic vesicles. The low pH of the endosome induces an unfolding of the HA molecule which exposes the hydrophobic amino terminus of HA2. Fusion of HA2 with endosome membrane then occurs (Kielian and Junerwirth, 1990).

5.4.2 Arenavirus Fusion

The molecular mechanisms by which arenaviruses fuse with endosomal membranes are not well characterized. From the above discussion, however, it is hypothesized that the fusion domain lies in the glycoproteins of PV, GP1 and GP2. GP1 may be responsible for interaction with cell membranes and endocytosis of virions into acidic vesicles due to the observation that it is a peripheral protein on the membrane of LCM virions (Burns and Buchmeier, 1991). GP2 is hypothesized to contain the characteristic hydrophobic domain which mediates fusion of PV membrane with the endosome membrane. This is based on the recent observation that GP1 and GP2 appear to combine in the form of native homotetramers to form the glycoprotein spike of arenaviruses and that GP2 is an integral membrane protein which is partly shielded by GP1 (Burns and Buchmeier, 1991; 1993). In support of this, it has recently been demonstrated that a hydrophobic domain conserved between Lassa virus, LCMV, TAC, and PV spanning 23 amino acids in Lassa virus GP2 may contain

fusogenic activity (Glushkova et al., 1992). A synthetic peptide corresponding

to this region was capable of inducing fusion of liposome membranes. Fusion

activity occurred at pH 4.5-5.5 indicating the requirement for low pH. Further

investigation into arenavirus fusion must be undertaken to conclusively

determine if GP2 is involved in the fusion process. In particular, experiments

examining fusion of purified or recombinant GP1 and GP2 or purified arenavirus

particles with liposomes could be undertaken.

5.5 HOST CELL FUNCTIONS IN PV REPLICATION

5.5.1 The Effects of Actinomycin D

As discussed in Chapter 4, arenavirus replication is inhibited by the

cellular transcription inhibitor, actinomycin D. These data were used to

strengthen the hypothesis that PV initiates transcription by cap snatching and

also helps to explain the apparent requirement for nuclear function in arenavirus

replication. However, there may be additional host cell functions required for

arenavirus replication that are also susceptible to inhibition by actinomycin D.

Actinomycin D inhibits DNA polymerase 1 and DNA polymerase 2-mediated

transcription of host cell genes. The 18S, 5.8S, and 28S rRNA genes are

transcribed by DNA polymerase 1, while those genes transcribed by DNA

polymerase 2 include most other precursor mRNAs termed heterogenous

nuclear RNA (hnRNA) that are processed into mature mRNAs (Zubay, 1983).

Thus, ribosomal RNAs and proteins as well as other host cell mRNAs and

proteins may be required for the replication of PV. To date there has only been

evidence to suggest an association of ribosomal components with the replication of negative stranded RNA viruses. Indeed, NS$_s$ protein of Uukuniemi bunyavirus is associated with the 40S ribosomal subunit in a baculovirus expression system (Simmons et al., 1992), and Germiston and La Cross bunyaviruses require an active 40S ribosomal subunit for viral polymerase activity (Vialat and Bouloy, 1992; Bellocq and Kolakofsky, 1987; Bellocq et al., 1987). The 40S ribosomal subunit contains the rRNA as well as a number of proteins. Interestingly, the 40S ribosome subunit is involved in translation initiation (Moldave, 1985). These studies in the bunyavirus system indicate the requirement for ongoing translation in in vitro transcription systems and suggest that the 40S ribosomal subunit prevents transcription termination and allows bunyavirus polymerase to continue transcribing to generate antigenomic molecules (Vialat and Bouloy, 1992). It should be noted that despite the suggestions that ribosomal RNAs and proteins may be required for arenavirus replication in infected cells, the ribosomes that are found within arenavirus particles are not required for virions to be infectious. This is based on the observation that when PV is passaged in cells that contain a temperature sensitive mutation for ribosome function, the virions which are produced contain the ts mutant ribosomes and are still capable of replication at the non-permissive temperature (Leung and Rawls, 1977). In addition, arenavirus particles produced in cells treated with low concentrations (50 ng/ml) of actinomycin D do not contain 28S and 18S rRNAs but are still infectious

(Pederson et al., 1971; Carter et al., 1973). This finding most likely distinguishes incorporation of ribosomes into virions during packaging from a possible requirement for cellular ribosomes during viral replication. In order to elucidate potential effects of cellular mRNAs in PV replication an assessment of the effect of the DNA polymerase 2 specific inhibitor, α-amanitin, on PV replication would be required. There is only one study which has reported that α-amanitin together with amphotericin B inhibits PV protein synthesis in a 6 hour pulse-chase experiment (Harnish, 1982), suggesting that cellular mRNAs and proteins may be involved in PV replication.

The data presented in Chapters 2 and 3 suggest that host cell nuclear function is required for PV replication in THP-1 cells. This is mainly derived from the observation that treatment of THP-1 cells with Actinomycin D prior to stimulation with PMA and infection with PV greatly inhibits viral replication. Higher concentrations of actinomycin D also inhibited macrophage differentiation of PMA treated THP-1 cells. Accordingly, it is proposed that PMA treatment of THP-1 cells results in the expression of factors that are prerequisite for PV replication and differentiation of the cells to macrophages.

CHAPTER 4

5.6 NONTEMPLATED BASES ON PV S RNAs

5.6.1 Universality of PV Transcription and Replication Strategies

Cloning of PV S RNA termini revealed the presence of nontemplated bases beyond the known 5' end. As discussed in Chapter 4, these extra bases

presumably represent the products of PV transcription and replication initiation. The mechanisms involved in the generation of the nontemplated bases are predicted to occur in all cell types that are infected by PV. The mechanisms involved are hypothesized to be generated by the PV polymerase complex and are therefore virus specific. Thus, in PV infected PMA treated THP-1 cells, the same mechanisms of transcription and replication initiation are believed to occur as in PV infected BHK cells. However, untreated THP-1 cells do not support PV replication and the earliest detectable point at which the restriction lies is at primary transcription of NP mRNAs. Note, however, that the studies performed in chapters 2 and 3 cannot discount potential components of the restriction that occur during uncoating of viral RNA or after viral transcription and replication. Thus, in contrast to transcription and replication which is a virus specific event, the restriction of PV replication is a host cell specified event that is removed by differentiation of THP-1 cells to macrophages with PMA. It is conceivable that part of the restriction may relate to a host cell factor that facilitates initiation of transcription but is lacking in untreated THP-1 cells. Alternatively the restriction may involve a host cell factor that inhibits PV transcription initiation and this factor is downregulated during PMA induced differentiation of THP-1 cells. Whether or not this putative host cell factor is associated with the polymerase complex is not known. As will be discussed, there is some precedence for host cell proteins as components of RNA dependent RNA polymerases in other RNA viruses.

5.6.2 Nontemplated G Nucleotide on PV S Genomes and Antigenomes

In the initial cloning studies of PV and Lassa S RNAs there was ambiguity at the 5′ termini of these molecules. It was demonstrated that the 5′ termini of PV and Lassa arenavirus S RNA genomes and antigenomes contained an extra G nucleotide (Auperin et al., 1984b, 1986). When the 5′ ends of TAC S RNAs were cloned, an extra G nucleotide was found beyond the predicted 5′ terminus of TAC S genomes and antigenomes (Raju et al., 1990). To explain the mechanism of generation of this extra nucleotide, a novel mechanism of genome replication initiation was forwarded (Garcin and Kolakofsky, 1990). The model assumes that a purine nucleotide is used for initiation in keeping with the fact that all known viral polymerases initiate with purine. Because of this assumption, the model postulates that TAC polymerase initiates replication with the nucleotide GTP at position +2 on the 3′ end of PV genomes and antigenomes, and base pairs with the C base found at this position. The exact 5′ terminus is defined as +1 and any bases downstream of this base (ie 5′-3′) are preceeded by a "+" sign, while those bases upstream are preceed by a "-" sign. Upon incorporation of the next base in the growing transcript (based on TAC S RNA sequence, this would be a C), the polymerase slips backward by two bases such that the initiating pppG now appears to be nontemplated and is found at position -2. Because most of the 3′ termini of arenavirus RNAs end with the same sequence (3′ GCG...) the model may be applicable to arenavirus replication initiation in general. This model can be thought of as a form of

pseudo-templated synthesis (Garcin and Kolakofsky, 1990). An in vitro replication system has also been established to further substantiate the mechanism of initiation. It has been shown that the dinucleotide GpC is capable of priming TAC S RNA genome and antigenome synthesis to a higher level as compared with other primers (Garcin and Kolakofsky, 1992). When GpC is used as a primer, genomes and antigenomes extend to position -1 and the base found at this position is invariably a G nucleotide.

5.6.3 Cap Snatching for PV Transcription Initiation?

It was suggested in Chapter 4 that PV may initiate transcription by a cap snatching mechanism. Alternatively, the PV polymerase may generate the nontemplated bases using short, cellular derived oligoribonucleotides. The cap structure could then be added by virus-encoded guanylytransferase and methyltransferase activities. The PV polymerase could also synthesize the short 5' extensions in the absence of a template and then modify the 5' terminus by guanylylation and methylation as described above. Thus, the data in Chapter 4 cannot formally exclude these possibilities. However, there is evidence from other arenavirus systems that suggests that the mechanism of arenavirus transcription initiation involves cap snatching. This is based on the observation that TAC GPC and NP mRNAs contain nontemplated bases at their 5' termini and that a proportion (approximately 30%) of the S mRNAs are selectable with anticap antiserum (Garcin and Kolakofsky, 1990). In addition, the 5' termini of LCMV NP and GPC mRNAs possess 1 to 7 nontemplated

nucleotides that contain a methyl cap structure (Meyer and Southern, 1993).

Further evidence for the existence of such a mechanism for arenavirus

transcription initiation is provided by studies which have examined the effect

of certain antiviral compounds on arenavirus replication (Andrei and De Clercq,

1990). Inhibitors of the enzyme S-Adenosylhomocysteine (AdoHcy) Hydrolase

have been examined for inhibition of Junin and TAC replication in Vero cells

(Andrei and De Clercq, 1990). AdoHcy is required to hydrolyze S-

adenosylhomocysteine which is produced when S-adenosylmethionine (SAM)

donates its methyl group to a number of cellular acceptors for methyl groups

which is catalyzed by methyltransferase. S-adenosylhomocysteine must be

hydrolysed because its presence is inhibitory to the methyltransferase reaction.

A number of cellular products require methylation for proper activity including

the 5' cap structure of mRNAs. Figure 4 depicts the steps in the formation of

the cap structure. Because S-adenosylmethionine is the principal methyl group

donor for these reactions, it could be envisaged that disruption of this process

by specific inhibitors would disrupt the methylation of the 5' cap structure of

mRNAs. Thus, the use of inhibitors of AdoHcy could be predicted to inhibit the

replication of viruses which require the presence of 7 methylguanosine on the

5' ends of virus mRNAs. This is exactly what is observed in the case of

Vaccinia virus (poxviridae), Rhabdoviruses and Paramyxoviruses. These viruses

encode their own methyltransferase activities and the viral mRNAs which are

produced upon infection by these viruses have 5' methylguanosine caps

$pppN_1N_2N_3 \cdots$

RNA triphosphatase

$\rightarrow P_i$

$ppN_1N_2N_3 \cdots$

GTP

mRNA guanylyltransferase

$\rightarrow PP_i$

$G(5')pppN_1N_2N_3 \cdots$

mRNA (guanine-7-) methyltransferase

S-Adenosylmethionine (methyl donor)

S-Adenosylhomocysteine

$m^7G(5')pppN_1N_2N_3 \cdots$

mRNA (nucleoside-2'-) methyltransferase

S-Adenosylmethionine

S-Adenosylhomocysteine

$m^7G(5')pppN_1mN_2N_3 \cdots$

Transport to cytoplasm

mRNA (nucleoside-2'-) methyltransferase

S-Adenosylmethionine

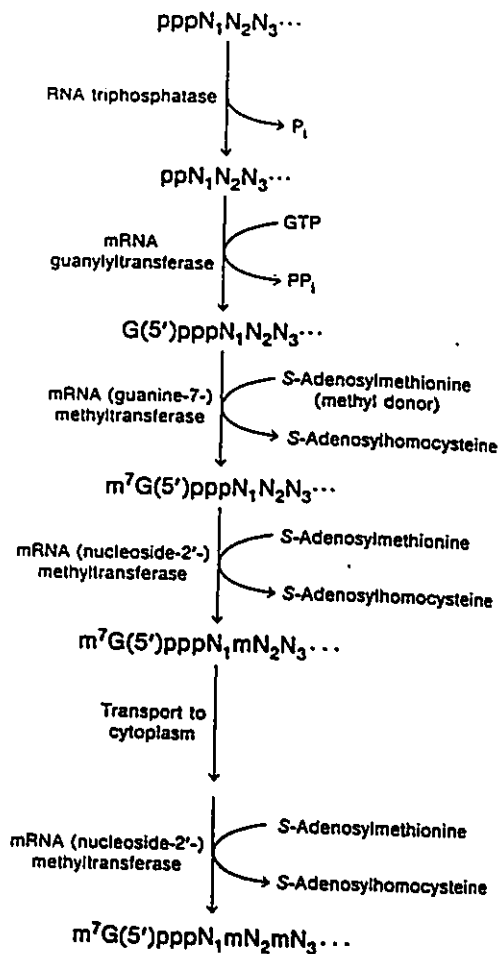S-Adenosylhomocysteine

$m^7G(5')pppN_1mN_2mN_3 \cdots$

FIGURE4 : The proposed mechanism of 5' 7-methylguanosine cap formation on cellular mRNAs. Note the involvement of methyl transfer from the methyl group donor, S-Adenosylmethionine in 3 reactions. Note also that S-Adenosylhomocysteine is an end product of 3 reactions. Its presence is inhibitory to the methyltransferase reaction. From Zubay (1983).

(DeClercq, 1987). In the study which examined the replication of TAC and Junin virus in Vero cells in the presence of AdoHcy inhibitors it was found that viral replication was severely limited (Andrei and De Clercq, 1990). By analogy with the virus replication cycles which are inhibited by AdoHcy inhibitors, it is suggested that arenavirus replication requires the production of 7-methylguanosine caps. Thus, these data also strengthen the hypothesis that arenavirus transcription initiation involves the stealing of the 5' cap structure from cellular mRNAs. Furthermore, the studies reported in this thesis and the studies outlined above indicate that transcription of GPC and NP mRNAs initiates at the exact 3' terminus of the viral RNA template.

5.6.4 Cap Snatching in Other Negative Strand Viruses

Cap-snatching has been well characterized in the influenza virus system (reviewed in Krug, 1981) and bunyavirus system (Bouloy, 1991; Kolakofsky and Hacker, 1991). The viral RNA dependent RNA polymerase possesses an endonuclease activity which recognizes the cap and cleaves the cellular mRNA at a defined distance downstream. For influenza virus, the host cell mRNA is cleaved 10-13 nucleotides from the cap (Krug, 1981) while cleavage occurs at 13-18 nucleotides from the cap in bunyavirus (Bouloy et al., 1990; Ihara et al., 1985; Bishop et al., 1983). For influenza virus, it has been shown that cap recognition and endonucleolytic cleavage is mediated by the influenza virus PB2 protein which is part of the polymerase complex (Ulmanen et al., 1981, 1983; Blaas et al., 1982; Braam et al., 1983; Nichol et al., 1981). The bunyavirus

polymerase has also been shown to possess a cap-dependent endonuclease activity (Patterson et al., 1984; Jin and Elliot, 1993). These capped mRNA termini then serve as primers and are extended by PB1 protein. It has also been shown in in vitro transcription and translation systems that capped globin mRNA primers are transferred to both influenza virus and bunyavirus mRNA 5' termini (Bouloy et al., 1978; Plotch et al., 1979; Shaw and Lamb, 1984; Vialat et al., 1992). Thus, the predictions of the cap snatching model have been experimentally verified for influenza virus. However, such studies have not yet been performed on arenaviruses.

5.6.5 Functions of Cap Snatching

The acquisition of a 7-methylguanosine cap by the PV polymerase serves a number of functions. First, as discussed above, the cap structure plus the additional nontemplated nucleotides serves as a primer for viral transcription. Second, the cap also serves to direct ribosomes to assemble on the nascent mRNA transcript and thereby permit preferential translation of virus proteins. Binding to the cap is mediated by eukaryotic initiation factor 4E (eIF-4E), which is a component of the 40S ribosomal preinitiation complex (reviewed in Moldave, 1985). The interaction of eIF-4E with the cap structure may therefore help to explain the apparent requirement for ongoing translation in negative strand virus in vitro transcription systems (see below). Third, the presence of the cap also serves to increase the half-life of virus mRNAs by protecting the transcript from 5' exonucleolytic activity. In this respect, the

cap structure has been shown to confer resistance to cellular nucleases (Furuichi et al., 1977; Shimotohno et al., 1977).

5.6.6 Implications of Cap Snatching

If cap snatching is proven to be the mechanism by which arenaviruses initiate transcription, then this reaction might be a susceptible target for the design of antiviral compounds. Indeed, certain uncapped ribonucleic acid polymers have been shown to inhibit the cap snatching reaction of influenza virus (Krug, 1981). As discussed above, inhibitors of S-adenosylhomocysteine hydrolase inhibit the replication of arenaviruses. The design and use of such inhibitors would certainly assist in the characterization of arenavirus transcription initiation and could be of great therapeutic value in the control of arenavirus induced illness. The great advantages of AdoHCy hydrolase inhibitors are the lack of cytotoxicity as assessed by cell morphology and DNA synthesis when tested at concentrations 10-40 times higher than the concentrations which effectively inhibit arenavirus replication in vitro (Andrei and De Clercq, 1990).

5.7 A MULTICOMPONENT PV POLYMERASE

The PV polymerase and all arenavirus polymerase molecules are hypothesized to consist of multiple subunits. In this regard the polymerases of all negative strand viruses studied to date consist of at least 2 or 3 proteins. For example, the polymerase of Rhabdoviruses and Paramyxoviruses consist of L protein (the RNA dependent RNA polymerase) and P protein which is a

transcription factor (Banerjee et al., 1991). The polymerase of Influenza virus (Orthomyxoviridae) consists of the PA, PB1 and PB2 protein. PA is the polymerase, while PB2 is involved in cap snatching and PB1 in extension of capped primers (Krug, 1981). Recent studies with TAC arenavirus indicate that Z protein is required for in vitro transcription and replication (Garcin et al., 1993). Thus, Z protein may also be a component of the TAC polymerase machinery. This is strengthened by zinc-finger binding motifs found in the Z protein amino acid sequence. Such motifs are found in the class of transcription factors termed zinc-finger binding proteins (Evans and Hollenberg, 1988). In addition, host cell proteins may be a component of the arenavirus polymerase complex, by analogy with the bacteriophage, $Q\beta$, and the plant RNA bromovirus, brome mosaic virus (BMV). The RNA dependent RNA polymerase of $Q\beta$ consists of five protein subunits. Four of the subunits are derived from E. coli and one subunit is virus-encoded. The host cell components include the ribosomal protein S1 (Groner et al., 1972), the translation elongation factors EF Tu and EF Ts (Blumenthal et al., 1972), and a newly cloned host factor-1 (Kajitani and Ishihama, 1991). S1 is a component of the prokaryotic 30S ribosomal subunit of E.Coli and is thought to facilitate the interaction between the $Q\beta$ polymerase and $Q\beta$ RNA (Blumenthal and Carmichael, 1979). EF Tu normally functions in protein synthesis as a transfer RNA (tRNA) binding factor. Because the 3' end of $Q\beta$ RNA has the capacity to form secondary structure resembling tRNA, EF Tu has been hypothesized to

bind the 3' end of Qβ RNA and hence bring the Qβ polymerase into the correct position on the viral template (Blumenthal and Carmichael, 1979). More recently, the translation factor eIF-3 has been shown to be stably associated with the RNA dependent RNA polymerase of BMV (Quadt et al., 1993). eIF-3 is required for ribosome dissociation and the formation of the eukaryotic protein synthesis 40S preinitiation complex (Moldave, 1985). Therefore, the association of host cell derived translation factors with the RNA dependent RNA polymerases of Qβ and BMV is suggestive of common feature of replication by positive strand RNA viruses. By extension, the replication of arenaviruses and other negative strand RNA viruses may involve the association of cellular translation factors with the viral polymerases or other virus proteins. This prediction is based on several pieces of evidence. First, as discussed above, there is a requirement for ongoing translation in in vitro bunyavirus transcription systems which reflects the need for an intact 40S ribosomal subunit (Vialat and Bouloy, 1992; Bellocq and Kolakofsky, 1987; Bellocq et al., 1987). Second, an association between NS$_x$ protein of Uukuneimi bunyavirus and the 40S ribosomal subunit has been documented (Simmons et al., 1992). Third, in arenavirus infected cells, ribosomes accumulate in areas of viral replication (Bishop, 1990). Fourth, arenavirus mRNAs contain capped, methylated structures at their 5' end which presumably enhances ribosome association onto the mRNA. Fifth, the protein synthesis preinitiation complex consists of a capped mRNA associated with the 40S ribosomal subunit and

various translation initiation factors. The initiation factors which are of direct relevance to this hypothesis are eukaryotic initiation factors eIF-2, eIF-3 and eIF-4E which are involved in initiator methionine-tRNA binding, association of the 40S subunit with the mRNA, and in cap recognition, respectively (reviewed in Moldave, 1985). Therefore, it is predicted that the translation initiation factors eIF-2, eIF-3 and eIF-4 or others may be components of the arenavirus polymerase either by direct physical incorporation into the polymerase or indirectly through mediating polymerase interactions with other viral proteins. The putative functions of the translation initiation factors in PV replication could be multiple. For instance, the assembly of the 40S preinitiation complex onto a nascent viral mRNA molecule could facilitate the release of newly transcribed mRNA from the viral RNA template. Alternatively, the GTP nucleotide required for arenavirus genome and antigenome replication initiation may be provided by eIF-2 which binds GTP (Moldave, 1985). Because of its role in binding to the 5' 7-methylguanosine cap, eIF-4E could be involved in the cap snatching reaction. Admittedly, this model cannot determine if any of the suggested functions of translation initiation factors in arenavirus replication are encoded within the virus itself.

An intriguing conceptual issue arises regarding the nature of the polymerase or L protein during its transcriptive and replicative modes. It could be argued that these modes are identical and that the switch from transcription to replication is mediated by other viral proteins such as the concentration of

soluble NP. Alternatively, though not mutually exclusive, the different modes could arise as a result of changes in the concentrations of components of the transcriptase complex. Although such issues cannot be clarified until the advent of a suitable in vitro transcription and replication system using recombinant L protein, it can be stated (as this thesis and other studies indicate) that the initiation of transcription and replication appear to be different based on the observation that the 5′ termini of mRNAs and full length genomes and antigenomes appear to be different. This suggests that there may be clear differences between transcriptive and replicative arenavirus polymerases or that the 5′ terminus of an RNA determines whether a mRNA or full length RNA molecule is formed. In this regard it has been demonstrated that the presence of a 5′ terminal cap structure inhibits influenza virus antitermination (Beaton and Krug, 1986).

As indicated in Chapter 3, a partial clone of approximately 600 nucleotides corresponding to the L RNA of PV has been generated in our laboratory. Efforts are currently underway to complete the entire sequence of PV L RNA. At present, 4.8 kb of L RNA have been cloned. When this reagent becomes available, it will provide valuable information with respect to the genes encoding the RNA polymerase of PV (L protein) and the as yet the unidentified Z protein of PV. With the cloning of L RNA, a number of experiments can be performed to examine the function of the proteins encoded by PV. For instance, an in vitro transcription and replication system could be established

using recombinant L, NP, Z, GP1, and GP2 proteins in the presence of purified or recombinant PV S and L RNAs and host cell derived cell extracts. This system would permit an evaluation of the contributions of PV proteins to the activities observed in infected cells. The activities that could be assessed would include the putative endonucleolytic cleavage of cellular mRNAs and the snatching of 7-methylguanosine caps to initiate PV transcription, initiation of PV replication by dinucleotide priming and polymerase slippage, the regulation of the transition from transcription to replication (termed antitermination), and the involvement of host cell derived proteins in PV replication. In particular, the role of THP-1 cell derived factors could be evaluated by assessing PV replication in vitro in the presence of cell extracts derived from untreated THP-1 cells and PMA treated THP-1 cells. In addition, the establishment of an in vitro transcription and replication system would permit studies involving the complementation of temperature sensitive mutants of PV (Shivaprakash et al., 1988).

The cloning of the PV L protein will also reveal aspects of the structure of an RNA dependent RNA polymerase. It will be of interest to determine if PV L protein possesses various motifs conserved among RNA dependent polymerases. In this respect, the L proteins of TAC and LCMV have been shown to possess 4 motifs that appear to be conserved between other negative and positive strand virus RNA dependent RNA polymerases and retrovirus RNA dependent DNA polymerases (Poch et al., 1989). The 4 conserved motifs are

hypothesized to constitute a prerequisite "polymerase module" involved in positioning the polymerase on the RNA template and the activity of the polymerase (Poch et al., 1989). In each of the 4 motifs, there is an invariant aspartic acid, glycine, or lysine amino acid residue. Interestingly, the invariant amino acids are placed at or very near turn structures of the protein, suggesting a conserved general structure for many RNA polymerases. In particular, LCMV and TAC L proteins display regions of amino acid identity over the sequences Asp-His-Ser-Lys-Trp-Gly-Pro (1172-1178 in LCMV L protein, 1189-1195 in TAC L protein) (Poch et al., 1989). The aspartic acid underlined indicates the invariant residue in conserved motif 1. Similarly, in motif 2, the sequence Asp-Met-Gly-Gln-Gly-Ile-Leu-His-Asn is conserved in amino acid positions 1270-1278 in LCMV L and 1288-1296 in TAC L proteins. Again, the invariant glycine amine acid in this domain is underlined. In motif 3, the sequence Tyr-Thr-Ser-Ser-Asp-Asp-Gln is conserved between the two L proteins (amino acids 1301-1307 and 1311-1317 in LCMV and TAC L proteins, respectively), while in motif 4, the sequence Ala-Glu-Phe-Lys-Ser-Arg is conserved (amino acids 1354-1369 and 1364-1369 in LCMV and TAC L proteins, respectively). It will be of interest to determine if PV L protein possesses these conserved motifs. Site-directed mutagenesis of these motifs would determine the relative role of these motifs in arenavirus polymerase functions.

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5.8 THE PV LIFE CYCLE

Based on the data presented in Chapters 1-3, the following represents
a hypothetical summary of the PV infection cycle. Upon binding to the receptor
for PV, virus is internalized into acidic endosomes. At present, the receptor for
PV and other arenaviruses in not known. The acidic environment of the
endosome induces conformational changes in the glycoprotein spike which
contains GP1 and GP2 and results in fusion of the viral membrane and
endosomal membrane. Fusion is hypothesized to be mediated by GP2. Fusion
results in the release of PV RNP into the cytoplasm where transcription of NP
and L mRNAs occurs. This is performed by the PV polymerase complex that
is present in the infectious virions. Undefined host cell factors either by direct
incorporation as a polymerase subunit or by indirect effects on virus proteins
may affect this process. Transcription is initiated at the 3' terminus of the viral
RNA template when the PV polymerase complex removes the 7-
methylguanosine cap and a few (2-9) nucleotides immediately downstream of
the cap from a cellular mRNA. The stolen cap serves as a primer for
transcription initiation which is extended by the PV polymerase using the S and
L RNAs as a template for synthesis. These capped mRNAs are translated by
the cellular ribosome machinery to generate NP and L proteins. As outlined in
the introduction, transcription antitermination then occurs which results in the
production of complementary S and L antigenomes. Initiation of S and L
antigenome synthesis may involve an entirely different mechanism than cap

snatching. One mechanism may be priming by a dinucleotide GpC to generate the single nontemplated G nucleotide at the 5' end of these RNA species. Antigenomes then serve as templates for GPC and Z (not yet demonstrated for PV) transcription which is also initiated by cap snatching. These mRNAs are translated to generate GPC and Z proteins. GPC is translated on the rough endoplasmic reticulum and enters the lumen of the ER by a signal sequence (Burns and Buchmeier, 1993). GPC is then transported in vesicles from the ER to the golgi apparatus where cleavage of GPC to GP1 and GP2 occurs (Wright et al., 1990). At later times post infection, virus assembly is initiated and the progeny virions leave the infected cell by budding from the plasma membrane.

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