# EXPRESSION OF ADENOVIRUS TUMOR ANTIGENS IN E. COLI

# EXPRESSION OF ADENOVIRUS TYPE-5 E1B TUMOR ANTIGENS IN ESCHERICHIA COLI

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

December, 1983

MASTERS OF SCIENCE (1983) McMASTER UNIVERSITY
(Biology) Hamilton, Ontario

TITLE: Expression of Adenovirus type-5 E1b Tumor Antigens in *Escherichia coli*

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NUMBER OF PAGES: x, 102

ABSTRACT

The Ad5 E1B antigens of MW 58000 and 19000 are known to be involved in oncogenic transformation of mammalian cells. To obtain sufficient quantities of these proteins for biochemical studies on their mechanism of action, I have attempted to express the Ad5 E1B genes in *Escherichia coli*. Using the strategy developed by Guarente *et al.* (1980), I have constructed plasmids which have the transcriptional and translational controls of the *E. coli* lac operon linked 5' of the 19K and 58K coding sequences. One plasmid was shown to synthesize high levels of a stable, immunoreactive 19K analogue consisting of 19K with 29 adenovirus-coded amino acid residues at its amino terminus. It appears that an adenovirus-coded ribosome-binding sequence is functional in directing translation of this protein. Synthesis of 58K was not demonstrated, perhaps the result of protein instability in *E. coli*. However, immunoreactive proteins which may correspond to the amino terminal region of 58K were demonstrated.

ACKNOWLEDGEMENTS

I wish to thank Dr. F.L. Graham for his guidance, support, and patience throughout this work. I am most grateful to P. Rotsaert, Y. Haj-Ahmad, D. Bautista, R. McKinnon and E. Arpaia for lively and most helpful discussions. I am indepted to D. Rowe for Ad5 tumor antisera, L. Guarente for expression vectors pLG400 and pGL101, H. Heyneker for *E. coli* strain D1210, D. Bautista and D. Holmyard for photography, and K. McArthur for carefully typing this thesis. Lastly, I am grateful that my wife Jane maintained her patience, interest and confidence during the course of my studies.

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# INTRODUCTION

## 1.1 Adenovirology

### 1.1.1 Adenoviruses

Adenoviruses are non-enveloped particles with icosahedral symmetry (Horne *et al.*, 1959) containing linear double-stranded DNA genomes of molecular weight (MW) 20-25 x $10^6$ daltons (van der Eb and van Kesteren, 1966; Green *et al.*, 1967; van der Eb *et al.*, 1969). At least 35 distinct human serotypes have been identified (Beladi, 1972; Keller *et al.*, 1977; Stadler *et al.*, 1977), many of which are important etiological agents of human respiratory disease (Hilleman, 1957; Parrot, 1960; Hope-Simpson and Higgins, 1969). Interest in these viruses increased dramatically after Trentin and coworkers (1962) demonstrated that human adenovirus type 12 (Ad12) induced malignant tumors following inoculation of newborn hamsters. Since then, adenoviruses have been studied intensely in an effort to understand the molecular basis of mammalian cell transformation and tumorigenesis.

### 1.1.2 Adenovirus Tumor Biology

Following the observations of Trentin *et al.* (1962), numerous human serotypes were evaluated and found to have oncogenic potential in a variety of rodent species (Huebner *et al.*, 1962, 1965; Yabe *et al.*, 1964; Girardi *et al.*, 1964; Rabson *et al.*, 1964; Larsson *et al*, 1965; Pereira *et al.*, 1965; Trentin *et al.*, 1968). Not all of the human serotypes were tumorigenic and oncogenicity varied amongst those which

were. Based on the efficiency of tumor induction and latancy, human serotypes were classified as highly oncogenic, weakly oncogenic, or non-oncogenic (Huebner, 1967; Trentin *et al.*, 1968; Green, 1970). A comprehensive classification scheme based on additional criteria such as hemagglutination, epidemiology, DNA sequence homology, and viral DNA GC content is presented in Table 1.

Despite differences in oncogenicity, all human serotypes evaluated to date are capable of transforming primary rodent cells in culture (McBride and Weiner, 1964; Freeman *et al.*, 1967 abc; Casto, 1968; Gilden *et al.*, 1968; McAllister and McPherson, 1968; McAllister *et al.*, 1969ab). Cell lines established by transformation with oncogenic serotypes are often capable of inducing tumors following injection into syngeneic newborn or weanling animals (Kitamura *et al.*, 1964; Freeman *et al.*, 1967bc; Strohl *et al.*, 1967; Casto, 1969). In contrast, cells transformed by non-oncogenic serotypes generally fail to induce tumors in syngeneic animals. However some cell lines established through transformation by non-oncogenic serotypes are able to induce tumors in immunosuppressed newborn animals (Gallimore, 1972; McDougall *et al.*, 1975). In addition, Ad2 and Ad5 transformed hamster cells are oncogenic in non-immunosuppressed hamsters (Williams 1973; Graham *et al.*, 1974a; Lewis *et al.*, 1974), thereby indicating a degree of oncogenic potential even for non-oncogenic serotypes.

Historically, the highly oncogenic Ad12 and non-oncogenic Ad2 and Ad5 have been studied most intensely. Consequently, most of the information surveyed in this section pertains directly to the above serotypes. However, the overwhelming similarities between different

Table 1

HUMAN ADENOVIRUS HOMOLOGY CLASSES

| Class | Representative Serotypes | Hemag-glutina-tion Group * | % DNA Homology + | % G + C | Oncogenicity in Rodents | Target Tissue | Epidemiology |
|---|---|---|---|---|---|---|---|
| A | 12, 18, 31 | IV | a 48-69%<br>b 8-20%<br>c 50-80% | 48% | high | gastrointestinal tract | cryptic gastrointes-tinal infection |
| B | 3, 7, 11, 21 | I | a 89-94%<br>b 9-20%<br>c 50-80% | 51% | weak | pharynx lungs (upper & lower respiratory tract) hemorrhagic cystitis (lower urinary tract) conjunctivits (eye) | acute epidemic infection |
| C | 1, 2, 5, 6 | III | a 99-100%<br>b 10-16%<br>c 50-80% | 58% | nil | pharynx (upper respiratory tract) | latent throat infection; cryptic gastrointestinal infection |
| D | 8, 9, 19 | II | a 94-99%<br>b 4 -23% | 58% | nil | keratoconjunctivitis (eye) | acute epidemic infection |
| E | 4 | III | | 58% | nil | upper respiratory tract | |
| F | EA | | | | nil | gastrointestinal tract | enteritis-associated enteric infection |

* I. Complete agglutination of monkey erythrocytes; II. Complete agglutination of rat ethryocytes; III. Partial agglutination of rat erthrocytes; IV. Minimal agglutination response.

\+ a. Homology of members of same group; b. Homology of members of different groups; c. DNA sequence homology of members of different groups (0-4.5 map units and 15-17 map units).

Reprinted from Sambrook et al. (1981)

serotypes suggest that current information applies to human adenoviruses in general; at least in broad outline.

### 1.1.3 Adenovirus Transforming Region

The search for the adenovirus transforming principle was accelerated following the advent of gene transfer via the calcium phosphate technique (Graham and van der Eb, 1973). This approach enabled researchers to transform primary rodent cells with naked viral DNA and thereby define regions of the adenovirus genome involved in transformation. It was shown that restriction endonuclease fragments consisting of the left-end approximately 10% of the Ad2 and Ad5 genomes contained the viral functions necessary and sufficient for transformation of primary rodent cells (Graham *et al.*, 1974ab; van der Eb *et al.*, 1977). This is consistent with the observation that adenovirus-transformed cells usually contain only a portion of the viral genome, with the left-end 12-14% generally present (Sharp *et al.*, 1974a; Gallimore 1974; Sambrook *et al.*, 1974; Flint *et al.*, 1976: Johansson *et al.*, 1977, 1978).

To elucidate the mechanism of adenovirus transformation, the left-end of the genome has been extensively characterized.

### 1.1.4 Transforming Genes

The left-end transforming region contains E1, one of five regions which are transcribed before the onset of viral DNA replication during lytic infection (Phillipson *et al.*, 1974; Sharp *et al.*, 1974b; Smiley *et al.*, 1978). The DNA sequence of Ad5 E1 has been determined (van Ormondt *et al.*, 1978, 1980; Matt and van Ormondt, 1979; Matt *et al.*, 1980; Bos *et al.*, 1981) and the

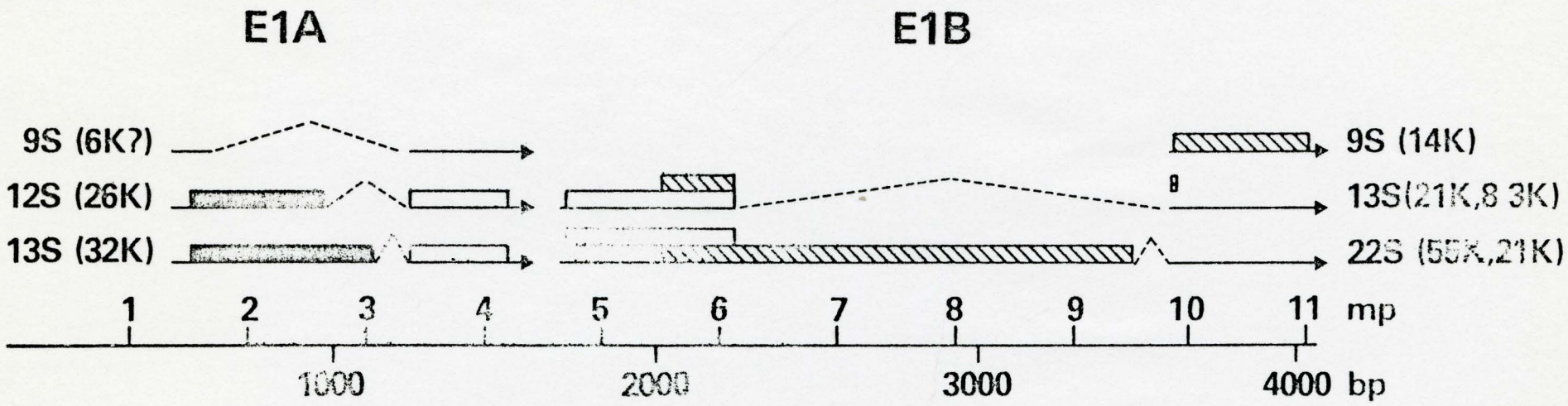
transcriptional patterns have been identified (Berk and Sharp, 1978; Chow *et al.*, 1977; Perricaudet *et al.*, 1979, 1980; Kitchingman and Westphal, 1980). The E1 transcripts and predicted protein coding regions are shown in figure 1.

Region E1 consists of two independently promoted transcription units: E1A extending from 1.3 to 4.4 map units and E1B from 4.6 to 11.1 map units (Berk and Sharp, 1977; Chow *et al.*, 1977). E1A encodes three mRNA species which are 5' and 3' coterminal but differ in their slicing patterns. The 12S and 13S messages are synthesized early in infection and code for proteins of predicted MW 26 kilodaltons (Kd) and 32 Kd respectively.

Three mRNA species are expressed from E1B. The 13S and 22S messages are expressed early in infection and can be detected in most transformed cell lines (Sharp *et al.*, 1975; Flint *et al.*, 1975, 1976). The 22 S message is functionally polycistronic, encoding proteins of predicted MW 21 Kd and 55 Kd from different but overlapping reading frames (Bos *et al.*, 1981). The 13S message encodes the 21 Kd protein as well as the amino terminal region of 55 Kd. The 21 Kd and 55 Kd proteins have observed MWs of 19K (Schrier *et al.*, 1979; Jochemsen *et al.*, 1981) and 58K (Levinson and Levine, 1977ab; Lassam *et al.*, 1979a) respectively as determined by SDS-polyacrylamide gel electrophoresis. To avoid confusion, the E1B antigens will be referred to by their observed MWs; 19K and 58K.

To determine which of the E1 functions are involved in transformation, restriction endonuclease fragments containing various portions of E1 have been assayed for transforming ability.

Figure 1. Early region I of Ad2 and Ad5 showing spliced mRNA species which have been detected and their polypeptide coding sequences (reprinted from Graham, 1983). r strand open reading frames are represent by open, closed and hatched bars for reading frames 1, 2, and 3 respectively. Theroretical molecular weights of E1 proteins are given in parenthesis beside their corresponding mRNAs.


E1A
E1B
9S (6K?)
12S (26K)
13S (32K)
9S (14K)
13S(21K,8 3K)
22S (55K,21K)
1
2
3
4
5
6
7
8
9
10
11
mp
1000
2000
3000
4000
bp

Transformation of primary rodent cells by fragments containing the entire E1 region result in transformed cells which are indistinguishable from those transformed by virions (Graham *et al.*, 1974b; Shiroki *et al.*, 1977; Yano *et al.*, 1977; Mak *et al.*, 1979; van der Eb *et al.*, 1979). Morphologically similar transformants also result when cells are transformed by smaller restriction fragments (e.g. Ad5 Hind III -G) representing E1A plus the region of E1B encoding 19K and the amino-terminal half of 58K (Graham *et al.*, 1974a, Schrier *et al.*, 1979). This suggests that 58K is not required for morphological transformation by DNA (Rowe *et al.*, 1984).

The smallest left-end restriction fragments capable of transformation contain E1A exclusively (e.g. Ad5 HpaI-E). E1A transformants tend to be fibroblastic rather than epitheloid, grow slower and reach lower saturation densities than the "fully" transformed cells described above (Dijkema *et al.*, 1979; Shiroki *et al.*, 1979a; Houweling *et al.*, 1980). The terms "immortalized" and "partially transformed" are used to describe E1A transformants.

Based on the above studies, it appears that both E1A and at least the portion of E1B encoding 19K are required for the establishment and maintenance of the transformed state. Although apparently dispensable in DNA-mediate transformation, the E1B 58K function is required for transformation by virions. Ad5 host range II (hrII) mutants were useful in determining this. This class of mutants map between 6.1 and 8.5 map units, within sequences retained by the E1B message but spliced from the 13S message (Galos *et al.*, 1980). HrII mutants fail to synthesize the 58K protein (Lassam *et al.*, 1979b) and express normal amounts of 19K (Rowe and Graham, 1983). On several cell types hrII

mutants are transformation defective (Graham *et al.*, 1978), although DNA purified from hrII virions transforms primary rodent cells with efficiencies which are comparable to that of DNA from wild-type virions (Rowe and Graham, 1983). The nature of this discrepancy as well as the molecular basis by which the E1 proteins are active in transformation are unclear. From the results with hr mutants and DNA, it is thought that expression of E1A "immortalizes" cells and that combined expression of at least one E1B function (19K) is required to establish and maintain a "fully" transformed state (Graham, 1983).

### 1.1.5 Properties of E1B Tumor Antigens

The characterization of E1 tumor antigens has been a slow process, yielding little more than general physical properties so far.

The 19K antigen has been shown to be membrane-associated in both infected and transformed cells (Persson *et al.*, 1982). However, there is no direct evidence that 19K is a surface antigen. As membrane-associated antigen, 19K may contribute to the transformed phenotype by inducing changes at the cell surface. Such alterations could be responsible for the lack of contact inhibition in growth, epithelial morphology or anchorage independence of adenovirus-transformed cells.

In DNA-mediated transformation assays, it has been observed that in the absence of 19K expression, transformed BRK cells (transformed by E1A) appear unable to form colonies in Joklik's medium supplemented with HS (R. McKinnon, personal communication). This suggests that 19K provides a function, such as a growth factor, which is present in FCS but is either absent or blocked in horse serum.

19K has also been implicated in the host immune response to adenovirus-transformed cells. The region encoding 19K has been linked to the ability of Ad12-transformed hamster cells to immunize rats against tumor induction by Ad12 virions (Shiroki *et al.*, 1979b). Therefore, 19K may function as a component of adenovirus TSTA (tumor-specific transplantation antigen) and in this capacity may be involved in determining tumorgenicity. McAllister *et al.* (1969a) proposed that cells transformed by non-oncogenic serotypes are unable to induce tumors in immunocompetent animals because they are more immunogenic than cells transformed by oncogenic serotypes. Consistent with this view is the observation that TSTA of highly-oncogenic Ad12 cross-reacts antigenically with that of weakly-oncogenic Ad7 but not non-oncogenic Ad5 (Ankerst and Sjorgren, 1970).

In summary, 19K is membrane-associated and may be a component of adenovirus TSTA, properties which may contribute to the morphology, growth characteristics and oncogenic potential of adenovirus-transformed cells. Clearly the capacity with which 19K acts in cellular transformation and tumorigenesis merits further investigation.

As with 19K, the function of 58K is unknown. 58K is phosphorylated both in infected and transformed cells (Ross *et al.*, 1980; Gaynor *et al.*, 1982; Malette *et al.*, 1983). 58K is found in both cytoplasmic and nucleoplasmic subcellular fractions, accumulating preferentially in the nucleus late in infection (Rowe *et al.*, 1983). Fluorescent antibody staining with a 58K-specific monoclonal antibody confirmed these results (Sarnow *et al.*, 1982a).

In a variety of adenovirus-transformed cells, 58K forms a

complex with a cellular 54K protein which is identical or closely related to a cellular protein found associated with large T antigen in simian virus-40 (SV40) transformed cells (Lane and Harlow, 1982; Sarnow *et al.*, 1982b). Similarly, in Epstein-Barr virus (EBV) transformed cells a cellular 54K protein is found complexed with EBV nuclear antigen (Luka *et al.*, 1980). A cellular 54K protein is found in elevated quantities in other viral and chemically transformed cells (Lane and Crawford 1979; Linzer and Levine, 1979; DeLeo *et al.*, 1979; Lane and Harlow, 1982) perhaps indicating a common mechanism for transformation by different agents. In addition, Ad5 58K has been shown to be associated with protein kinase activity in both infected and transformed cells (Branton *et al.*, 1979, 1981; Lassam *et al.*, 1979c). Using antisera from hamsters bearing Ad5-induced tumors, immune precipitates containing 58K were shown to phosphorylate the IgG heavy chain, or exogenous histone protein. Synthesis of kinase activity followed the same kinetics as 58K synthesis and was reduced in cells infected with hrII mutants (Branton *et al.*, 1979). In contrast to the tyrosine-specific kinase of avian sarcoma viruses (Hunter and Sefton, 1980), the 58K-associated kinase was specific for serine and threonine (Branton *et al.*, 1981). These studies did not establish whether kinase activity was intrinsic to 58K or merely a consequence of an associate cellular kinase in the immune precipitates. Ideally, a purified preparation of 58K would be needed to determine this. Following this rational, numerous viral tumor antigens have been purified. For example, SV40 large T antigen (Livingston *et al*, 1974) and Ad2 19K (Persson *et al.*, 1982) have been purified from virus infected cells. In an alternative

approach several viral tumor antigens have been expressed in *Escherichia coli* either as native or fusion polypeptides. Amongst these are the SV40 small t (Roberts *et al.*, 1979a; Jay *et al.*, 1981; Thummel *et al.*, 1981; Derom *et al.*, 1982; Gheysen *et al.*, 1982; Bikel *et al.*, 1983) polyoma middle T antigen (Palme *et al.*, 1983), the Ad5 E1A 13S mRNA product (Jones *et al.*, 1983), Ad2 E1A and E1B proteins (Harter and Ko, 1983) and Ad12 E1B 19K (Fukui *et al.*, 1983).

I have attempted to express the Ad5 E1B 19K and 58K antigens in *E. coli*. This approach was adopted to obtain sufficient quantities of these proteins for the assessment of biological activities involved in oncogenic transformation.

## 1.2 Expression of Eukaryotic Genes in *E. coli*

### 1.2.1 Principles of Expression

To express a eukaryotic gene in *E. coli*, the gene (or its corresponding cDNA) must be provided with regulatory sequences which direct efficient transcription and translation in *E. coli*. Numerous expression protocols have been developed, all of which provide ways of overcoming fundamental differences between eukaryotic and prokaryotic transcriptional and translational control sequences.

### 1.2.2 Transcriptional Control Sequences

In both prokaryotes and eukaryotes the DNA-dependent RNA polymerase recognizes specific sequences (promoters) which control the fidelity and frequency with which a gene is transcribed. Though functionally similar, prokaryotic and eukaryotic promoter sequences differ significantly such that the prokaryotic polymerase generally

will not transcribe from eukaryotic promoters. With few exceptions (Struhl *et al.*, 1980; Struhl and Davis, 1981), efficient expression of eukaryotic genes in *E. coli* has been achieved only when the gene has been placed under the control of a strong *E. coli* promoter.

For expression of foreign genes in *E. coli* it is advantageous to use a regulated *E. coli* promoter to direct transcription. Transcription from such promoters can be repressed and induced, thereby modulating the level of transcription. In particular, constitutively high levels of transcription are undesirable in cases where the foreign protein may be toxic to *E. coli*. Regulated promoters can be used to demonstrate toxicity and determine sub-lethal expression levels. In this manner toxicity of vesicular stomatitis virus glycoprotein G was demonstrated (Rose and Shafermann, 1981) and sub-lethal levels of lambda bacteriophage CII expression were determined (Shimatake and Rosenberg, 1981). In addition, regulated transcription circumvents plasmid instability due to high levels of transcription interfering with plasmid DNA replication (Remaut *et al.*, 1981). There are several strong regulated *E. coli* and bacteriophage promoters commonly used for expression of foreign genes in *E. coli*. These include the $P_L$ promoter of bacteriophage lambda, and the trp and lac, promoters of *E. coli*.

### 1.2.3 Translational Control Sequences

To achieve efficient gene expression it is necessary to provide not only a strong promoter, but also an efficient translational apparatus. Although the process of translation is essentially identical in prokaryotes and eukaryotes, again the sequences which control the

initiation of translation are quite dissimilar.

In eukaryotes, functional initiation codons are generally closest to the 5' end of mRNA and the sequence GNNA$\overset{A}{}$UGG is thought to provide optimal initiation by eukaryotic ribosomes where AUG is the initiation codon (Kozak, 1981).

The sequences which control ribosome binding and initiation of translation in prokaryotes have been studied more intensively. Through examination of several different mRNA species, Shine and Dalgarno (1974) proposed that a sequence (now termed the Shine and Dalgarno sequence) just upstream of the initiation codon could base-pair with complementary sequences near the 3' end of 16S rRNA. Ribosome binding sequences from over 150 bacterial and phage mRNAs have since been analyzed (Steitz, 1980; Gold *et al.*, 1981) and with few exceptions, they contain sequences which are to varying degrees complementary to the 3' end of 16S rRNA.

Simplistically, a prokaryotic ribosome binding site can be viewed as an initiation codon, a Shine and Dalgarno sequence (SD), and appropriate spacing between the two (Gold *et al.*, 1981).

Specific interaction between mRNA and 16S RNA has been supported by several studies: i) translation of mRNA *in vitro* can be inhibited by short oligonucleotides which contain SD sequences (Tanaguchi and Weissman, 1978; van der Laken *et al.*, 1980); ii) direct interaction between the 3' end of 16S rRNA and mRNA has been demonstrated *in vitro* (Steitz and Jakes, 1975; Steitz and Steege, 1977); iii) mutations which disrupt mRNA.rRNA complementarity reduce translational

efficiency *in vivo* (Dunn *et al.*, 1978; Schwartz *et al.*, 1981; Singer *et al.*, 1981).

Although the interaction of rRNA with complementary sequences in mRNA has been clearly established, its importance in ribosome binding and initiation of translation should not be overemphasized. There are examples of prokaryotic mRNAs which are efficiently translated *in vivo* yet contain no obvious SD sequences (Gold *et al.*, 1981, Smiley *et al*, 1982). Interestingly, the lambda CI mRNA made in a lysogen under the control of the $P_{RM}$ promoter has its 5' end beginning at the initiation codon (Ptashne *et al.*, 1976). There are no sequences upstream of the initiation codon yet the CI message is translated *in vivo*. Presumably regions other than the SD domain must play a role in ribosome binding. This view is supported by experiments in which an oligonucleotide complementary to the 3' end of 16S rRNA was used to inhibit formation of the 30S rRNA . mRNA binary complex. Backendorf *et al.* (1980) found that the oligonucleotide was inhibitory only if added before or simultaneously with mRNA. The oligonucleotide failed to displace mRNA from preformed binary complexes, indicating interactions beyond the SD domain were involved.

Computer analysis of numerous prokaryotic mRNAs has been used to define a consensus ribosome-binding sequence. Stormo *et al.* (1982) compared sequences of 123 mRNAs to 198 theoretical ribosome-binding sites. Theoretical ribosome binding sites were found by screening an extensive *E. coli* mRNA sequence library, searching for AUG codons having SD sequences located 5-9 nucleotides upstream. To their knowledge, initiation from these sites did not occur *in vivo*.

Computer analysis was used to define differences between translationally functional mRNAs (gene group) and the theoretical control group. With the exception of the SD domain, nucleotides were essentially random at positions surrounding the theoretical initiation codons of the control group. In contrast, four non-random domains were identified in the functional gene group. These were located between -21 and +13, where 0 is the first nucleotide of the AUG codon.

In a similar study, Scherer *et al.* (1980) compared the sequence of 78 prokaryotic mRNAs by aligning each sequence by the known initiation codon, allowing a ±2 nucleotide shift to accommodate varying distances between SD sequences and initiation codons. They found a preferential domain extending from -20 to +18 and suggested that a region 46-48 nucleotides in length may direct translation. The proposed sequence is shown in Table 2 (Scherer *et al.*, 1980).

In summary, the prokaryotic translational apparatus consists of an initiation codon and a SD sequence located 7±2 nucleotides upstream. In addition, ribosome binding is probably influenced by other sequences both 3' and 5' of the initiation codon.

### 1.2.4 Optimizing Expression of Eukaryotic Genes in *E. coli*

All the existing expression strategies share the common feature of introducing a strong prokaryotic promoter in front of the gene to be expressed. Generally, high levels of transcription are easily obtained whereas translational efficiency usually limits the efficiency of synthesis of eukaryotic proteins in *E. coli*. Consequently, the factors which determine translational efficiency have been studied

intensely. These include: i) the distance separating the SD sequence from the initiation codon; ii) sequences 5' and 3' of the initiation codon; iii) mRNA secondary structure and iv) codon usage.

The distance separating the SD sequence from the initiation codon is a crucial determinant of translational efficiency. In most mRNAs 7±2 nucleotides separate the 3' end of the SD sequence from the first nucleotide of the initiation codon (Stormo *et al.*, 1982).

As discussed above, the sequences 5' and 3' of the initiation codon are probably involved in ribosome binding and translation. Exemplifying this, Fukui *et al.* (1983) reported dramatic differences in Ad12 19K expression from recombinant plasmids which differed only in sequences immediately 3' of the initiation codon.

Several lines of evidence suggest that mRNA secondary structure can have profound effects upon translational efficiency. Roberts *et al.* (1979a) found that small differences in the 5' untranslated leader region resulted in up to 20 fold differences in cro expression. Iserentant and Fiers (1980) proposed that mRNA secondary structures might account for the observed differences in cro expression. mRNA secondary structure may impair translation by masking the SD sequence (Hall *et al.*, 1982; Saito and Richardson, 1981), the initiation codon (Iserentant and Fiers, 1980) or both (Gheyson *et al.*, 1982). Secondary structure may also enhance translation by bringing a distant SD sequence close to the initiation codon. This has been hypothesized for the case of T4 bacteriophage gene 38 (Gold *et al.*, 1981). Still, it remains unknown to what extent mRNA secondary structure affects prokaryotic translation in general.

Lastly, codon usage may be a limiting factor in prokaryotic translation. Codon usage in *E. coli* mRNAs appears to correlate with tRNA availability. This correlation is stronger for those genes which are expressed at high levels, perhaps indicating a selective pressure to optimize use of the available tRNA species (Ikemura, 1981ab). Grosjean and Fiers (1982) proposed that use of certain codons may modulate translation. The "modulator codons" have corresponding tRNAs which are very minor species in *E. coli*. Use of these codons is almost exclusively avoided in *E. coli* genes which are expressed efficiently, yet are employed and in some cases even preferred in regulatory genes and genes which are expressed to a lesser degree. As with secondary structure, the extent to which codon usage can limit translation is an area requiring further study.

In summary, optimization of eukaryotic gene expression in *E. coli* relies on successfully mimicking the features of prokaryotic mRNA.

### 1.2.5 Experimental Design

The strategy I have employed for expression of the Ad5 19K and 58K antigens in *E. coli* was developed by Guarente *et al.* (1980). This method provides a systematic approach for optimizing expression in *E. coli*. Briefly, plasmids are constructed which consist of a region encoding the amino terminal portion of the eukaryotic protein linked, with reading frame in phase, to a fragment encoding β-galactosidase. The work of Beckwith and colleagues has established that fusions can be made to the amino terminus of lacZ and the resulting fusion protein

retains β-galactosidase activity (Bassford *et al.*, 1978). A promoter fragment from the *E. coli* lac operon is then introduced at various positions upstream of the fusion gene's initiation codon. The promoter fragment contains the lac uv5 promoter, lac operator, and SD sequence. Promoter insertion creates a hybrid translational apparatus, consisting of the lac SD sequence linked to the initiation codon of the eukaryotic gene. Promoter placements which optimize the distance between the SD sequence and initiation codon should result in efficient expression of an enzymatically active β-galactosidase hybrid protein. The protein coding sequences of the eukaryotic gene are then reconstituted, bringing the entire gene under the transcriptional and translational controls of the lac operon.

# MATERIALS AND METHODS

## 2.1 Bacterial Strains

*E. coli* strains NK5031 ($F^-$, lac Z M5265 Su111$^+$, nal$^R$) and D1210 ($F^-$, hsd S20, recAB, ara-14, proA2, galK2, rpsL20, xyl-5, mtl-1, supE55, λ-, lac $i^q$) were used. Strain NK5031 bears a deletion of lac Z (Guarente *et al.*, 1980) and was used to identify clones containing β-galactosidase-expressing plasmids. Strain D1210 overproduces the lac represser (Sadler *et al.*, 1980) and was used in the construction and characterization of 19K and 58K expresser plasmids.

## 2.2 Bacterial Culture Techniques

### 2.2.1 Liquid Culture

Liquid culture was at 37°C with constant agitation. Luria-Bertani (LB) broth (per liter: 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g NaCl, 1 g glucose) was used unless otherwise stated. Ampicillin (Bristol Labs) was added to a final concentration of 40 μg/ml. when bacteria contained plasmids expressing β-lactamase.

### 2.2.2 Solid Culture

Solid culture was at 37°C in inverted plastic petri dishes (Fischer) containing agar-based media. Luria agar (LB broth plus 1.9% agar w/v) and MacConkey agar were used. Ampicillin was added to a final concentration of 40 μg/ml after the autoclaved media had cooled to 55°C. Plates were incubated overnight at 37°C prior to use.

2.2.3 Culture Storage

1.0 ml aliquots of overnight liquid cultures were diluted 1:1 in 40% glycerol (v/v) and frozen at -70°C in sterile 3.0 ml glass vials. Viable cells could be recovered by scratching the frozen surface of the stock culture with a sterile inoculation loop.

2.3 Transformation of *E. coli* with plasmid DNA.

The calcium chloride technique of Mandel and Higa (1970) was used to transform *E. coli* with plasmid DNA. An overnight liquid culture was diluted 1/100 in LB broth and grown to $OD_{660}$ 0.4-0.6. The culture was transfered to 50 ml corning tubes and centrifuged for 5 minutes at 4000 rpm (2720 x g) in an MSE centrifuge. The supernatant was aspirated and the cells were resuspended in 20 ml ice cold $CaCl_2$-Tris (75 mM $CaCl_2$, 5 mM Tris pH 7.6). The cell suspension was incubated on ice for 20 minutes and then centrifuged at 4000 rpm (2720 x g) for 5 minutes. The supernatant was aspirated and the cells were resuspended in 1 ml ice-cold $CaCl_2$-Tris solution. Plasmid DNA (1 µg) was added and after 1 hour incubation on ice, the cells were heat shocked at 42°C for 2 minutes and diluted into 8 ml prewarmed (37°C) LB broth. The culture was incubated at 37°C without agitation for 30 minutes and 10 fold serial dilutions in LB broth were prepared. 0.3 ml aliquots from each dilution were plated on selective media (Luria agar or MacConkey agar plus 40 µg/ml ampicillin). The plates were incubated upright at 37°C for 30 minutes and then inverted. Colonies were generally detectable after 12-16 hours incubation.

## 2.4 Purification of Plasmid DNA from *E. coli*

### 2.4.1 Small Scale Analytical Preparation

The method of Birmboim and Doly (1979) was used for rapid isolation of plasmid DNA from *E. coli*. This is an alkali extraction procedure, exploiting the relative resistance of covalently closed plasmid DNA to mild alkali.

Colonies were picked with wooden applicator sticks and inoculated into 2.5 ml LB broth containing ampicillin. Cells were cultured for 4 hours and chloramphenicol was added to a final concentration of 50 µg/ml. The culture was amplified overnight and 1.5 ml was transfered to a 1.5 ml eppendorf tube. Cells were centrifuged for 15 seconds and the supernatant was aspirated. The cell pellet was suspended in 100 µl lysozyme solution (5 µg/ml lysozyme in a solution of 50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 7.8). This solution was incubated on ice for 20 minutes and 200 µl freshly prepared alkaline-SDS solution (0.2 N NaOH, 1% SDS) was added. After 5 minutes at room temperature, the solution became clear and viscous, indicating cell lysis. 150 µl ice cold 3 M sodium acetate (pH 4.8) was added and the mixture was incubated on ice for 1 hour and precipitated high MW nucleic acids were removed by centrifugation for 5 minutes. The supernatant was transferred to a clean 1.5 ml eppendorf tube and precipitated with 2 volumes 96% ethanol (-20°C). After 2 minutes centrifugation the DNA pellet was dissolved in 100 µl 0.1 M sodium acetate and ethanol precipitated once more. The pellet was dried at 37°C and dissolved in 50 µl 10 mM Tris pH 7.6, .1 mM EDTA (TE buffer) and stored at -20°C. 5-10 µl of this preparation was generally sufficient for restriction

endonuclease cleavage and analysis by agarose or polyacrylamide gel electrophoresis.

2.4.2 Large-Scale Plasmid Purification

A scaled-up version of the Birmboim and Doly (1979) procedure was used to prepare large amounts of plasmid DNA for CsCl banding. 300 ml liquid cultures were grown to $OD_{660}$ 0.4 to 0.6. Chloramphencol was added to a final concentration of 50 µg/ml and the incubation was continued overnight. The remainder of the procedure was identical to the small-scale method except 10 ml lysozyme, 20 ml alkaline-SDS and 15 ml 3 M sodium acetate were used. After 2 ethanol precipitations, the pellet was dried at 37°C and dissolved in 7.0 ml TE (pH 7.6) buffer. This preparation was then CsCl banded to purify the plasmid DNA.

2.4.3 CsCl Banding of Plasmid DNA

8.4 g CsCl and 0.5 ml ethidium bromide solution (6 mg/ml) were added to the DNA solution described above. The solution was transferred to a Beckman ultracentrifuge tube, layered with parafin oil and capped. The sample was centrifuged for 48 hours at 35000 rpm, 11°C in a Beckman type 50 rotor. The plasmid band was visualized with UV light and extracted by puncturing the centrifuge tube with a hypodermic needle. The ethidium bromide was extracted by adding 2 volumes of CsCl-saturated isoamyl alcohol and aspirating the upper phase. This was repeated 3 times or until the pink colour had disappeared from the aqueous phase. The aqueous phase was transferred to a dialysis sac and dialyzed for 12 hours against 2 changes of TE (pH 7.6) buffer. To concentrate the DNA, aquacide (Calbiochem) was layered onto the sac

and left at room temperature for 1 hour. Generally 1 mg of plasmid DNA (1 µg/µl) could be prepared from 300 ml of culture using this procedure.

## 2.5 Enzyme Reactions

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase and Exonuclease III were purchased from Bethesda Research labs or New England Biolabs. S1 nuclease was purchased from Sigma.

### 2.5.1 Restriction Endonucleases

Restriction endonuclease reactions were conducted at 37°C for 4-12 hours. Generally 1 unit of enzyme was sufficient for complete digestion of 1 µg plasmid DNA. A buffer containing 50 mM NaCl, 10 mM Tris (pH 7.5), 10 mM $MgCl_2$ and 1 mM dithiothreitol (DTT) was used for all reactions. Enzymes were inactivated by heating at 65°C for 15 minutes.

### 2.5.2 T4 DNA Ligase

T4 DNA ligase catalyzes the formation of a phosphodiester bond between adjacent 3'-OH and 5'-P termini in DNA (Weiss, 1968). Ligation reactions were conducted at 15°C for 12 hours. Generally 2-4 units of enzyme were used per reaction. The reaction buffer contained 0.66 M Tris (pH 7.6), 50 mM $MgCl_2$, 50 mM DTT and 10 mM ATP.

### 2.5.3 T4 Polynucleotide Kinase

This enzyme catalyzes the transfer of the γ-phosphate of ATP to a 5'-OH terminus in DNA (Richardson, 1971). Using T4 polynucleotide kinase, synthetic KpnI linkers were kinased prior to ligation. In a

total reaction volume of 20 µl, 0.05 µg linker DNA was kinased using 10 units enzyme in a buffer containing 0.5 mM ATP, 0.2 mg/ml BSA, 6.7 mM DTT, 0.67 mM $MgCl_2$. The reaction mixture was incubated at 37°C for 5 hours.

2.5.4 Exonuclease III

This enzyme catalyzes a stepwise 3'→5' removal of 5' mononucleotides from double-stranded DNA carrying a 3'-OH end (Weiss, 1976). Digestion rates of 60-70 bp per minute were achieved when 50 µg linearized plasmid DNA (10.6 Kb) were reacted with 50 units of enzyme at 37°C in a 100 µl reaction volume. The reaction buffer consisted of 6.6 mM Tris (pH 7.4) 6.6 mM $MgCl_2$, 6.6 mM β-mercaptoethanol and 60 mM NaCl. Exonuclease III reactions were stopped by adding an equal volume of 2X nuclease S1 buffer to the reaction mixture.

2.5.5 Nuclease S1

Nuclease S1 degrades single-stranded DNA (Vogt, 1973) to give 5' phosphoryl mono- or oligonucleotides. Reactions were conducted at 37°C for 1 hour in a buffer containing 30 mM sodium acetate (pH 4.5), 0.5 mM $ZnSO_4$, 0.1 M NaCl, and 0.1% SDS. 150 units enzyme was used to treat 50 µg of ExoIII treated plasmid. Mild S1 digestions were conducted at room temperature for 15 minutes using 5-10 units enzyme per reaction. Under these conditions digestion of double-stranded sequences was shown to be minimal.

Nuclease S1 reactions were stopped by adding an equal volume of TE saturated phenol to the reaction. The aqueous phase was ethanol precipitated twice and the pellet was dried and dissolved in TE buffer.

2.6 β-galactosidase Assay

β-galactosidase is an enzyme which hydrolyzes β-D-galactosides. It can easily be measured with chromogenic substrates such as O-nitrophenol-β-D-galactoside (ONPG). β-galacotidase converts ONPG which is colourless, into galactose and O-nitrophenol. O-nitrophenol is yellow and can be measured easily at 420 nm. The method of Miller (1972) was used to assay β-galactosidase activity.

Overnight LB broth cultures were used to inoculate fresh media containing $10^{-2}$ M isopropyl thiogalactoside (IPTG). The cultures were grown for 2 hours and the $OD_{660}$ was determined. 0.5 ml culture was added to an equal volume of Z buffer (0.06 M $Na_2HPO_4.7H_2O$, 0.04 M $NaH_2PO_4.H_2O$, 0.01 M KCl, 1 mM $MgSO_4.7H_2O$, 0.05 M β-mercaptoethanol). To make the cells permeable to ONPG, 2 drops chloroform and 1 drop 0.1% SDS were added and mixed by vortexing. The reaction mixture was equilibrated at 28°C before addition of 200 µl ONPG (Sigma) solution (2 mg/ml).

The time taken for the reaction mixture to turn yellow was recorded and the reaction was stopped by adding 0.5 ml 1 M $Na_2CO_3$. The $OD_{550}$ and $OD_{420}$ were determined and β-galactosidase units were calculated using the formula:

$$\beta\text{-galactosidase units} = 1000 \times \frac{OD_{420} - OD_{550}}{t(\text{min}) \times v(\text{ml}) \times OD_{660} \text{ of cell culture}}$$

MacConkey indicator plates were used to identify $lac^+$ recombinants. This differential medium has lactose as a sole carbohydrate source and neutral red as an indicator of acid production resulting from lactose fermentation. $lac^-$ colonies appear white, whereas $lac^+$

colonies are pink or red.

## 2.7 Electrophoresis of DNA

### 2.7.1 Agarose Gel Electrophoresis of DNA

1.2% (w/v) agarose gels were cast between glass plates (15 cm X 17 cm X 0.2 cm) mounted in a vertical gel apparatus. Agarose was prepared in tris-acetate electrophoresis (TAE) buffer (0.4 M Tris-acetate, 0.002 M EDTA). DNA samples, (generally 20 µl), were mixed with 5 µl loading buffer (20% glycerol, 2% SDS, 0.5% bromphenol blue) and run at 2 v/cm. After electrophoresis for 10-15 hr 100 µl ethidium bromide solution (6 mg/ml) was added to 400 ml TAE and the gel was stained for 5 minutes. Gels were photographed by a mounted polaroid camera using high speed Kodak type 57 film under UV light with a Toshiba monochromatic (red) filter.

### 2.7.2 Polyacrylamide Gel Electrophoresis of DNA

Polyacrylamide gel electrophoresis (PAGE) was used to analyze small DNA framents(< 500 bp). Gels were cast between glass plates as above. 5% gels were prepared from a stock solution of 50% by weight of acrylamide and 1.67% by weight of N, N'-bis-methylene acrylamide (bis). Gels were polymerized chemically by the addition of .025% tetramethyl-ethylenediamine (TEMED) and ammonium persulfate. Gels were run, stained, and photographed as with agarose gels.

### 2.7.3 Isolation of DNA Fragments from Polyacrylamide Gels

DNA restriction fragments were isolated from polyacrylamide gels using the procedure of Maxam and Gilbert (1977). DNA was

visualized by ethidium bromide staining and the desired band was excised from the gel using a sharp scapel. The acrylamide slice was placed in a 1.5 ml Eppendorf tube and mixed in 600 µl elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS) using the plunger of a tuberculin syringe. The solution was incubated O/N at 37°C and the acrylamide pelleted by centrifugation for 5 minutes. The supernatant was collected and the DNA precipitated with ethanol twice. The DNA pellet was dried and dissolved in TE buffer. DNA prepared in this way could be claved with restriction enzymes or ligated efficiently with T4 ligase.

## 2.8 Tumor Antisera

Tumor antisera were obtained from D.T. Rowe. AE antiserum was generated by injecting hamsters with the 954.1 hamster cell line. These cells had been transformed with HindIII digested Ad5 DNA (Rowe *et al.*, 1983). The AE antiserum reacts strongly with 19K and has relatively low avidity towards 58K.

14b antiserum was produced by injecting hamsters with 14b cells. These are Ad-5 transformed hamster cells which contain the left-end 40% of the Ad5 genome (Sambrook *et al.*, 1974). 14B antiserum recognizes both 19K and 58K (Lassam *et al.*, 1979b).

## 2.9 Preparation of *E. coli* Extracts

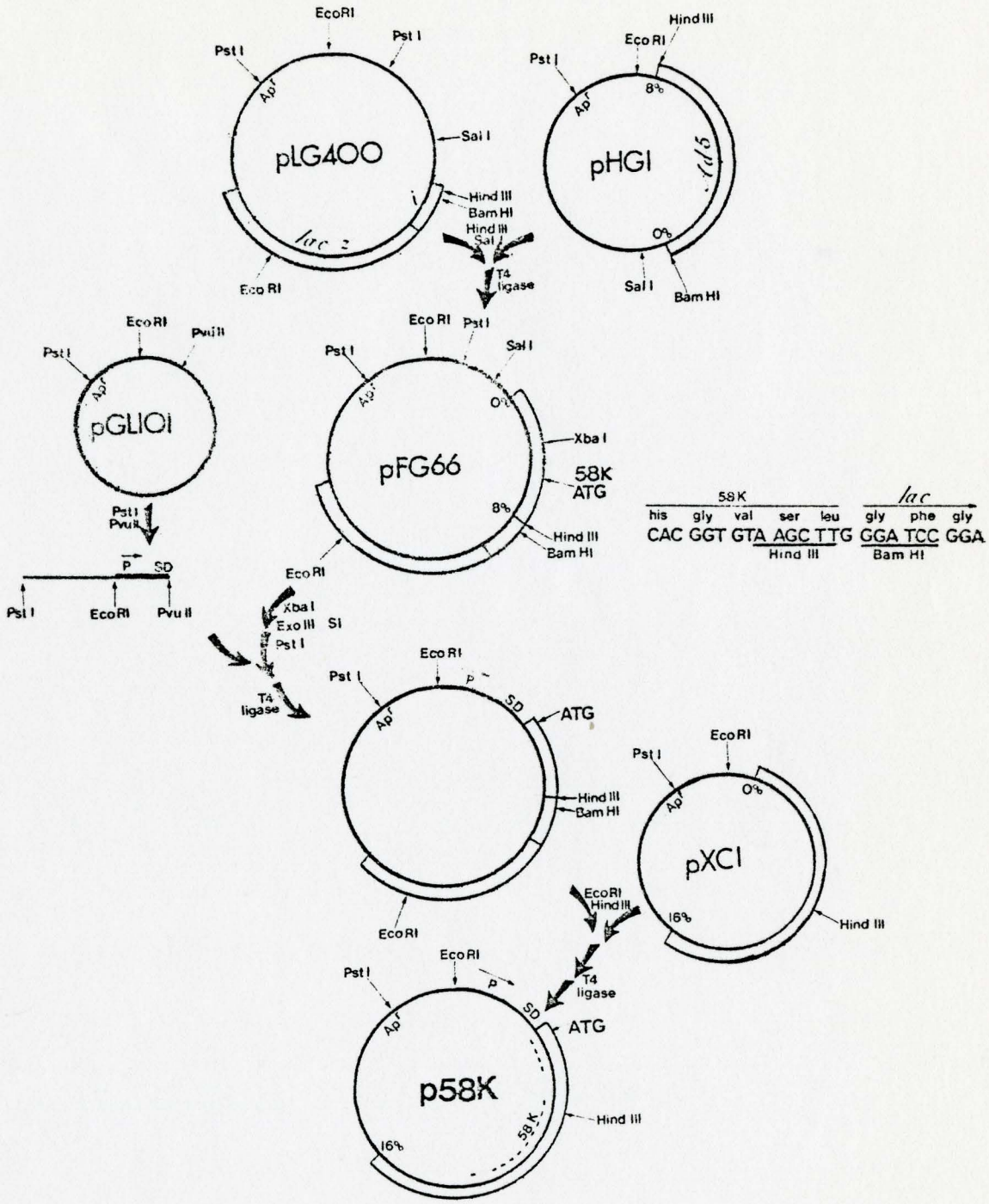
Cells were cultured using a procedure similar to that of Neidhardt *et al.* (1980). *E. coli* D1210 were grown to $OD_{660}$ 0.2 in M9 medium (Miller, 1972) plus casamino acids. Chloramphenicol was added to a final concentration of 20 µg/ml and the incubation was continued

overnight. The culture was divided into 2 fractions, one uninduced and one induced. To the induced sample, IPTG was added to a final concentration of $10^{-2}$M and incubated for 3 hours. 1 ml of each culture was pelleted and resuspended in M9 medium lacking $MgSO_4$.100 µCi $^{35}$S-methionine (Amersham) was added to each culture and IPTG ($10^{-2}$M) to the induced culture. Cells were labeled for 20 minutes, then pelleted and resuspended in 1 ml RIPA buffer (50 mM Tris pH 7.5, 0.2% SDS, 1.0% Triton X100, 1.0% deoxycholate, 100 mM NaCl, 2 mM phenyl methyl sulfonyl fluoride). Cells were lysed by sonication on ice for 1.5 minutes (30 second bursts at 1 minutes intervals on Biosonik III, setting 30). Cell debris was removed by centrifugation and the samples were stored at -70°C.

2.10 Immune Precipitation of Ad5 Antigens from *E. coli* Extracts

Tumor antigens were immune precipitated using the protein A-Sepharose technique of Schaffhausen *et al.* (1978) as modified by Lassam *et al.* (1979b). Reaction mixtures usually contained 20 µl antiserum (preabsorbed against unlabeled *E. coli* D1210 cell extract), 100 µl protein A-Sepharose beads, 200 µl $^{35}$S-methionine labeled extract, and RIPA buffer to a total volume of 1 ml. Samples were incubated overnight at 4°C with constant mixing and then the Sepharose beads were pelleted and washed 3 times with 100 mM Tris (pH 7.5) containing 250 mM LiCl and 0.1% 2-mercaptoethanol (v/v). 100 µl loading buffer (0.1% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.0005% bromphenol blue, 0.0625 M Tris, pH 6.8) was added to the beads and the mixture was boiled for 2 minutes to release the immune complexes from the beads. The beads were removed

Figure 2. Original strategy adopted from Guarenete *et al.* (1980) for the construction of 58K expression plasmids. See text for additional details.

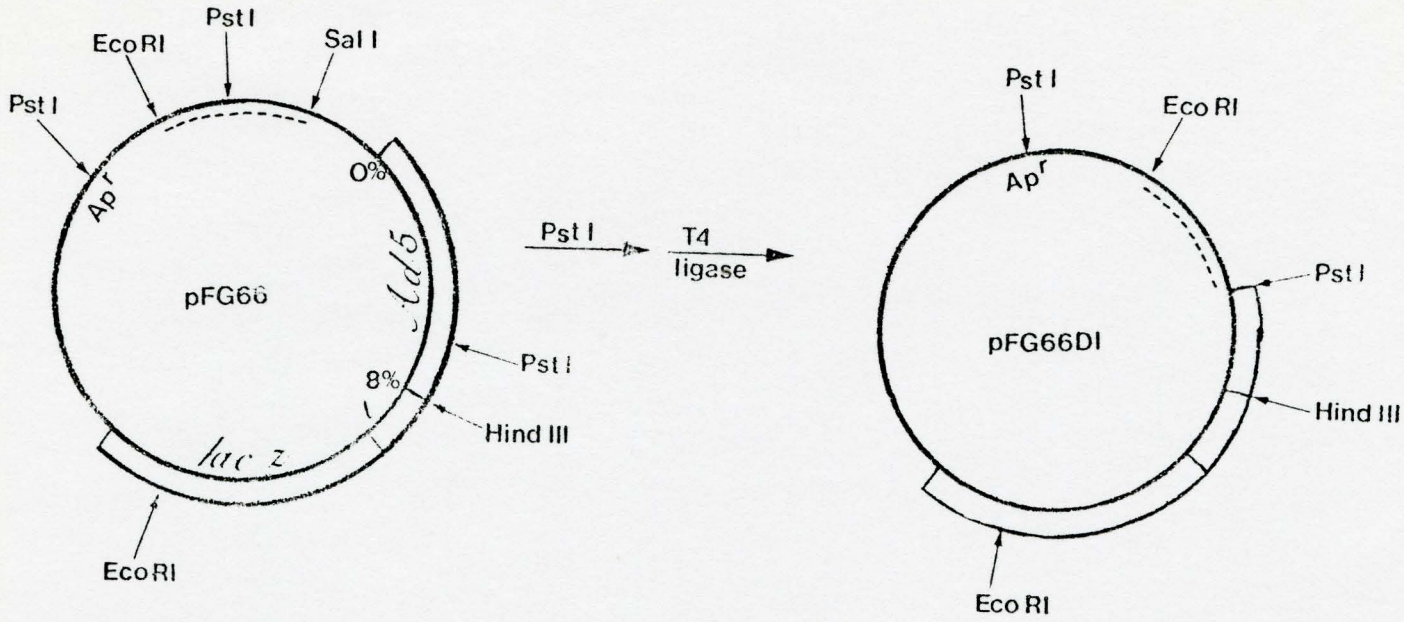

EcoRI
Pst I
Ap
pLG400
Sal I
Hind III
Bam HI
lac z
i
pHGI
Hind III
EcoRI
8%
rds
0%
Sal I
Bam HI
T4 ligase
pGLIOI
Pvu II
P
SD
pFG66
Xba I
58K
ATG
Exo III
S1
58K
his gly val ser leu gly phe gly
CAC GGT GTA AGC TTG GGA TCC GGA
Hind III
Bam HI
lac
pXCI
16%
p58K

NK5031 transformed with pFG66 were $lac^+$ on MacConkey indicator plates. The level of β-galactosidase activity was quantitatively assayed (Miller, 1972) and found to be approximately 800 units. This undoubtedly would interfere with subsequent assays in which β-galactosidase activity provides an indirect measure of gene expression under the control of the lac promoter. To determine which sequences were responsible for β-galactosidase expression from pFG66 we constructed a series of plasmids, derivatives of pFG66 bearing deletions of Ad5 sequences. These plasmids were assayed for β-galactosidase activity. The following section describes the construction and characterization of one particular plasmid, pFG66D1.

### 3.1.2 Construction and Characterization of pFG66D1

Deletion derivatives of pFG66 were made by partial digestion of pFG66 with PstI followed by *in vitro* ligation. Figure 3 shows the construction of pFG66D1. This plasmid had all but 300 bp of Ad5 sequences deleted, however it retained levels of β-galactosidase activity comparable to pFG66. This suggested that the Ad5 sequences remaining in pFG66D1 were directing transcription and translation. These sequences were examined for possible sites from which translation could initiate. Four ATG triplets in phase for 58K and β-galactosidase were found. In addition, we also found that there is an in phase ATG triplet within lacI sequences just upstream of the β-galactosidase gene. Alternate initiation codons such as GUG, UUG, AUU and AUA can function in *E. coli* (reviewed in Kozak, 1983). However, initiation from alternate codons is generally inefficient (Kozak, 1983) and unlikely to result in the levels

Figure 3. Construction of pFG66 deletion derivative, pFG66D1. The dashed sequences represent lambda phage DNA, Ad5 and lac sequences are indicated, and the remaining sequences are of pBR322 (Bolivar *et al.*, 1977).


Pst I
Eco RI
Pst I
Sal I
Pst I
Ap r
0%
pFG66
Ad5
8%
Pst I
Hind III
lac z
Eco RI
Pst I
T4
ligase
Pst I
Eco RI
Ap r
Pst I
pFG66DI
Hind III
Eco RI

of β-galactosidase expression observed for pFG66. Therefore, only in phase ATG triplets were considered candidates for translational initiation sites. The five possible initiation codons and their surrounding sequences are shown in table 2. The initiation codon at position 2727 is the most probable candidate. Just 5 bp upstream is a 4 bp sequence which is complementary to the 3' end of *E. coli* 16S rRNA. In addition, the sequences 5' and 3' to 2727 resemble those of the "consensus" ribosome binding sequence proposed by Scherer *et al.* (1980).

The Ad5 sequences of pFG66D1 were also analyzed for prokaryotic transcriptional promoter sequences. The model sequence proposed by Scherer *et al.* (1978) was the sole criterion for identifying putative promoters. Table 3 shows the Ad5 sequence which most closely resembled the model. The predicted 5' end of the transcribed RNA would correspond to position 2698, 29 bp upstream of the predicted ribosome binding site.

Having tentatively identified the sequences responsible for β-galactosidase expression from pFG66D1 (and presumably pFG66 also) plasmids were constructed which had these sequences deleted. In the following section I describe the protocol for generating these deletions, while maintaining a unique restriction site downstream of the 58K initiation codon for subsequent reconstitution of the E1b coding sequences.

Table 2. Candidate Sites for Initiation of Translation in pFG66.

2679

1. GATAGGGTGGCCTTTAGATGTAGCATGATAAATATGTGGCCGGGGGT

2688

2. GCCTTTAGATGTAGCATGATAAATATGTGGCCGGGGGTGCTTGGCAT

2709

3. AATATGTGGCCGGGGGTGCTTCCGATGGACGGGGTGGTTATTATGGA

2727

4. CTTGGCATGGACGGGGTGGTTATTATGAATGTAAGGTTTACTGGCCC

5. CCCCGCGCGTTGGCCGATTCATTAATGCAGCTG

2019

6. TGCTTTTTTGAGTTTTATAAAGGATAAATGGAGCGAAGAAACCCATCTGAG

5' 3'
AACCUGCGGUUGGAUCACCUCCUUA 16S rRNA

Brosius *et al.*, 1978

Shine and Dalgarno, 1974

TTTTAAAAATTAAGGAGGTATATTATGAAAAAAATTAAAAAACTCAA
AA T A ATA A CTC G

Scherer *et al.*, 1980

Sequences 1-6 represent the antisense strand written 5'→3'. ATG initiation codons in phase with 58K and β-galactosidase in pFG66 are boxed and the nucleotide position is indicated. Nucleotide positions in agreement with the sequence of Scherer *et al.* (1980) are underlined and sequences which are complementary to the 3' end of 16S rRNA are overlined. Sequence 5 lies within the lacI portion of pFG66. The overlined nucleotides in 16S rRNA most frequently participate in ribosomal binding to natural *E. coli* mRNA (Kozak, 1983).

Table 3. Putative Ad5 Promoter of pFG66D1

```
                                                  2687
                                                  -10
Ad5   5' GGAGGATAGGGTGGCCTTTAGATGTAGCATGATAAATATGTGGCCGGG 3'

                                                -10
lac     ACCCCAGGCTTTACACTTTATGCTTCCGGGTCGTATGTTGTGTGGAAT

                                          -10
tacI    GAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCG


        acc-t-gttGTTGAcATTTtt----ttggcGGTTATATTg---cCAT
                 t    a    a             ATA a       g
```

Scherer *et al.*, 1978

The antisense strands of the putative Ad5 promoter and the lac (Dickson *et al*, 1974) and tacI (de Boer *et al.*, 1983) promoters are presented. The -10 position is given with reference to the 5' end of the transcribed RNA (+1). An exact base to base correspondence between the model sequence of Scherer *et al.* (1978) and the promoters is indicated by thick (capital letters) and thin bars (small letters). In the model sequence of Scherer *et al.* (1978) the capital letters represent bases which appear to have high functional significance and are conserved in most promoters.

3.1.3 Construction of lac$^-$ derivatives of pFG66

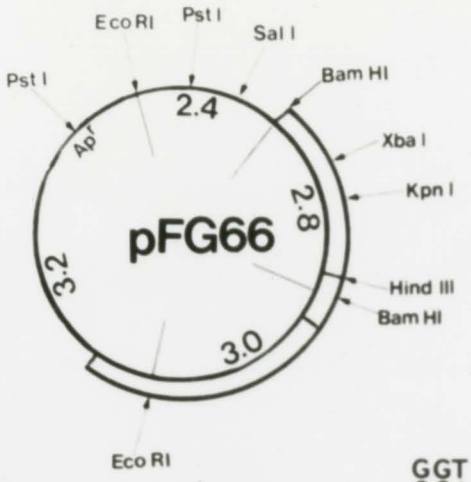
In figure 4 the approach used to construct lac$^-$ plasmids containing 58K-β-galactosidase or 19K-β-galactosidase hybrid genes is outlined.

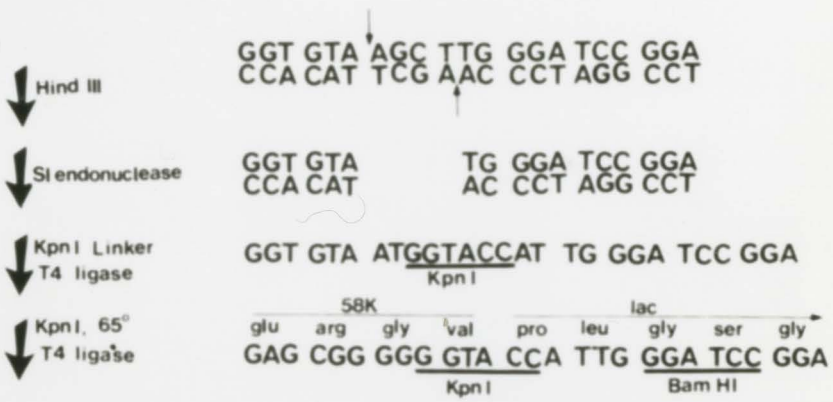
pFG66 was linearized by digestion with HindIII which cuts at the junction of Ad5 and lac sequences. Limited nuclease S1 treatment was used to remove the HindIII single-stranded overhangs (figure 4). A synthetic KpnI decamer linker was phosphorylated and inserted by blunt-end ligation, creating a KpnI recognition site at the Ad5-lac junction. Digestion with KpnI followed by *in vitro* ligation resulted in the deletion of Ad5 sequences 2048-2800. This includes the region thought to contain transcriptional and translational control sequences. Transformation of lac$^-$ *E. coli* NK5031 on MacConkey indicator plates provided rapid screening of lac$^-$ recombinants.

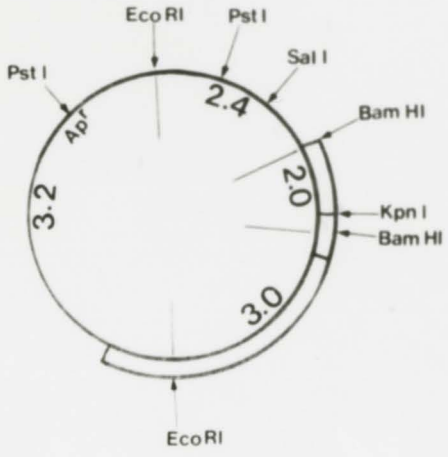
Plasmids were extracted from lac$^-$ colonies and analyzed for the presence of the KpnI restriction site. Out of sixty-six plamids, I found four which had the KpnI site and the desired deletion. These were designated p24, p29, p32 and p36. The remaining plasmids had restriction patterns similar to pFG66 but lacking the HindIII site. These presumably resulted from blunt end ligation of the S1-treated HindIII cut, and are lac$^-$ due to a frameshift.

Since nuclease S1 can result in limited digestion of double-stranded DNA sequences, it was decided to determine if this had occurred during the construction of p24, p29, p32, and p36. The plasmids were analyzed for the presence of a unique BamHI site in the lacI sequences. If three or more bases of double-stranded DNA had been removed by

Figure 4. Construction of $lac^-$ deletion derivatives of pFG66. The predicted sequences resulting from each step of the protocol are shown along with agarose gel analysis of EcoRI-BamHI restricted pFG66, p24, p29, p32 and p36.


EcoRI
PstI
SalI
PstI
BamHI
2.4
Ap
XbaI
KpnI
2.8
pFG66
3.2
HindIII
BamHI
3.0
EcoRI


GGT GTA AGC TTG GGA TCC GGA
CCA CAT TCG AAC CCT AGG CCT
HindIII
SI endonuclease
GGT GTA TG GGA TCC GGA
CCA CAT AC CCT AGG CCT
KpnI Linker
T4 ligase
GGT GTA ATGGTACCAT TG GGA TCC GGA
KpnI
KpnI, 65°
T4 ligase
58K
lac
glu arg gly val pro leu gly ser gly
GAG CGG GGG GTA CCA TTG GGA TCC GGA
KpnI
BamHI


EcoRI
PstI
SalI
PstI
2.4
Ap
BamHI
2.0
KpnI
3.2
BamHI
3.0
EcoRI


EcoRI, BamHI
24
29
32
36
FG66
4.4 kb
3.2
3.0
2.8
2.4
2.0

nuclease S1, the BamHI recognition site would be lost (figure 4). I found that only one plasmid (p29) had retained the BamHI site, confirming that double-stranded sequences were lost during the S1 treatment. If no sequences had been lost, the resulting plasmid would be in phase for 58K expression (figure 4). However, since double-stranded sequences were lost in at least 3 plasmids, the possibility of reading frame shifts had to be considered. In table 4 I have listed the predicted shifts in reading frames resulting from nuclease S1 digestion of double-stranded sequences. Resulting plasmids could have any one of three Ad5 reading frames in phase with β-galactosidase. In addition, start or stop codons could be introduced in certain instances.

The reading frames of the four plasmids could be determined either directly by nucleotide sequencing or indirectly by mapping the locations of the lac promoter in β-galactosidase expressing plasmids. I chose the second approach, which involves promoter insertion throughout the Ad5 sequences upstream of the β-galactosidase gene. If the plasmid were in phase with 58K, β-galatosidase expressing plasmids should have the promoter upstream of the 58K initiation codon (2019). Similarly plasmids in phase with 19K should have the promoter upstream of the 19 initiation codon (1714). In principle, β-galactosidase expressing plasmids should not result from promoter insertion into plasmids which have the closed reading frame in phase with β-galactosidase.

### 3.1.4 Promoter Insertion

The general protocol for promoter insertion is outlined in

Table 4. Predicted Shifts in Reading Frames Resulting From Nuclease S1 Treatment

| Number of Bases Digested by S1 | Nucleotide Sequence | Reading Frame in Phase with lacZ |
|---|---|---|
| 0 | GGG GTA CCA TTG GGA TCC GGA GCT<br>KpnI BamHI | 58K |
| 1 | GG GGT ACC T▽ATG GA TCC GGA GCT | CRF, (closed reading frame) |
| 2 | G GGG TAC CAT TG▽GA TCC GGA GCT | 19K |
| 3 | GGG GTA CCA TGG▽TGA TCC GGA GCT | 58K |
| 4 | GG GGT ACC TGGG▽ATA TCC GGA GCT | CRF |
| 5 | G GGG TAC CAT TGGGA▽TCC GGA GCT | 19K |
| 6 | GGG GTA CCA TGGGAT▽TCC GGA GCT | 58K |
| 7 | GG GGT ACC TGGGATC▽ATC GGA GCT TGG | CRF |
| 8 | GGGG TAC CAT TGGGATCC▽GGA GCT TGG | 19K |
| 9 | GGG GTA CCA TGGGATCCG▽TGA GCT TGG | 58K |
| 10 | GG GGT ACC TGGGATCCGG▽ATA GCT TGG | CRF |
| 11 | G GGG TAC CAT TGGGATCCGGA▽GCT TGG | 19K |
| 12 | GGGG GTA CCA TGGGATCCGGAG▽TCT TGG | 58K |

Sequences represent the antisense strand written 5'→3' in the β-galactosidase reading frame. Nuclease restriction sites are underlined and deleted bases are indicated. In phase ATG codons or nonsense codons are boxed.

figure 2. The plasmids were linearized at the unique XbaI site (1339). A progressive exonuclease III (ExoIII) digestion followed by nuclease S1 treatment was used to digest from the XbaI site to the 19K or 58K initiation codons. The DNA was then digested with PstI which interprets the β-lactamase gene.

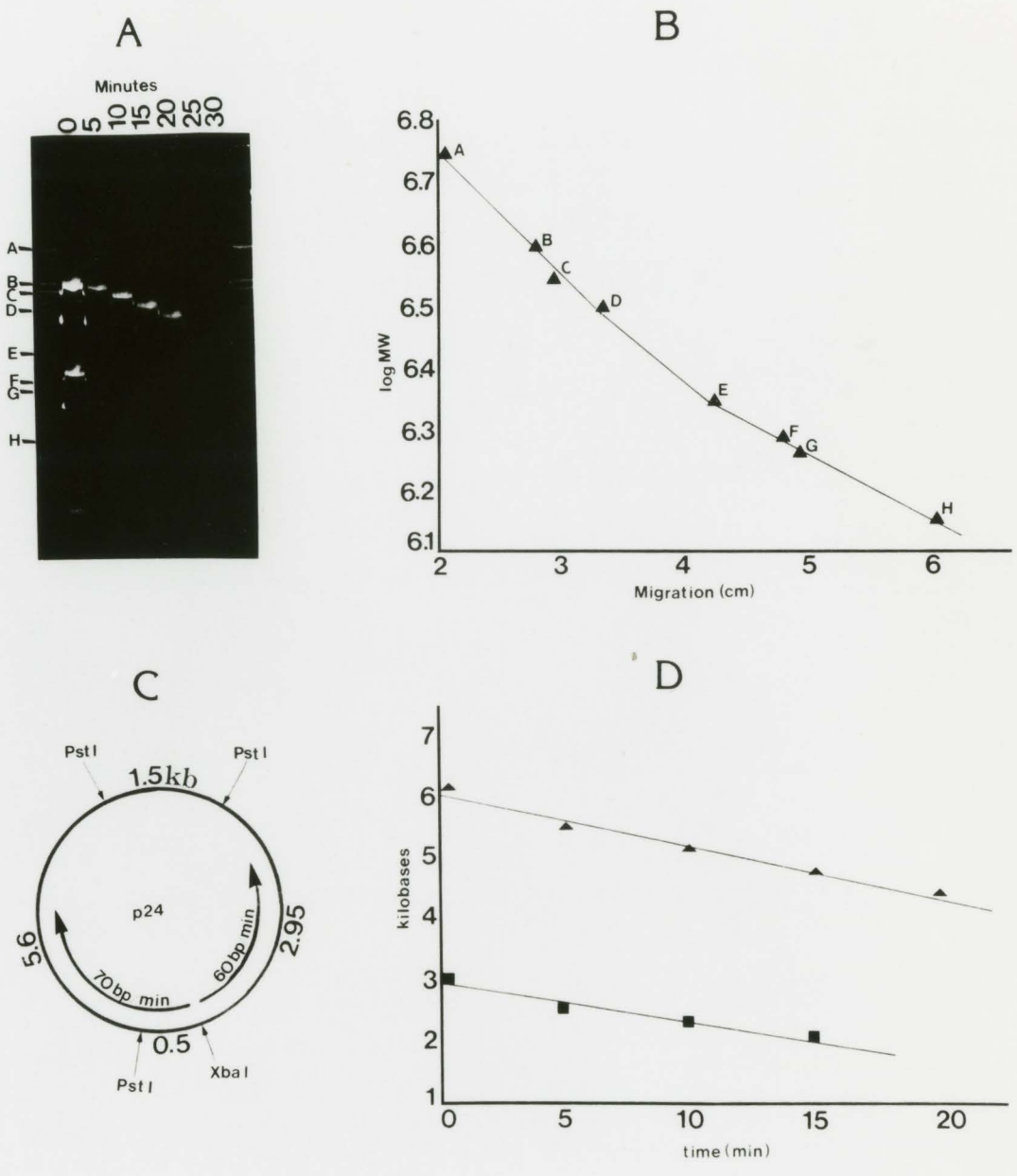
From pGL101 (Guarente *et al.*, 1980) a PstI-PvuII restriction fragment carrying the lac uv5 promoter was isolated. Ligation into the above DNA resulted in reconstitution of the β-lactamase gene and promoter insertion at positions determined by the end-point of the ExoIII digestion. Promoter placements which optimized the spacing between the SD sequence carried on the promoter fragment and the initiation codon should express high levels of β-galactosidase activity.

Because promoter location was dependent upon the extent of the ExoIII digestion, preliminary experiments were conducted to determine the rate of digestion by ExoIII under defined conditions. Figure 5 shows the result of an experiment using p24 DNA linearized with XbaI, and then digested with ExoIII for varying lengths of time followed by S1 and PstI digestions. Under those conditions, ExoIII proceeded at approximately 60-70 bp per minute.

### 3.1.5 Promoter Placement for Expression of 58K

As discussed previously, the reading frames of the 4 plasmids were not known. Therefore promoter insertion for 58K expression was attempted for each plasmid. Based on the predicted rate of digestion, ExoIII reactions were run for nine minutes, thereafter aliquots were removed at five second intervals and pooled in S1 buffer to stop the

Figure 5. Determination of the rate of DNA digestion by Exonuclease III. A) Agarose gel of p24 DNA restricted with XbaI and digested with ExoIII for 0-30 minutes followed by nuclease S1 and PstI digestions. Marker fragments A-H are of Ad5 DNA restricted with HindIII. B) Graph showing relationships between marker fragment sizes and migration on agarose gel. C) Map of p24 showing the calculated rates of digestion by ExoIII. D) Graph used to calculate the rates of ExoIII digestion. Solid triangles represent the sizes of fragments used to calculate the counter-clockwise digestion rate and solid squares represent the sizes of fragments used to calculate the clockwise digestion rate.


A
Minutes
0 5 10 15 20 25 30
A
B
C
D
E
F
G
H
B
6.8
6.7
6.6
6.5
6.4
6.3
6.2
6.1
log MW
2 3 4 5 6
Migration (cm)
C
Pst I
Pst I
1.5kb
p24
5.6
2.95
70bp min
60bp min
0.5
Pst I
Xba I
D
7
6
5
4
3
2
1
kilobases
0 5 10 15 20
time (min)

reaction. Under these conditions, digestion end points should have ranged from position 1850 to beyond the 58K initiation codon. Following promoter insertion as described above, $lac^-$ *E. coli* NK5031 were transformed and $lac^+$ recombinants were isolated off MacConkey indicator plates.

Promoter insertion into p24 resulted in $lac^+$ recombinants at a frequency of $\sim 10^{-2}$. As described in the next section, these plasmids all had promoters which mapped either upstream of the 58K initiation codon or within the lacI sequences. $Lac^+$ recombinants resulting from promoter insertion into p29, p32, or p36 all contained the promoter within the lacI sequences and expression probably resulted from insertion immediately upstream of the in phase ATG codon in lacI. The results suggest that p24 was probably in phase for 58K, whereas p29, p32 and p36 had either the closed reading frame or 19K in phase with β-galactosidase. Experiments conducted to establish the reading frames of p29, p32 and p36 are described in a later section.

### 3.1.6 Mapping of Promoter Position in p24

A total of 45 $lac^+$ colonies were isolated by promoter insertion into p24 and designated as p24EX1-p24EX45. Double digestions with PstI and KpnI were conducted to determine the approximate locations of the inserted promoter (results not shown). Twenty-seven plasmids had lost the KpnI site, indicative of promoter insertion within lacI sequences and translation initiation from the in phase initiation codon in lacI. The remaining insertions all mapped upstream of the KpnI site (2048) and presumably the 58K initiation codon (2019).

The promoter position of several p24EX plasmids were subsequently mapped using a variety of restriction enzymes. Double digestions with EcoRI and PvuII were used to map p24EX3, 9, 15, 16 17, 20, 24, 27, and 39. PvuII cuts at the junction of lacI and lacZ sequences (Guarente *et al.* , 1980), approximately 100 bp 3' to the KpnI site of p24. EcoRI cuts at the 5' end of the lac promoter fragment. Double digestion with EcoRI and PvuII produces a diagnostic fragment which contains the lac promoter, Ad5 sequences from site of promoter insertion to KpnI, and the lacI sequences. Sizing of this fragment was used to estimate the promoter locations (figure 6). With the exception of p24EX20, the EcoRI-PvuII results were in agreement with results using PstI-KpnI analysis. PstI-KpnI analysis mapped the promoter of p24EX20 to approximately position 1970 in Ad5 whereas analysis with EcoRI-PvuII mapped the promoter 100 bp 3' of that position within the lacI sequences. However, the promoter could not have been positioned within lacI because the KpnI site had been demonstrated in p24EX20. To clarify this descrepancy a derivative of p24EX20 (p24EX20.82) was analyzed with several restriction endonucleases. p24EX20.82 has only one predicted PvuII site yet analysis with PvuII revealed an additional PvuII site located approximately 450 bp away from the predicted PvuII site (figure 7b). Further analysis with PvuII in combination with other enzymes showed that this additional PvuII site was located at or near the junction of the lac promoter and Ad5 sequences (figure 7b). The promoter fragment ends with $^{5'}$CAG$^{3'}$ and therefore must have integrated at $^{3'}$CTG$^{5'}$ to create this PvuII site. Only three such sites exist in

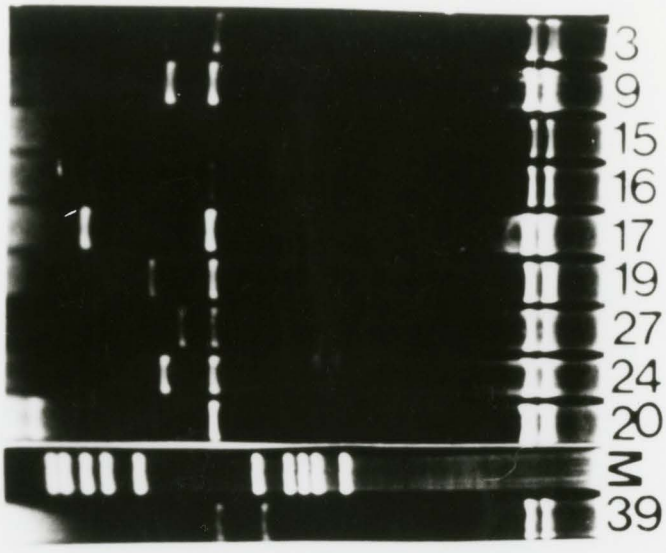
the 100 bp immediately upstream of the 58K initiation codon, clustered at positions 1978, 1985 and 1988. Integration at these sites would result in promoter placements 40, 34, and 31 bp respectively upstream of the 58K initiation codon (figure 7b). HaeIII analysis of p24EX20.82 confirmed that the promoter of p24EX20 was located 30-50 bp upstream of the 58K initiation codon and was used to confirm the promoter locations in p24EX27 and p24EX39 (figures 7a and 8).

The p24EX20 mapping results provided an accurate reference point to which promoter positions of other p24EX plasmids could be compared. The majority of p24EX plasmids had the promoter positioned upstream with respect to that of p24EX20. However, four plasmids (p24EX15, 16, 17, 30) had promoters which mapped closer to the 58K initiation codon than p24EX20. A comprehensive summary of the promoter mapping results is shown in figure 9.

### 3.1.7 β-galactosidase Expression from p24EX Plasmids

To quantitate the level of gene expression, β-galactosidase activity was assayed by the method of Miller (1972). The results are presented in Table 5. The levels of activity varied greatly depending on promoter location. Promoter position should determine the spacing between the promoter's SD sequence and the 58K initiation codon, which in turn should dictate the efficiency of translation. In contrast, I found that levels of activity were not dependent upon the promoter being in close proximity to the initiation codon. In fact, promoters located as far as 210 bp upstream of the 58K initiation codon (p24EX39) expressed higher levels of activity than those immediately upstream of

Figure 6. Polyacrylamide gel analysis of p24EX plasmids digested with EcoRI and PvuII. Marker fragments are of pBR322 digested with HaeIII. The diagnostic fragment is indicated with an asterisk.


3
9
15
16
17
19
27
24
20
M
39

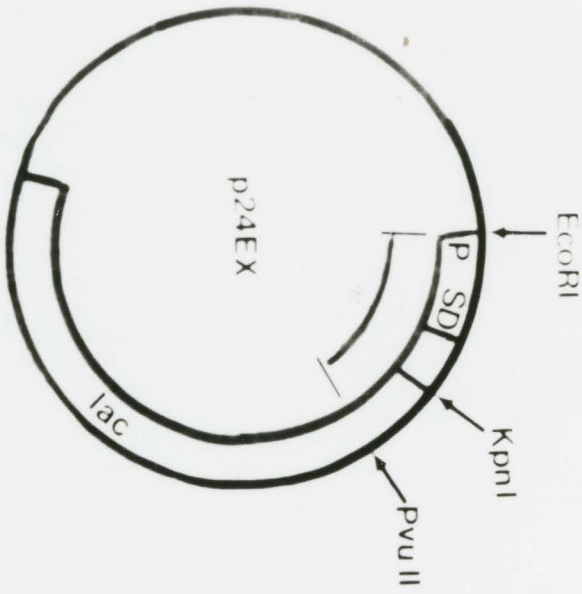
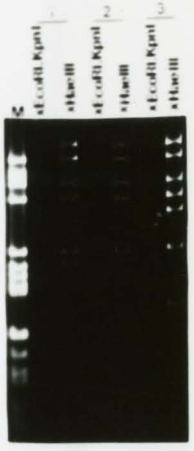
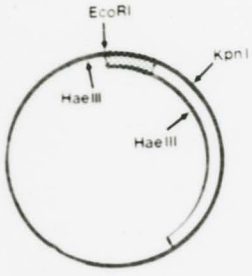
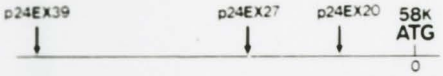

p24EX
P
SD
lac
EcoRI
KpnI
PvuII

Figure 7. Detailed restriction analysis of p24EX plasmid derivatives. A) Polyacrylamide gel analysis: lanes 1, p24EX27.82; lanes 2, p24EX20.82; lanes 3, p24EX39.82; markers, pBR322 x HaeIII. B) Agarose gel analysis of p24EX20.82: lane 1, PvuII; lane 2, PstI PvuII; lane 3, PvuII EcoRI KpnI; lane 4, PvuII KpnI; marker, Ad5 x HindIII. Fragment sizes are given in base-pairs (bp). The three potential sites for promoter insertion in p24EX20 are shown.

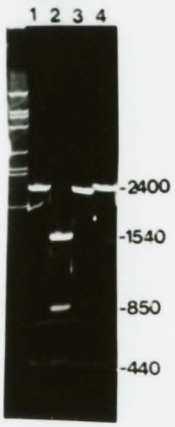
# A

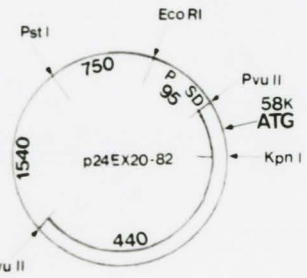



EcoRI
Kpn I
Hae III
Hae III


p24EX39
p24EX27
p24EX20
58K
ATG
0

# B


1 2 3 4
2400
1540
850
440


Pst I
Eco RI
750
Pvu II
SD
58K
ATG
1540
p24EX20-82
Kpn I
440
Pvu II

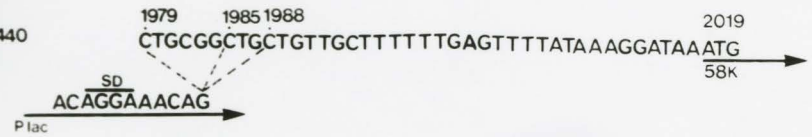

1979
1985 1988
2019
CTGCGGCTGCTGTTGCTTTTTTGAGTTTTATAAAGGATAAATG
58K
SD
ACAGGAAACAG
Plac

Figure 8. Promoter mapping of p24EX plasmid derivatives by polyacrylamide gel analysis. Lane 1, pBR322 x HaeIII; lane 3, p24EX39.82 x HaeIII; lane 5, p24EX20.82 x HaeIII; lane C, p24EX27.82 x HaeIII. The diagnostic HaeIII fragment is indicated with an asterisk and the approximate promoter locations as determined by the unique HaeIII fragments are shown. Fragment sizes are given in bp.

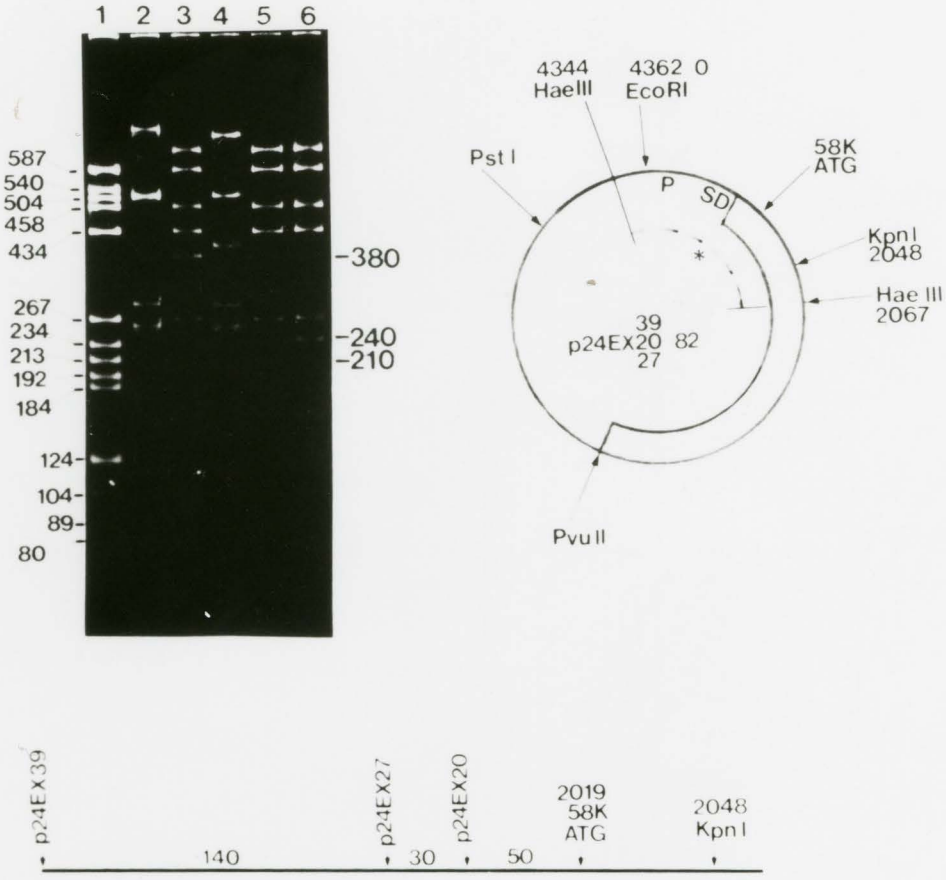

1 2 3 4 5 6
587
540
504
458
434
267
234
213
192
184
124
104
89
80
-380
-240
-210
4344
HaeIII
4362 0
EcoRI
Pst I
58K
ATG
P
SD
Kpn I
2048
Hae III
2067
39
p24EX20 82
27
Pvu II
p24EX39
140
p24EX27
30
p24EX20
50
2019
58K
ATG
2048
Kpn I

Figure 9. A comprehensive summary of the p24EX mapping results. Positions of p24EX20, 27 and 39 were determined using EcoRI-PvuII (figure 6), EcoRI-KpnI (figure 7) and HaeIII (figures 7 and 8) analysis. Relative promoter positions of the remaining p24EX plasmids were based on EcoRI-PvuII analysis (figure 6) or PstI-KpnI analysis (data not shown).

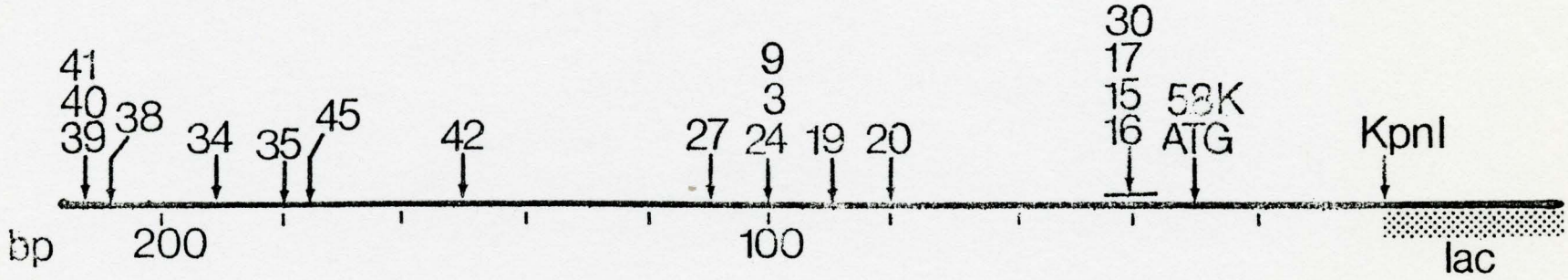

41
40
39
38
34
35
45
42
9
3
27
24
19
20
30
17
15
16
53K
ATG
KpnI
bp
200
100
lac

Table 5. Levels of β-galactosidase Activity from p24EX Plasmids

| Plasmids | β-galactosidase Activity (mean ± SD) |
|---|---|
| p24EX3 | 748 ± 79 |
| p24EX9 | 367 ± 49 |
| p24EX15 | 99 ± 15 |
| p24EX16 | 304 ± 77 |
| p24EX17 | 228 ± 43 |
| p24EX19 | 598 ± 96 |
| p24EX20 | 558 ± 51 |
| p24EX24 | 754 ±106 |
| p24EX27 | 352 ± 51 |
| p24EX39 | 429 ± 46 |

β-galactosidase activity is expressed in units as defined by Miller (1972). Each value represents the mean of four independent replicates ± the standard deviation.

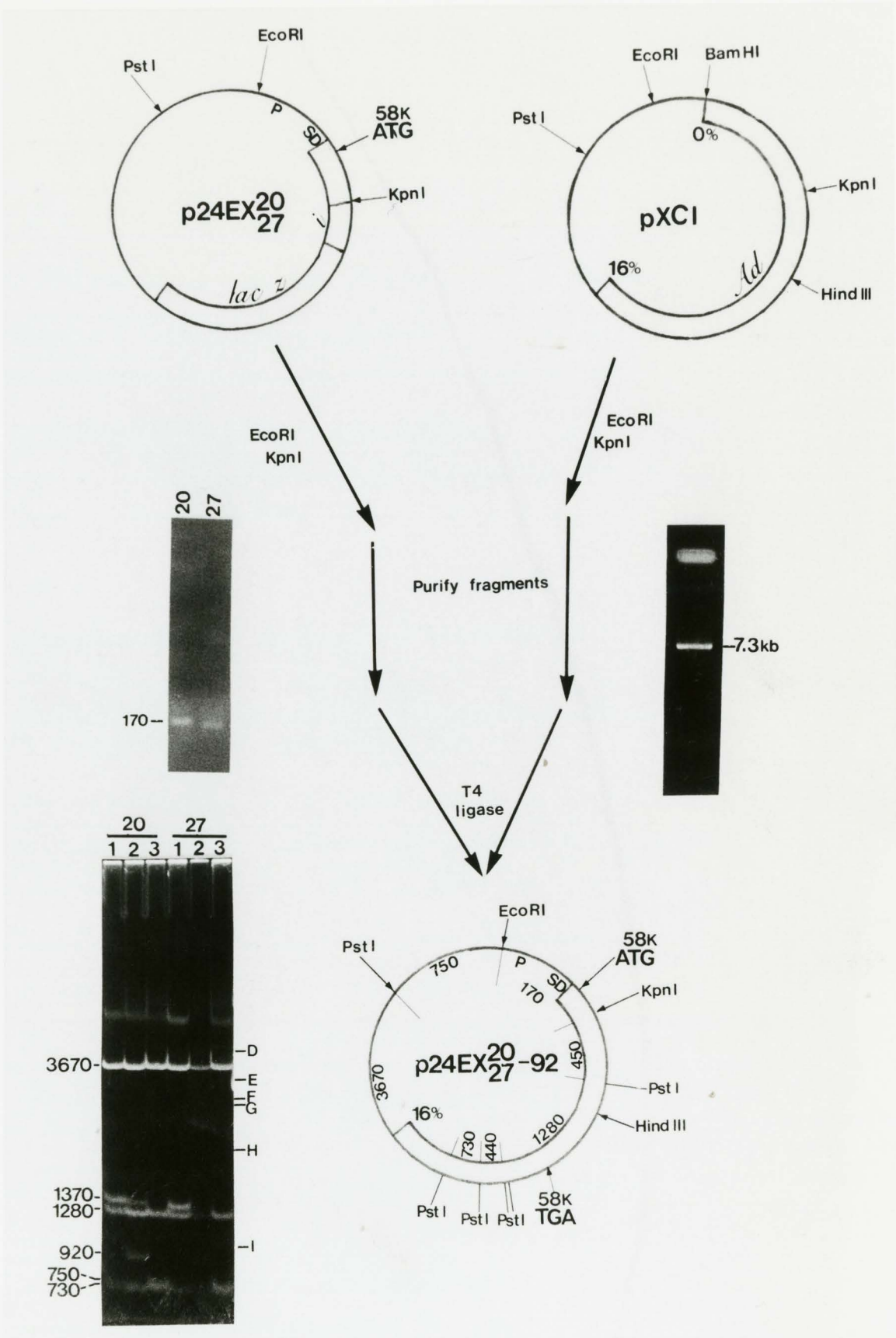
the initiation codon (p24EX15, 16, 17). Roberts *et al.* (1979b) obtained similar results from inserting the lac promoter-SD fragment varying distances upstream from cro sequences which contained the native cro ribosome binding sequences. Similarly, these results could be explained if there were a functional ribosome binding site in the Ad5 sequences surrounding the 58K initiation codon. Candidate sequences are shown in Table 2. Three base-pairs upstream of the 58K initiation codon, there is a 4 bp SD sequence. Although the distance separating this sequence from the initiation codon is less than optimal (7 ± 2 bp), translation could conceivably initiate from this site. In addition, sequences both 5' and 3' to the ATG triplet resemble the "consensus" ribosome binding sequence of Scherer *et al.*, (1980) (Table 2). Initiation of translation therefore may be directed by an adenovirus-encoded ribosome binding sequence.

3.1.8 Reconstitution of 58K Coding Sequences of p24EX Plasmids

The final step in the construction of 58K-expressing plasmids is the substitition of sequences encoding the carboxyl portion of 58K for the lac sequences in a p24EX plasmid. The approach used is outlined in figure 10.

From pXC1 (McKinnon *et al.*, 1982) an EcoRI-KpnI restriction fragment was isolated. This contained the β-lactamase gene, plasmid origin of replication, and 58K coding sequences from KpnI (2048) to 3510 (TGA). Similarly, EcoRI-KpnI fragments containing the lac promoter, 58K initiation codon, and 58K coding sequences up to the KpnI site (2048) were isolated from p24EX20, p24EX27, and p24EX39. These

Figure 10. Procedure for the reconstitution of the 58K coding sequences of p24EX20 and p24EX27. Agarose gel analysis of restriction fragments isolated from polyacrylamide gels are shown as well as agarose gel analysis of the reconstituted plasmids p24EX20.92 and p24EX27.92: 1, PstI; 2, PstI KpnI; PstI KpnI EcoRI; marker, Ad5 x HindIII. Fragment sizes given in bp.


EcoRI
Pst I
P
SD
58K
ATG
Kpn I
p24EX20 27
i
lac Z
EcoRI
Bam HI
Pst I
0%
Kpn I
pXCI
16%
Ad
Hind III
EcoRI
Kpn I
EcoRI
Kpn I
20
27
170
Purify fragments
7.3kb
T4
ligase
20
27
1 2 3 1 2 3
3670
1370
1280
920
750
730
D
E
F
G
H
I
EcoRI
Pst I
750
P
SD
170
58K
ATG
Kpn I
p24EX20 27 -92
3670
450
Pst I
16%
730
440
1280
Hind III
Pst I
Pst I
Pst I
58K
TGA

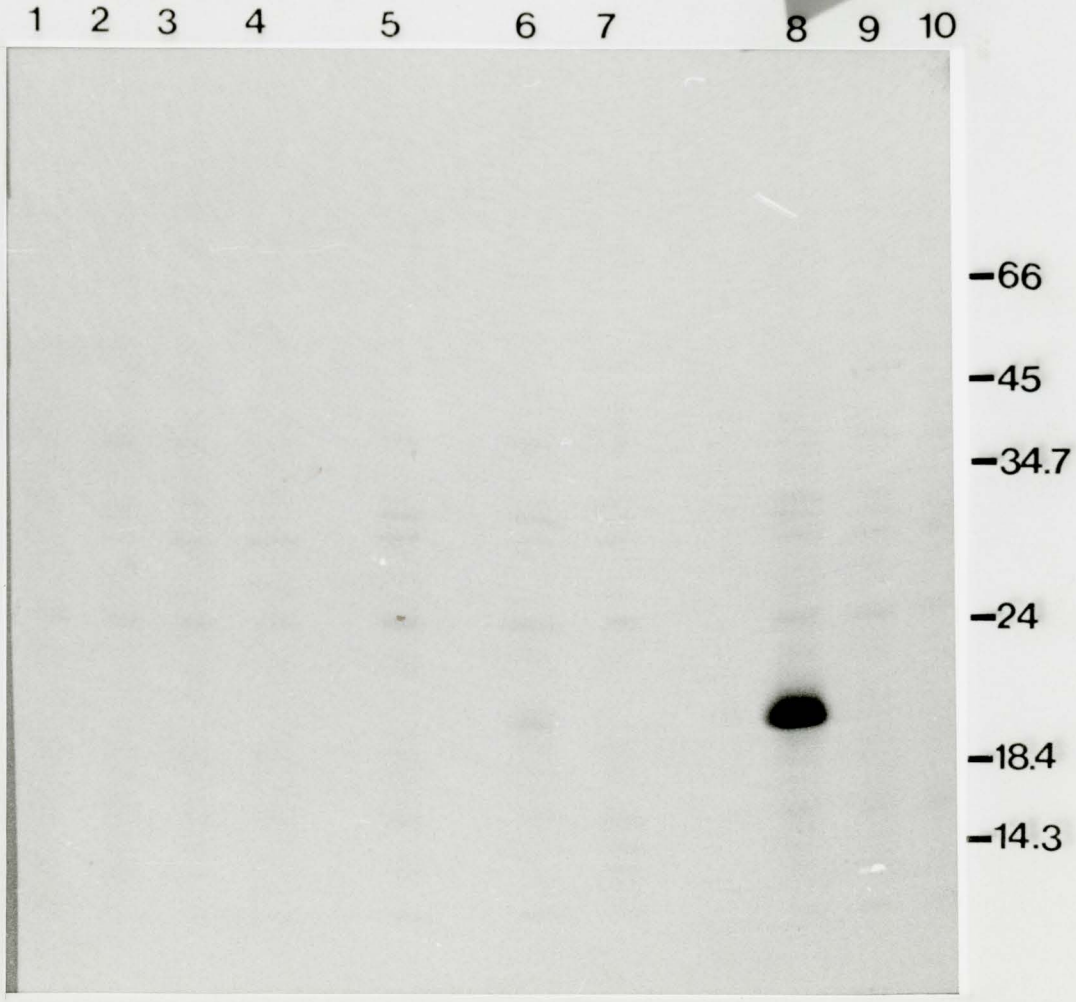
fragments were ligated *in vitro* to the pXC1 fragment and a lac $i^q$ strain of *E. coli* (D1210) was transformed. This strain overproduces the lac repressor (Sadler *et al.*, 1980). In the absence of a suitable inducer (lactose, IPTG), transcription from the lac promoter is inhibited. This avoided selection against the construction of plasmids which express 58K in the event that such expression might be deleterious. Products of the reconstituted plasmids were later analyzed by induction with the gratuitous inducer IPTG.

Recombinants which resulted from the reconstitution experiment were analyzed with several restriction endonucleases. The predicted restriction map of reconstituted p24EX20 and p24EX27 corresponded to the results of the restriction analysis (figure 10), thereby verifying the identities of reconstituted p24EX20 and p24EX27. Similar analysis confirmed successful reconstitution of p24EX39 (results not shown). The reconstituted plasmids were designated p24EX20.92, p24EX27.92 and p24EX39.92.

3.1.9 Evaluation of Expresser Plasmids for Production of 58K

To determine if the above plasmids directed synthesis of 58K in *E. coli*, $^{35}$S-methionine labelled cell extracts were prepared from transformants cultured with or without induction (IPTG). These were immune precipitated using antisera from hamsters bearing Ad5-induced tumors or normal hamsters. Figure 11 (lanes 1-4) shows an autoradiograph of immune precipitates from p24EX20.92 and p24EX27.92 run on a denaturing SDS-polyacrylamide gel. Clearly, no inducible, plasmid-encoded, tumor-specific protein of MW 58K was detected. Similarly, no such protein

Figure 11. Autoradiograph of $^{35}$S-methionine labeled *E. coli* D1210 extracts immune precipitated and run on a 14% SDS-polyacrylamide gel. Samples 1-4 were immune precipitated with a mixture of AE and 14b antisera. Lane 1, p24EX20.92 uninduced; lane 2, p24EX20.92 induced; lane 3, p24EX27.92 uninduced; lane 4, p24EX27.92 induced. Lane 5, *E. coli* D1210 (no plasmid) induced and immune precipitated with a mixture of AE and 14B antisera. Lane 6, p24EX21.83, uninduced, AE and 14b; lane 7, p29EX21.83, induced, normal hamster antiserum; lane 8, p24EX21.83, induced, AE and 14b, lane 9, p29EX27.91, uninduced, AE and 14b; lane 10, p29EX27.91, induced, AE and 14b. Molecular weight standards are given in kilo daltons (kdaltons).


1 2 3 4 5 6 7 8 9 10
66
45
34.7
24
18.4
14.3

was detected from p24EX39.92 (result not shown). At present it is not known whether the failure to detect 58K was due to: i) instability of the transcript, ii) instability of the 58K protein in *E. coli*, iii) low avidity of the tumor serum for 58K produced in *E. coli* or iv) levels of 58K synthesis too low for detection by immune precipitation. Evidence in support of 58K being sensitive to protease degradation in *E. coli* will be discussed later.

## 3.2 19K Expression in *E. coli*

### 3.2.1 Promoter Placement for Expression of 19K

As discussed previously, the plasmids p29, p32 and p36 could have 19K in phase with β-galactosidase. To determine this, the promoter was positioned at points upstream of the 19K initiation codon in these three plasmids. Plasmids were linearized by digestion with XbaI and ExoIII digestions were run for 5-8 minutes. The predicted endpoints of the ExoIII digestion ranged from positions 1600-1800. Due to a PstI site at 1833, downstream of the 19K initiation codon (1714), a partial PstI digestion was required prior to promoter insertion so that a proportion of the molecules would be cut within the β-lactamase gene but not at position 1833 in Ad5. This step lowered the efficiency of promoter insertion, in fact only 36 $lac^+$ recombinants were isolated from p29 in 3 attempts. All attempts using p32 and p36 failed to yield $lac^+$ colonies, suggesting that these plasmids probably had the closed reading frame in phase with β-galactosidase. As described in the following section, all of the p29EX plasmids which contained the lac promoter, had it positioned upstream of the 19K initiation codon. This suggested

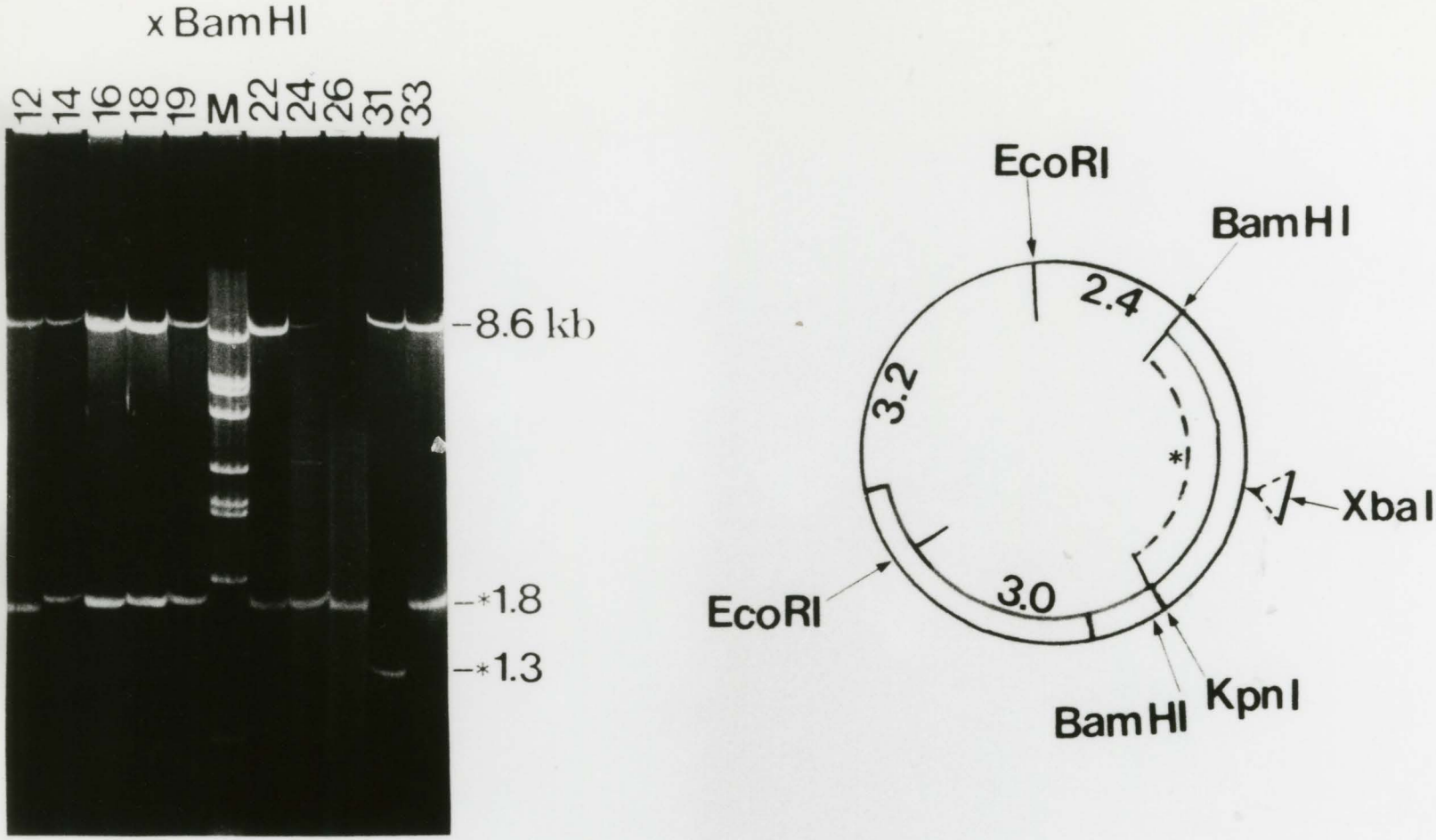
that p29 had 19K in phase with ß-galactosidase.

3.2.2 Mapping of Promoter Position in p29

The $lac^+$ recombinants derived from p29 were designated p29Ex1-p29EX36. Promoter positions were mapped using EcoRI-KpnI double digestions in analyses similar to those used previously to locate the site of promoter insertion relative to the 58K initiation codon. Nine plasmids had the promoter positioned upstream of the 19K initiation codon. Surprisingly, the remaining plasmids did not contain the promoter fragment. Analysis of these "promoterless" expressers with BamHI showed that they were essentially identical to p29 with the exception of a small deletion of Ad5 sequences (figure 12). p29 contains ∿ 2050 bp of Ad5 sequences flanked by BamHI sites. All of the "promoterless" expressers retained the BamHI sites (figure 12) but were missing the XbaI site (result not shown). Sizing of the BamHI fragments (figure 12) showed the 26 plasmids had lost approximately 200 bp and one had lost 750 bp of Ad5 sequences. Presumably these plasmids represent deletions around the XbaI site, resulting from XbaI digestion followed by ExoIII-S1 treatment and blunt-end ligation. Although "promoterless", these simple deletion derivatives of p29 apparently express the 19K-ß-galactosidase hybrid protein. However, levels of expression were quite low (see next section), so further characterization was not justified. Presently we do not understand the basis for expression from these plasmids, although possible explanations will be discussed.

During the course of the mapping experiments another complication

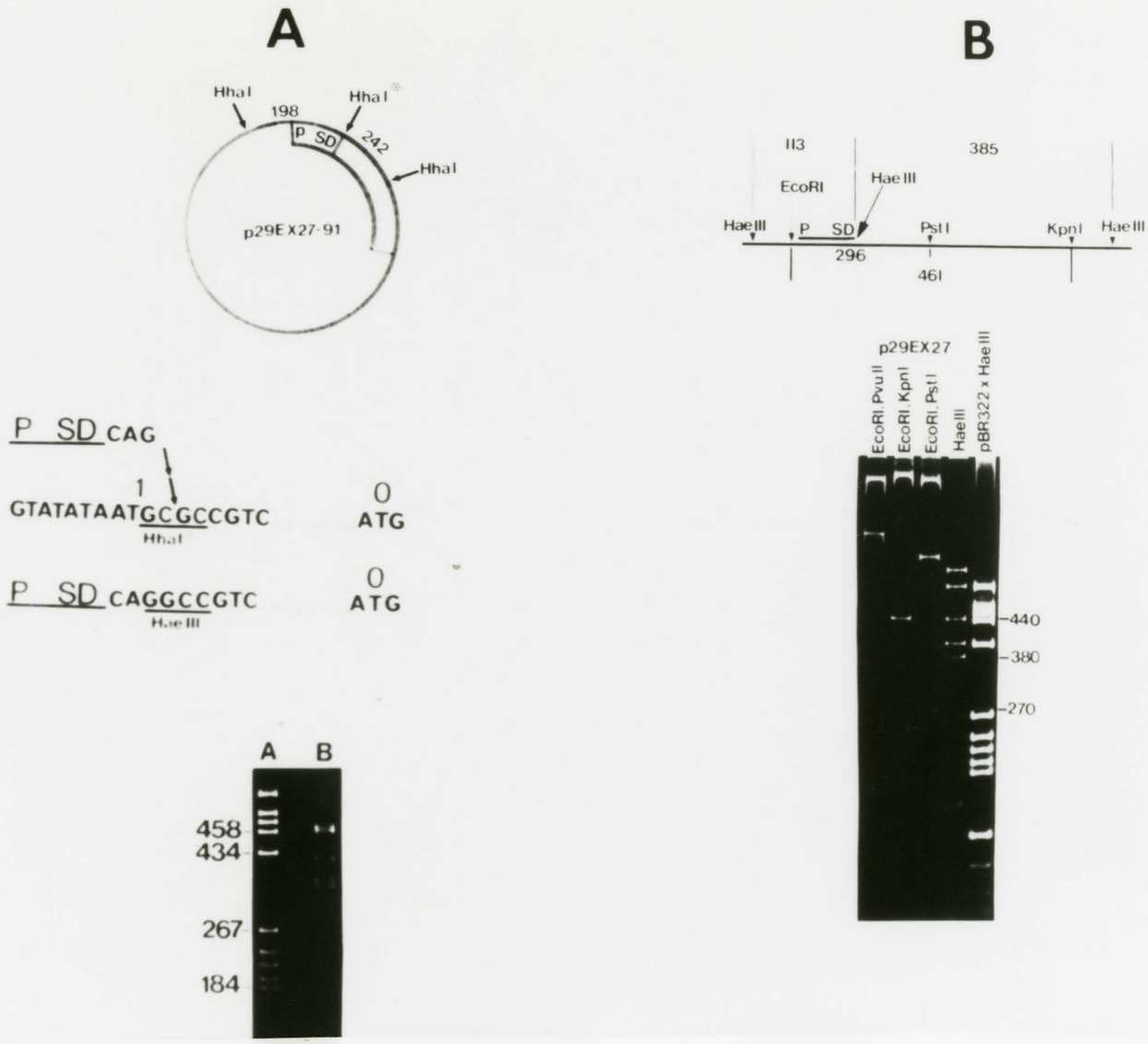
Figure 12. Agarose gel analysis of "promoterless" p29EX plasmids. The diagnostic BamHI fragment is indicated with an asterisk.


x BamHI
12
14
16
18
19
M
22
24
26
31
33
-8.6 kb
-*1.8
-*1.3
EcoRI
BamHI
2.4
3.2
*
XbaI
EcoRI
3.0
BamHI
KpnI

became evident. Within the 100 bp immediately 5' to the 19K initiation codon (1714), there are two in phase initiation codons which extend the 19K reading frame in the 5' direction. Utilization of the initiation codon at 1627 [-2] or 1678 [-1] would result in expression of 19K analogues having 29 or 12 additional amino acids respectively at the amino terminus of 19K. Preliminary mapping showed that 8 of 9 plasmids had the promoter situated upstream of the initiation codon at 1627 [-2]. The remaining plasmid, p29EX27, had the promoter upstream of either the initiation codon at 1678 [-1] or the authentic 19K initiation codon [0].

To determine the exact location of the p29EX27 promoter, a more detailed restriction analysis was conducted (figure 13). Using EcoRI-KpnI digestion, a diagnostic fragment of ∿ 440 bp was observed. This corresponded to a promoter positioned at ∿ 1693. PstI-EcoRI digestion resulted in a 270 bp fragment which corresponded to a promoter positioned at 1658. Digestion with HaeIII produced a diagnostic band at ∿ 380 bp. This was not consistent with the EcoRI-KpnI or PstI-EcoRI results above. HaeIII should produce a unique fragment of ∿ 530 bp if the promoter were situated at 1650. To reconcile this discrepancy, the hypothesis was adopted that a HaeIII site had been created at the junction of promoter-Ad5 sequences. This would result in 2 fragments from HaeIII digestion, migrating at 385 bp and 113 bp. Although the 113 bp fragment was not resolved (its presence was not anticipated), the 380 bp band observed could correspond to the 385 bp fragment predicted above. To create a HaeIII site [$^{5'}$GGCC$^{3'}$], the promoter fragment (ending in $^{5'}$CAG$^{3'}$) would have to integrate at

Figure 13. Restriction analysis using polyacrylamide gel electrophoresis to map the promoter position in p29EX27. A) HhaI restriction map showing the predicted HhaI site at the junction of promoter-Ad5 sequences (asterisk) and the consequence of promoter insertion at the HhaI site. Polyacrylamide gel analysis: lane A, pBR322 x HaeIII; lane B, p29EX27.91 x HhaI. B) Detailed polyacrylamide gel restriction analysis of p29EX27.91 showing the predicted restriction map. Fragment sizes given in bp.


A
HhaI
198
HhaI
P SD
242
HhaI
p29EX27-91
P SD CAG
1
GTATATAATGCGCCGTC
HhaI
0
ATG
P SD CAGGCCGTC
HaeIII
0
ATG
A B
458
434
267
184
B
113
385
EcoRI
HaeIII
HaeIII
P SD
PstI
KpnI
HaeIII
296
461
p29EX27
EcoRI PvuII
EcoRI KpnI
EcoRI PstI
HaeIII
pBR322 x HaeIII
440
380
270

$5'_{GCC}{}^{3'}$. Examination of Ad5 sequences from 1580 to 1820 revealed only one GCC sequence located at position 1682. Integration at this site would not only create a HaeIII site, but eliminate a HhaI site at 1679 (figure 13a). Analysis with HhaI produces several restriction fragments of predicted size. If the HhaI site at position 1679 were present diagnostic fragments of 198 bp and 242 bp should result (figure 13a). Elimination of this site should produce a fragment of 440 bp. The predicted fragments of 198 bp and 242 bp were not observed, yet a fragment migrating slightly faster than the 434 bp marker fragment was observed (figure 13a). In conjunction with the HaeIII analysis, the elimination of the HhaI site at position 1679 strongly suggests that the promoter of p24EX27 was located at position 1682, just 32 bp upstream of the authentic 19K inititaion codon.

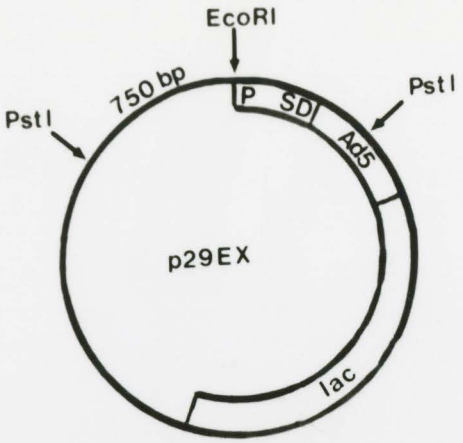
Promoter position of the remaining p29EX plasmids were mapped by EcoRI-PstI digestions (figure 14). A summary of the p29EX promoter mapping is given in figure 14.

### 3.2.3 β-galactosidase Expression from p29EX Plasmids

Table 6 shows the levels of β-galactosidase activity assayed for the p29EX plasmids. The 27 "promoterless" plasmids all expressed at low levels (100-250 units). Seven of the eight plasmids which had the promoter upstream of [-2] expressed high levels of activity (950-2200 units), the remaining plasmid however expressed only 150 units. High levels of activity were observed for p29EX27 (1300 units) which has its promoter upstream of [0] or [-1].

With the exception of p29EX17, high levels of activity were

Figure 14. Polyacrylamide gel analysis of PstI-EcoRI digested p29EX plasmids and their promoter position designations relative to the 19K initiation codons. Marker, pBR322 x HaeIII. Fragment sizes given in bp.


EcoRI
750 bp
PstI
PstI
P
SD
Ad5
p29EX
lac


13
17
20
21
23
25
27
M
587
540
458
434
267

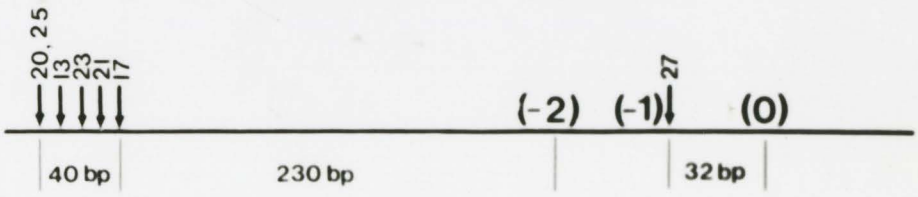

20, 25
13
23
21
17
(-2)
(-1)
27
(0)
40 bp
230 bp
32 bp

Table 6. Levels of β-galactosidase Activity from p29EX Plasmids

| Plasmid | β-galactosidase Activity (mean ± SD) |
|---|---|
| p29EX7 | 150 ± 11 |
| p29EX8 | 165 ± 23 |
| p29EX9 | 230 ± 16 |
| p29EX10 | 155 ± 14 |
| p29EX11 | 147 ± 9 |
| p29EX12 | 193 ± 18 |
| p29EX13* | 1099 ± 87 |
| p29EX14 | 236 ± 18 |
| p29EX15 | 135 ± 16 |
| p29EX16 | 148 ± 10 |
| p29EX17* | 85 ± 9 |
| p29EX18 | 216 ± 15 |
| p29EX20* | 1085 ±234 |
| p29EX21* | 2220 ±217 |
| p29EX22 | 176 ± 15 |
| p29EX23* | 1342 ±166 |
| p29EX24 | 203 ± 18 |
| p29EX25* | 954 ±186 |
| p29EX26 | 205 ± 14 |
| p29EX27* | 1299 ±142 |
| p29EX28 | 210 ± 16 |

β- galactosidase activity is expressed in units as defined by Miller (1972). Each value represents the mean of four independent replicates ± the standard deviation.

Plamids containing the lac promoter are indicated by asterisks.

observed regardless of the spacing between the promoters' SD sequence and the initiation codons. As will be shown later, we have evidence suggesting that translation initiates at [-2] for p29EX21 which has its promoter ∿ 250 bp upstream of [-2]. Presumably translation also initiates at [-2] in the other p29EX plasmids which have the promoter upstream of [-2].

Table 7 lists the in phase 19K initiation triplets ([+1], [0], [-1] and [-2]) and their surrounding sequences. The [-2] initiation sequence is similar to the ribosome binding sequences proposed by Scherer *et al.* (1980) and contains a SD sequence 8 bp upstream of [-2]. Therefore initiation of translation from [-2] appears to be mediated by an adenovirus-encoded ribosome binding sequence.

Translation in p29EX27 could also depend on an adenovirus-encoded translational control sequence. The promoter's SD sequence is 36 bp upstream of [0] and probably is not involved in initiation of translation. Translation could initiate from either the [0] AUG codon or from a downstream [+1] GUG codon. Both sequences are shown in Table 7. At present, it is not known from which codon(s) translation initiates in p29EX27. However, because [-2] and [-1] were excluded in p29EX27, this plasmid was considered a possible candidate for expression of the authentic 19K antigen. Consequently, p29EX27 and p29EX21 were chosen to construct plasmids which contain the entire 19K coding sequences.

### 3.2.4 Reconstitution of the 19K Coding Sequences

The procedure used to reconstitute the 19K coding sequence was

Table 7. Candidate Sites for Initiation of Translation in p29

[-2]
1627

1. ATCTAAGTTTAATAAAGGGTGAGATAATGTTTAACTTGCATGGCGTGTTAAATGGGG

[-1]
1678

2. AATGGGCGGGGCTTAAAGGGTATATAATGCGCCGTGGGCTAATCTTGGTTAC

[0]
1714

3. CGTGGGCTAATCTTGGTTACATCTGACCTCATGGAGGCTTGGGAGTGTTTGGAA

[+1]
1750

4. CTTGGGAGTGTTTGGAAGATTTTTCTGCTGTGCGTAACTTGCTGGAACAGAGCTC

5' AACCUGCGGUUGGAUCACCUCCUUA 3'  16S rRNA

TTTTAAAAATTAAGGAGGTATATTATGAAAAAAATTAAAAAACTCAA
AA T A ATA T CTC G

Scherer *et al.* (1980)

Sequences 1-4 represent the antisense strand written 5'→3'. ATG or GTG initiation triplets in phase with 19K are boxed and the nucleotide position is indicated. Nucleotide positions in agreement with the sequence of Scherer *et al.* (1980) are underlined and sequences which are complementary to the 3' end of the 16S rRNA are overlined.

as described in section 3.1.8 with the exception that pXC78 was used instead of pXC1. pXC78 was constructed from pXC1 as shown in figure 15. This plasmid contains the Ad5 sequences from 1574 (HpaI) to 2487 (PvuII) and as a result the EcoRI-KpnI fragment isolated from pXC78 contained only the Ad5 sequences from 2048 (KpnI) to 2487 (PvuII). This was done to avoid constructing plasmids which contain both the complete 19K and 58K coding sequences. The 19K coding regions of p29EX21 and p29EX27 were reconstituted and the resulting plasmids were designated p29EX21.83 and p29EX27.91 respectively. Restriction analysis with HaeIII as described previously verified their predicted structures (figure 16).

3.2.5 Evaluation of Expresser Plasmids for Production of 19K

$^{35}$S-methionine labelled extracts of *E. coli* D1210 transformants were analyzed as described earlier. Figure 11 shows that a protein of MW 22K was produced by p29EX21.83. Synthesis of 22K was repressed in *E. coli* D1210 (lac $i^q$) and was increased $\sim$ 40 fold after induction by IPTG, indicating 22K is a product of the lac promoter transcript. *E. coli* D1210 containing no plasmid failed to synthesize 22K, indicating that synthesis of 22K is plasmid-specific. Finally, non-immune hamster serum failed to immune precipitate 22K, suggesting 22K is virus-specific. Figure 17 illustrates that the 22K protein comigrated with a major *E. coli* protein species. To estimate the amount of 22K relative to total cellular protein in the extract microdensitometer traces were made of the uninduced and induced cell extracts (figure 17, lanes 1 and 2 respectively). These were compared and the induced peak of 22K was estimated to be $\sim$ 3% of the total protein present in the extract.

Figure 15. Construction of pXC79 and pXC1. pXC1 contains Ad5 sequences from 0% - 16%; pXCd1A from 4.5% to 16%; pXC78 from 4.5% to 7.1%.

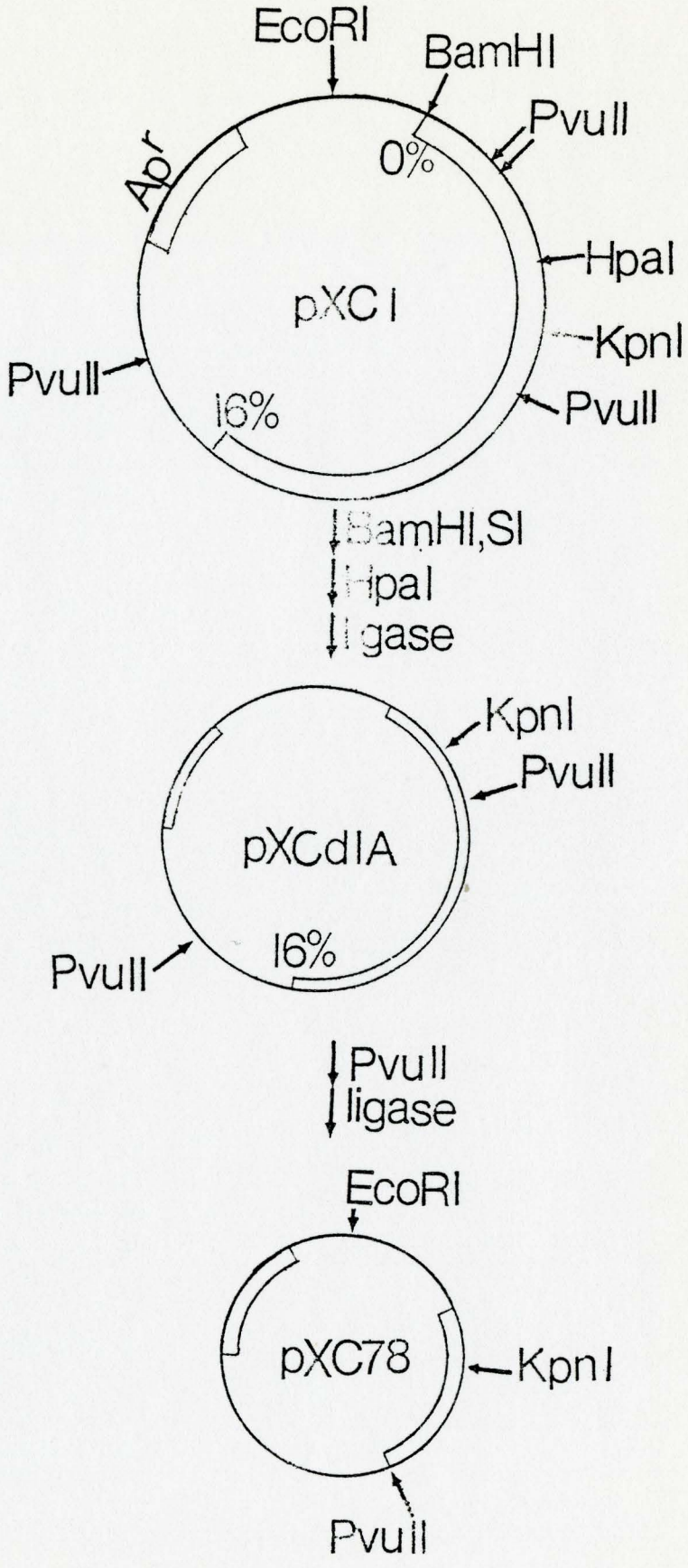
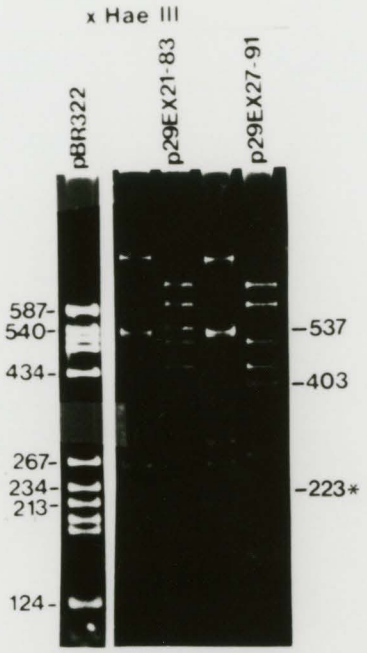
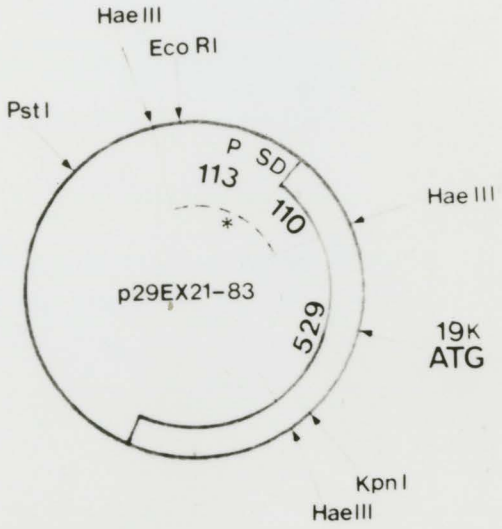
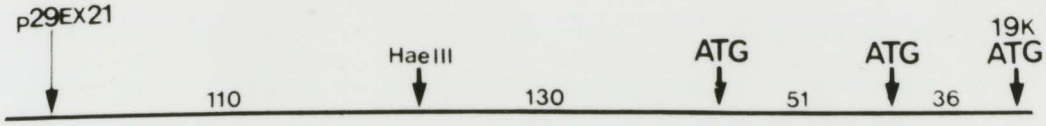

EcoRI
BamHI
PvuII
0%
Ap^r
HpaI
pXC1
KpnI
PvuII
16%
PvuII
BamHI,SI
HpaI
ligase
KpnI
PvuII
pXCd1A
16%
PvuII
PvuII
ligase
EcoRI
pXC78
KpnI
PvuII

Figure 16. Restriction analysis of p29EX21.83 and p29EX27.91. The diagnostic fragment is indicated with an asterisk and the promoter position of p29EX21.83 is shown. Fragment sizes given in bp.


x Hae III
pBR322
p29EX21-83
p29EX27-91
587-
540-
434-
267-
234-
213-
124-
-537
-403
-223*


Hae III
Eco RI
Pst I
P
SD
113
110
Hae III
p29EX21-83
529
19K
ATG
Kpn I
Hae III


p29EX21
Hae III
ATG
ATG
19K
ATG
110
130
51
36

The promoter of p29EX21.83 is ∿ 250 bp upstream of the [-2] initiation codon, making all three in phase initiation codons available for translation. Figure 18 shows that the protein expressed from p29EX21.83 is larger than the 19K antigen from Ad5 infected cells. Its molecular weight corresponded to that of a protein initiating from [-2] rather than from [-1].

Figure 17 shows that in addition to the 22K protein, 5 protein species of lower molecular weights were detected in relatively low quantitites from p29EX21.83. Each was subsequently shown to be inducible, plasmid-encoded and specifically immune-precipitated by tumor antiserum (figure 18). Their observed molecular weights were 21K, 19K, 17K, 15K and 13K (figure 18). It is tempting to speculate that the 21K and 19K species correspond to translational products of the 19K reading frame initiated from [-1] and [0] respectively. However, data in support of this is lacking.

Figures 17 and 18 show that the 17K and 15K polypeptides were synthesized by both p29EX21.83 and p29EX27.91 whereas the 22K, 21K, 19K and 13K polypeptides were synthesized exclusively by p29EX21.83. Because the 17K and 15K polypeptides were plasmid-encoded and inducible, they are probably products of the lac promoter transcript. As common products of p29EX21.83 and p29EX27.91 translation must have initiated from [0] in the 19K reading frame or from downstream initiation codons in either the 19K or 58K reading frames.

Using different antisera it was observed that the 22K protein of p29EX21.83 was antigenically different from the 17K and 15K polypeptides of p29EX21.83 and p29EX27.91. The two antisera employed (AE

Figure 17. Autoradiograph of $^{35}$S-methionine labeled samples analyzed on a 14% SDS-polyacrylamide gel. Lane 1, p29EX21.83 uninduced extract; lane 2, p29EX21.83 induced extract; lane 3, p29EX21.83 uninduced, immunoprecipitated with AE and 14b; lane 4, p29EX21.83 induced, AE and 14b; lane 5, p29EX27.91 uninduced, AE and 14b; lane 6, p29EX27.91 induced, AE and 14b; lane 7 KB cells infected with wt Ad5, AE and 14b. Molecular weight standards are given in kdaltons. Arrowheads indicate the positions of 22K, 21K, 19K, 17K, 15K and 13K.

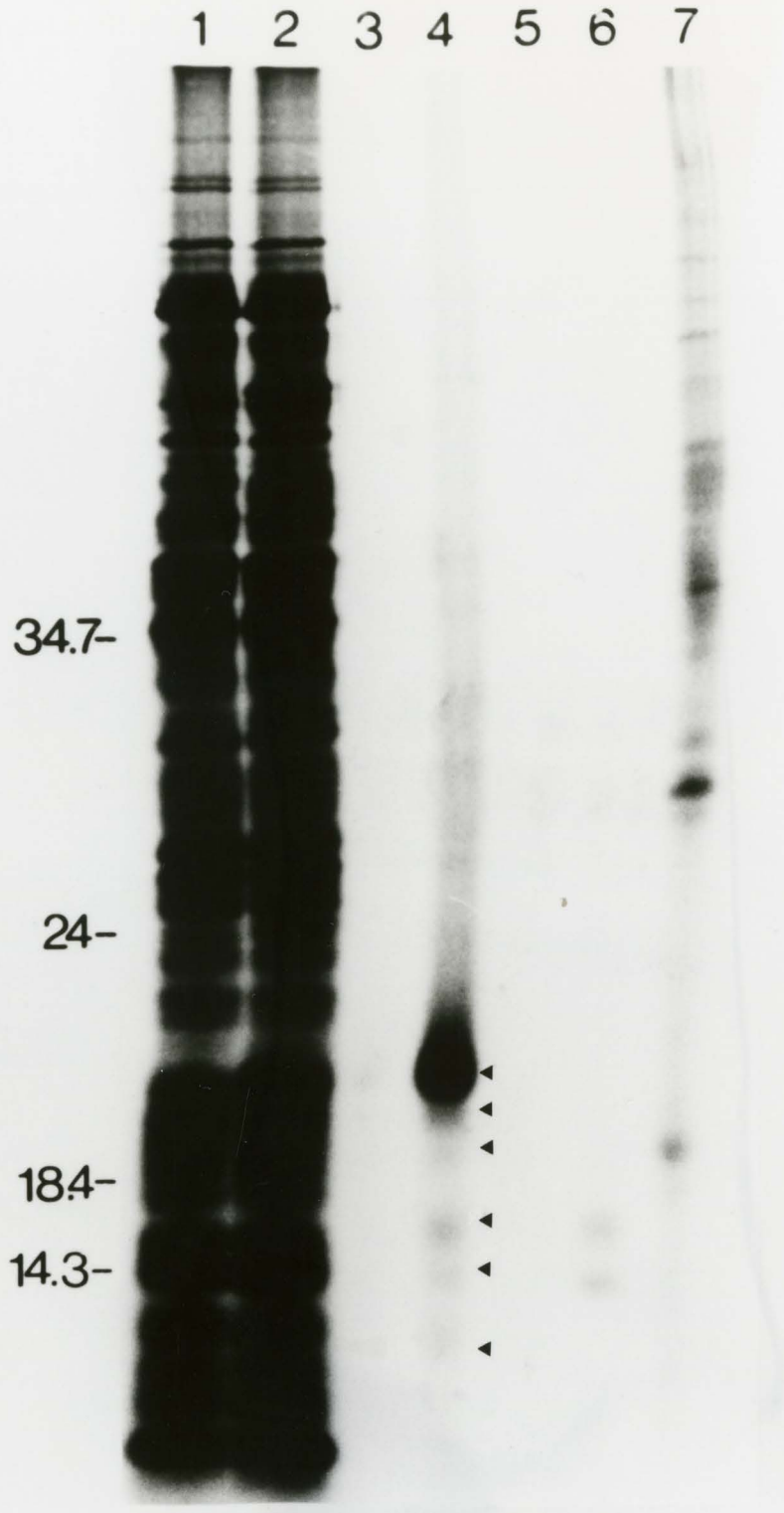

1 2 3 4 5 6 7
34.7-
24-
18.4-
14.3-

Figure 18. Autoradiograph of $^{35}$S-methionine labeled immune precipitates analyzed on a 14% SDS-polyacrylamide gel. Lane 1, p29EX21.83, uninduced, AE and 14b; lane 2, p29EX21.83, induced, normal hamster antiserum; lane 3, p29EX21.83, induced, AE and 14b; lane 4, p29EX21.83, induced, 14b; lane 5, *E. coli* D1210 (no plasmid), induced, AE and 14b; lane 6, p29EX27.91, uninduced, AE and 14b; lane 7, p29EX27.91, induced, normal hamster antiserum; lane 8, p29EX27.91, induced, AE and 14b; lane 9, KB cells infected with wt Ad5, AE; lane 10, KB-Ad5, normal hamster antiserum; lane 11, KB-Ad5, AE and 14b; lane 12, KB-Ad5, 14b. Molecular weight standards are given in kdaltons. Arrowheads indicate the positions of 22K, 21K, 19K, 17K, 15K and 13K.

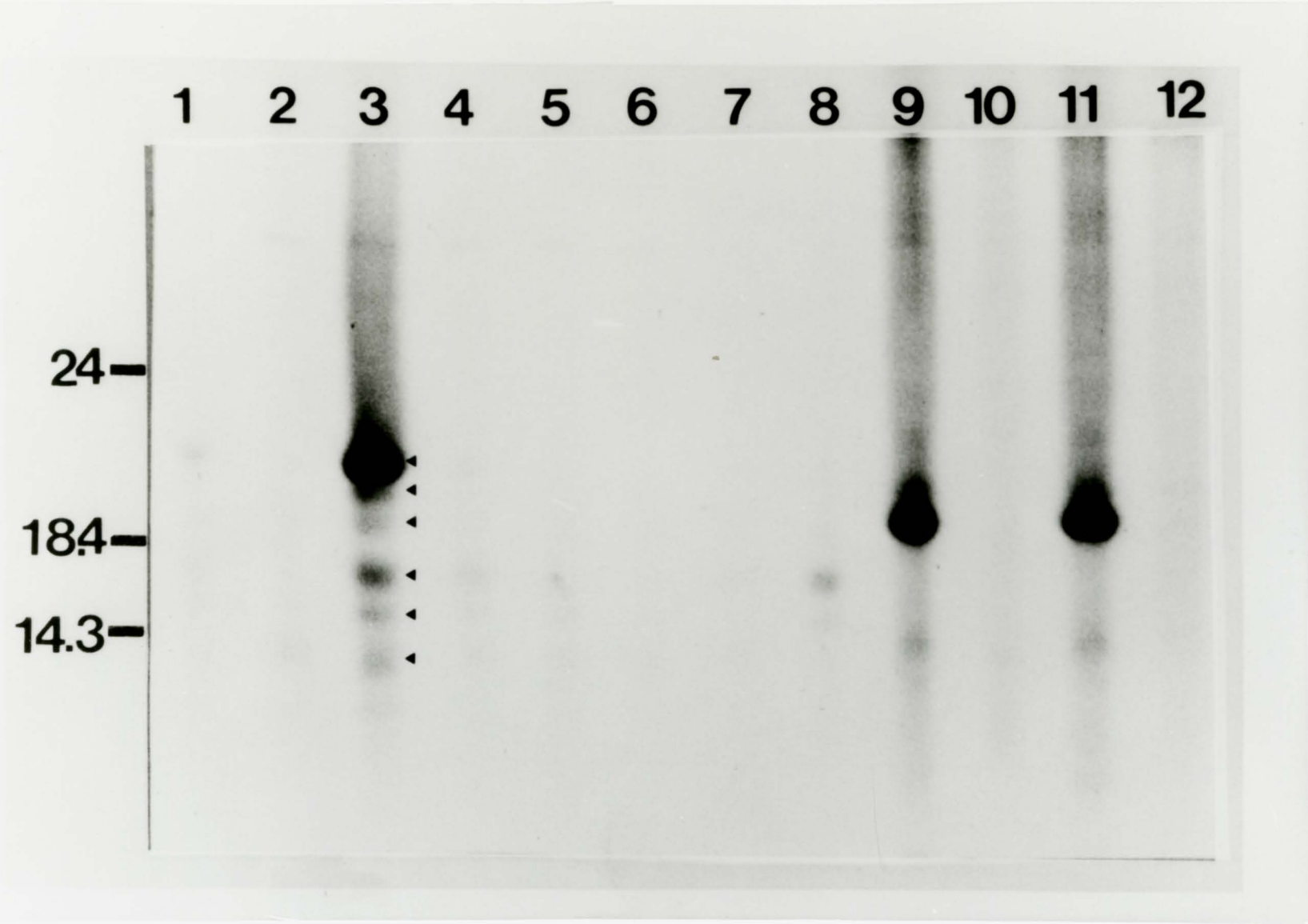
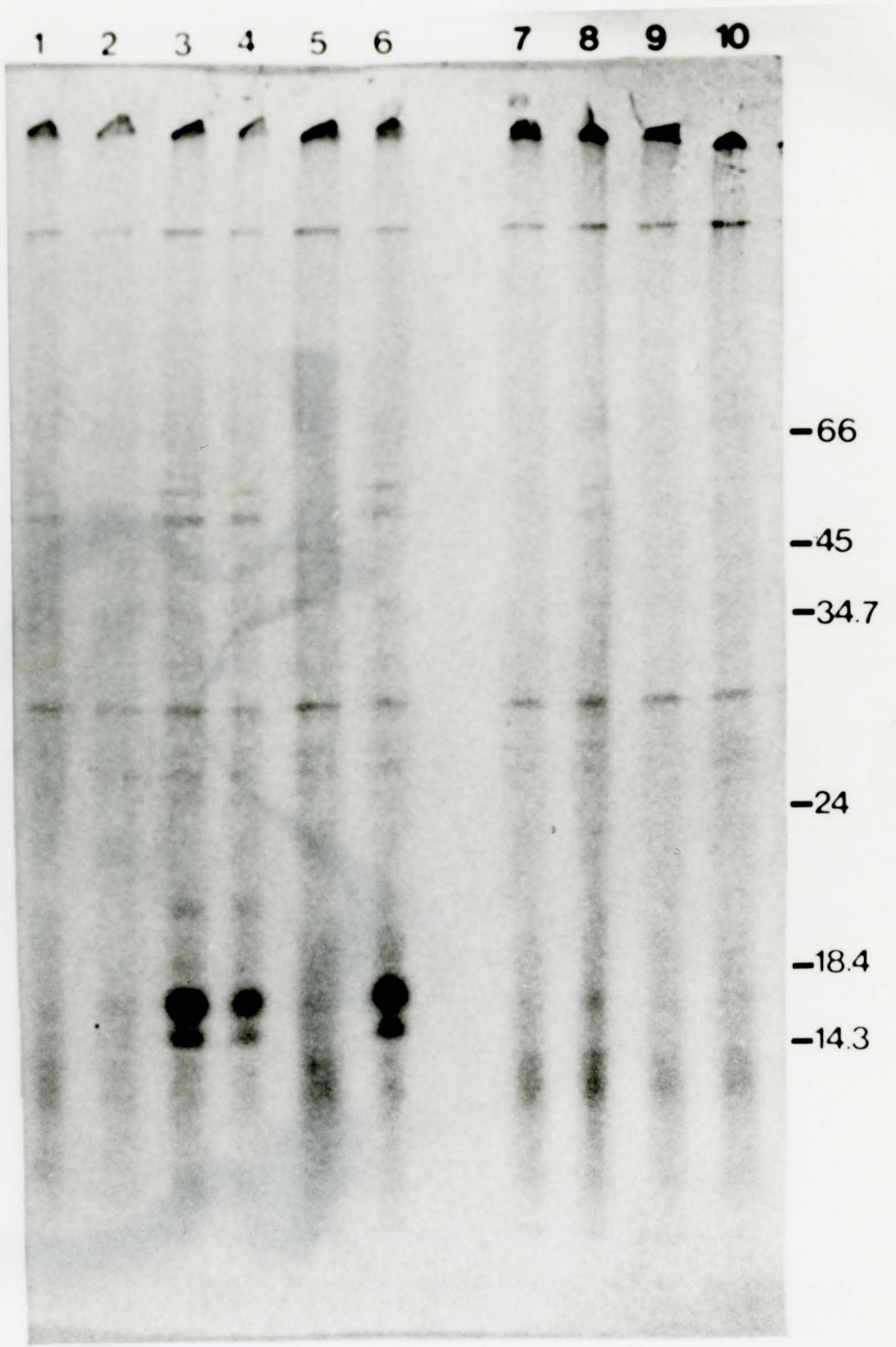

1
2
3
4
5
6
7
8
9
10
11
12
24
18.4
14.3

Figure 19. Authoradiograph of $^{35}$S-methionine labeled immune precipitates analyzed on a 14% SDS-polyacrylamide gel. All samples were immune precipitated using 14b antiserum unless otherwise stated. Lane 1, *E. coli* D1210 (no plasmid), induced, normal hamster antiserum; lane 2, p29EX21.83, uninduced; lanes 3 and 4, p29EX21.83, induced; lane 5, p29EX27.91, uninduced; lane 6, p29EX27.91, induced; lane 7, p24EX20.92, uninduced; lane 8, p24EX20.92 incuded; lane 9, p24EX27.92, uninduced; lane 10, p24EX27.92 induced. Molecular weight standard are given in kdaltons.


1
2
3
4
5
6
7
8
9
10
66
45
34.7
24
18.4
14.3

and 14B) show differential avidities towards the 58K and 19K antigens of viral-infected cells. AE antiserum shows strong avidity towards 19K and relatively weak specificity for 58K whereas the converse is true of 14b antiserum (see Materials and Methods). Figure 19 shows that 14b antiserum recognizes the 17K and 15K polypeptides but reacted relatively weakly with the 22K protein of p29EX21.83. This is in contrast to results obtained using AE antiserum which reacted very strongly with the 22K protein and recognized the 17K and 15K polypeptides as well (figure 17). If the 17K and 15K polypeptides were antigenically similar it would be expected that 14b antiserum would recognize the 22K protein to a degree similar to that observed with AE antiserum. The conclusion drawn from this experiment is that the 17K and 15K polypeptides are antigenically unrelated to the 22K protein and therefore are probably not products of the 19K reading frame. The lac promoter transcripts of p29EX21.83 and p29EX27.91 contain both 19K and 58K open reading frames. The 58K open reading frame extends from the 58K initiation codon into pBR322 sequences and encodes an amino-terminal portion of 58K of predicted MW 17,800 daltons. This could correspond to the 17K polypeptide of p29EX21.83 and p29EX27.91. Initiation of translation in the 58K reading frame from a downstream GUG codon at 2079 would produce a polypeptide of predicted MW 15,600 daltons, perhaps corresponding to the 15K polypeptide of p29EX21.83 and p29EX27.91.

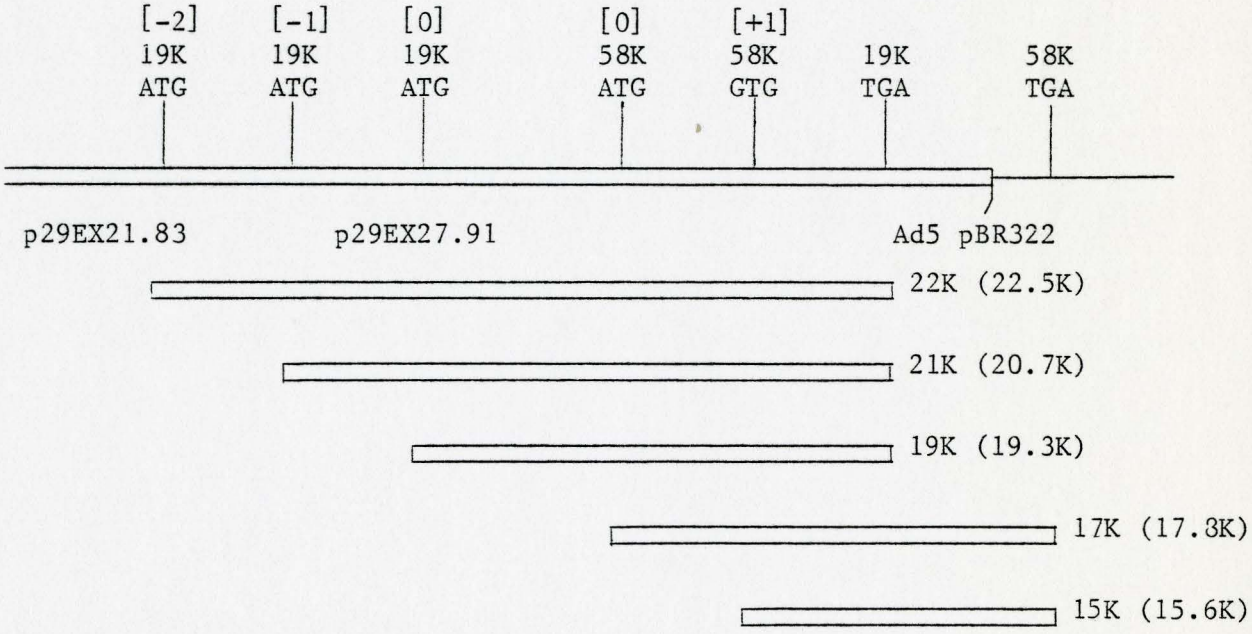
Lastly, a 13K protein and smaller species were produced from p29EX21.83. These polypeptides shared the same antisera specificity as the 22K protein and were not detected from p29EX27.91. Although apparently related to the 22K protein, these polypeptides do not

correspond to any specific 19K reading frame. They may be degradation products of the p29EX21.83 19K analogue proteins.

A summary of the various polypeptides produced from recombinant plasmids is shown in Table 8. Based on the considerations above, the polypeptides were assigned to either the 19K or 58K reading frame.

Table 8. Polypeptides Produced by Recombinant Plasmids

| Observed Polypeptide See Figure 17 | Plasmid Detected | Plasmid Not Detected | Tentative Assignment |
|---|---|---|---|
| 22K<br>21K<br>19K | p29EX21.83 | p29EX27.91<br>p24EX20.92<br>p24EX27.92<br>p24EX39.92 | 19K reading frame initiating from [-2], [-1], and [0] respectively. |
| 17K<br>15K | p29EX21.83<br>p27EX27.91 | p24EX20.92<br>p24EX27.92<br>p24EX39.92 | 58K reading frame initiating from [0] and [+1] respectively. |
| 13K<br>< 13K | p24EX21.83 | p24EX27.91<br>p29EX20.92<br>p29EX27.92<br>p24EX29.92 | 19K reading frame. Breakdown products of 22K or 21K. |




Tentative assignment of polypeptides produced from p29EX21.83 and p29EX27.91. Observed MWs are indicated and predicted MW are given in parentheses. The promoter positions of p29EX21.83 and p29EX27.91 are indicated. In phase start codons relative to the authentic 19K and 58K start codons [0] are shown as well as nonsense codons in the 19K and 58K reading frames.

# DISCUSSION

## 4.1 Complications in Retrospect

In the preceding section I have described attempts to express the Ad5 E1B 58K and 19K tumor antigens in *E. coli*. During the course of this study, several complications worthy of discussion were encountered. These included various anomalies of the E1B genes as well as problems intrinsic to the system employed.

The strategy we used (Guarente *et al.*, 1980) as well as other expression systems, rely strongly on the assumption that eukaryotic and prokaryotic genes are significantly different. As previously discussed, the transcriptional and translational control sequences of eukaryotes differ from those which control prokaryotic gene expression. However, due to the simplicity and degeneracy of prokaryotic transcriptional and translational control sequences, they could exist fortuitously in eukaryotic genes.

I have shown that a functional prokaryotic promoter exists within the 58K coding sequences. This is not without precedent, as others have reported functional prokaryotic within eukaryotic sequences (Westphal, 1970; Struhl *et al.*, 1980; Struhl and Davis, 1981).

Functional prokaryotic transcription terminators can also occur in eukaryotic genes. A fortuitous terminator in the 5' untranslated region of the Herpes Simplex type 1 thymidine kinase gene has been described (Becker *et al.*, 1983). An analogous situation 5' of the 19K coding sequences will be discussed later in this section.

Lastly, functional prokaryotic ribosome-binding sequences can occur in eukaryotic genes. Several eukaryotic genes have been expressed in *E. coli* having translation controlled by a fortuitous *E. coli* ribsome binding sequence (Emtage *et al.*, 1980; Struhl *et al.*, 1980; Kit *et al.*, 1981; Alton *et al.*, 1982). Fortuitous ribosome-binding sequences can direct translation from upstream or downstream initiation codons relative to the gene's authentic initiation codon. I have demonstrated fortuitous initiation from an upstream initiation codon in the 19K reading frame.

In addition to the above, several features of the expression protocol (Guarente *et al.*, 1980) made this method difficult to apply to expression of E1B antigens. Promoter insertion relies on digesting with ExoIII-S1 or Bal31 up to the gene's initiation codon from a unique upstream restriction site. In this study, ExoIII-S1 digestions were initiated from a unique XbaI site located 400 bp and 700 bp upstream of the 19K and 58K initiation codons respectively. Controlling the fidelity of the ExoIII digestion over such distances proved difficult. In the construction of 58K expression vectors, the ExoIII digestions extended beyond the 58K initiation codon into the lac sequences. This resulted in efficient translation initiating from an in phase initiation codon in lacI. Efficient use of this strategy would require a unique restriction site at a relatively short distance upstream of the gene's initiation codon.

In addition, the portion of the eukaryotic gene represented in the β-galactosidase fusion gene should not contain any PstI sites downstream of its inititiation codon. These necessitated the use of partial PstI digestions, significantly reducing the efficiency of promoter

insertion. Therefore, restriction sites both 3' and 5' of the genes initiation codon dictate the degree of efficiency with which the method of Guarente *et al.* (1980) can be used.

In retrospect, the use of cDNA corresponding to the 22S mRNA would have alleviated several of the above complications. cDNA could have been cloned into a plasmid vector which provides unique restriction sites in close proximity to the 5' end of the cDNA. Most importantly, cDNA would not contain the two upstream 19K initiation codons [-1] and [-2] because transcription of the 22S message initiates downstream of those codons (Perricaudet *et al.*, 1979, 1980). Clearly, the probability of creating ribosome-binding sites at the authentic 58K and 19K initiation codons would have been greater had cDNA been employed.

4.2 Expression of 58K

At present, it is not clearly understood why synthesis of 58K in *E. coli* could not be demonstrated. The plasmids from which the putative 58K expressors were derived contained the lac promoter upstream of the 58K-β-galactosidase coding sequences. Mean levels of β-galactosidase expression from thse recombinants (p24EX20, p24EX27, p24EX39) ranged from 350 to 550 units which corresponds to approximately 1000-4000 molecules per cell (Guarente *et al.*, 1980). These expression levels were lower than desired and may be attributed to any of the following: i) inefficient initiation of translation, ii) poor codon usage, or iii) low specific activity of the hybrid protein or instability of hybrid.

Translation probably initiates through a fortuitous SD sequence in the Ad5 sequences immediately upstream of the 58K initiation codon.

It is possible that initiation from this sequence is inefficient and elongation may be retarded due to poor codon use since of the eleven 58 codons present in the transcript, three are "modulator codons" whose corresponding tRNAs are very minor species in *E. coli* (Grosjean and Fiers, 1982). Lastly, low levels of β-galactosidase activity may be the consequence of reduced specific activity of the 58-β-galactosidase hybrid protein relative to β-galactosidase. This is unlikely because several proteins have been linked to the amino terminus of β-galactosidase with little or no reduction of enzymatic activity resulting (Bassford *et al.*, 1978).

Regardless of the reasons for low activity, expression of 58K from reconstituted plasmids (p24EX20.92, p24EX27.92, p24EX39.92) should have been demonstrable assuming 58K was expressed to the same degree as the 58K-β-galactosidase hybrid protein. The failure to demonstrate 58K synthesis in *E. coli* may be a consequence of: i) instability of the transcript, ii) premature termination of the transcript, iii) low avidity of the tumor antisera for 58K produced in *E. coli*, iv) inefficient translation of 58K or v) instability of the 58K protein in *E. coli*.

The stability of the 58K transcript was not investigated. Presumably mRNA synthesis initiates from the lac promoter, procedes through the 58K coding sequences, and terminates at the terminator of the pBR322 tetracycline resistance gene. To determine the stability of this transcript in *E. coli*, S1 mapping (Berk and Sharp, 1978) would have to be conducted with and without induction by IPTG. In this manner, Fukui *et al.*(1983) were able to show that inefficient expression of Ad12 19K was not due to mRNA instability. S1 mapping could also establish whether

full length or truncated transcripts are made. If a fortuitous terminator exists within the 58K sequences, it would probably occur downstream of the 19K coding sequences because the complete 19K coding sequences appeared to be translated from the plasmid p29EX21.83. Termination beyond the 19K coding sequences would result in truncated 58K polypeptides. These were not observed from reconstituted p24EX clones, suggesting that premature termination was not occuring.

If a stable, full length 58K transcript were synthesized and translated, failure to detect the 58K product may be due to low avidity of the tumor antisera. This is unlikely because several different antisera were employed, each capable of immune precipitating 58K from Ad5-infected cells.

If translation of the 58K sequences was less efficient than that of the 58K-β-galactosidase hybrid gene, synthesis of 58K might be difficult to demonstrate by SDS-PAGE analysis. Poor codon usage could lower the efficiency of translation. Approximately one-tenth of the amino acids of 58K are coded by "modulator codons". However, the extent to which codon use can actually limit translational efficiency is not known.

Lastly, 58K may be sensitive to degradation by *E. coli* proteases. Several studies have indicated that foreign proteins may be unstable in *E. coli* (Itakura *et al.*, 1977; Fraser and Bruce, 1978; Derynck *et al.*, 1980; Shine *et al.*, 1980; Davis *et al.*, 1981; Edman *et al.*, 1981). Reduced labelling times (<2 minutes) were used in an attempt to detect undegraded 58K in whole cell lysates analyzed by SDS-PAGE. 58K was not observed, nor could it have been easily detected due to cellular proteins

which migrated at or near 58K (results not shown).

Although 58K could not be demonstrated in *E. coli* extracts (p24EX20.92, p24EX27.92, p24EX39.92), polypeptides which may correspond to the amino terminus of 58K were synthesized from p29EX21.83 and p29EX27.91. Because these polypeptides could be detected only by immune precipitation, their level of synthesis could not be estimated. It is conceivable that the amino terminal portion of 58K is stable whereas the complete 58K protein is sensitive to *E. coli* proteases. Ad2 58K synthesized in *E. coli* is reportedly unstable (M. Harter, personal communication), lending support to the interpretation that 58K was unstable in the expression system utilized in the present studies as well.

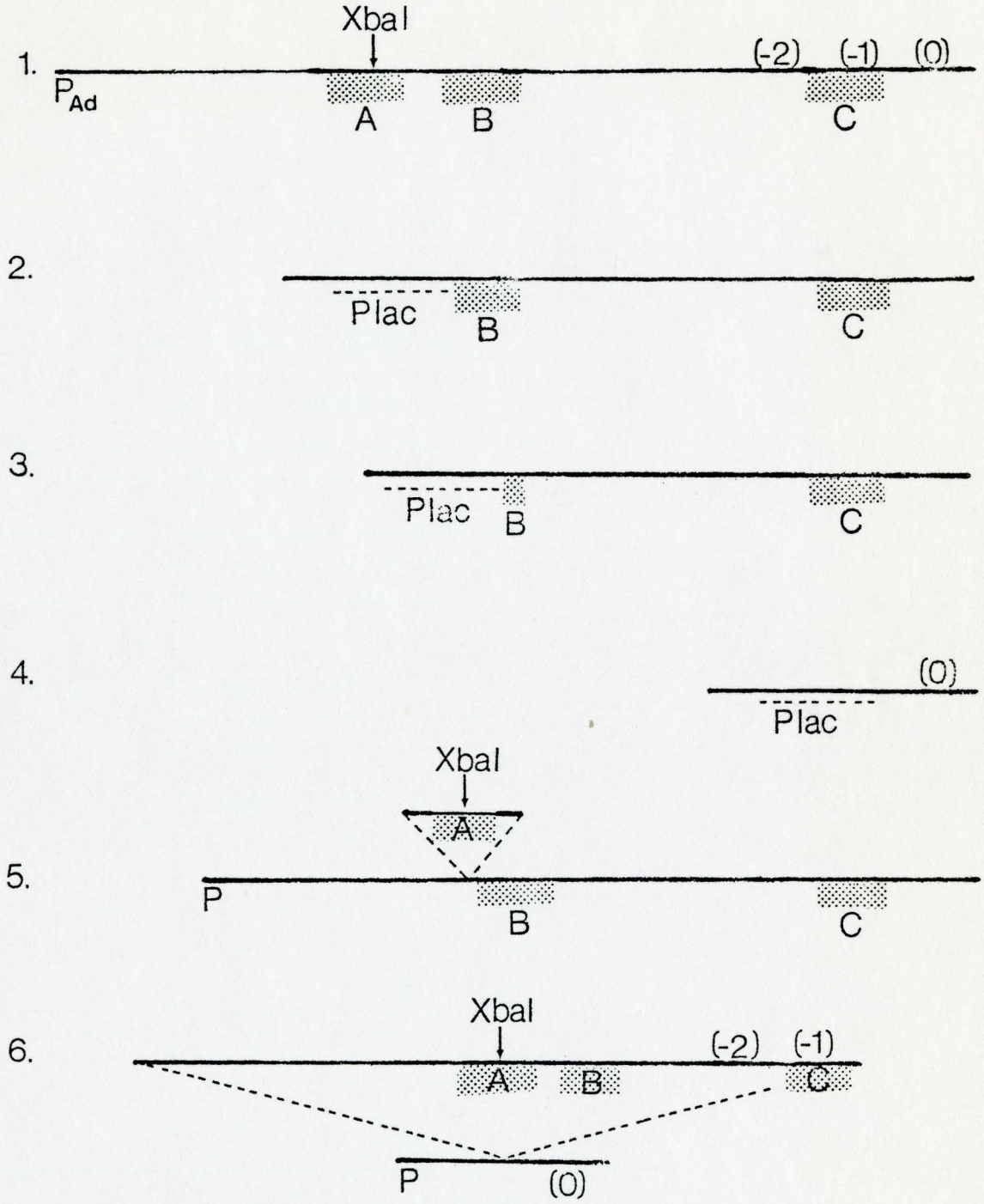
4.3 Expression of 19K

As was the case with 58K, synthesis of the authentic 19K antigen was not demonstrated. Although only nine different promoter positions resulted from promoter insertion, it was interesting that no promoters were mapped between 1400 and 1680. Either the ExoIII digestions did not extend into these sequences or promoter insertion into these sequences resulted in plasmids which failed to express the 19K-β-galactosidase hybrid protein. The fact that one plasmid (p29EX27) had its promoter, positioned at 1682 and one "promoterless" expresser (p29EX31) resulted from ExoIII digestion up to approximately position 1700 argue against the former. In support of the latter, I propose that a transcription terminator exists in the Ad 5 sequences immediately upstream of [-1] and that inclusion of sequences around position 1400 in the transcript inactivate the terminator through mRNA secondary structures. Indeed the formation

of one secondary structure (antiterminator) excludes the formation of terminator structures in several bacterial operons (Yanofsky, 1981; Stroynowski and Yanofsky, 1982). This hypothesis is consistent with several observations. Promoters positioned upstream of 1400 express levels of β-galactosidase activity from 950-2200 units, whereas insertion at 1400 resulted in low expression (150 units). This could be interpreted to mean that insertion at position 1400 partially eliminates the "anti-terminator" sequence so the terminator can function to a degree. Promoters positioned upstream of 1400 transcribe the "antiterminator" sequence, thereby inactivating the downstream terminator. Numerous "promoterless" expressers were isolated, the majority having ExoIII end-points from 1400 to 1450, and one at approximately 1700. Again deletions extending into the sequences between ∿ 1450 and 1700 were not observed. Perhaps these plasmids express β-galactosidase activity because they either maintain the "antiterminator" or delete both the "antiterminator" and the terminator. Because these expressers did not contain the lac promoter, there must be a weak promoting sequence upstream of the "antiterminator". If so, p29 should express levels of β-galactosidase activity similar to those of the "promoterless" expressers. p29 transformants were $lac^-$. Perhaps the sequences around the XbaI site, which are present in p29 and absent in the "promoterless" expressers, can terminate transcription. A model depicting the hypothesis is presented in figure 20. mRNA secondary structure analysis is needed to substantiate the model.

From p29EX21.83, efficient synthesis of a 22K protein was demonstrated. Translation probably initiates from [-2], producing a 19K protein having 29 additional amino acids at its amino terminus. This

Figure 20. Model depicting proposed mRNA structures which may influence transcription of the 19K coding sequences. Sequences A and C represent transcription terminators and sequence B inactivates the terminator structure of sequence C by forming a stable structure whose existence precludes formation of the terminator at sequence C. $P_{Ad}$ represents a proposed weak prokaryotic promoter sequence located in Ad5 sequences upstream of XbaI. Line 1, p29; Line 2, p29EX21; Line 3, p29EX17; Line 4, p29EX27; Line 5, p29EX12; Line 6, p29EX31. Levels of expression from the above plasmids are given in Table 6.


1.
XbaI
(-2)
(-1)
(0)
$P_{Ad}$
A
B
C
2.
Plac
B
C
3.
Plac
B
C
4.
(0)
Plac
5.
XbaI
A
P
B
C
6.
XbaI
(-2)
(-1)
A
B
C
P
(0)

conclusion is based on the following observations: i) p29EX21.83 has the lac promoter upstream of [-2], the initiation site most closely resembling a functional ribosome binding site, ii) p29EX27.91 has the promoter downstream of [-1] and did not synthesize the 22K protein, indicating 22K translation initiates from either [-2] or [-1], iii) in comparison to the 19K from Ad5-infected cells, the *E. coli* 22K protein appeared approximately 3000 daltons larger by SDS-PAGE, iv) the only other open reading frame included in the p29EX21.83 transcript is that of 58K which encodes an amino terminal peptide of predicted MW 17800 daltons. This reading frame is shared by both p29EX21.83 and p29EX27.91, indicating that the 22K is not a product of the 58K reading frame.

The plasmid p29EX27.91 was considered a good candidate for expression of the authentic 19K because the promoter was situated directly upstream of the [0] initiation codon and p29EX27 expressed high levels of β-galactosidase activity. However, tumor antisera failed to detect a 19K protein from p29EX27.91 extracts. Two smaller proteins of MW 18K and 16K were detected and appeared antigenically unrelated to the 22K protein and presumably the 19K protein as well. These proteins may correspond to products of the 58K reading frame, initiating from the 58K AUG codon and the closest downstream GUG codon. Expression in the 58K reading frame would not contribute to the level of β-galactosidase activity observed for p29EX27 (1300 units) because β-galactosidase is in phase with the 19K reading frame. Therefore 1300 units or 5000-10000 molecules per cell of 19K-β-galactosidase were synthesized from p29EX27. High levels of activity suggest efficient translation initiating from an in phase initiation codon. Efficient translation from the [0] initiation

codon is unlikely because 36 bp separate the promoter's SD sequence from [0]. The sequences immediately upstream of [0] do not contain a SD sequence, nor do they resemble a "consensus" ribosome binding sequence (Table 7). Also, there is no obvious mRNA secondary structure which would bring the promoter's SD sequence in close proximity to [0]. There is however an in phase GUG codon (designated [+1]) located downstream of [0]. It has a short SD sequence 12 bp upstream and shows some similarity to the ribosome binding sequence of Scherer *et al.* (1980) (Table 7). Translation initiating from this codon would produce a protein which is approximately 1300 daltons smaller than 19K. This could correspond to the 17K protein immune precipitated from p29EX27.91 extracts. However, because the 17K protein appeared antigenically unrelated to the 22K protein, it is unlikely that 17K corresponds to a protein initiated at [+1].

At present, the mechanism by which protein is translated from p29EX27 is not clearly understood. Efficient translation initiating from either in phase initiation codons ([0] or [+1]) is difficult to reconcile. Regardless of how and where translation initiates, expression of the predicted 19K protein was not detected from p29EX27.91. Several possibilities could account for this. Because the 22K protein of p29EX21.83 was synthesized efficiently and detected easily using tumor antisera, it is unlikely that failure to detect 19K was due to premature termination of transcription, poor codon usage, or low serum avidity.

Alternatively, the 19K protein may be sensitive to degradation by *E. coli* proteases. As a corollary, the 29 additional amino acids of

22K could act to stabilize the 19K analogue. Although fusions between eukaryotic and prokaryotic coding sequences are thought to produce stabilized products in *E. coli* (Itakura *et al.*, 1977; Goeddel *et al.*, 1979; Davis *et al.*, 1981), it is not clear that 29 amino acids would be sufficient to stabilize 19K.

The explanation I favour is that p29EX27.91 does not direct synthesis of 19K because translation does not initiate from [0]. The parental plasmid (p29EX27) expressed high levels of β-galactosidase activity yet based on its predicted sequence there did not appear to be a suitable ribosome-binding sequence from which translation could initiate. I propose that translation from p29EX27 initiates from the ATG triplet within lacI and consequently β-galactosidase is expressed from p29EX27 but 19K is not synthesized from p29EX27.91. Using similar expression vectors, Gray *et al.* (1982) constructed numerous fusions between various eukaryotic sequences and lacI-Z. Many of the clones produced fusion proteins having greater MWs than the lacI-Z protein. In addition, several different fusions were shown to produce only the lacI-Z protein. The authors proposed that the lacI-Z protein resulted from degradation of larger fusion proteins although they did not discount the possibility that the lacI-Z protein resulted from translation initiating at or near the junction of eukaryotic and lacI-Z sequences. Translation could initiate from a suitable initiation codon near the 3' end of the eukaryotic sequences or downstream from the ATG codon within lacI.

p29EX27 may be analogous to the fusions described by Gray (1982) which produced only the lacI-Z protein. The observation that numerous eukaryotic-lacI-Z fusions produced only the lacI-Z protein

(Gray *et al.*, 1982) may represent a common artifact of this expression strategy.

4.4. Significance and Future Experiments

Although efficient expression of the authentic 58K and 19K antigens was not achieved efficient synthesis of a stable, immune reactive, 19K analogue was demonstrated. The 19K analogue (22K) can be used in a variety of experiments to investigate the function of 19K. 22K is immune reactive with tumor antisera and therefore probably is capable of eliciting an immune response against the authentic 19K antigen. Animals can be immunized using 22K purified from *E. coli* to generate a 19K specific antiserum and perhaps monoclonal antibodies against 19K.

In addition, the 22K protein can be used to determine if 19K is a component of Ad5 TSTA. Hamsters can be immunized with 22K to determine if an immune response against 19K can elicite a TSTA response in hamsters which have been inoculated with Ad5-transformed syngeneic cells. Quantitative analysis of the protective effects of immunization could provide direct evidence for 19K being a component of adenovirus TSTA.

Finally, it would be very interesting to use the 19K analogue made in *E. coli* to assess any effects it may have on the growth of E1A-transformed cells. As discussed previously, BRK cells transformed with E1A appear unable to form colonies in Joklik's medium supplemented with horse serum but can grow in medium containing FCS (R. McKinnon, personal communication). The 19K analogue could be used to supplement the selective media to determine whether 19K could enable cells transformed by E1A alone to grow in Joklik's plus horse serum.

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