NORTHERN BLOT HYBRIDIZATIONS OF RNA FROM AD 5 TRANSFORMED CELL LINES

NORTHERN BLOT HYBRIDIZATIONS OF RNA FROM AD 5 TRANSFORMED CELL LINES

By

LORRAINE DE TINA DAVISON, B.Sc.

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AUTHOR: Lorraine De Tina Davison, B.Sc. (McMaster University)

SUPERVISOR: Professor S.T. Bayley

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ABSTRACT

Researchers have tried to identify the viral factor involved in making a transformed cell oncogenic in newborn hamsters. Although studies on the viral proteins from transformed cells failed to show a relationship to oncogenicity, this study intended to identify and map viral mRNAs from these transformed cell lines in order to test the same relationship.

Northern blot hybridizations were used to study the Ad5 E1 region specific mRNAs from ten transformed cell lines. These cell lines had been transformed with either virus, total restriction endonuclease digested viral DNA, or specific fragments of viral DNA from the left hand end of the genome. All of these lines were selected for their varying oncogenicity in newborn hamsters, and for the size of the transforming fragment. Ad5 DNA fragments inserted into recombinant plasmids were used as the probes for detection of mRNAs, providing tools for mapping transformed cell mRNAs to specific regions of the Ad5 genome.

The results failed to show a relationship between E1 region mRNA production and oncogenicity, but did reveal unusual mRNA transcription patterns from most of the cell lines. All the transformed cell lines tested appeared to have only an E1B 22s mRNA as the major mRNA from that region. An E1A/E1B cotranscript was identified as well as viral/cell chimeric mRNAs. These chimeras were due to either run-off transcription or to preinitiation in cellular sequences.

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Ad 2, 5, 12	adenovirus types 2, 5 and 12
α-MEM	alpha-minimum essential medium
b	nucleotide base
BSA	bovine serum albumin
CH	cycloheximide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
G + C	guanine + cytosine
hr	host range mutant
l,r strand	leftward and rightward transcribed strand
mRNA	messenger ribonucleic acid
M	molar
MOPS	morpholinopropanesulfonic acid
mu	map unit
NDS	1,5-naphthalenedisulfonate
NP40	nonidet p40
PI	post infection
PVS	polyvinylsulfuric acid potassium salt
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
Tris	tris (hydroxymethyl) aminomethane
ts	temperature sensitive
wt	wild type

INTRODUCTION

The family of adenoviruses

Adenoviruses are small DNA viruses capable of infecting many animals and have been isolated from a number of species: human, simian, bovine, canine, murine and avian (Pereira et al., 1963). Human adenoviruses comprise the largest family with thirty-five different serotypes described so far (Wadell et al., 1980; Ginsberg, 1979). The discovery by Trentin et al., (1962) that human adenovirus serotype 12 (Ad12) could induce tumors in newborn hamsters stimulated research into the mechanisms of tumorigenicity. Adenovirus has also proved to be a useful model for studies on gene expression and its regulation.

Human adenoviruses have been grouped into six different subgroups, A, B, C, D, E, and F, based upon: the % G+C content of their genomic DNA, their oncogenicity in rodents, the degree of DNA homology, their hemagglutination of monkey and rat erthrocytes and their restriction endonuclease maps (Wadell et al., 1980; Flint, 1980). Adenovirus serotype 2 (Ad2) and Ad5 belong to group C adenoviruses, and are highly related in their DNA content and restriction maps. There is between 99-100% DNA homology in these two serotypes. They also share similar abilities in transforming rodent cells, and in failing to induce tumors in newborn hamsters. These two serotypes have been studied extensively and are the basis of most studies of the molecular biology of adenoviruses.

The genomic organization of Ad2/Ad5

The adenovirus 2 or 5 genome consists of a linear double stranded DNA molecule, about 36,000 base pairs in length, which has both DNA strands available for transcription at early and late times in the lytic cycle. The early and late phases of infection are separated temporally by viral DNA replication, which occurs several hours after virus penetration into a permissive cell (Tooze, 1981; Philipson, 1979).

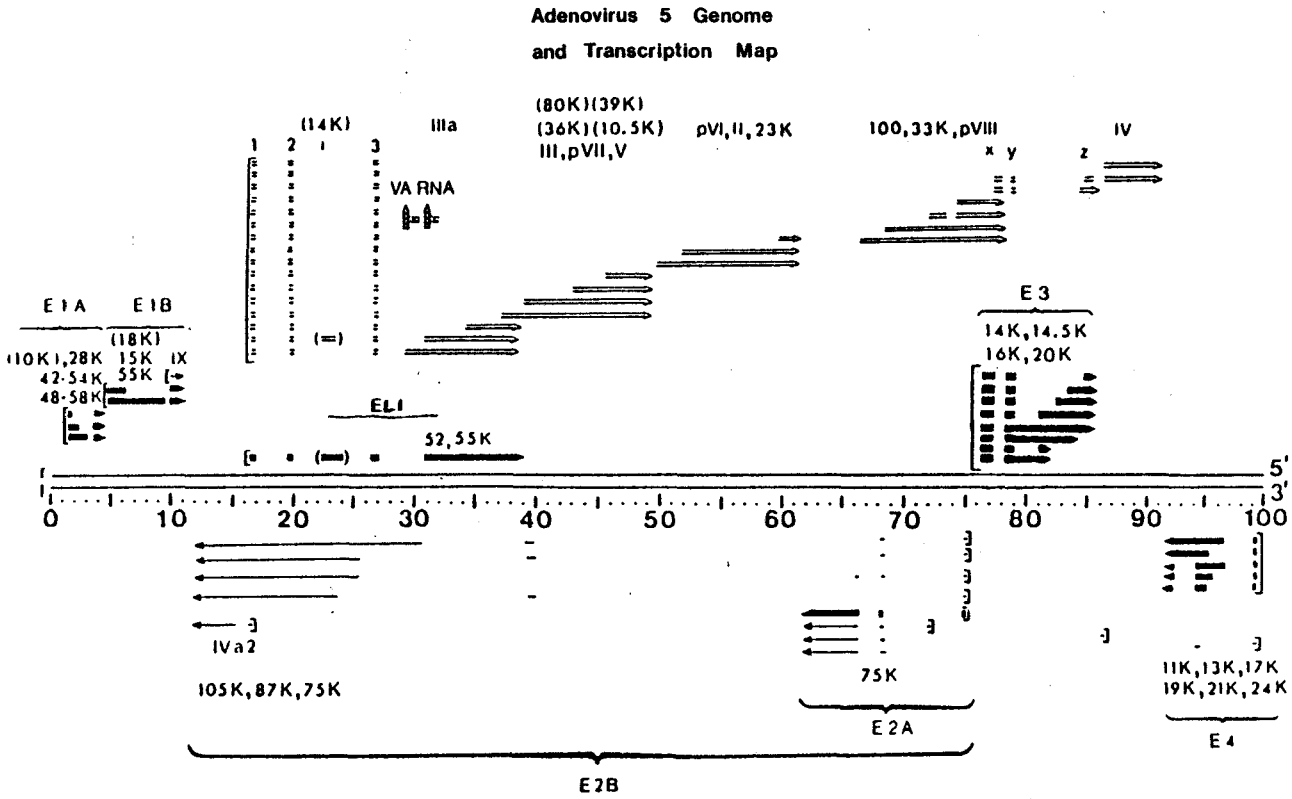
The genome consists of an r (right) and l (left) strand, representing the direction of transcription along the genome. The process of mRNA mapping to the genome was accomplished by hybridization of RNAs to separated strands of restriction fragments of adenoviral DNA (Sharp et al., 1974b; Pettersson et al., 1976; Smiley and Mak, 1978). This was improved by the study of DNA and mRNA hybrids with electron microscopy (Chow et al., 1977; Chow and Broker, 1978; Kitchingman et al., 1977; Kitchingman and Westphal, 1980; Chow et al., 1979). Further studies used S1/endonuclease VII digestion of DNA:mRNA hybrids followed by gel electrophoresis of the DNA fragments to map splicing sites of adenovirus mRNA (Berk and Sharp, 1978).

There are seven early transcription units located in five regions of the adenoviral genome, E1A, E1B, E2A, E2B, E3, E4, and EL1 (Figure 1). All these units encode mRNA families which differ in splicing patterns and enable a sequence of DNA to encode a number of different polypeptide products. Also, each unit except E2B has its own promoter. This has been demonstrated by ultraviolet transcriptional mapping (Berk and Sharp, 1977a; Wilson et al., 1979), hybridization

FIGURE 1: Transcription map of Adenovirus 2

The organization of the Ad2 genome is depicted in this drawing (reprinted from Pettersson and Akusjarvi, private communication). Thick lines represent mRNAs which are expressed early after infection prior to DNA replication, and the thin lines represent mRNAs from intermediate and late times after infection. The arrowheads show the location of the 3' ends of mRNAs and the brackets indicate promoter location. Five families of mRNAs inititated at the major late promoter (16.5 mu) share 3' coterminal ends.

Ad2 and Ad5 share 99% homology in DNA sequence and their transcription maps are virtually identical.


Adenovirus 5 Genome
and Transcription Map
(80K)(39K)
(36K)(10.5K)
III,pVII,V
pVI,II,23K
100,33K,pVIII
IV
(14K)
IIIa
VA RNA
E1A
E1B
(18K)
(10K),28K
15K
IX
42-54K
55K
48-58K
E3
14K,14.5K
16K,20K
E1.1
52,55K
5'
3'
0
10
20
30
40
50
60
70
80
90
100
IVa2
105K,87K,75K
75K
E2A
E2B
11K,13K,17K
19K,21K,24K
E4

of viral restriction fragments to nascent nuclear RNA transcripts (Evans et al., 1977), mapping of viral mRNA 5' cap structures to DNA sequences of adenovirus (Ziff and Evans, 1980), and in vitro transcription of viral DNA restriction endonuclease fragments, (Fire et al., 1981).

At intermediate times of infection transcription units coding for polypeptides IX and IVa2 are independently expressed from their own promoters (Pettersson and Mathews, 1977; Wilson et al., 1979; Persson et al., 1979).

After viral DNA replication, the major late promoter at about 16.5 map units (mu)[1] generates a primary transcript of 28,000 nucleotides (Evans et al., 1977). An mRNA is generated by endonucleolytic cleavage of the transcript at one of a number of possible sites, with transcription occuring beyond this site to the end of the genome (about 99 mu) (Fraser et al., 1979). The 3' end undergoes polyadenylation at the nearest AAUAAA site (Nevins and Darnell, 1978). Following poly-adenylation, splicing occurs creating an array of mRNA families and adding an untranslated 5' leader RNA segment to all late products from this promoter. This shared leader sequence of 203 nucleotides is a combination of sequences from map co-ordinates 16.5, 19.6, and 26.6. The result of both differential splicing patterns and different 3' terminal ends is a set of five 3' co-terminal mRNA families as seen in Figure 1.

1. a map unit = 1% of the genome length
   0 mu is located at the extreme left end of the genome,
   100 mu at the right end.

Gene expression and its regulation

a) The early phase

During the early phase of lytic infection early transciption units actively transcribe viral mRNAs from 25 to 35% of the adenoviral genome (Wold et al., 1977; Tibbetts and Pettersson, 1974). There are at least 20 different mRNAs expressed before viral DNA replication (Chow et al., 1979; Stillman et al., 1981; Chow et al., 1980).

The early regions are expressed in a pattern with respect to the time and rate of mRNA initiation from different promoters (Berk et al., 1979, Nevins et al., 1979, Ziff, 1980). Transciption activity can first be detected in the E1A region and the major late promoter's EL1 region about 1 hour post infection (PI) (Nevins et al., 1979; Shaw and Ziff, 1980; Nevins and Wilson, 1981). Transcription reaches a maximum in the E1A region at 6 hours PI. Transcription from regions E1B, E3 and E4 begin 1 hour later with the E3 and E4 regions reaching a maximum at 3 hours PI and then declining in rate. The E1B transcription rate appears to be constant throughout infection (Wilson and Darnell, 1981). The E2 region begins last of all the early regions at 3 hours PI, reaching a maximum at 7 hours (Nevins et al., 1979).

Various factors appear to influence the accumulation of mRNA in the cytoplasm including the rate of initiation and the stability of mRNA. Cells infected by host range mutants (Berk et al., 1979), and deletion mutants (Jones and Shenk, 1979a) with lesions in region E1A contain very low levels of viral mRNAs from early regions E1B, E2, E3,

and E4. Therefore, a product encoded by the E1A region enhances the accumulation of early viral mRNAs in the cytoplasm. The poor accumulation of early region mRNAs is believed to be due to lower levels of transcription but this block can be overcome with a high multiplicity of infection or the use of protein synthesis inhibitors (Nevins, 1981). Addition of anisomycin to cells before infection with E1A mutants can result in early gene expression from all promoters (Nevins, 1981; Lewis and Mathews, 1980). Protein synthesis inhibitors were postulated to be limiting the production of a cellular repressor of transcription. Nevins (1981) proposed that the mechanism of E1A function is to inactivate an unstable cellular factor that inhibits early adenovirus transcription. Further studies on the role of an E1A function on early viral transcription have not confirmed these findings but still suggest a regulatory role for the E1A gene product (Cross and Darnell, 1983; Gaynor and Berk, 1983).

The addition of protein synthesis inhibitors after viral infection appeared to increase the stablility of early mRNA (Harter et al., 1976; Lewis and Mathews, 1980; Cross and Darnell, 1983). A 9s mRNA from region E1A normally not detected until intermediate times of infection can be detected earlier with the additon of cycloheximide, a protein synthesis inhibitor (Spector et al., 1980). This indicates that regulation of adenovirus mRNA can occur at a post-transcriptional level.

Other mechanisms of regulation for gene expression exist. The DNA binding protein 72K, which is expressed early in lytic infection, appears to repress the expression of region E4 mRNA as well as

destabilizing all early RNA, including its own mRNA (Babich and Nevins, 1981), demonstrating autoregulation.

b) The intermediate and late phase

The expression of viral genes changes during and after viral DNA replication. The RNA transcription pattern of the E2 region alters, with initiation of mRNAs occuring at a new promoter used exclusively after DNA replication (Chow et al., 1979) and intermediate promoters begin their transcription.

Some regulation of mRNA occurs at the level of cytoplasmic stability. Early in infection E1 mRNAs have short cytoplasmic half-lives of 6 to 10 minutes, but after replication of DNA all but the 22s E1B mRNA, demonstrate a longer half-life leading to greater accumulation of these mRNAs in the cytoplasm (Wilson and Darnell, 1981). This changing relative abundance of the 22s and 13s mRNA of E1B (Spector et al., 1978; Wilson and Darnell, 1981) appears to require the synthesis of late proteins in order to occur (Babich and Nevins, 1981).

The greatest change in expression occurs with the initiation of mRNAs from the major late promoter. At early times in the lytic infection, transcription from this region is terminated at about 40 mu to produce two mRNAs (see Figure 1, the EL1 region), encoding the L1 52-55k proteins (Chow et al., 1980; Akusjarvi and Persson, 1981). After viral DNA replication, transcription proceeds along the r strand to about 99 mu. The most abundant protein products are encoded by this major late promoter block. This shift of transcription activity

in the late phase appears to depend upon viral DNA replication. If DNA replication is blocked by a DNA synthesis inhibitor or by a replication defective genetic mutant, most late mRNA products are not formed (Carter and Ginsberg, 1976; Chow et al., 1979).

Introduction to transformation

The discovery by Trentin et al. (1962) that Ad 12 could induce sarcomas when inoculated into newborn hamsters initiated a great deal of study into adenovirus' interaction with rodent cells. Researchers found that a variety of adenovirus serotypes could induce tumors in rodents, and were also capable of transforming cultured cells into a potentially oncogenic state (Trentin et al., 1962; Huebner, 1967; Green, 1970).

For a certain serotype of adenovirus, a host cell might be permissive, semi-permissive or non-permissive for lytic infection. Cells which are non-permissive, or semi-permissive for a virus are susceptible to transformation by that virus.

Group A adenoviruses are classified as highly oncogenic, inducing tumors readily in newborn hamsters. The most commonly used member of this group is Ad12 which has been used extensively by researchers. The infection of hamster cells by Ad12 is abortive, failing to replicate viral DNA or transcribe viral late regions (Doerfler, 1968, 1969; Mak, 1975) but cells become transformed. The transformed cell lines induced by Ad12 often contain many integrated genomic copies (Mak et al., 1979, Green et al., 1976; Ibelgaufts et al., 1980; Groneberg et al., 1977).

Ad 2 and Ad 5, Group C non-oncogenic viruses, can induce tumors, but only in immunoincompetent rodents. These viruses can replicate in hamster cells, but with a reduced yield of infectious virus compared to human cells (Williams, 1973). Group C transformed rodent cell lines usually contain viral genomes with large deletions (Flint et al., 1975; Flint and Sharp, 1976; Visser et al., 1979). Since these viruses are semipermissive in hamster and rat cells (Gallimore, 1974), only genomes which have a defect blocking the lytic cycle could transform rather than lytically infect, so that defective genomes are selected for in this semi-permissive situation (Schaller and Yohn, 1974). Consistent with this idea temperature sensitive mutants like Ad5 ts125, which are defective in replication, fail to transform cells at permissive temperatures where replication can occur but they do act to transform cells at non-permissive temperatures. Ad5 ts125 induced transformed cells contain multiple full length mutant genomes (Dorsch-Hasler, et al., 1980; Ginsberg et al., 1974; Mayer and Ginsberg, 1977).

Integration appears to play a key role in the establishment of transformed cells. Adenoviral transformed cells contain integrated viral sequences and not episomal copies of viral DNA (Flint et al., 1976; Gallimore et al., 1974; Johansson et al., 1978; Sambrook et al., 1974; Sambrook et al., 1980; Westin et al., 1982). The method of integration is not understood but it has been established that transformation depends on cellular DNA synthesis (Casto, 1973).

The transforming region

Analysis of adenovirus transformed cell lines revealed that only the left hand portion of the genome was always maintained, although other regions of the genome were sometimes present as well. One line of Ad2 transformed rat cells showed the presence of as little as the left 14% of the genome (Gallimore et al., 1974), and while other Ad2 transformed rat cell lines and Ad5 transformed hamster cell lines had additional viral information present, they always retained this left region (Gallimore et al., 1974; Sambrook et al., 1974; Sharp et al., 1974a).

The development of the calcium phosphate technique (Graham and Van der Eb, 1973) to permit entry of DNA into cells directly without the aid of virus, allowed discrete portions of the Adenovirus genome to be tested for the ability to transform. Using Ad5 DNA fragments, Graham et al. (1974a,b) demonstrated the unique transforming ability of the left 8% of the genome. These results were confirmed with other adenovirus serotypes using the transfection technique (Dijkema et al., 1979; Mak et al., 1979, Shiroki et al., 1979).

Transfection using the left 4.5% of Ad5 was able to "immortalize" rat cells. However, these cells were less efficiently transformed and were different from fully transformed cells in morphology and other "classic" transformation features (Houweling et al., 1980). This study showed that although the left 4.5% of the Ad5 genome might be sufficient to immortalize rat cells, some function was missing to provide a fully transformed state.

Many researchers have concentrated their efforts on the

transforming region in an effort to understand the roles that protein products from this region play in normal lytic infection, in transformation, and in oncogenesis. Studies on the mRNA expression of transformed cell lines have shown that these cells appear to express early phase mRNAs, as though expression of the virus has been arrested at its pre-replicative stage (Flint et al., 1975, 1976; Ortin et al., 1976). We will review the transforming region early mRNAs and encoded proteins.

mRNAs and proteins encoded by the transforming region

a) Region E1A

Region E1a encodes three early mRNA species of about 1100, 900 and 600 nucleotide bases (b) (13s, 12s, and 9s) as determined by Northern blot hybridization analysis of polyadenylated cytoplasmic mRNA (Van den Elsen et al., 1983). The three E1a mRNAs are processed from the same initial transcript to share common 5' and 3' terminal ends, a common 3' splice site, but different 5' splice sites (see Figure 2) (Berk and Sharp, 1978; Chow et al., 1979).

Nucleotide sequencing of adenovirus DNA and c-DNA (Perricaudet et al., 1979; van Ormondt et al., 1980; Gingeras et al., 1982) predicted that the two largest E1A mRNAs would direct the synthesis of 32k and 26k polypeptides. These polypeptides were predicted to share amino and carboxy terminal ends, but were expected to differ in an internal 46 amino acid sequence due to mRNA splicing differences.

In vitro translation of E1A selected mRNA has identified several

protein products. The E1A 12s and 13s mRNAs each encode two (Halbert et al., 1979; Lewis et al., 1979; Esche et al., 1980) or three proteins (Harter and Lewis, 1978; Smart et al., 1981). These proteins range in size from 35 to 57k and prove to be highly related by tryptic peptide analysis. The size discrepancies between the predicted and observed protein weights have not been explained, nor has there been a clear explanation for the multiple products arising from the same mRNA.

There has been some difficulty with immunoprecipitation studies of E1A proteins due to their low production in infection and the failure to obtain antisera specific for these proteins. Researchers using this technique succeeded in isolated E1A specific proteins from lytically infected cells. Four or more proteins ranging in size from 35 to 55k have been identified (Wold and Green, 1979; Green et al., 1979; Schrier et al., 1979). Another study analyzed E1A viral proteins by immunoprecipitation with an anti-serum raised against a peptide suggested to be the amino-acid sequence shared by all E1A proteins at the common carboxy-terminal end. Rowe et al. (1983a) using KB cells infected with Ad5 mutant pm975 (synthesizing no 900 b mRNA) and hr 1 (synthesizing a truncated 1100 b mRNA), demonstrated two major and one minor E1A proteins were synthesized by each of these E1A mRNAs. Three proteins were encoded by the 1100 b (13s) mRNA, the 52k, 48.5k and 37.5k proteins and the 900 b (12s) mRNA encoded the 50k, 45k and 35k proteins. They were able to assign these products to E1A mRNAs due to the nature of the viral mutant defect.

It is not clear what functions are served by the E1A region in

lytic infection. Montell et al. (1982) have shown that the 900 b mRNA from E1A is not necessary for viral replication to occur in regular cultured fibroblasts, but does function in the replication of virus in growth arrested cells (Montell et al., 1984). They constructed viruses with mutations in E1A which synthesized either the E1A 1100 b (pm975 mentioned earlier) or 900 b (dl 1500) mRNAs only and demonstrated that the virus required the 1100 b mRNA, but not the 900 b mRNA in order to replicate in tissue culture cells, but that both E1A mRNAs must be present for a virus to replicate in cells with a low serum environment.

b) Region E1B

From region E1B early in the lytic cycle, two mRNAs have been commonly detected, a 2500 b and a 1000 b mRNA (22s and 13s) (Halbert et al., 1979; Esche et al., 1980; van den Elsen et al., 1983; Lupker et al., 1981). As in the case of region E1A mRNAs, the two E1B mRNAs are processed from the same initial transcript to share common terminal ends and the same 3' splice site but differ in the location of the 5' splice site (see Figure 2).

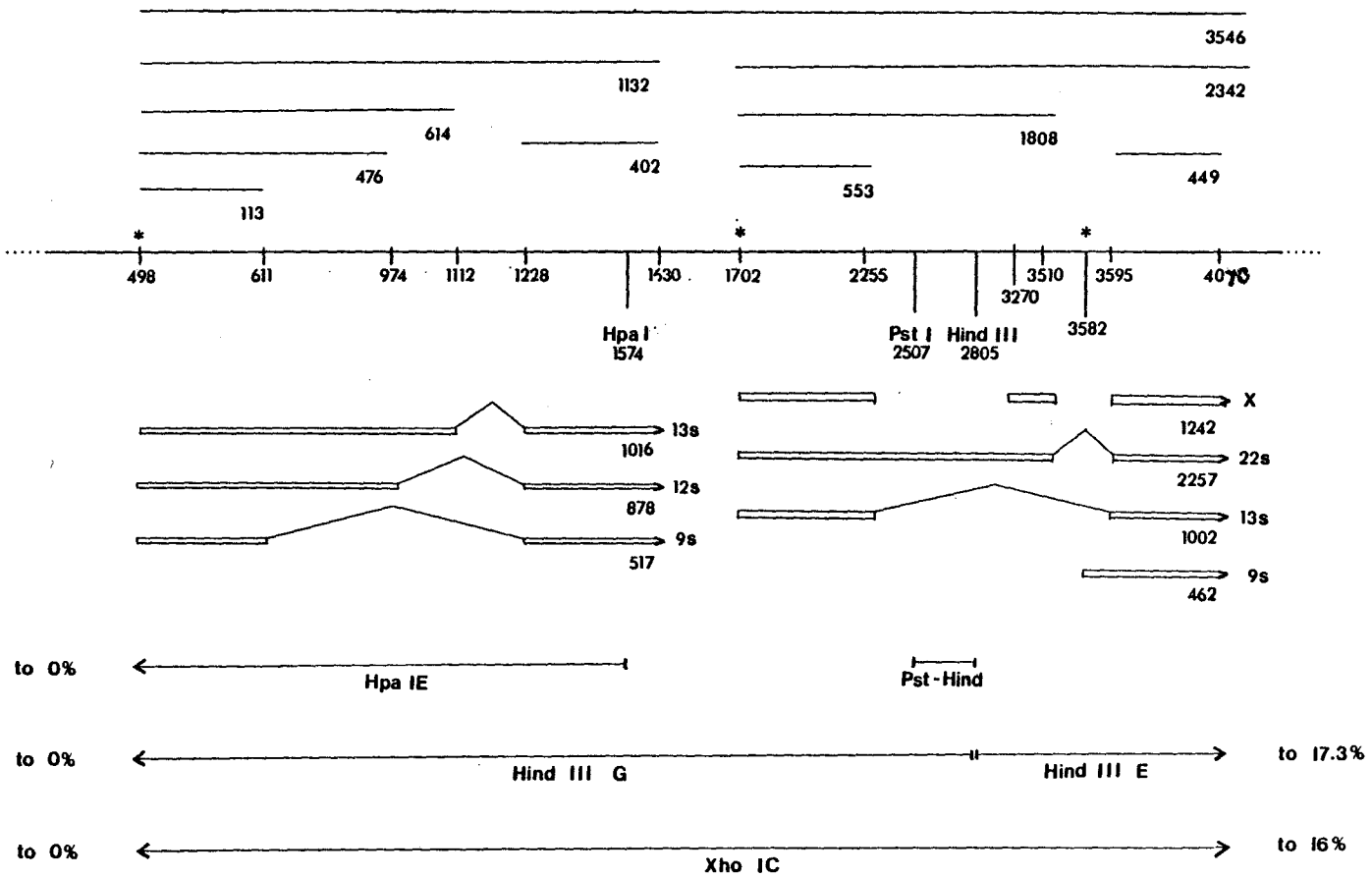
The larger 2500 b mRNA can encode two proteins a 58k and a 19k from different AUG start sites and in different reading frames (Esche et al., 1980; Bos et al., 1981; Lupker et al., 1981), so that they do not share tryptic peptides (Anderson et al., 1984). The 13s mRNA can encode only the 19k protein. The 19k protein is associated with the membrane of infected or transformed cells and is not phosphorylated (Persson et al., 1982; Rowe et al. 1983b). The 58k protein is a

FIGURE 2: Transcription map of the transforming region of Adenovirus 5

The mRNA map of the left end of Adenovirus 5 shows the size of the spliced mRNA regions in relation to the Adenovirus genome and restriction sites. The Ad5 genome maps (in nucleotides) relevant restriction endonuclease sites (Hpa I, PstI, Hind III) promoter sites (*), 3' terminal ends of mRNAs (arrowheads), and the donor and acceptor splice sites for each mRNA. The typical mRNA map for region E1 is shown below this with the nucleotide sizes of the different mRNAs from both E1A and E1B (determined without poly-A tails). At the bottom of the figure the viral DNA fragments used for hybridization are shown to demonstrate their specificities for various E1A or E1B mRNAs.

X The E1B 1242 b mRNA has been postulated from protein sequences (Anderson et al. (1984)

Note that the map is not drawn to scale


3546
1132
2342
614
1808
476
402
449
553
113
498
611
974
1112
1228
1430
1702
2255
3270
3510
3595
Hpa I
1574
Pst I
2507
Hind III
2805
3582
X
1242
13s
1016
22s
2257
12s
878
13s
1002
9s
517
9s
462
to 0%
Hpa IE
Pst-Hind
to 0%
Hind III G
Hind III E
to 17.3%
to 0%
Xho IC
to 16%

phosphoprotein, and is either associated with or is a protein-kinase in vivo. Immunoprecipitates of 58k from infected or transformed cells, can phosphorylate 58k or an exogenous substrate (Lassam et al., 1979, Branton et al., 1979, 1981).

Both of the 19k and 58k proteins are thought to be important to transformation because the sera from tumor-bearing animals show specificity to them (Rowe et al., 1984) and almost all transformed cells produce these two proteins.

An E1B 18k protein has been detected from infected cells using in vitro translation and immunoprecipitation (Green et al., 1979; Lupker et al., 1981; Downey, 1983). This protein is unrelated to the 19k protein but does share tryptic peptides with the 58k protein (Matsuo et al., 1982; Downey, 1983). Only recently has another E1B mRNA been surmised from an E1B protein sequence. This E1B specific mRNA is 1250 b long, and shares 5' and 3' coterminal ends with the 22s and 13s mRNAs but has two introns, one similar to the 22s mRNA and another 230 nucleotides upstream of the 22s intron site (see Figure 2, mRNA-x) (Anderson et al., 1984). This mRNA encodes a 155 amino acid residue polypeptide which is approximately 18k in size (Anderson et al., 1984). Van den Elsen et al. (1983) may have detected this same mRNA in infected cells when they found an mRNA capable of encoding both the 18k and the 19k protein. It is not clear if the mRNA is an early or late mRNA, since Anderson et al. (1984) used protein synthesis inhibitors to accumulate a large amount of mRNA for study, which sometimes causes an earlier accumulation of late mRNA (Spector et al., 1980). Van den Elsen et al. (1983) described their mRNA from E1B as a

late mRNA.

Another protein from E1B about 16k in size has been made *in vitro* after hybridization selection of mRNA to this region, and it shares amino terminal peptides with 58k and the 18k described above (Anderson *et al.*, 1984). It is not clear from which mRNA the 16k is encoded.

c) Unidentified open reading frames

From sequencing studies of viral DNA Gingeras *et al.* (1982) identified two unassigned open reading frames that exist in the leftmost region of the l strand in Adenovirus 2. These two open reading frames could encode a 14k and a 23k protein from nucleotide 2290 to 2005 and from 1715 and 1196, respectively. A polyadenylation hexanucleotide signal AAUAAA at 1.2 mu on the l strand was also detected which could be used by an mRNA in this region. An open reading frame in the E1 region from the l strand could be important since the possible encoded proteins may function in transformation.

Katze *et al.* (1982) has reported a 20s mRNA from the l strand in Ad2 infected cells using hybridization selection of mRNAs to the E1 region. *In vitro* translation of this mRNA resulted in a 14k protein.

Genetic studies on transformation

Genetic studies allow certain questions to be asked with respect to the fuctions involved in transformation. Due to the nature of the information in the E1 region of Adenovirus, where overlapping mRNAs encode different proteins, there have been difficulties in determining the role of these proteins in transformation. Many mutations which

occur in region E1 must affect the sequences of multiple mRNAs, and may affect all the encoded protein products translated from those sequences, thereby making it difficult to assign function to a particular protein.

In order to be able to grow mutants with defects in E1 functions, 293 cells were established by transforming human embryonic kidney cells with Ad5 DNA (Graham et al., 1977) which expresses E1 functions (Aiello et al., 1979) and therefore could be used in order to isolate host range (hr) mutants with lesions in this region. Functions encoded by region E1 could now become non-essential for Ad5 replication. These hr mutants fail to replicate or replicate poorly in HeLa or KB cells. The Adenovirus host range mutants Group I and II (Harrison et al., 1977), from E1a and E1b respectively (Frost and Williams, 1978), failed to transform rodent cells, but the E1a host range mutants could cause an abortive transformation in baby rat kidney cells (Graham et al., 1978). This result is consistent with the idea that E1a gene products are required for immortalization of the transformed phenotype (Graham et al., 1978).

Solnick and Anderson (1982) have shown that an E1A mutant hr440 which produces no 900 b (12s) mRNA and a truncated 1100 b (13s) mRNA is defective for transformation. Carlock and Jones (1981) constructed a mutant with an altered E1A 1100 b (13s) mRNA but otherwise normal genotype with respect to the 900 b (12s) and 600 b (9s) mRNAs. This mutant was defective in transformation of rat embryo and baby rat kidney cells, demonstrating that an 1100 b (13s) mRNA product is critical for transformation.

Ho et al. (1982) described a cold sensitive mutant which mapped to the E1A 1100 b (13s) mRNA and was defective for the maintenance of transformation and for viral replication at the non-permissive temperature. Babiss et al. (1983a) further showed that a number of altered sites could generate this phenotype, which was dependent on a defect in the 1100 b (13s) mRNA, demonstrating the requirement for an E1A 13s mRNA gene product in the maintenance of transformation.

Montell et al. (1984) have determined that both the E1A 1100 b and 900 b (13s and 12s) gene products are required for complete transformation. Using mutants of Ad2 which lack either the 1100 b or 900 b mRNA due to mutations in the donor splice sites of these two mRNAs, they have shown that neither virus can fully transform a rat cell line CREF. However, both viruses are capable of inducing a partially transformed phenotype. This indicates that both E1A gene products must be available for complete transformation to occur.

The fact that neither the Adenovirus 5 Hpa 1E fragment (see Figure 2)(Houweling et al., 1980), nor the Adenovirus 12 E1A region (Shiroki et al., 1979) alone can fully transform primary rat embryo cells, indicates that some E1B function is required as well as E1A. Previous work using host range and deletions mutants with lesions in E1B had already shown that an E1B and E1A function were required to transform rodent cells with virions (Graham et al., 1978; Jones and Shenk, 1979a).

McKinnon et al. (1982) demonstrated that the 5' region of E1B plays an essential role in transformation. Ad5 DNA with insertions in the 5' end of the E1B region encoding the 19k antigen failed to

transform primary rodent cells. This is consistent with the observation by Matsuo et al. (1982) that all transformed cell lines they examined expressed a 19k antigen. Recently Chinnadurai (1983) made mutants of 19k which fail to induce transformation in a rat embryo cell line 3Y1. One of these mutants contained a single amino acid change in the amino terminal end of the protein, indicating that this region of the 19k protein is essential for the induction of transformation.

The requirements for transformation are less stringent if viral DNA rather than virions are used for transformation. In this instance host range Group II mutants with defects in the E1B gene (Galos et al., 1980) can transform cells (Rowe et al., 1983c).

Viral mRNAs expressed in transformed cells

A number of Ad2 transformed cell lines have been studied for their mRNA expression. The method used originally was to use fragments of viral DNA for saturation hybridization of labeled cytoplasmic mRNA. All the lines studied appeared to have mRNA from the left hand 14% of the genome (Flint et al., 1975, 1976; Sharp et al., 1974b) and these mRNAs appeared to be similar to the early phase mRNAs of a productive infection (Flint et al., 1975). In vitro translation of viral specific mRNA from transformed cells shows protein products similar to those seen in lytic infection (Lewis and Mathews, 1981; Lewis et al., 1976; Esche et al., 1980; Saito et al., 1983; Jochemsen et al., 1981).

Recently techniques with an increased sensitivity for the

detection of viral cytoplasmic mRNAs have been used for analysis of adenovirus transformed cells: S1 nuclease mapping and Northern blot analysis. These studies indicate that transformed cells do not always express the E1 region mRNA in a similar pattern to that of early lytic infection. Their findings will be discussed in the context of our own results using Northern blot analysis of the E1 region of Ad5 transformed cell lines.

Aim of this study

We wished to analyze transformed cell lines for their viral mRNA production in order to determine if oncogenicity of a cell line[2] was related to either the abundance or type of mRNA being produced. We had available to us a number of lines transformed with different regions of Ad5 DNA: 0% to 7.8 %, 0% to 16% or 0% to 43%. Different types of DNA were also used: wt virus and hr mutants. As well these lines varied in their oncogenicity, some being highly tumorigenic, others moderate and others non-tumorigenic.

These cell lines were made available to us by Dr. F.L. Graham (McMaster University) and all but one line was isolated in his laboratory. The cell lines were all derived from primary hamster kidney cells, and studies on their protein production and oncogenicity in syrian hamsters had been done (see Table 1, all data from Rowe et al., 1984). Three lines had been transformed with the Xho IC fragment of Ad5 (see Figure 2), two with Hind III digests of Ad5, one

2. relative oncogenicity determined by number and rate of tumors formed in syrian hamsters inoculated by transformed cell lines (see Table 1).

TABLE 1. Tumorigenicity of Ad5-transformed hamster cells*

cell line	transformed by virus/fragment	integrated sequences hybridizing Ad5 Hind III fragments	Number of E1 inserts	Tumor induct'n potent'l[a]	specificity of Hamster anti-tumor sera vs. Ad5 antigens			Ad5 Tumor antigens detected		
					58	19	48-52	58	19	48-52
983.2	Ad5 Xho 1C	G,E	2	++++	++		+	+	+	+
972.2	Ad5 Xho 1C	G,E	2	NT				+	+	+
972.3	Ad5 Xho 1C	G,E	4	-	++			+	+	+
954.21	Ad5 Hind III digest	G,E	4	-				+	+	NT
954.1	Ad5 Hind III G	G	1	+++	+	++	+	-	+	+
945.C1	Ad5 Hind III digest	G,E	1	+++		+	+	-	+	+
1019.3	hr50 Xho 1 digest	NT	NT	+++			+	-	-	
1019.2	hr6 Xho 1 digest	NT	NT	+					+	
1019.1	hr6 Xho 1 digest	NT	NT	+		+			+	
14b[w]	ts14 virus	left 43%[f]	5.5[f]	++++	++					

a 5 x 10 cells injected subcutaneously into newborn Syrian hamsters

NT not tested

f (Flint et al., 1976)

w (Williams, 1973)

* This table reproduces data from Rowe et al. (1984).

with Hind III G fragment of Ad5 and three with the Xho IC fragment of Host range Group II mutants. A final line established by Dr. J.F. Williams (1973) was transformed with sheared Ad5 ts14 DNA. All pertinent information is displayed in Table 1.

In order to analyze the viral mRNA production of these cell lines we used the Northern blot technique and probed with various plasmid clones containing Ad5 sequences from different regions of the Ad5 genome (see probes on Figure 2). These probes would allow a survey of the E1 region, allowing us to identify all E1 mRNAs or specifically E1A mRNAs, the 22s E1B mRNA, or mRNAs from the 3' end of the E1B region.

MATERIALS

α-MEM	Grand Island Biological Co.(GIBCO)
Antibiotic-antimycotic Solution	GIBCO
BSA	Sigma Chemical Co.
Calf serum	GIBCO
DNase I	Worthington Biochemical Corp.
Horse Serum	GIBCO
Lysozyme	Worthington Biochemical Corp.
Naphthalene disulphonate	Eastman Kodak Co.
Newborn calf serum	GIBCO
Nonidet P-40	Bethesda Research Laboratories
Lux dishes	Flow Laboratories
Mycostatin suspension	GIBCO
(^{32}P) -dCTP	New England Nuclear
Penicillin-Streptomycin Solution	GIBCO
Poly (U)-Sepharose 4B	Pharmacia
Polyvinylsulphate (K+ salt)	Eastman Kodak Co.
Restriction Endonucleases	Boeringher Mannheim or Bethesda Research Laboratories
SDS	Serva Feinbiochemical Co.
Tris-HCl	Sigma
Trypsin solution	GIBCO

METHODS

Ad5 transformed cell lines

Table 1 lists the Ad5 transformed hamster cell lines investigated in this study. Table 1 also shows the origin of the DNA used to transform each of the cell lines.

The 14b cell line was derived from hamster embryo fibroblasts transformed using Ad5 ts14 DNA (Williams, 1973). These cells contained 5.5 copies of the left 43% of viral DNA (Flint et al., 1976).

The remaining lines were all isolates of baby hamster kidney cells transformed with various Ad5 or Ad5 host range DNA fragments. These were gifts of Dr. F.L. Graham of McMaster. The cell lines can be grouped according to the type of restriction fragment used for the transformation namely: Ad5 Xho 1 C for lines 983.2, 972.2, and 972.3; Ad5 Hind III digests for 954.21 and 945.C1; Ad 5 Hind III G for cell line 954.1 and Xho 1 digests of hr50 and hr6 DNA for 1019.3, and for 1019.2 and 1019.1 respectively. The table also indicates the number of inserts of viral DNA where known, and the antigens detected in these cell lines (Rowe et al., 1984).

Maintenance of KB and transformed cells

KB cells were maintained as a monolayer culture in 150 mm plastic dishes (Lux) with Eagle's minimum essential alpha medium (α-MEM), 5% calf serum, 1% penicillin-streptomycin solution or 1% antibiotic-antimycotic solution, and 0.5% mycostatin suspension. Cells were

grown at 37°C in 5% CO_2 and subcultured three times a week with trypsin.

Transformed cell lines were maintained in the same way as KB cells except that 1) medium was supplemented with 5% horse serum or 10% newborn calf serum instead of 5% calf serum and 2) subculturing was done using versene (140 mM NaCl, 40 mM Na_2HPO_4, 25 mM KCl, 1 mM KH_2PO_4, 1 mM glucose, 0.5 mM EDTA.Na salt) not trypsin.

Infection of KB cells by Ad5

Ad5 virus was originally obtained from Dr. F.L. Graham (McMaster University). It was purified for use from infected cell lysates by a method adapted from Lawrence and Ginsberg (1967) and was previously titered by C. Evelegh.

The KB cell line was originally isolated from a human epithelial carcinoma (Eagle, 1955). Confluent KB cell monolayers in Lux dishes were infected with Ad5 at a multiplicity of 50 plaque forming units per cell. The virus was absorbed for 1 to 1.5 hours in 5 ml growth medium. 20 ml of medium per plate was then added after absorption was complete. When cycloheximide was used to inhibit protein synthesis it was added to a final concentration of 25 ug/ml in the 20 ml of medium used after absorption (Harter et al., 1976).

Extraction of Cytoplasmic RNA

The method used for the extraction of cytoplasmic RNA from transformed or KB infected cells was from Wheeler et al. (1977).

Four plates of confluent monolayers were harvested for each RNA

extraction. The plates were washed with Tris-saline solution and the cells were suspended by a 5 minute incubation with either trypsin or versene. The cells were pelleted by centrifugation at 1000 rpm for 5 minutes at 4° C, washed again in Tris-saline, repelleted and resuspended in 20 ml of a 1% (v/v) NDS solution. The cells were incubated for 10 minutes at 4° C, and then NP40 detergent was added to a final concentration of 1% (v/v), to lyse the plasma membranes. Nuclei were pelleted by centrifugation at 2500 rpm for 5 minutes at 4° C. The supernatant was poured off and it was made 0.5% with SDS and 100 ug/ml with PVS. Protein was removed from this solution by 3 sequential phenol extractions at room temperature. An equal volume of phenol-chloroform-isoamyl alcohol (50:50:1) was used for each extraction. The resulting solution was then extracted 2 times with an equal volume of chloroform-isoamyl alcohol (50:1). Phase separation was achieved by centrifugation at 2500 rpm for 5 minutes at room temperature, and the proteinaceous layer was always avoided when removing the aqueous solution.

RNA was precipitated by the addition of a 2 fold volume of cold 95% ethanol and 20 mM potassium acetate to the solution, and standing overnight at -20° C. RNA was pelleted at -20° C by centrifugation at 8,000 rpm for 20 minutes. The pellet was washed 2 times in an ethanol: 0.2 M NaCl solution (2:1). The RNA pellet was resuspended in sterile water and the concentration was estimated by A_{260} assuming 1 absorbance unit corresponds to 42 ug/ml.

Recipe for Tris-saline

NaCl	8 g/l
Tris	3 g/l
glucose	1 g/l
KCl	0.34 g/l
Na_2HPO_4	0.1 g/l

pH to 7.4 with HCl

Buffers used in purifying Poly-(A) mRNA

Buffer I	Buffer II	Buffer III
700 mM NaCl	500 mM NaCl	10 mM Tris pH 7.5
50 mM Tris pH 7.5	50 mM Tris pH 7.5	10 mM EDTA pH 7.6
10 mM EDTA pH 7.6	10 mM EDTA pH 7.6	90% (v/v) Formamide
25% (v/v) Formamide[3]	50% (v/v) Formamide	0.2% (w/v) SDS

3 Formamide was purified and deionized on AG 501-X8 mixed bed resin

Purification of polyadenylated mRNA

The method used for mRNA purification is essentially that of Lindberg and Persson (1974).

0.4 g of Poly-(U)-Sepharose was swelled for 1 hour at room temperature in a 1 M NaCl solution. The Sepharose beads were poured into a sterile 10 ml syringe containing a glass fibre filter base to create a 1 ml bed. The bed was washed with Buffer I and then the RNA sample was applied. All buffer solutions were kept at 4°C before use.

Purified cytoplasmic RNA was dissolved in 5 ml of sterile water and then added to 5 ml of Buffer 2x I. This solution was applied to the washed column, allowed to run out and then reapplied 2 times. The column and bound mRNA were then washed with 25 ml of Buffer I and 30 or more ml of Buffer II. During these and subsequent washes the optical density of the solution from the column was measured at 260 nm for the presence of any RNA. When the optical density reached 0 the ribosomal RNA had been removed from the column and the mRNA was then eluted by the addition of 6 ml of Buffer III. The optical density of these fractions were measured to determine complete elution of mRNA. The eluate was added to two volumes of -20° C ethanol and made 0.5 M in ammonium acetate. The mRNA was centrifuged at 40,000 rpm for 4 hours at 4° C, to precipitate the mRNA. The mRNA was then resuspended in a small quantity of sterile water, the A_{260} was taken to determine the mRNA concentration and the sample was stored at -70° C until used.

Northern Blot Analysis

a) RNA electrophoresis

Cytoplasmic RNA and Poly-(A) mRNA samples were fractionated by electrophoresis in denaturing agarose-formaldehyde gels. The method of Nevins and Wilson (1981) as modified by Lai Fatt (1983) was used. The agarose was melted in the MOPS buffer and cooled to 60°C; formaldehyde was added, and the solution was mixed and poured into the gel apparatus. 1 or 2 OD units (equivalent to 40 to 80 ug) of each RNA sample was suspended in 50% deionized formamide, 1 x MOPS buffer (100 mM MOPS, 5 mM Na acetate, 1 mM EDTA) and 2 M formaldehyde and incubated at 65°C for 5 minutes. The sample was adjusted to 1% ficoll, 0.1% SDS, and 0.005% bromophenol blue and loaded onto a horizontal 1% agarose gel (16.5 cm x 17.0 cm x 0.6 cm) with 2 M formaldehyde and 1 x MOPS buffer. Electrophoresis was for 12 hours at 35 volts with the continuous circulation of the tank buffer (1 x MOPS buffer and 2 M formaldehyde).

Size markers used were ribosomal RNA from *E. coli* donated and prepared by S.T. Bayley (McMaster University) and total KB cell cytoplasmic RNA. The sizes of the rRNAs were obtained from Nordstrom *et al.* (1979) but were also checked against known mRNA markers as discussed in the results section.

b) RNA gel blot hybridizations

RNA agarose gels were soaked in a solution of 0.5 M sodium acetate with 1 ug/ml ethidium bromide for 30 minutes to stain

ribosomal RNA markers then destained. Gel washes and transfers were done according to Southern (1975, 1979). Twenty minute washes in 0.5 M sodium hydroxide, were followed by two 30 minute washes in a buffer of 0.5 M Tris pH 8.0 and 3 M NaCl. The gel was blotted to a nitrocellulose filter soaked in 20 x SSC and transferred from 4 to 12 hours. The RNA was baked to the filter by heating to 80°C for 2 hours. Ribosomal bands were illuminated by ultra-violet light and marked for later size reference.

Prehybridization of the filter was for at least 6 hours in prehybridization buffer at 42°C. Hybridization was for 12 to 18 hours in hybridization buffer at the same temperature. The method of hybridization is basically that of Southern (1979) with modifications by Wahl et al. (1979) in terms of preincubation and hybridization buffers. The addition of 10% dextran sulfate to the hybridization procedure increased the rate of hybridization.

The filter was washed in 2 x SSC and 0.1% SDS, for three 10 minute washes. The filter was dried and exposed to X-ray film for various lengths of time at room temperature with an intensifying screen. The film was developed and the rRNA size markers were marked onto the film itself. Using bottom illumination the mRNA and rRNA bands were measured for their migration from the loading well which could be discerned on the autoradiogram. The migration was measured to an accuracy of 0.5 mm to the center of the RNA band but due to limited resolution of the band the centerpoint could be +/- 1 mm.

Recipes for solutions used in Northern blot hybridizations

Prehybridization Buffer		Hybidization Buffer	
750 mM	NaCl	750 mM	NaCl
75 mM	Na citrate	75 mM	Na citrate
50 mM	Na phosphate buffer pH 6.5	20 mM	Na phosphate buffer pH 6.
5 x	Denhardt's solution	1 x	Denherdt's solution
50% (v/v)	deionized formamide	50% (v/v)	deionized formamide
250 ug/ml	salmon sperm DNA	250 ug/ml	salmon sperm DNA
		10%	sodium dextran sulfate
			(^{32}P)-labeled DNA probe

1 x Denhardt's Solution

0.02% (w/v)	Bovine serum albumin
0.02% (w/v)	polyvinyl pyrrolidone
0.02% (w/v)	ficoll

Preparation of the Northern blot hybridization probes

a) Source of DNA probes

E. coli containing pBR322 plasmids with insertions of Ad5 fragments were available from Dr. F.L. Graham (McMaster University) who generously donated cloned strains of *E. coli* containing these recombinant pBR322 plasmids: pHG-1 (containing the Hind III G fragment of Ad5, from 0-7.8%); pXC-1 (containing the Xho IC fragment from 0-15.5%); 994-5 (containing the Hind III E fragment, from 7.8-17.3%); 985-10 (containing the Hind III H fragment from 32.4-38.3%); pHE-1 (containing the Hpa IE fragment from 0-4.5%). In addition, during the present study, a small region of Ad5 sequences from 7.1 to 7.8%, or the Pst 1-Hind III, fragment was cloned into a pBR322 plasmid called pHG-1PH, in order to distinguish between the two major E1B mRNAs (see Figure 2). The appendix carries details of the construction of this plasmid.

b) Plasmid purification

A 10 ml overnight culture of bacteria was diluted 1:100 in Luria broth and grown overnight at 37°C. Chloramphenicol (50 ug/ml) was added when the broth reached an optical density of 0.7, for the amplification of plasmid DNA.

DNA was extracted using the method of Birnboim and Doly (1979) except that one litre of cells were harvested and volumes of lysozyme, alkaline-SDS, 3 M sodium acetate pH 4.8 and Tris-sodium acetate solutions were increased accordingly to 10 ml, 20 ml, 15 ml and 10 ml

respectively.

The final DNA pellet was air dried and resuspended in 7 ml of Tris-EDTA pH 7.5, 0.5 ml ethidium bromide (4 mg/ml), and 7.5 g caesium chloride. The DNA mixture was centrifuged at 35,000 rpm for 48 hours at 15°C to achieve isopycnic banding. The plasmid DNA band was removed and the ethidium bromide was extracted in three washes of an isoamyl alcohol solution which was saturated with a 1.54 g/ml caesium chloride solution. The DNA was dialyzed overnight against 0.1 x SSC, and for 6 hours against 10 mM Tris-1 mM EDTA solution. The DNA solution was lyophilized and resuspended in 0.5 ml of water. To confirm the type of plasmid harvested approximately 1 to 0.5 ul (equivalent to about 1 ug) of this DNA was used for restriction endonuclease analysis on acrylamide gels described in the appendix.

c) Nick translation of DNA

DNA was labeled for hybridization experiments with α-^{32}P labeled dCTP, by the nick-translation method of Maniatis et al. (1975) as modified by Weinstock et al. (1978), and Redfield (1981).

In general, 0.5 ug of DNA was nicked by incubation with 0.15 ng of DNAse I for 10 minutes at 37°C, in 50 ul of nick translation buffer (50 mM Tris pH 7.9, 10 mM β-mercaptoethanol, 5 mM MgCl , 100 ug/ml Bovine serum albumin, 10 uM of dATP, dGTP, and dTTP). The DNA was incubated for 1 hour at 12°C with 50 uCi of ^{32}P-labeled dCTP and 6 units of E. coli DNA polymerase I. The mixture was adjusted to 0.1 M EDTA to stop the reaction and protein was removed by phenol extraction. DNA was separated from unreacted molecules by gel

filtration through a Sephadex G-50 column. The DNA was concentrated by ethanol precipitation. Label incorporation ranged from 50% to 90%, with a specific radioactivity of 2 to 8 x 10 cpm/ 0.5 ug. Radioactivity was measured using a liquid scintillation counter.

Preparation of recombinant plasmid pHG-1PH

a) Transformation and initial selection of bacteria

One ug of both pBR322 and pHG-1 DNA was digested with 2 units of Pst 1 and Eco R1 enzymes for 3 hours at 37°C in 25 ul of digestion buffer (100 mM NaCl, 50 mM Tris pH 7.5, 6 mM MgCl, 100 ug/ml BSA). The enzymes were then inactivated by heating at 65°C for 15 minutes. 10 ul of ligation buffer (10x = 500 mM Tris pH 7.7, 200 mM DTT, 100 mM $MgCl_2$, 10 mM ATP), and 2 units of T_4 ligase (BRL) were added to the DNA mixture at a final volume of 100 ul, and incubated overnight at 4°C.

A 5 ml culture with an A_{550} = 0.45-0.55 of E. coli strain HB101 in Luria broth was pelleted and resuspended into 100 ul of transfection buffer (75 mM $CaCl_2$, 5 mM Tris pH 7.6). The cells incubated with the ligated DNA in transfection buffer for 60 minutes at 4°C. The bacteria were then heat shocked at 42°C for 2 minutes, and the E. coli bacteria were added to Luria broth for 1 hour at 37°C, in a shaking water bath (Ullrich et al., 1977). The bacteria were then plated onto Luria agar with tetracycline (10 ug/ml) in a dilution series. After incubation at 37°C for 24 hours, colonies were picked from these plates and then duplicate plated onto Luria agar plates with either tetracyline (10 ug/ml) or ampicillin (20 ug/ml). Twenty

four hours later colonies which had grown on tetracycline plates but had failed to grow on ampicillin plates were selected for growth in Luria broth overnight, at 37°C in shaking water bath. Each culture was screened with the Birnboim technique.

b) Birnboim method of purification of plasmid DNA for screening purposes

For screening purposes, plasmid DNA was purified using the method of Birnboim and Doly (1979), except that 1.5 ml of each 5 ml overnight culture was extracted.

Selected pBR322 recombinant clones were grown for 18 hours in 5 mls of Luria broth (5 g/l yeast extract, 5 g/l NaCl, 10 g/l tryptone, 1 g/l glucose) with tetracycline (10 ug/ml). 1.5 ml of each culture was pelleted into an eppendorf tube and the supernatant was removed by aspiration. Each cell pellet was resuspended in 100 ul of lysozyme solution (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA, 2 mg/ml Lysozyme), and incubated to weaken the cell walls. The bacteria were lyzed by adding 200 ul of an alkaline-SDS solution (1% SDS, 0.2 N NaOH) and mixing. Incubation for 1 hour at 0°C caused precipitation of high molecular weight DNA, RNA and protein-SDS complexes. The solution was centrifuged and the supernatant which contained only small closed circular plasmid DNA, and small RNA was collected.

The DNA was precipitated by adding 1 ml of cold ethanol to 400 ul of the supernatant, and standing at -70°C for 30 minutes. The DNA was centrifuged and pelleted, then resuspended in 100 ul of a Tris-sodium acetate buffer and reprecipitated in 2 volumes of cold ethanol at

-70°C for 30 minutes. The DNA was pelleted, air dried and suspended in 50 ul of water. 10 ul of this was sufficient for visualizing fragments of a restriction endonuclease digest by gel electrophoresis.

c) DNA gel electrophoresis

The method of Maniatis et al. (1975) for polyacrylamide gel electrophoresis of DNA was used to size restriction fragments. A 7% polyacrylamide gel was made (7% w/v acrylamide, 0.093% w/v bis, 10% w/v ammonium persulphate, 0.06% w/v TEMED) in electrophoresis buffer (40 mM Tris pH 7.8, 5 mM sodium acetate, 1 mM EDTA)). Sample buffer (1 mM EDTA, 5% w/v sucrose, 0.1% w/v SDS, 0.02% v/v bromophenol blue) was added to each sample and electrophoresis was for 3 hours at 150 volts. The gel was placed into an ethidium bromide solution (1 ug/ml) and the DNA fragments were visualized with short wave ultra-violet light. Polaroid type 57 film was used to record the fluorescent bands.

d) Restriction endonuclease digestion

Restriction endonucleases used were obtained from Bethesda Research Laboratories and Boeringer Mannheim. All DNA digests were done in a Tris-acetate buffer (66 mM potassium acetate, 33 mM Tris pH 7.9, 10 mM magnesium acetate, 0.5 mM DTT, 0.1 mg/ml BSA) developed by O'Farrell et al. (1981). For each ug of DNA, 10 ul of digestion buffer and 2 units of enzyme were incubated for 12 hours at 37°C.

RESULTS

Method of Analysis

Generally the transformed cell line RNAs were analyzed using the following procedure. Total cytoplasmic RNA was extracted from monolayers of a transformed cell line. The RNA was precipitated in ethanol, concentrated by centrifugation and dissolved in water. The concentration of RNA was estimated by the absorbance of the solution at 260 nm (nanometers). One or two optical density equivalents (or about 40 to 80 ug) of RNA were loaded onto a formaldehyde-agarose denaturing gel, and electrophoresed. RNA markers were run in adjacent lanes; both KB cell total RNA and E. coli rRNA were used. After electrophoresis, the gel was stained for 30 minutes in an ethidium bromide solution, then destained, washed twice for 20 minutes in an alkaline solution, and twice for 30 minutes in a high salt buffer. The gel was blotted to nitrocellulose paper, the rRNA bands marked on the blot, and the blot was hybridized to a particular ^{32}P labeled plasmid DNA. The blot was then exposed to X-ray film for various lengths of time in order to achieve a suitable exposure of the RNA bands, where the bands were clearly visible but not over developed on the film. Exposure took from 10 hours to 2 weeks in different experiments.

Calibration of Agarose-formaldehyde gels

To determine the size of viral mRNAs an accurate measure of size must be incorporated into the Northern blot hybridization procedure. In order to ensure that the viral mRNA sizes could be determined by

comparison to the mobility of ribosomal RNA markers in our denaturing gels, a calibration experiment was undertaken. Since KB cell rRNA and E. coli rRNA were available, and were of a suitable size for comparison to the viral E1 mRNAs, they were chosen to be tested for their mobility.

These ribosomal markers were electrophoresed in lanes adjacent to a labeled RNA preparation containing VSV mRNA of known lengths (Rose and Gallione, 1981; Gallione et al., 1981), which was prepared in the following way. Between 3 to 6 hours after a vesicular stomatitis virus infection of mouse L cells, host cell mRNA synthesis is shut off by viral factors and viral mRNAs of discrete lengths are synthesized in copious amounts (Batt-Humphries et al., 1979). Incubation of these cells with ^{32}P-orthophosphate for 3 hours at that time exclusively labels VSV mRNA. Using this procedure, total cytoplasmic RNA was extracted from cells 5 hours PI and run on denaturing agarose-formaldehyde gels, alongside KB cell rRNA (28s,18s) and E. coli rRNA (23s,16s).

A portion of the gel containing the ribosomal markers was washed in an ethidium bromide solution to stain these bands, then the whole gel was blotted to nitrocellulose, and the blot exposed to X-ray film (see Figure 3). This Figure shows a variety of RNA bands, particularly the mRNAs for proteins G, N, M and NS. Four minor RNA bands migrate higher in the lane, highest is the genomic RNA, then the mRNA for L protein (very blurred and faint), and two RNA bands which appear to be defective interfering particles in the 3000 b range. The log nucleotide length was plotted against the migration of the mRNAs and rRNAs (Figure 4). Using this procedure the rRNA markers would

FIGURE 3: Calibration of VSV-infected L mouse cell total cytoplasmic RNA with KB cell rRNA and E. coli rRNA

A confluent monolayer of L cells were infected with VSV (Indiana strain) at an MOI of 100 in minus-phosphate medium. At 3 hours post-infection, 1 mcurie of ^{32}P-orthophosphate in 5 mls of minus-phosphate medium replaced the existing medium. At 5 hours post-infection, cells were harvested for cytoplasmic RNA. 0.5 ug of RNA was electrophoresed beside E. coli RNA and total cytoplasmic KB cell RNA under standard conditions. Part of the gel was stained in ethidium bromide for binding to RNA, then the gel was washed and transfered as usual. The rRNA bands were visualized with UV light and marked with ^{14}C ink. XAR film was exposed to the blot, and then developed. The migration of the RNA bands was measured, and graphed vs log nucleotide length in Figure 4.

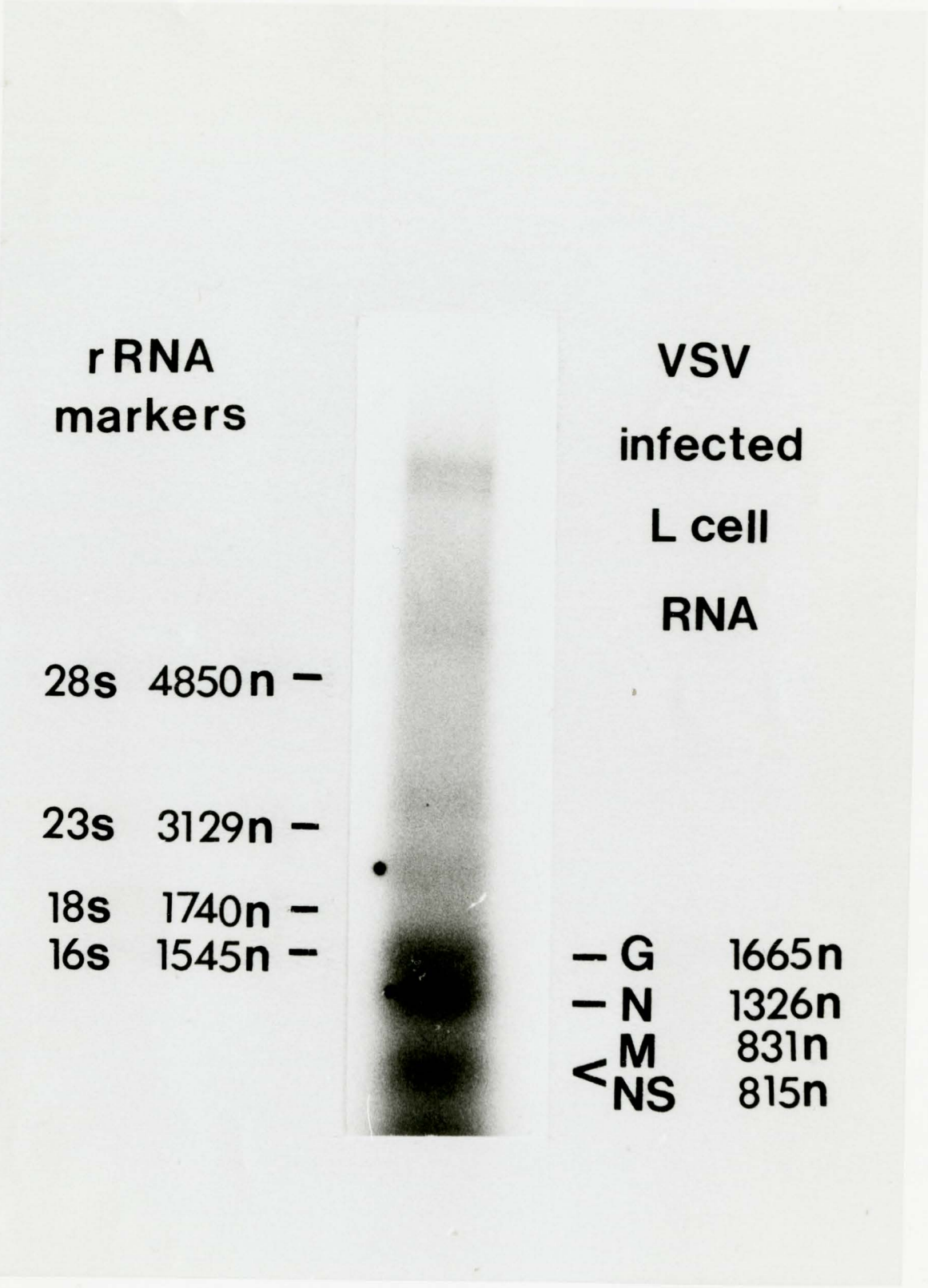

rRNA
markers
28s 4850n
23s 3129n
18s 1740n
16s 1545n
VSV
infected
L cell
RNA
G 1665n
N 1326n
M 831n
NS 815n

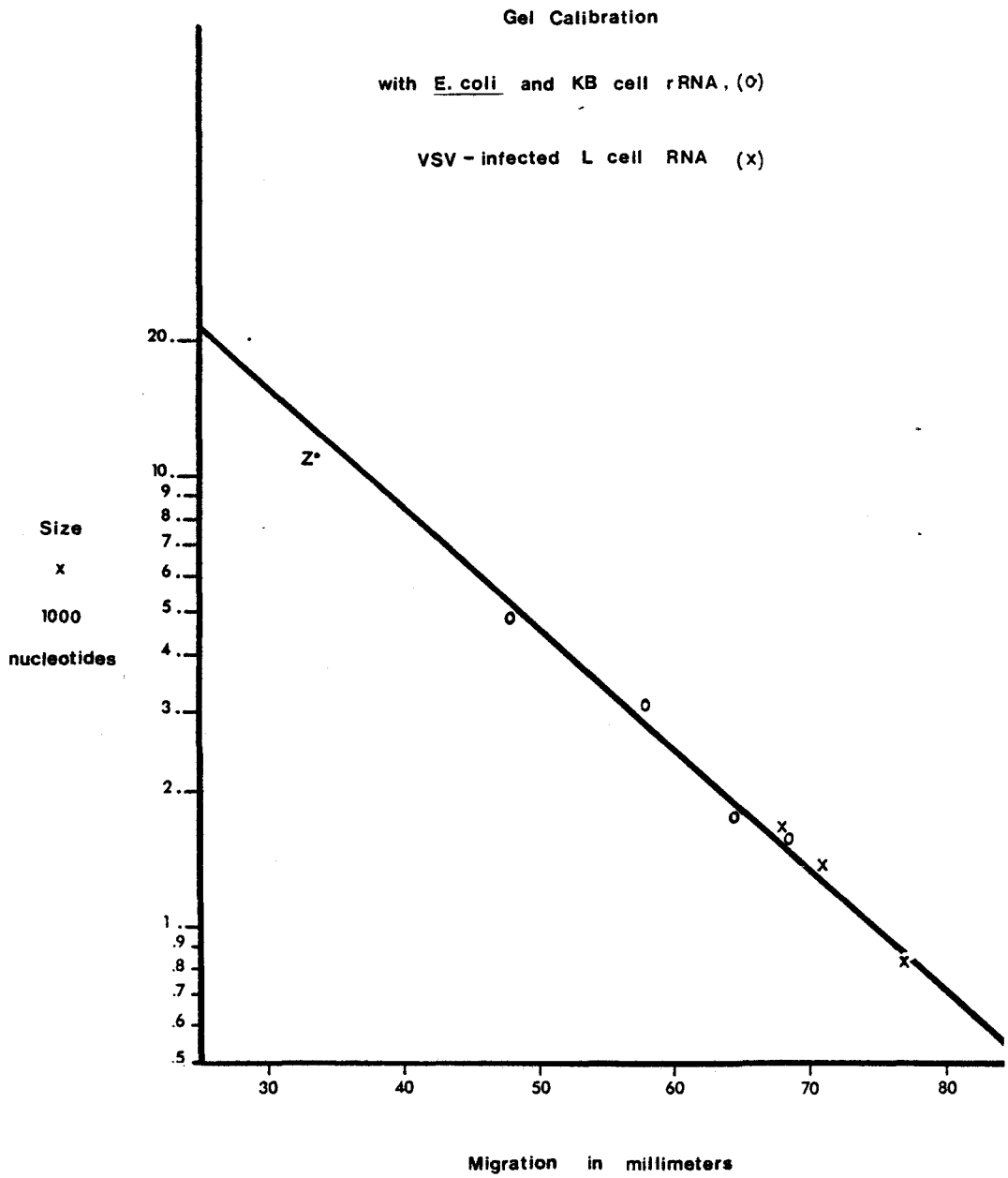
FIGURE 4: Migration of VSV mRNA, KB cell rRNA and *E. coli* rRNA versus the log of nucleotide length in a denaturing agarose-formaldehyde gel

The migration of both mRNA and rRNA of known nucleotide length was measured to determine if rRNA could be used accurately for mRNA size markers in denaturing agarose-formaldehyde gels.

X - VSV mRNAs : mRNA (G protein) 1665 nucleotides
mRNA (N protein) 1326 nucleotides
mRNA (M and NS proteins)
average 823 nucleotides

Z - VSV genome 11,000 nucleotides;
migrated at 13,000 nucleotides

0 - KB cell rRNAs : 28s 4850 nucelotides
18s 1740 nucleotides
0 - *E. coli* rRNAs : 23s 3065 nucleotides
16s 1545 nucleotides


Gel Calibration
with E. coli and KB cell rRNA, (O)
VSV - infected L cell RNA (x)
Size
x
1000
nucleotides
20.
10.
9.
8.
7.
6.
5.
4.
3.
2.
1.
.9
.8
.7
.6
.5
30
40
50
60
70
80
Migration in millimeters

give a good calibration of molecular size for mRNAs at least in the range of 4850 to 800 nucleotides. The VSV genome (11,000 b in length) detectable in Figure 3 as the highest visible band, was overestimated by 2,000 b by calibrating with rRNA bands (see Figure 4). This indicates that only mRNAs of about 4850 b and less could be accurately estimated by comparison to the rRNA markers. Fortunately, the sizes of viral mRNAs expected from transformed cell lines were within this range so the rRNA markers were suitable for calibrating gels. Only these markers were used in subsequent gels for size determination.

As well as including size markers of rRNA in each gel, an internal control of cytoplasmic RNA from Ad5 infected KB cells (18 hour) was also run because it demonstrated a few Ad5 mRNAs of suitable size for comparison to transformed cells.

Northern Blot Analysis

In order to determine the size and type of mRNA transcription pattern occuring in transformed cells a series of probes were used. The results of these Northern blot analyses will be discussed in the sequence: 1) Xho IC which probes the entire E1 region, 2) Hind III G which will hybridize both the E1A and the 5' end of E1B, 3) Hind III E which will detect mRNAs from the 3' end of E1B, 4) Hpa IE which will hybridize E1A mRNAs exclusively and 5) Pst-Hind (pHG-1PH) which should detect certain E1B mRNAs.

The following sections together with Figures 5 to 12 and tables 2 to 4 describe the cytoplasmic RNA products detected from the Adenoviral transforming region of ten transformed cell lines and Ad5 infected KB cells. The RNA preparations shown in Figures 7 through 11

were of total cytoplasmic RNA not Poly (A) mRNA alone. Total cytoplasmic RNA was used in order to reduce the amount of time and material required in preparing samples from each line. In a few instances however where total RNA preparations proved to be of poor quality (eg. 972.2) earlier Northern blots containing Poly (A) mRNA preparations were used and the blot hybridized to Xho IC (see Figure 12). In another instance, 14b Poly (A) mRNA was available and was run beside 14b cytoplasmic RNA preparations for additional information (see Figures 5 and 6).

All the data presented were reproduced using different preparations of RNA from each cell line. The mRNA band measurements were obtained from the blots depicted in the photographs since these were the clearest available.

The Northern blot method has limitations with respect to detecting and analyzing mRNA. Although this technique can detect mRNAs complementary to the DNA probe being used, it does not allow for fine mapping or structural analysis of the mRNAs. As well, mRNA species contain variable lengths of 3' Poly (A) sequences which average more than 200 nucleotides (Sawicki et al., 1977; Brawerman, 1976). This will affect the resolution of the mRNA bands and affect the size determination of mRNAs. All data from this study will be expressed in terms of the additional Poly (A) 3' sequences.

a) Ad5 mRNAs detectable from infected cells

A battery of recombinant clones were used to detect viral mRNA from different regions of the left end of Adenovirus.

The hybridization blots depicted in Figure 5 and 6 show the viral

FIGURE 5: Cytoplasmic and poly (A) mRNA from 14b and Ad5 infected cells hybridized with Hpa IE, Hind III H and pHG-1PH

This figure combines three hybridizations with different DNA probes on preparations from either total (14b) or poly-A selected (14b mRNA) cytoplasmic RNA from 14b transformed cells or Ad5 infected KB cells (harvested at 18 hours or 8 hours + CH*). Ribosomal markers were run in neighboring lanes and the figure shows migration of the RNA bands. Electrophoresis and preparation of the blot hybridizations were as previously described. The probes hybridize to Adenoviral sequences: 0-4.5% (Hpa 1E), 7-7.8% (pHG-1PH = Pst-Hind), and 32.4-38.3% (Hind III H).

* CH signifies cycloheximide added to the infection after adsorption

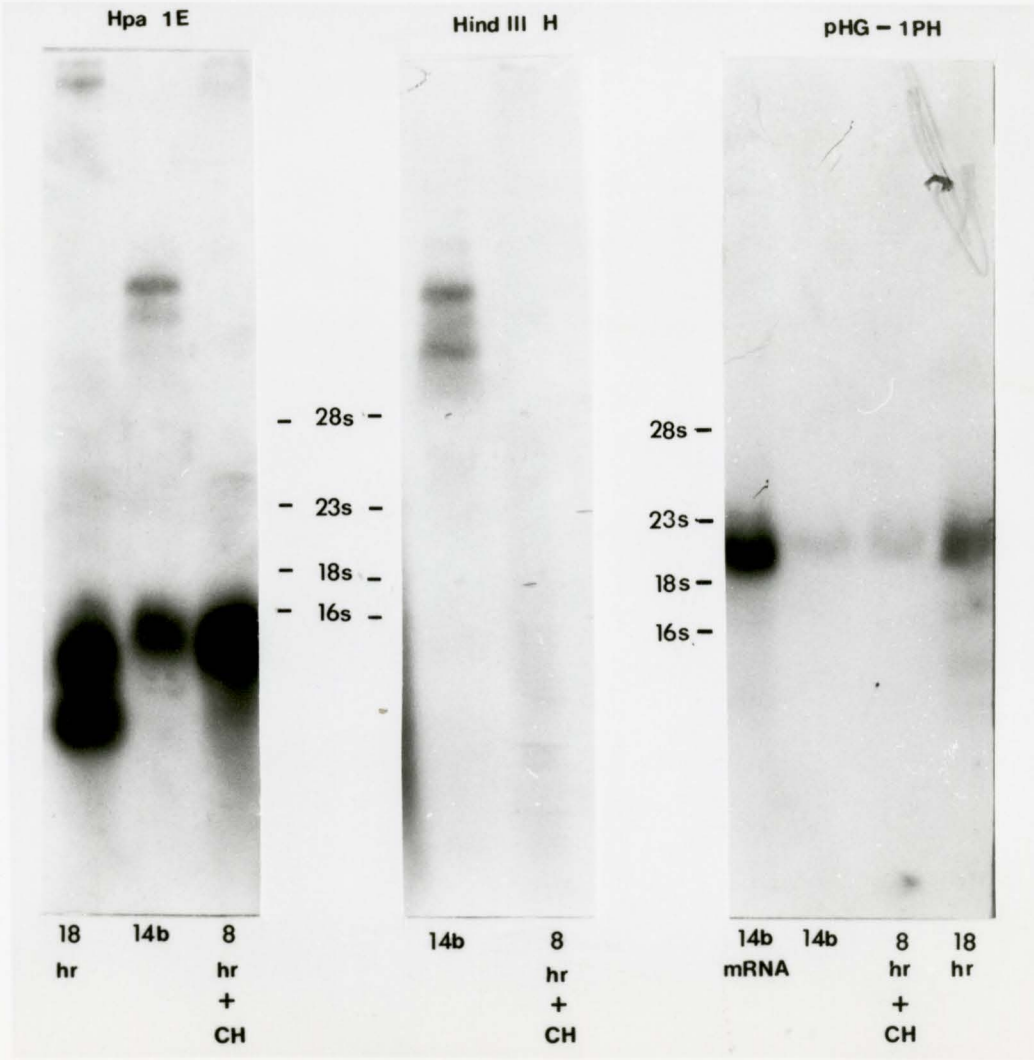
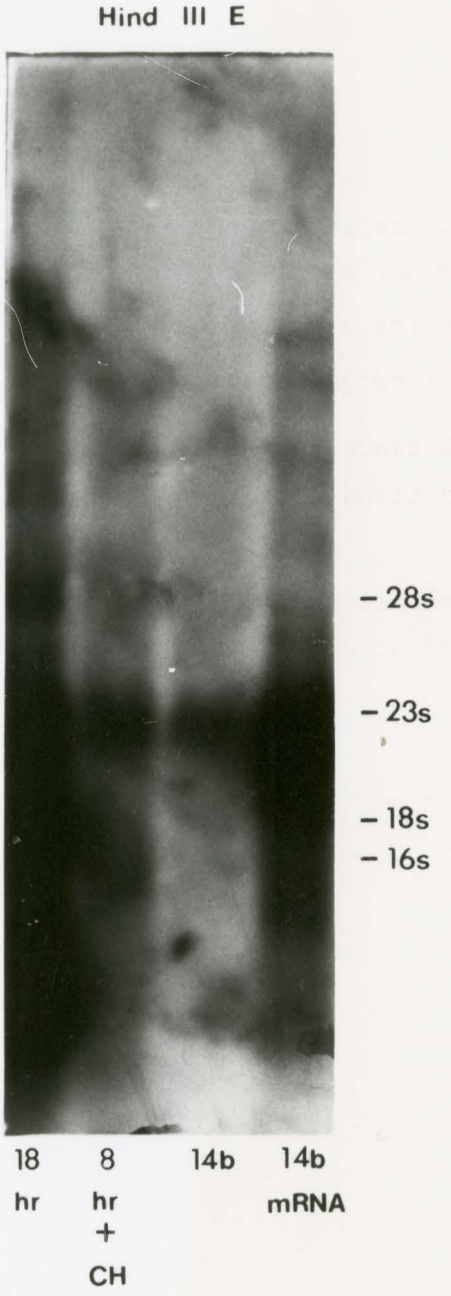

Hpa 1E
Hind III H
pHG – 1PH
28s
23s
18s
16s
18 hr
14b
8 hr + CH
14b
8 hr + CH
28s
23s
18s
16s
14b mRNA
14b
8 hr + CH
18 hr

FIGURE 6: Cytoplasmic and poly (A) mRNA from 14b and Ad5 infected cells hybridized with Hind III E

This figure shows the Hind III E hybridization of either total (14b) or poly-A selected (14b mRNA) cytoplasmic RNA from 14b transformed cells or Ad5 infected KB cells (harvested at 18 hours or 8 hours + CH*). Ribosomal markers were run in neighboring lanes and the figure shows migration of the RNA bands. Electrophoresis and preparation of the blot hybridizations were as previously described.

* CH signifies cycloheximide added to the infection after adsorption


Hind III E
– 28s
– 23s
– 18s
– 16s
18 hr
8 hr + CH
14b
14b mRNA

specific cytoplasmic RNA from KB cells infected with Adenovirus for 18 hours, and for 8 hours with the addition of cycloheximide hybridized with various probes.

The 8 hour plus cycloheximide infected KB cell cytoplasmic RNA demonstrated a single E1A mRNA of 1170 b (13s), and no 600 b (9s) mRNA (see Hpa IE probe - Figure 5). This preparation of RNA also demonstrated E1B-specific mRNAs of 2195 b (22s), detectable with the 22s specific probe Pst-Hind (pHG-1PH), as well as a 1360 b mRNA (13s) detected with the Hind III E probe (Figure 6). No mRNAs were detectable with the Hind III H probe.

The late infected KB cells demonstrated a variety of Ad5 mRNAs. In region E1A (detectable using the Hpa IE probe) there is a 1060 b and a 680 b mRNA (12s and 9s) . A larger 13s mRNA may be present but appears to be a minor mRNA at this time. Two very large RNA bands at the top of the lane of the Hpa IE probed blot may be contaminating nuclear transcripts but are more likely to be artifacts because of their position at the top of the gel (Figure 5). Information from the Hind III E probed blot (Figure 6) for this RNA preparation was not available due to the blots overexposure. With the Pst-Hind (pHG-1PH) probe, not only is the 2225 b (22s) mRNA detected (as expected) but two more size classes of mRNAs at 1670 b, and 1140 b. These might be fairly minor mRNAs previously undetected, being transcribed from either a postulated region of transcription from the right strand (Gingeras, 1982), or from some alternative E1B splicing pattern. Although a 13s mRNA from E1B might be expected it would not contain sequences in the Pst-Hind region (see Figure 2).

Since the 18 hour infected KB cells demonstrated the greatest

variety of mRNAs in these blots, this RNA preparation was used in the other larger Northern blot hybridizations as a comparison with transformed cell mRNAs.

b) Xho IC Ad5 sequences from 0 to 15.5%

There was considerable non-specific binding of the Xho IC probe to the KB cell rRNA. Pencil marks in Figure 7 indicate these bands in a few lanes.

The 18 hour infected KB cell band demonstrates the widest array of mRNA on this blot. A summary of the mRNA size classes is shown in Table 2. The largest size of mRNA is 6050 b, and probably encodes sequences from E2B region. The 1620 b mRNA may also be a 1700 b E2B mRNA encoding the protein IVa2 since it is very abundant at this time. The other mRNA size classes are from the E1 region and include a 2490 b (22s), 1145 b (13s and/or 12s mRNA*) and 725 b (9s).

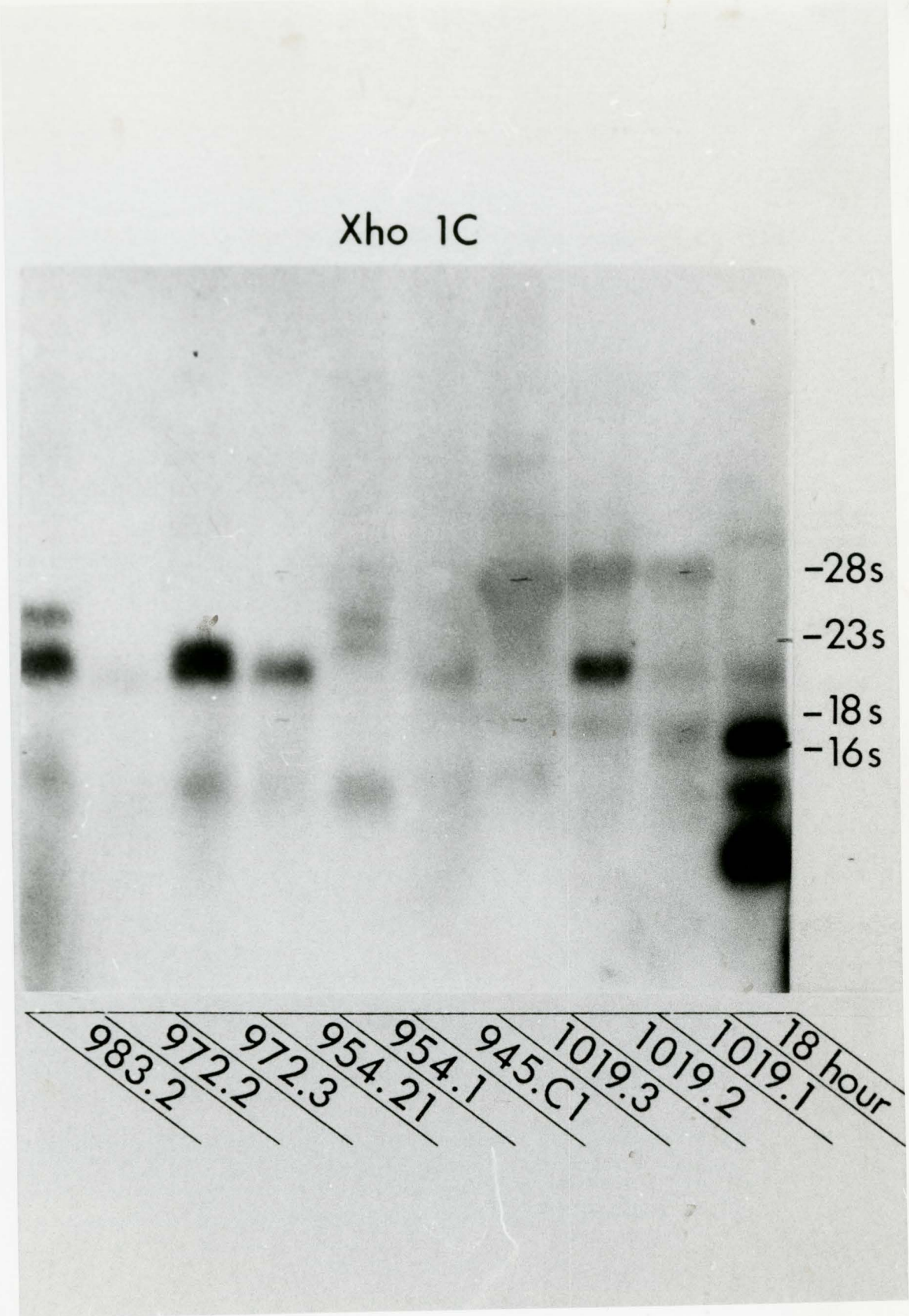
The transformed cell cytoplasmic RNA demonstrate small mRNAs migrating below the 16s rRNA marker (1545 b) from approximately 1180 to 1350 b in length and these probably constitute the E1 13s mRNAs. This mRNA size class is seen in 1019.3, 945.C1, 954.1, 954.21, 972.3, and 983.2, and comigrates with the 18 hour infected cell mRNA, although some size variation exists in each lane (see Table 2). There may or may not be a faint band of this size in the 972.2 lane but more information will be shown for this particular transformed cell line below.

* the 13s/12s mRNA band will be termed 13s from this point onward; the Discussion section will review this question.

FIGURE 7: Cytoplasmic RNA from transformed and Ad5 infected KB cells hybridized with Ad5 Xho 1C DNA

Samples were electrophoresed through an agarose-formaldehyde gel for 12 hours at 35 volts, then washed and blotted to nitrocellulose paper in the standard way. Ribosomal markers were visualized, and marked for later calibration. The blot was prehybridized for 1 hour, then hybridized for 18 hours with a recombinant pBR-322 clone containing the Ad5 Xho IC fragment (plasmid pXC-1). The blot was washed, dried and the ribosomal markers marked with ^{14}C ink. XAR film was exposed to the blot for a suitable period of time, and the migration of the mRNA and rRNA bands was measured. The mRNA size classes were determined by calibration with the known rRNA nucleotide lengths.

ribosomal RNA sizes	28s	4850 nucleotides
	23s	3065 nucleotides
	18s	1740 nucleotides
	16s	1545 nucleotides


Xho 1C
-28s
-23s
-18s
-16s
983.2
972.2
972.3
954.21
954.1
945.C1
1019.3
1019.2
1019.1
18 hour

The next largest size classes of cytoplasmic RNA are very faint and blurred and are in 1019.1 and 983.2 lanes just at and above the 16s rRNA marker (1545 b). This band is especially difficult to detect in 1019.1 due to the heavy 18s rRNA (1740 b) non-specific binding but migrates to a position of 1500 b. In cell line 983.2 the band is just above the 16s rRNA marker and is about 1670 b in length.

Above the 18s rRNA (1740 b) and below the 23s rRNA marker (3065 b) are the 22s cytoplasmic RNAs. These are detected in all lanes except 1019.3 and 954.1. The appearance of the 1019.3 lane is disturbing because there appears to be binding in a wide region below the 28s rRNA band (4850 b) and some streaks above this. Perhaps so much material was loaded onto this lane to get some viral specific binding to these probes that this has resulted in poor resolution and smearing of RNA.

The 954.1 and 983.2 cell lines exhibit larger than 22s cytoplasmic RNAs in the 3800 b (983.2) and 3700 b and 3000b (954.1) regions.

Note that the very large RNA size classes detectable as faint bands high in the lanes of 1019.3, and 954.1 have been listed, and will be discussed later.

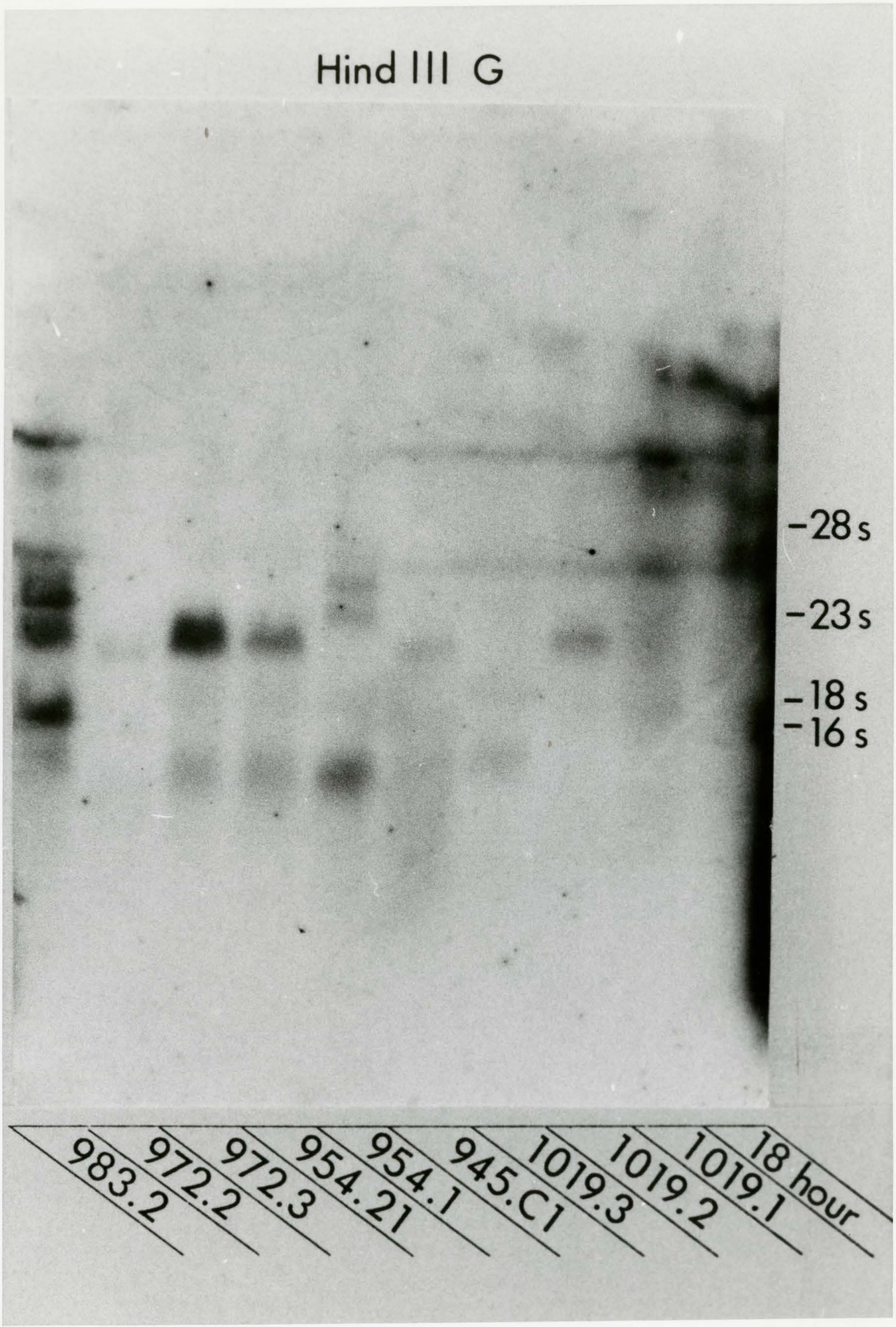
c) Hind III G Ad5 sequences from 0 to 7.8%

This probe should bind to both E1A mRNAs and the 5' end of E1B mRNAs as shown in Figure 2. In Figure 8 the hybridization of the nitrocellulose blot was not very intense, and there was extensive non-specific binding along the right edge and in two bands across the top half of the blot. These markings obscure the 18 hour infected KB cell

FIGURE 8: Cytoplasmic RNA from transformed cells and Ad5 infected KB cells hybridized with Ad5 Hind III G DNA

The samples were hybridized to a recombinant pBR322 clone containing the Ad5 Hind III G fragment (plasmid pHG-1). The viral mRNA map and the position of this fragment relative to it are depicted in Figure 2.

ribosomal RNA sizes	28s	4850 nucleotides
	23s	3065 nucleotides
	18s	1740 nucleotides
	16s	1545 nucleotides


Hind III G
-28s
-23s
-18s
-16s
983.2
972.2
972.3
954.21
954.1
945.C1
1019.3
1019.2
1019.1
18 hour

and the 1019.1 lane.

Briefly, all the mRNAs detected with Xho IC probe are still visible except for the 1019.1 2400 b mRNA, indicating that all the mRNAs detected are from the E1 region. The smallest cytoplasmic RNA size classes detectable with this probe are below the 16s marker (1545 b), and are about 1100 to 1150 b long. This band represents the E1 13s mRNA size class already detected with the Xho IC probe. This mRNA is found in 954.1, 954.21, 972.3, and 983.2 transformed cell lines. Cell line 1019.3 also exhibits a small cytoplasmic RNA at about 1245 b.

The most common size class of cytoplasmic RNA in these lanes is 2410 to 2550 b in length, and present in 1019.2, 945.C1, 954.21, 972.3, 983.2 and very faintly detectable in line 972.2. Most of these 22s cytoplasmic RNAs have been detected previously in the Xho IC hybridization. The 1019.3 line fails to display any distinct mRNA in this region despite broad hybridization in the Xho IC probed blot (Figure 7).

Other interesting size classes of cytoplasmic RNA from these cell lines are found in line 954.1 and 983.2. Cell line 954.1 contains a pair of cytoplasmic RNAs in the 3600 and 2950 b regions while line 983.2 contains a 1525 b RNA, and a large 3650 b mRNA, which may have another band above it, but this region is obscured by non-specific binding of the probe to the nitrocellulose blot.

d) Hind III E Ad5 sequences from 7.8 to 17.3%

This probe should hybridize to all E1B mRNAs since both the 22s and 13s mRNAs contain 450 b from this region (Figure 2). This probe

would hybridize to the 9s mRNA from E1B which is responsible for protein IX. As well, mRNAs from region E2B also complement about 2200 b of this DNA fragment.

The Ad5 18 hour infected KB cell RNA (in Figure 9) shows three distinct mRNAs at and below the 16s level (1545 b). The highest band of 1500 b may be the mRNA encoding protein IVa2 from the E2B region, which is about 1700 b in length (Bos et al., 1981). An 1150 b mRNA may represent the E1B 13s mRNA product which increases in amount and stability compared to the 22s mRNA product during late infection. The most abundant mRNA is about 530 b and may represent the 700 b mRNA encoding protein IX. The 22s mRNA is not detectable in this blot. Other markings high on the blot may be non-specific hybridization to the paper, or minor mRNA products.

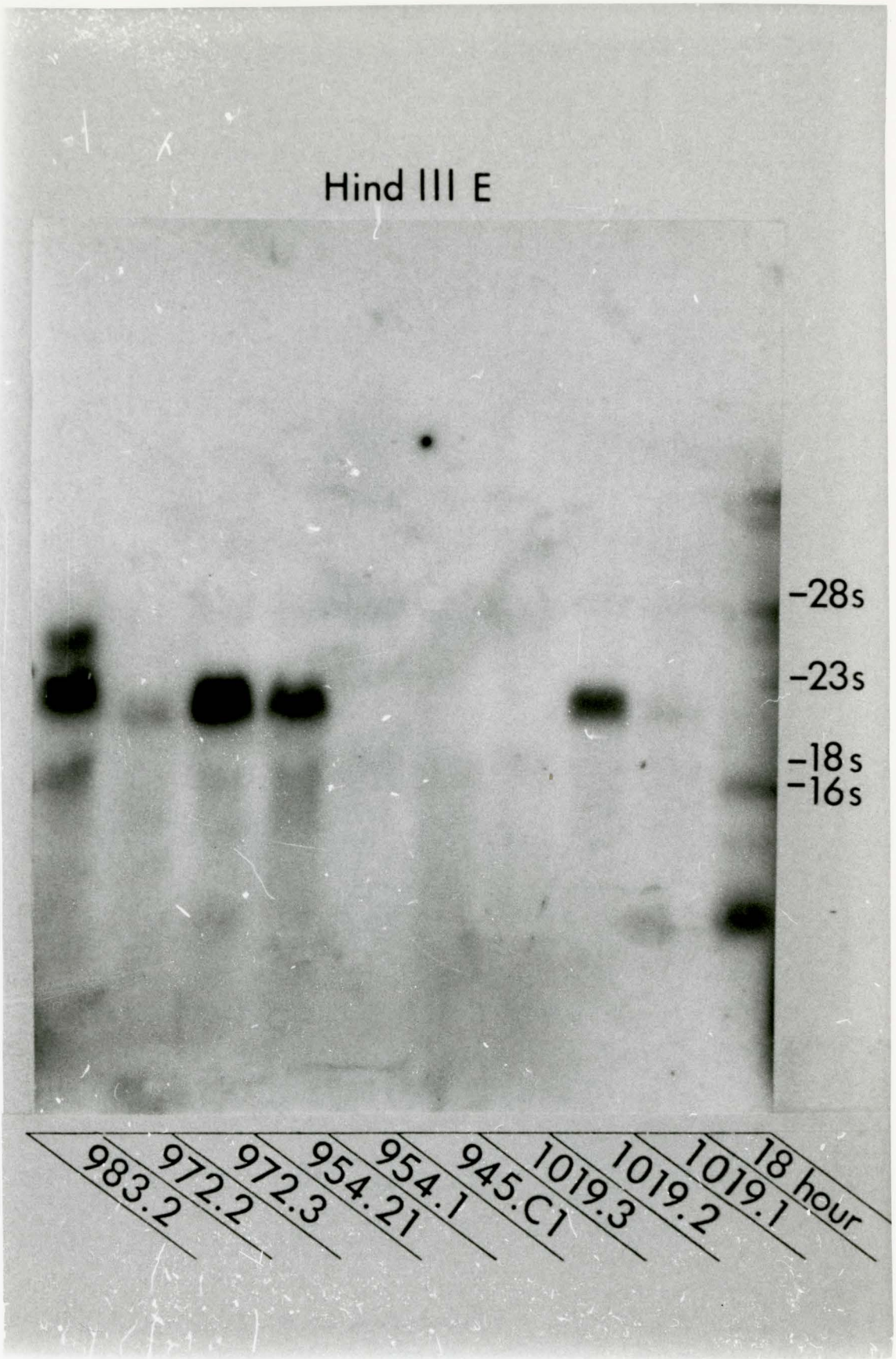
Clearly the most abundant species in the transformed cell lines is a broad band of mRNA in the 2370 b to 2500 b region. Cell lines 1019.1, 1019.2, 954.21, 972.3, 972.2, and 983.2 all share this mRNA which may be the 22s E1B mRNA (2457 b). In two of the lines, 972.3 and 983.2, the band is intense and may represent two discrete bands. The only other additional bands from these cells lines are both in 983.2 and constitute major mRNA products. These are a 3850 b and a 1650 b mRNA, which will be discussed again in the next section.

None of the transformed cell lines have any detectable E1B 13s mRNA despite long exposures, but even if some 13s mRNA did exist, it would be a fairly minor product compared to the abundant 22s mRNA. Note that cell lines 1019.3, 945.C1, and 954.1 show no traces of any hybridizing mRNA. Line 954.1 was transformed with the Adenovirus Hind III G fragment only, so this line will not show any sequences from the

FIGURE 9: Cytoplasmic RNA from transformed cells and Ad5 infected KB cells hybridized with Ad5 Hind III E DNA

The samples were hybridized to a recombinant pBR322 clone containing the Ad5 Hind III E fragment (plasmid 994-5). The viral mRNA map and this Ad5 fragment is shown in Figure 2.

ribosomal RNA sizes	28s	4850 nucleotides
	23s	3065 nucleotides
	18s	1740 nucleotides
	16s	1545 nucleotides


Hind III E
-28s
-23s
-18s
-16s
983.2
972.2
972.3
954.21
954.1
945.C1
1019.3
1019.2
1019.1
18 hour

3' end of E1B. One might expect hybridization to mRNAs from 1019.3 or 945.C1 since these lines contain integrated Adenoviral sequences from Hind III G and E (see Table 1) and 22s-like mRNAs are apparent from the Xho IC (line 1019.3 and 945.C1) and Hind III G (945.C1) probed blots. However the failure to detect these mRNAs with the Hind III E probe indicates that transcription from the 3' end of E1B either does not occur or is very minor.

e) Hpa IE Ad5 sequences from 0 to 4.5%

This probe should hybridize to E1A region mRNAs (see Figure 2) and to any transcripts from the 1 strand of the Hpa IE region.

In Figure 10, the RNA preparation from 18 hour infected KB cells shows 2 diffuse bands migrating at 1000 b and 650 b. These are likely to be the 13s mRNA and the 9s mRNAs. All of these transcripts would be present at this time of infection (Spector et al., 1980; Wilson and Darnell, 1981).

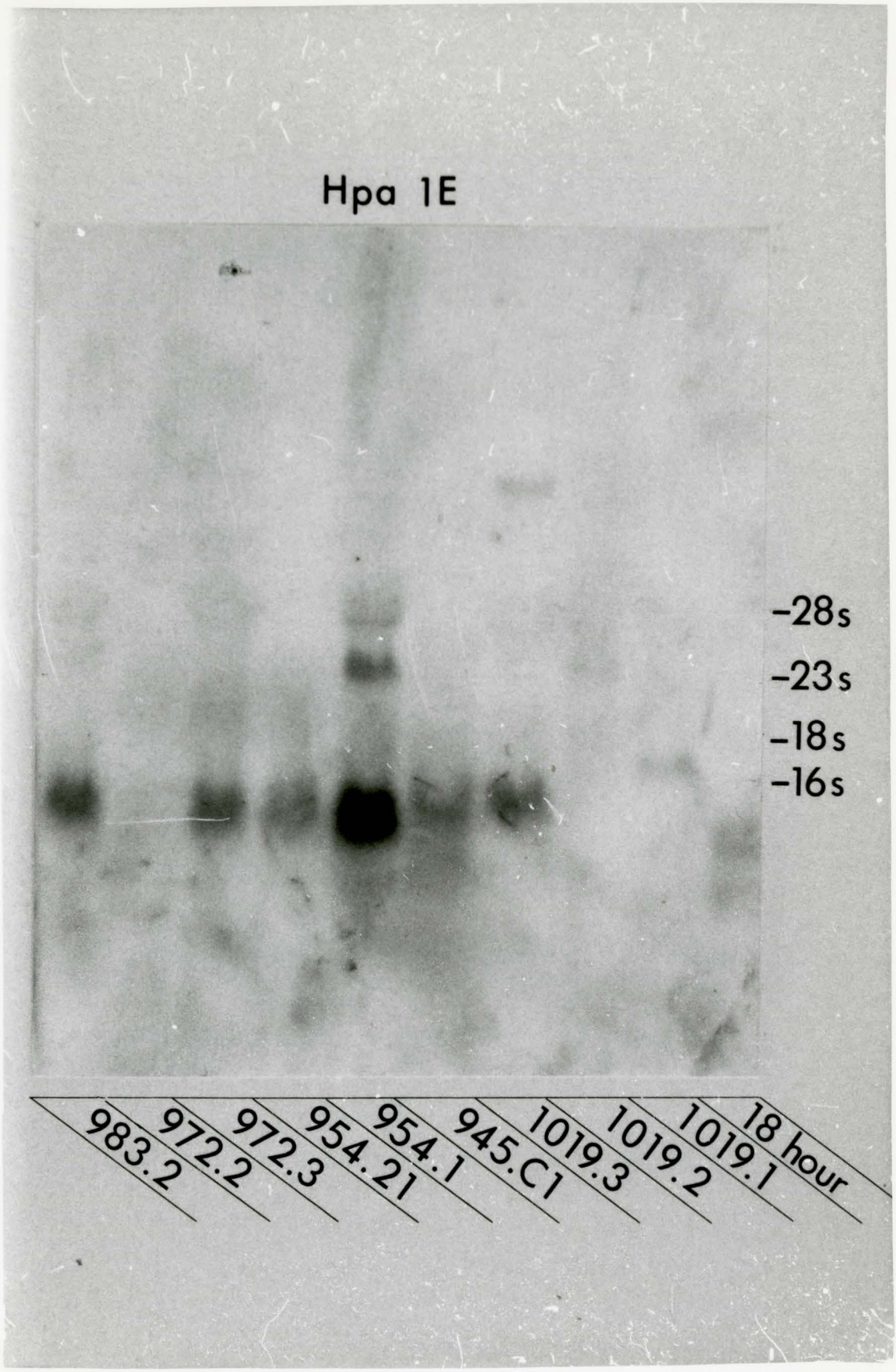
The major product of the transformed cell lines appears to be the 13s (1200 b) mRNA, present in six of nine lines (1019.3, 945.C1, 954.1, 954.21, 972.3, and 983.2). Other bands of interest are of larger sizes, notably (from right to left across the Figure): a 1640 b mRNA in 1019.1, a 3300 b mRNA in 1019.2, a 12,000 b mRNA in 1019.3, and a strong band at 3150 b mRNA in 954.1.

Some spots of non-specific hybridization appear notably in the ribosomal 28s rRNA of the 954.1 line. It is not clear if a band actually does migrate in this region or not, but since the band runs with the 28s rRNA marker and is not migrating with the previously detected 3700 b mRNA from Figures 7 and 8, it will be disregarded.

FIGURE 10: Cytoplasmic RNA from transformed cells and Ad5 infected KB cells hybridized with Ad5 Hpa 1E DNA

The samples were hybridized to a recombinant pBR322 clone containing the Ad5 Hpa I E fragment (plasmid pHE-1). The viral mRNA map and this Ad5 fragment is shown in Figure 2.

ribosomal RNA sizes	28s	4850 nucleotides
	23s	3065 nucleotides
	18s	1740 nucleotides
	16s	1545 nucleotides


Hpa 1E
-28s
-23s
-18s
-16s
983.2
972.2
972.3
954.21
954.1
945.C1
1019.3
1019.2
1019.1
18 hour

Hybridization of the DNA probe occuring in the 954.1 and 945.C1 cell lines in the region below the 1200 b mRNA could be due to specific hybridization of degraded E1A mRNA, or smudging caused by non-specific binding to the nitrocellulose paper in this place. These spots are dismissed from the analysis because of their diffuseness; only clearly hybridizing bands are included in tables.

f) Pst-Hind Ad5 sequences from 7.1 to 7.8%

This probe would hybridize to the E1B 22s mRNA, but not the 13s mRNA (see Figure 2).

In Figure 11, the most common mRNA detected with this probe is the 22s mRNA, migrating below the 23s marker (3065 b) in cell lines 1019.2, 954.21, 972.3, and 983.2. This mRNA ranges in size from 2400 b to 2950 b. Although it is very difficult to detect it in the photograph, cell lines 1019.1, 972.2 and the 18 hour infected KB cell line also contain a small amount of this mRNA. The E1B 22s mRNA is not abundant in the late phase of infection. It is not clear why a variation exists in the size of this mRNA in lines 983.2 and 972.3. There could possibly be a pair of mRNAs migrating together, as seen in the previous blot probed with Hind III E DNA in each of these cell lines.

Again the unusual size classes of mRNA are seen in 983.2 (3875 b), and in cell line 954.1 (3875 b and 3200 b) and are definitely E1B specific. These mRNAs are well over the size of the 22s mRNA seen in lytic infections, and at least 400 b longer than the mRNA detected from Ad5 18 hour infected KB cells. This might indicate that 954.1 cells, which have been transformed with Ad5 Hind III G DNA,

FIGURE 11: Cytoplasmic RNA from transformed cells and Ad5 infected KB cells hybridized with Ad5 Pst-Hind DNA (pHG-1PH)*

The samples were hybridized to a recombinant pBR322 clone containing the Ad5 Pst-Hind fragment (plasmid pHG-1PH). The viral mRNA map and the position of this fragment relative to it are depicted in Figure 2.

ribosomal RNA sizes	28s	4850 nucleotides
	23s	3065 nucleotides
	18s	1740 nucleotides
	16s	1545 nucleotides

* see appendix for construction

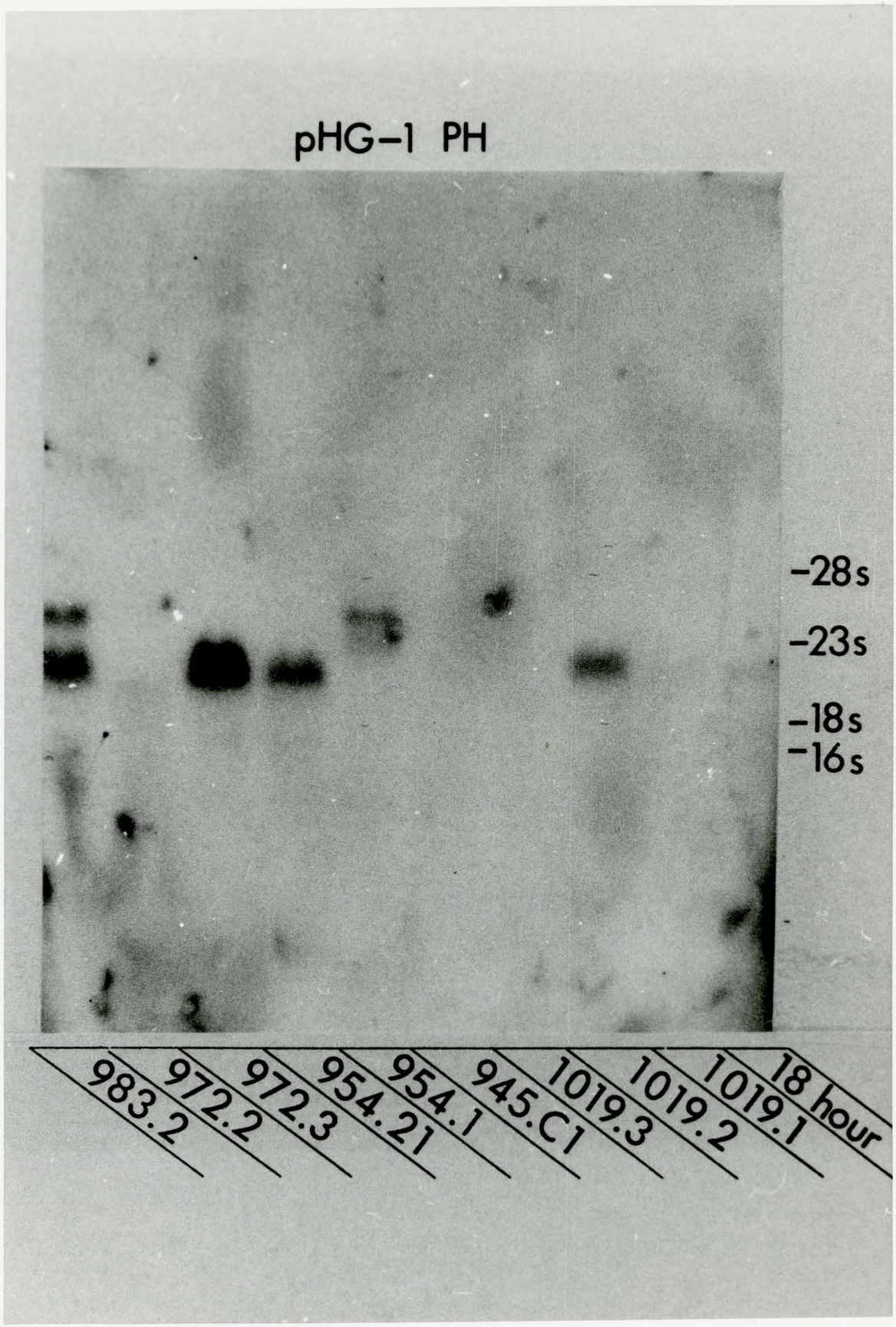

pHG-1 PH
-28s
-23s
-18s
-16s
983.2
972.2
972.3
954.21
954.1
945.C1
1019.3
1019.2
1019.1
18 hour

TABLE 2 Transformed and infected cell cytoplasmic mRNAs hybridizing with Ad5 fragments

Cell line	mRNA[1] average	Hpa I E	pHG-I PH	Hind III E	Hind III G	Xho I C	Present E1A	Present in E1B[2]
18 hour	6050					6050		
	2550		2600			2490		*
	1550			1500		1620		*
	1120	1060		1150		1145	*	*
	700	680				725@	*	
	630			530		725@		*
1019.1	2470		2600	2400		2400		*
	1570	1640			1500		*	
1019.2	3300	3300					*	
	2570		2650	2500	2550	2570		*
1019.3	11,000	12,000				10,000	*	
	7,000					7,000		
	1260	1250			1245	1280	*	
945.C1	2480				2410	2550		*
	1200	1200					*	
954.1	17,000					17,000		
	3725		3875		3600	3700		*
	3075	3150	3200		2950	3000	*	*
	1160	1200			1100	1180	*	
954.21	2500		2600	2450	2410	2550		*
	1200	1250			1150	1210	*	
972.3	2860		2950	2800	2700	3000		*
	2515		2500	2600	2410	2550		*
	1225	1250			1140	1280	*	
972.2	2405		2400	2370	2300	2550		*
	1280	A*					*	
983.2	3800		3875	3850	3650	3800		*
	2950		2950	2900	2950	3000		*
	2590		2500	2600	2550	2700		*
	1615			1650	1525	1670		*
	1250	1250			1150	1350	*	

1 the average of the nucleotide lengths from the blots as listed
2 any mRNA present in the Hpa IE blot was assigned to E1A, others to E1B
A* this information on size from figure 12, and discussed in section h
other information is shown in figures 7 through 11
@ these two listings are from the same band

contain viral-cell chimera mRNAs where the viral DNA has integrated into the host DNA. In the case of 983.2 cells which were transformed by an Xho IC fragment from Ad5, no such explanation can be made without assuming that some of the 3' sequences from E1B have been lost at one of the sites of integration.

Analysis of Transformed Cell Line mRNAs

a) Cell line 1019.1

This cell line was transformed with an Xho I digest of hr 6 Ad5 DNA, a Group II mutant with a defect in the E1B region. The 1019.1 line does not make detectable E1B 58k antigen and is interesting to test for the presence of any E1B mRNAs.

Although the 1019.1 preparation that was loaded onto the gels contained as much cytoplasmic RNA as other lanes, the intensity of viral mRNAs from this cell line was poor, and allowed detection of only two viral cytoplasmic RNAs. Other Northern blots were done with other RNA preparations of this cell line and always demonstrated the presence of only two mRNAs. An unusually large E1A specific mRNA of 1570 b was detected with both the large Xho IC probe (Figure 7) and the Hpa IE probe (0-4.5%) (Figure 10). An E1B specific mRNA of 2470 b was also detected using probes Xho IC, Hind III E and Pst-Hind. No E1B specific 13s mRNA was detectable.

b) Cell line 1019.2

These cells were transformed with the same DNA as the 1019.1 cells, and share the defect for 58k protein.

The cytoplasmic RNA preparation showed a very distinct mRNA band migrating at about the 2570 b region, which hybridized to all the E1B region probes (including the 22s specific mRNA probe), but not to the Hpa IE E1A probe. Faintly visible with the Hpa IE probe is an RNA band migrating at 3300 b, above the 23s rRNA marker (3065 b). This is a very minor band compared to the E1B mRNA, but the only one clearly visible.

c) Cell line 1019.3

This is the most interesting of the host range transformed cell lines because it is moderately tumorigenic, and yet does not appear to produce either E1B 58k or 19k (see Table1). An E1B product is postulated to be essential for transformation (Graham et al., 1974, 1978; Jones and Shenk, 1979; McKinnon et al., 1982; Chinnadurai, 1983). 1019.3 like the other host range transformed cell lines studied, was transformed by an Xho I digest of a Group II mutant; in this case hr 50 was used for transformation of rat kidney cells.

In the Hpa IE, Hind III G and Xho IC hybridized blots, an E1A 13s mRNA (1260 b) is detectable. Two other mRNAs (10,000 and 7,000 b) are detectable high in the lane of the Xho IC probed blot, and one of these is similar in size to an mRNA size class seen in the Hpa IE probed blot (12,000 b). The two values 12,000 b and 10,000 b may represent identical mRNAs since as discussed previously, accurate calibration of the RNAs is difficult above the 4000 b size. An mRNA of this size might be a virus/cell chimera.

A previous blot which had been used for studying the 972.2 cell line revealed some more information about the 1019.3 cells (see Figure

12). Poly-A selected cytoplasmic mRNA from this line was hybridized with an Xho IC probe and demonstrated two new mRNAs at values of above and below 22s. This preparation was not used in subsequent hybridization blots, and so only this limited information was available. Not only are these two 22s-like mRNAs unique to this preparation, but the other RNA size classes detected in the other blots are not discernable here. It is not clear why no other RNA bands are visible in this lane of Figure 12. Perhaps selection of poly-A mRNA has excluded these other RNAs due to their lack of tails or sensitivity to degradation. Clearly however, it is possible to suggest the presence of these mRNAs in the Xho IC probed blot (Figure 7), in the region below the 28s rRNA, where considerable yet blurry hybridization has occured. It is possible that this variation in preparations may be the result of some nuclear contamination occuring in the regular cytoplasmic RNA preparations, or it may be evidence of the stability of the certain size classes of mRNA.

There are at least 5 separate mRNAs present in this cell line but none can be assigned to the E1B region with the available information. There are two E1A specific mRNAs, a 1260 b and a larger 12,000 b present in Hpa IE hybridized blots. Another large mRNA appears in the Xho IC probed blot, but cannot be assigned accurately. As well the two 22s-like mRNAs visible in Figure 12 cannot be assigned but may represent a small quantity of viral mRNA only visible in that Figure due to a cleaner, less degraded preparation of mRNA.

d) Cell line 945.C1

This hamster derived cell line was transformed by a Hind III digest of Ad5 DNA. It is moderately tumorigenic and contains one copy of the Hind III G and E sequences as well as the right end of the Adenoviral genome (Table 1).

Although the RNA preparation appears to be slightly degraded in some hybridization blots (witness the binding of probe below the common 1200 b mRNA in the Hpa IE probed blot Figure 10), it demonstrates a substantial amount of mRNA present for hybridization.

Two major RNA size classes are present, a 1200 b E1A mRNA (12s, see Figure 10), and a 2480 b E1B mRNA (see Figure 7 and 8). The E1B 2480 mRNA does not display a typical 22s mRNA transcription pattern (Figure 2), since it does not hybridize to either the Hind III E or the Pst-Hind (pHG-IPH) probes (Figures 9, 11). Perhaps the pattern of transcription is similar to the typical E1B 13s mRNA which uses the first donor splice site in the E1B region, except that sequences from 3' end of the E1B region are not transcribed (Figure 14). Another alternative is that there is binding to the Pst-Hind (pHG-IPH) probe but it is so faint as to be undetectable. This is not very likely since comparison of the hybridization blots of Hind III G and Pst-Hind (pHG-1PH) (compare Figures 8 and 11) show that they are very similar in pattern, aside from the difference in this one lane. The best evidence for the alternative transcription pattern of the 2480 b E1B mRNA is the fact that in general, the normal 22s mRNA is bound equally well by either the Xho IC or the Hind III E probe (compare the 22s bands in Figures 7 and 9).

e) Cell line 954.1

This hamster cell line was transformed using the Hind III G fragment of Ad5 DNA, and contains only one copy of this fragment (see Table1). Since this Hind III restriction endonuclease site occurs in the middle of the E1B mRNA sequences, some effect was expected in the size of the mRNAs from this region.

Two mRNA size classes were detectable in the E1A region, an 1160 b mRNA (12s) and a much larger 3075 b mRNA (see Figure 10). This large mRNA size class was also detectable in the Xho IC, Hind III G and Pst-Hind (pHG-1PH) hybridized blots. Although it may simply be coincidental that the same size mRNA occurs in all these cases, it is more likely that the same mRNA is reading through from the E1A to the E1B sequences. Viral/viral chimera mRNA which contain sequences from two regions of the adenovirus genome have been demonstrated in transformed cells by Yoshida and Fuginaga (1980) and Sawada and Fuginaga (1980) and are a minor component of infected cells (Kitchingman et al., 1977; Saito et al., 1983).

An E1B 3725 b mRNA is detectable by the Pst-Hind (pHG-IPH) probe. This is most likely a runoff mRNA from the E1B sequences into cellular sequences where a poly-A addition site occurs, (see Figure 13).

A very large 17,000 b mRNA is detectable only with the Xho IC probe. This is probably either a small amount of contaminating nuclear RNA or it is a very large cellular mRNA which runs into viral sequences. There isn't enough information available to determine which is the case.

f) Cell line 954.21

This line was transformed using a Hind III digest of Ad5 DNA. It is not very oncogenic and has four Ad5 DNA insertions in cellular DNA.

The pattern displayed here is the most simple of all the transformed cell lines. There are two mRNA size classes, an E1A 1200 b (13s), and an E1B 2500 b (22s) mRNA. The pattern of hybridization of these mRNAs is normal, with the E1A mRNA hybridizing to Xho IC (0-16%), Hind III G (0-8%), and Hpa IE (0-4.5%) (see Figures 7,8 and 10). The E1B mRNA hybridizes to Xho IC (0-16%), Hind III G (0-8%), Hind III E (8-17.3%), and Pst-Hind (pHG-1PH 7-8%) (see Figures 7,8,9,and 11).

g) Cell line 972.3

The Xho IC fragment of Ad5 DNA was used to transform this cell line. Four copies of this fragment have been inserted into cellular DNA (see Table 1). This line produces the major E1 tumor antigens but is not highly tumorigenic in syrian hamsters. We would expect to see all the E1 mRNAs being produced in this cell line.

This cell line contains an E1A 1250 b mRNA (13s), hybridizing to the Hpa IE (0-4.5%), Hind III G (0-8%), and Xho IC (0-16%) probes. It is not absolutely clear if the E1B mRNAs (2515 and 2860 b) are indeed two separate mRNAs but the width of the band does give the appearance of two species in most of the hybridization blots, so measurements were taken from the presumed middle of each of the two bands. These E1B mRNA size classes each hybridize to Pst-Hind (pHG-1PH 7-8%), Hind III G (0-8%), Hind III E (8-17.3%), and Xho IC (0-16%) (see Table 2 for summary). In Figure 12, the poly-A selected cytoplasmic RNA

preparation made at an earlier time shows only a single band in this area of unknown size. Perhaps the second mRNA band from E1B indicates an unspliced nuclear RNA which contaminates that cytoplasmic preparation, or is a minor mRNA which is only apparent in better preparations.

h) Cell Line 972.2

This cell line was transformed with an Xho IC fragment from Ad5 DNA. It contains viral information hybridizing to Hind III G and E fragments, with 2 insertions being present.

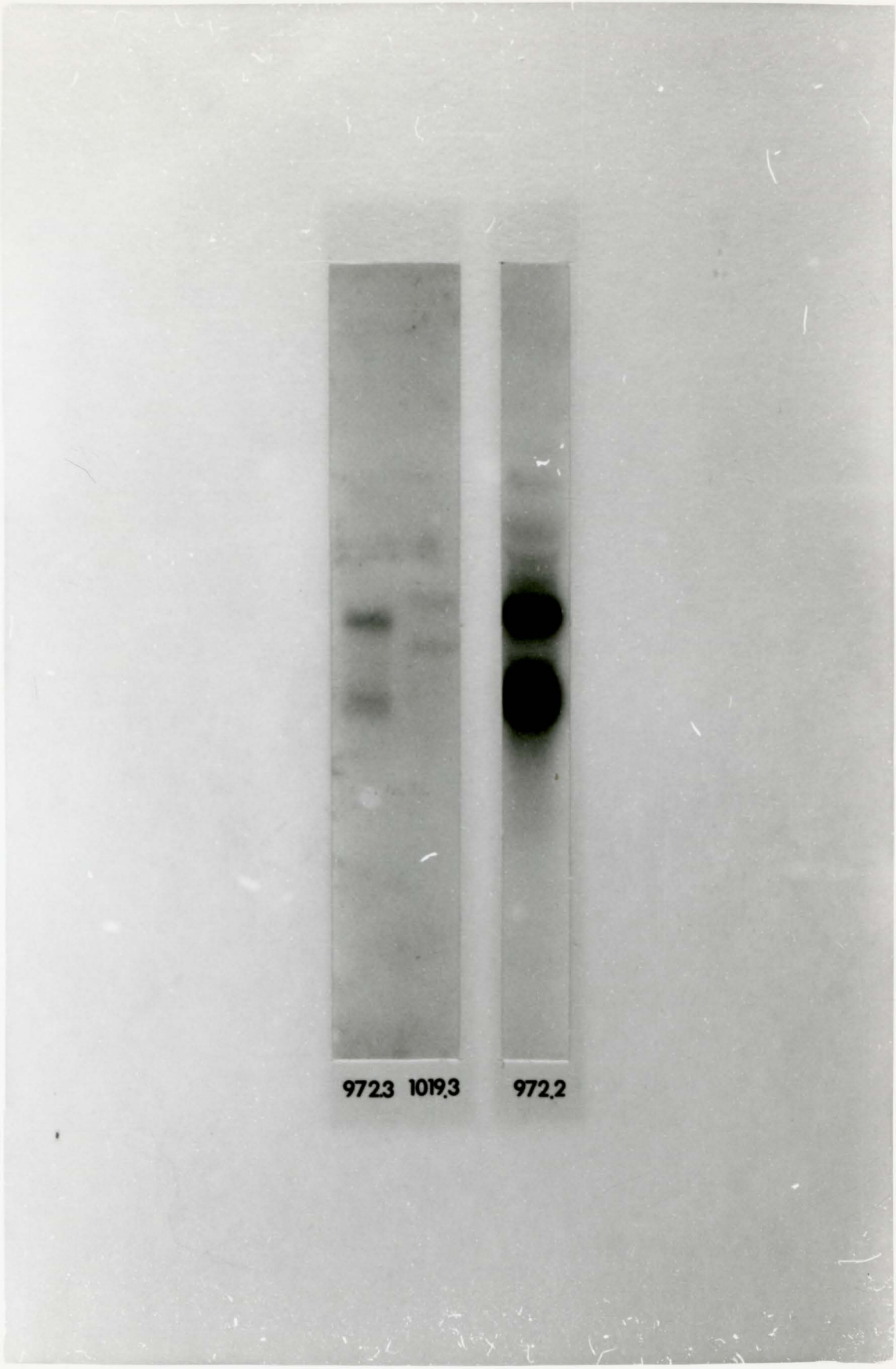
As stated earlier, the 972.2 RNA preparation used in the large blot hybridizations was not satisfactory, although a faint mRNA band was visible in the 2600 b region. Therefore an earlier Northern blot containing a previous preparation of 972.2 RNA was analyzed with Ad5 Xho IC DNA (Figure 12). Ribosomal RNA markers were not used for calibrating the mRNAs in this blot, so mRNA sizing was achieved by comparison between 972.2 and 972.3 mRNAs.

From our previous results with 972.3 RNA (section g), two mRNAs were sized at 2700 b and 1225 b (that is about 22s and 13s). Comparison of these mRNAs to 972.2 mRNA bands (Figure 12) indicates that the 22s cytoplasmic mRNA detected earlier from E1B and E1 region probes (Figures 7,8,9,11) are indeed present from these regions, and that a smaller RNA class also is present and probably contains the E1A 13s mRNA.

A number of bands visible above the 972.2 22s mRNA may be artifacts. The heaviness of the two major bands would indicate that these other bands are of minor importance, but larger than "normal"

FIGURE 12: Transformed cell Poly (A) mRNA hybridized to Xho IC

Poly-A selected mRNA from transformed cell lines were electrophoresed through an agarose-formaldehyde gel for 12 hours at 35 volts, then washed and blotted to nitrocellulose paper. Ribosomal markers were not used in this gel so that sizing must be inferred from the predominant mRNA size classes in the 972.3 cell line, which have been observed to migrate at 22 and 13s. The blot was hybridized to an Xho 1C probe and will illuminate all E1 mRNAs.


972.3
1019.3
972.2

cytoplasmic RNAs have been detected in previous hybridization blots. These could be nuclear RNAs which have been lost from the nucleus during extraction, non-specific binding to the nitrocellulose blot, or viral-cell mRNA chimeras.

i) Cell line 983.2

This cell line was transformed with the Ad5 Xho IC fragment, and unlike the previous two Xho IC transformed lines, is highly tumorigenic. It produces the major E1 antigens, 48-52k, 19k and 58k. The cell line contains integrated DNA sequences hybridizing to Hind III G (0-8%) and Hind III E (8-17.3%), and contains 2 insertions (see Table 1).

Five separate size classes of mRNA exist in this cell line. All of these mRNAs have been detected from other RNA preparations of the 983.2 cell line. There is a 1250 b mRNA (13s) from E1A, visible with Hpa IE (0-4.5%), Hind III G (0-8%), and Xho IC (0-16%). Of the other four mRNA bands the two middle bands are very similar in size and appear to be an overly broad band. Due to the breadth of the band two mRNAs were estimated to be migrating one above the other (2950 b and 2590 b). The 972.3 cell line also demonstrates a broad band which is more clearly separable into two mRNA bands.

Another mRNA of 1615 b hybridizes to Hind III G (0-8%), Hind III E (8-17.3%), and very faintly to Xho IC (0-16%). This may be an unusual form of the 13s mRNA of E1B, which utilizes the first donor splice site in that region, or as above, it may be an 1 strand mRNA, initiated by a cellular promoter in flanking sequences. In order to map the 1615 b mRNA further experiments must be undertaken using the

S1 nuclease technique with separated strands of Ad5 genome DNA. It would then be possible to determine if the l or r strand directs the synthesis of this mRNA and its viral sequences.

j) Cell Line 14b

Hamster embryo fibroblasts were transformed with Ad5 ts 14 virus (Williams, 1973), and the transformed cell line 14b was shown to contain sequences from the left hand 43% of the genome, with 5.5 copies inserted into the cellular DNA (Flint et al., 1976). Flint et al. demonstrated using mRNA:DNA saturation hybridization that transcription was occuring from Hpa I fragments E (0-4.5%) and C (4.5-25.5%).

Figure 5 and 6 are complex and so Table 3 should be used as a guide to the 14b cytoplasmic (14b) and Poly (A)-selected cytoplasmic (14b mRNA) mRNAs found in this figure. These hybridizations were not done from the same gel blot and the rRNA markers are in different areas of each blot.

The Hpa IE probed blot demonstrates a 1270 b mRNA from the 14b cell line with a comparable mRNA from 8 hr + CH (1170 b), and two smaller mRNAs from 18 hour infected cells (1060 and 680 b). Also visibile higher in the 14b lane are two mRNAs at 12,300 and 10,050 b in length. As mentioned earlier the very high molecular weight bands high in the lanes of 8 and 18 hour infected cells are probably artifacts.

The Pst-Hind fragment of pHG-1PH detects a 2225 b mRNA from 14b cells (total RNA), and possibly two mRNAs, 2900 b and 2110 b from 14b mRNA (poly (A) selected RNA). In the 8 hr + CH cells a 2195 b mRNA is

TABLE 3: Summary of 14b, 8 hour + CH and 18 hour infected cell mRNAs classes hybridizing to Ad5 DNA probes

Source of RNA		average mRNA size	0-4.5%	7-8%	8-17.3%	32.4-38.3%	Present in E1A[1]	E1B	other
KB	8 hr + CH a	2435		2195	2670				*
	b	1360			1360				*
	c	1170	1170					*	
KB	18 hr a	2225		2225					*
	b	1670		1670					*
	c	1140		1140					*
	d	1060	1060					*	
	e	680	680					*	
14b	a	15,500				15,500			*
	b	12,025	12,300			11,750	*		*
	c	9825	10,050			9600	*		*
	d	8100				8100			*
	e	6450				6450			*
	f	2450		2225	2670				*
	g	1270	1270					*	
14b mRNA @	f	3000*		2900	3000*				*
	f	2000*		2110	2000*				*

* indicates a large band encompassing this whole region
@ indicates that this preparation was poly-A selected for mRNA
1 determined by the presence of mRNAs in Hpa 1E (E1A), or Pst-Hind, Hind III G, Hind III E (E1B) probed blots
Other indicates presence of mRNAs in Hind III H probed blot

detectable. A major 2225 b mRNA is detected in 18 hr infected cells with minor bands at 1670 b and 1140 b. Therefore, the 14b cells appear to have at least one E1B 22s-like mRNA.

The autoradiograph of the Hind III E hybridized blot in Figure 6 was overexposed, and only a few bands can be distinguished. The 14b lane (total RNA) contains only a 2670 b mRNA, presumably the same mRNA as seen in the pHG-IPH probed blot. The 8 hr + CH lane contains two mRNAs at 2670 b and 1360 b as expected. The other lanes will not be discussed because of the difficulty in assigning bands although the poly (A) selected 14b mRNA does display a few large mRNAs in the same range as the Hpa IE hybridized blot.

The Hind III H probe was used to identify possible transcription from the right end of the integrated viral DNA. Since this cell line contains up to the left 43% of the Ad5 ts14 genome in multiple copies (Flint et al., 1976) we suspected that transcription could occur from a number of viral genes. It was remarkable to find a number of mRNAs, two of which were similar in size to mRNAs from the Hpa IE region. The two mRNAs will be considered identical since the deviation of 500 b from the overall size in the 10,000 b range is not significant. A possible variety of mRNA maps are depicted in Figure 17 to account for the large Hind III H mRNAs and common Hpa IE/Hind III H specific mRNAs. These arrangements include possible viral/cell chimeric mRNAs as well as unusual splicing patterns from E1A mRNAs.

DISCUSSION

Lack of E1B 13s mRNAs in transformed cell lines

One of the major findings of this study is that the major E1B mRNA in transformed cells is a 22s mRNA. No 13s mRNAs were observed and the only anomalous E1B mRNA of a non 22s pattern was a 1650 b mRNA made in 983.2 cells. Even the 14b cell line with its multiple gene products does not display any 13s mRNA from E1B. However, it is likely that the 1650 b mRNA in 983.2 cells is a 13s-like mRNA since it hybridizes to Hind III G and E fragments of DNA, but not to the Pst-Hind (pHG-1PH) sequences in the 7-8% region. It may be that this mRNA contains an extra splice site allowing for a longer mRNA in the same E1B region similar to the postulated 1250 b mRNA from Ad2 infected cells (Anderson et al., 1984).

Other researchers have also found that the major E1B transformed cell mRNA in Ad12 and Ad5 transformed cells is a 22s mRNA, due to the apparent lack of use of the first donor splice site (Sawada and Fuginaga, 1980; Shiroki et al., 1981; Saito et al., 1983; Van den Elsen et al., 1983). Other studies which do report multiple mRNAs from E1B are in 293 cells (Berk et al., 1979; Spector et al., 1980). Lewis and Mathews (1981) did not fractionate their E1B mRNAs but assumed that the 19k protein made by in vitro translation of viral fragment selected mRNA was encoded by a 13s mRNA. Other studies such as Jochemsen et al. (1981) and Esche et al., (1982) do not describe the mRNA species that were translated in vitro but report on the encoded products. Therefore all of the definitive studies have

demonstrated only a predominance of the E1B 22s mRNA in transformed cell lines, indicating perhaps a difference in the expression of the same adenoviral sequences in lytic and transformed cells.

Apparent lack of E1A 12s mRNAs in transformed cell lines

It is notable that this study did not detect any 12s mRNAs above the 13s mRNA background. Although the Northern blot hybridization method can detect the difference between a 13 and 12s mRNA in different lanes, it may not detect these bands as separate in the same lane. Migration of these bands is fairly distinct in Figure 5 in either 8 hour + CH or 18 hour infected KB cell mRNA lanes (8 hr + CH has 13s, 18 hr has 12 and 9s), but these lanes are separate. Therefore it would appear that the electrophoretic conditions used in this study cannot resolve the 13 and 12s mRNAs from the same sample. Another study using this procedure has failed to resolve these two mRNAs as well (S.T. Bayley personal communication).

Transcription patterns of Ad5 transformed cell lines

a) A cell line transformed with the Ad5 Hind III G fragment

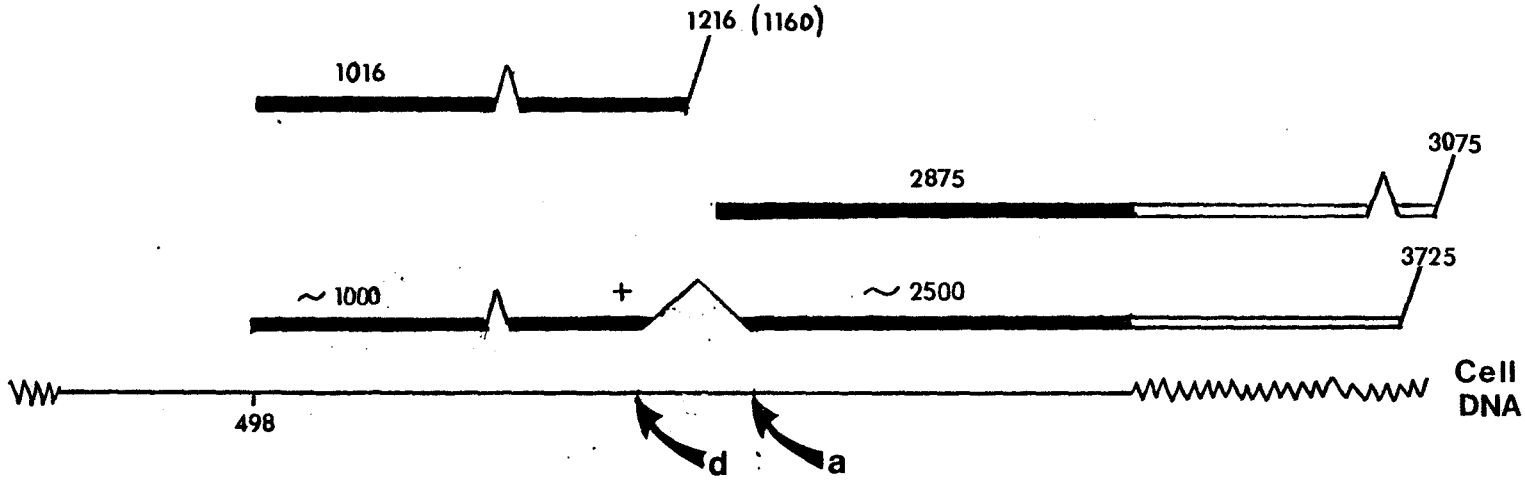
The 954.1 cell line was the only line analyzed which was transformed with the Hind III G fragment alone. All the other lines which were used in this study contained integrated viral sequences which hybridized to both Hind III G and E fragments. Due to the size of the fragment and the structure of the E1 genes (see Figure 2) the E1A and only the 5' part of the E1B gene would be present in the

FIGURE 13: Possible transcription map of 954.1 Ad5 mRNAs example of a cotranscript with E1A/E1B sequences

The E1A and E1B specific mRNAs of this cell line are depicted showing the size of the various viral sequences and the additional poly-A tail (200 b). The map of the mRNAs was adjusted to account for the size of mRNAs found in Northern blot hybridizations, and the size class of mRNA is indicated at the tail of the mRNA. If the mRNA was assumed to be "normal" in transcription pattern, two figures for mRNA size are indicated: the sequence determined mRNA size (see Figure 2) is indicated at the tail of the mRNA as well as our Northern blot determined size (in parentheses). The sequences normally expected in these mRNAs are dark; the unexpected sequences, either viral or cellular are light.

"d" and "a" indicate the possible donor and acceptor splice sites for an E1A/E1B cotranscript (Hashimoto et al., 1984)

954.1



transformed cell line. It is not suprising therefore that there are two distinct E1B "run off" mRNAs which must utilize neighboring cellular sequences for mRNA termination and poly-adenylation since these regulatory sequences are missing from the fragment (see Figure 13). This type of virus/cell chimera was demonstrated by Houweling et al. (1980) with Ad5 Hpa IE transformed rat cells. The E1A signals for poly-adenylation and mRNA cleavage occured beyond the fragment used for transformation (Matt and Van Ormondt, 1979) and so the E1A mRNAs from these transformed cells used cellular processing signals and produced overly large E1A mRNAs (16 to 22s). There have also been reports of this type of viral/cell chimeric mRNA from other researchers (Sawada and Fuginaga, 1980; Saito et al., 1983; Van den Elsen et al., 1983)

An interesting observation was the presence of an mRNA in 954.1 cells which appears to contain both E1A and E1B sequences. The mRNA may be initiated in E1A, run through E1B sequences, and terminate in cellular sequences beyond the fragment end (see Figure 13). There is no definite evidence that this observation is not the result of the coincidental expression of E1A and E1B specific mRNAs of the same size. However, other researchers have identified mRNAs of this sort from both transformed and infected cells. Cotranscription of this type is evident in a number of transformed cell lines where the E1A mRNA termination signals are not used and the transcript continues into E1B sequences and is normally processed there. Sawada and Fuginaga (1980) showed using S1 nuclease mapping of mRNAs from Ad12 transformed 3Y1 cells, that an E1A/E1B cotranscript was present

utilizing two splice sites in E1A and the 22s splice site in E1B. Saito et al.(1983) also observed this phenomenon with at least three Ad 12 transformed cell lines, with cycloheximide treated lytically infected cells, and very faintly in late infected cells. E1A/E1B cotranscripts have been observed in Ad 2 transformed (Chinnadurai et al., 1976) and Ad 2 infected cells (Buttner et al., 1976; Kitchingman et al., 1977; Berk and Sharp, 1978). Recently Hashimoto et al. (1984) demonstrated that these mRNAs can be translated into both E1B 19k protein and the E1A cluster of antigens. No reports of cotranscripts between viral genes have been made in Ad5 or 7 transformed cell lines, except where an E1A deletion mutant dl 313 (3' end of E1A deleted) was used to transform cells, and this type of cotranscript was expected (Esche et al., 1980; Shiroki et al, 1981).

Rowe et al. (1984) have shown that the proteins detected from the 954.1 cell line include the E1A proteins and the E1B 19k protein. Since the information present in the transforming fragment did not include sequences required to encode the E1B 58k it is not surprising that this protein was not detected. However, a viral/cell chimeric protein encoded by the "run off" mRNA may exist since anti-tumor sera derived from these cells do display specificity to the 58k protein.

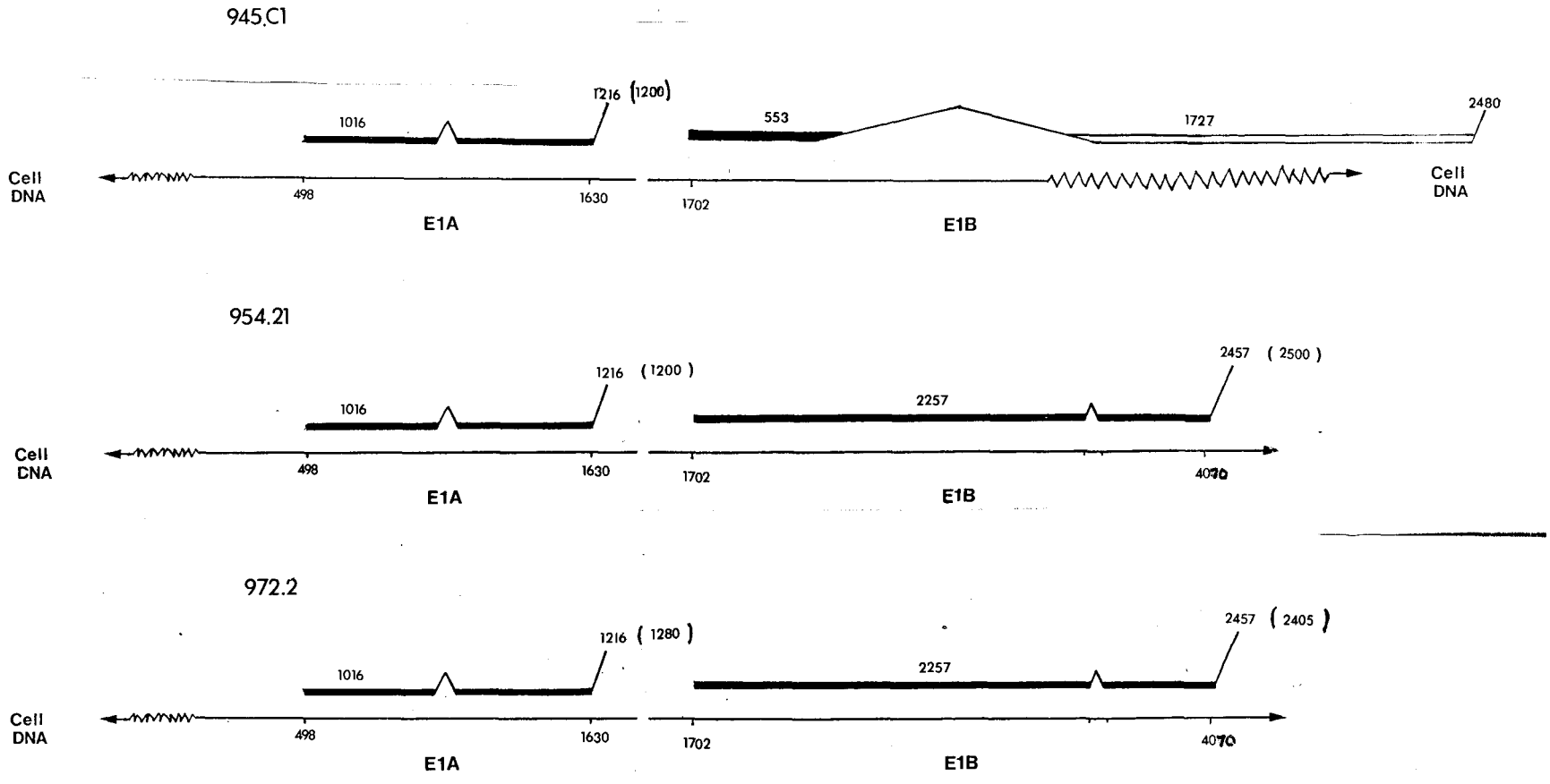
b) Cell lines transformed with Ad5 Hind III digested DNA

Two cell lines transformed with this type of DNA were analyzed: 945.C1 and 954.21. These lines share a common origin yet show different abilities to induce tumors in newborn hamsters. The 954.21 cell line demonstrated the most simple mRNA map pattern (Figure 14).

FIGURE 14: Possible transcription map of the Ad5 mRNAs of 945.C1, 954.21, and 972.2 cell lines

cell lines exhibiting "normal" sizes of viral mRNAs

The E1A and E1B specific mRNAs of these cell lines are depicted showing the size of the viral gene, the various mRNA sequences and the additional poly-A tail (200 b). The map of the mRNAs was adjusted to account for the size of mRNAs found in Northern blot hybridizations, and the size class of mRNA is indicated at the tail of the mRNA. If the mRNA was assumed to be "normal" in transcription pattern, two figures for mRNA size are indicated: the sequence determined mRNA size (Figure 2) is indicated at the tail of the mRNA as well as our Northern blot determined size (in parentheses). The sequences normally expected in these mRNAs are dark; the unexpected sequences, either viral or cellular are light.


945.C1
1216 (1200)
1016
553
1727
2480
Cell DNA
498
1630
1702
Cell DNA
E1A
E1B
954.21
1216 (1200)
1016
2457 (2500)
2257
Cell DNA
498
1630
1702
E1A
E1B
972.2
1216 (1280)
1016
2457 (2405)
2257
Cell DNA
498
1630
1702
E1A
E1B

The expected E1A 13s and E1B 22s mRNAs were present with the appropriate hybridization probes. The 945.C1 map also contained these mRNAs but the E1B 22s mRNA was absent from both the Hind III E and Pst-Hind specific probed blots. This absence suggested an mRNA using the first donor splice site in the E1B region and an acceptor splice site in cellular sequences resulting in a viral/cell chimeric mRNA. It would appear that the middle and 3' E1B interior sequences must be processed out of the 22s mRNA (Figure 14). Another possibility is that the integrated Hind III G and E sequences are not integrated in the proper viral orientation and the E1B sequences available do not include the Pst-Hind region, so that the E1B mRNA is a viral/cell chimera due to run through transcription. Evidence that this cell line does not contain a normal E1B mRNA is its failure to produce 58k antigen or anti-tumor sera with specificity against this antigen.

c) Cell lines transformed with the Ad5 Xho IC fragment

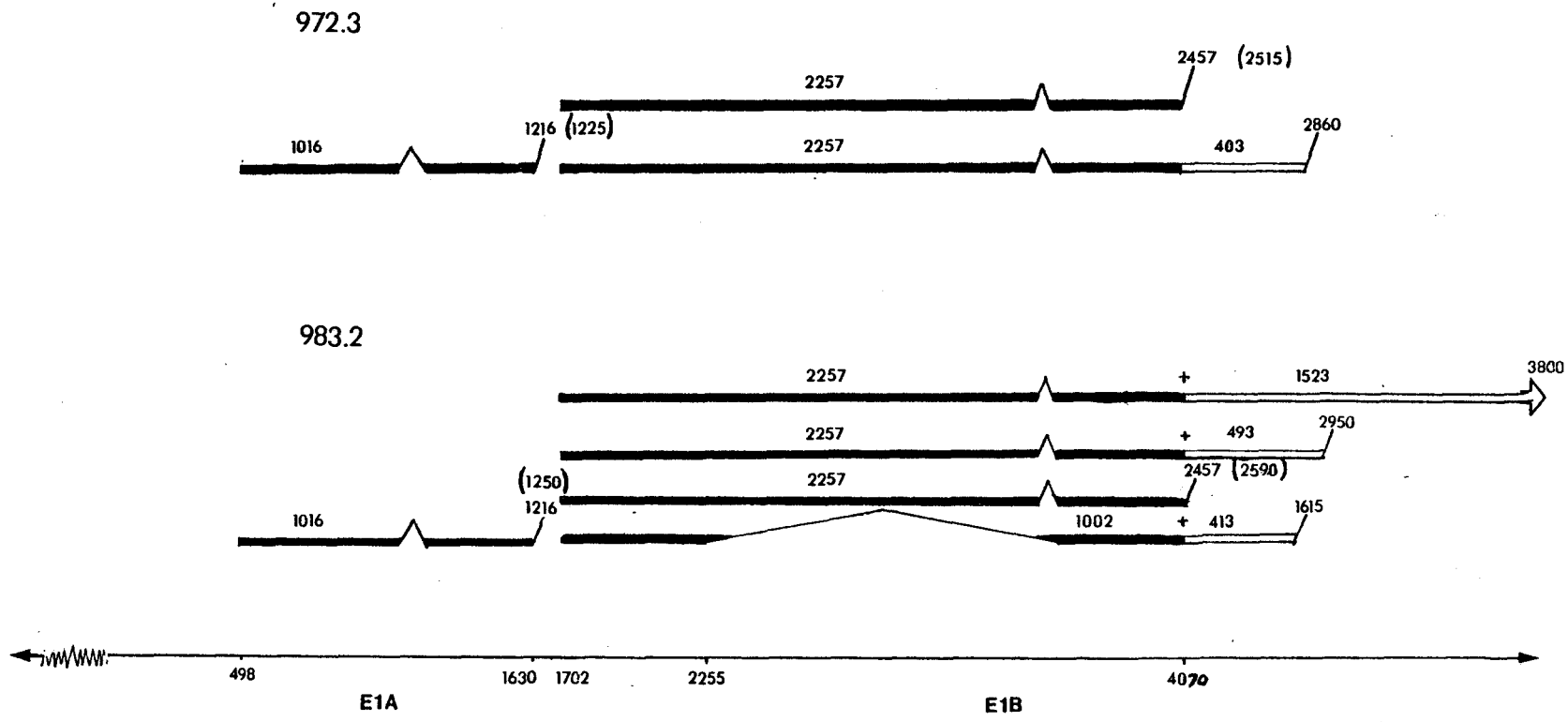
Three cell lines were transformed with this fragment: 972.3, 972.2, and 983.2. All of these lines produce the typical E1 antigens and yet they differ in their potential to induce tumors in newborn hamsters (Rowe et al., 1984).

The 972.2 cell line demonstrated the normal E1A 13s and E1B 22s mRNAs (Figure 14). The other two lines each demonstrated normal E1A mRNAs but multiple E1B mRNAs. Most of these E1B mRNAs ranged from the normal 22s to much longer E1B mRNAs. Figure 15 demonstrates possible transcription patterns which could result in mRNAs of this size. All of the unusual mRNAs may either fail to terminate and poly-adenylate

FIGURE 15: Possible transcription map of the Ad5 mRNAs of 972.3 and 983.2 cell lines

cell lines exhibiting normal E1A, abnormal E1B mRNAs

The E1A and E1B specific mRNAs of these cell lines are depicted showing the size of the viral gene, the various mRNA sequences and the additional poly-A tail (200 b). The map of the mRNAs was adjusted to account for the size of mRNAs found in Northern blot hybridizations, and the nucleotide size of mRNA is indicated at the tail of the mRNA. If the mRNA was assumed to be "normal" in transcription pattern, two figures for mRNA size are indicated: the sequence determined mRNA size (Figure 2) is indicated at the tail of the mRNA, as well as our Northern blot determined size (in parentheses). The sequences normally expected in these mRNAs are dark; the unexpected sequences, either viral or cellular are light.


972.3
2457
(2515)
2257
1216
(1225)
2860
1016
2257
403
983.2
2257
+
1523
3800
2257
+
493
2950
(1250)
2257
2457
(2590)
1216
1016
1002
+
413
1615
498
1630
1702
2255
4070
E1A
E1B

at the usual signal and terminate downstream (as depicted) or alternative splicing occurs thereby bypassing the viral signal to the same end. It is also possible that the E1B termination sequences may not be present since the extent of viral integrated sequences is not known.

An interesting mRNA of about 1600 b size was detected in 983.2 cells which may be evidence of the X mRNA from E1B region (Figure 2) or possibly an 1 strand mRNA encoding the open reading frames URF 10 and URF 11 (Gingeras et al., 1982).

d) Cell lines transformed with an Xho I digest of Group II mutant DNA

The Group II host range mutants as described earlier have mutations in their E1B gene so that 58k is not synthesized in lytic infection. It is not suprising therefore that the cell lines transformed by host range mutants of this group 1019.1, 1019.2 (hr6) and 1019.3 (hr50) also fail to produce detectable 58k protein. In fact 1019.3 cells fail to produce detectable 19k protein as well. The cell lines were chosen for their different abilities to induce tumors in newborn hamsters (Table 1) as well as their lack of E1B 58k.

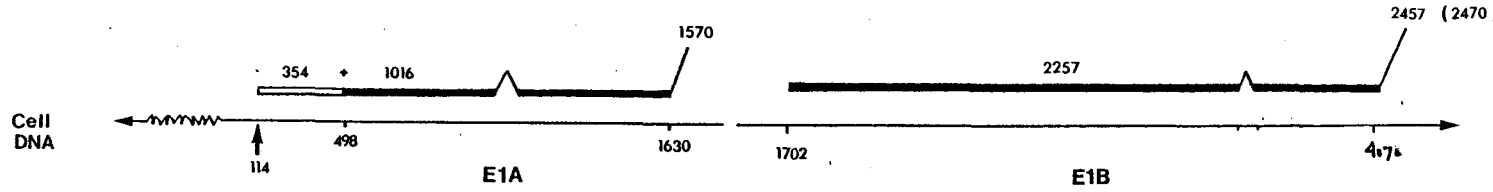
Cell line 1019.3 failed to demonstrate any E1B specific mRNA although some results suggested that minor mRNAs may have been present in preparations but at almost undetectable levels for this method. The other cell lines demonstrated the normal E1B 22s mRNA, but did contain unusual E1A mRNAs (see Figure 16). The 1019.1 mRNA is slightly larger than expected at 1570 b, and could have been the result of preinitiation of the mRNA in viral sequences just upstream

FIGURE 16: Possible transcription map of the Ad5 mRNAs from 1019.1, 1019.2, and 1019.3 cell lines

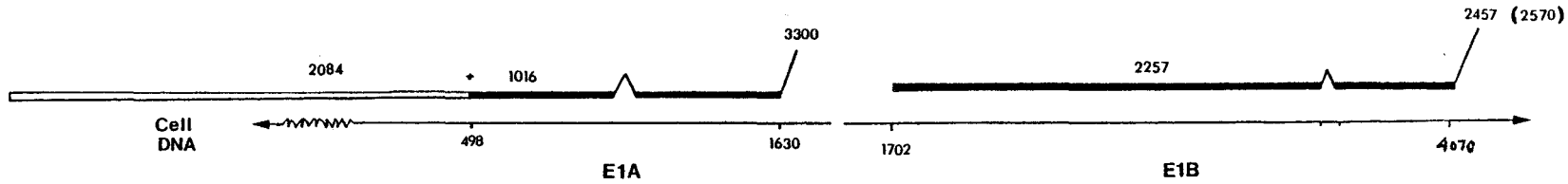
cell lines with abnormal E1A mRNAs

The E1A and E1B specific mRNAs of these cell lines are depicted showing the size of the viral gene, the various mRNA sequences and the additional poly-A tail (200 b). The map of the mRNAs was adjusted to account for the size of mRNAs found in Northern blot hybridizations, and the nucleotide size of mRNA is indicated at the tail of the mRNA. If the mRNA was assumed to be "normal" in transcription pattern two figures for mRNA size are indicated: the sequence determined mRNA size (Figure 2) is indicated at the tail of the mRNA, as well as our Northern blot determined size (in parentheses). The sequences normally expected in these mRNAs are dark; the unexpected sequences, either viral or cellular are light.

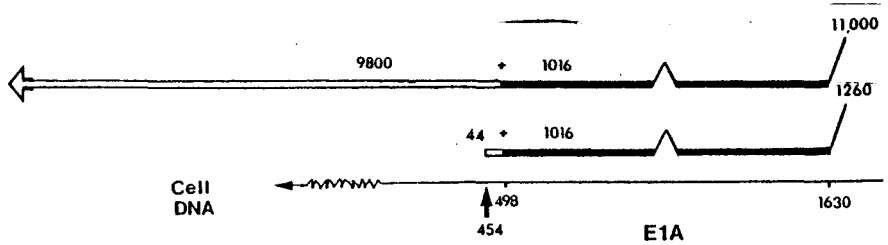
1019.1



1019.2



1019.3



of the usual promoter. Preinitiation of E1A mRNAs in Ad5 and 12 transformed cells has been shown using the S1 nuclease technique (Berk and Sharp, 1978). In both cases fragments of mRNA from the splice site to the 3' end were the same as in lytically infected cells, but the pre-splice fragment was of variable lengths (Sawada and Fuginaga, 1980; Saito et al., 1983; Van den Elsen et al., 1983). This phenomenon has been shown to occur in lytically infected cells as well although these mRNAs tended to be a minor mRNA component (Saito et al., 1981; Saito et al., 1983), so it is not an exclusive property of transformed cells.

Cell line 1019.2 has a very large E1A mRNA which might be the result of a promoter in cellular sequences well upstream of the viral genes. Virus/cell chimeras have been mentioned before but no researchers have detected Adenoviral sequences being promoted in this fashion. It is unlikely that a normal E1A initiation and splicing pattern could run on into cellular sequences since the cell line was transformed with an Xho I digest and should contain viral sequences downstream of the E1A region. The E1A and E1B sequences are probably side by side in this cell line necessitating a run-through mRNA to contain sequences from both these regions. There is no evidence that this is the case.

This cell line contains sequences from the right end of the virus (Downey et al., 1983). If a tandem insertion occured with the right and left sequences side by side, there could be an initiation in the E4 region DNA with a run through into the E1A sequences causing the overly large E1A 3300 b mRNA. This type of viral gene chimera has

been documented by Yoshida and Fuginaga (1980). In that study, a number of Ad 7 transformed rat cells were obtained using the Hind III I*J fragment (0 to 7.3%) and several transformed cell mRNAs with both E1A and E1B sequences were observed. All the cell lines examined contained mRNAs whose 5' ends are E1B sequences and whose 3' ends are E1A sequences. These mRNAs were transcribed by extension of E1B mRNAs into E1A sequences of viral DNA fragments joined in a tandem array in these transformed cell lines. Similar results were obtained in Ad 12 Hind III G transformed cells (Sawada and Fuginaga, 1980). As described earlier with viral/cell chimeric mRNAs, the regulatory signals in the viral gene which would normally terminate and polyadenylate the mRNA are missing from the transforming fragment and so this type of viral/viral chimeric mRNA occurs.

e) A cell line transformed by Ad5 ts 14 virus

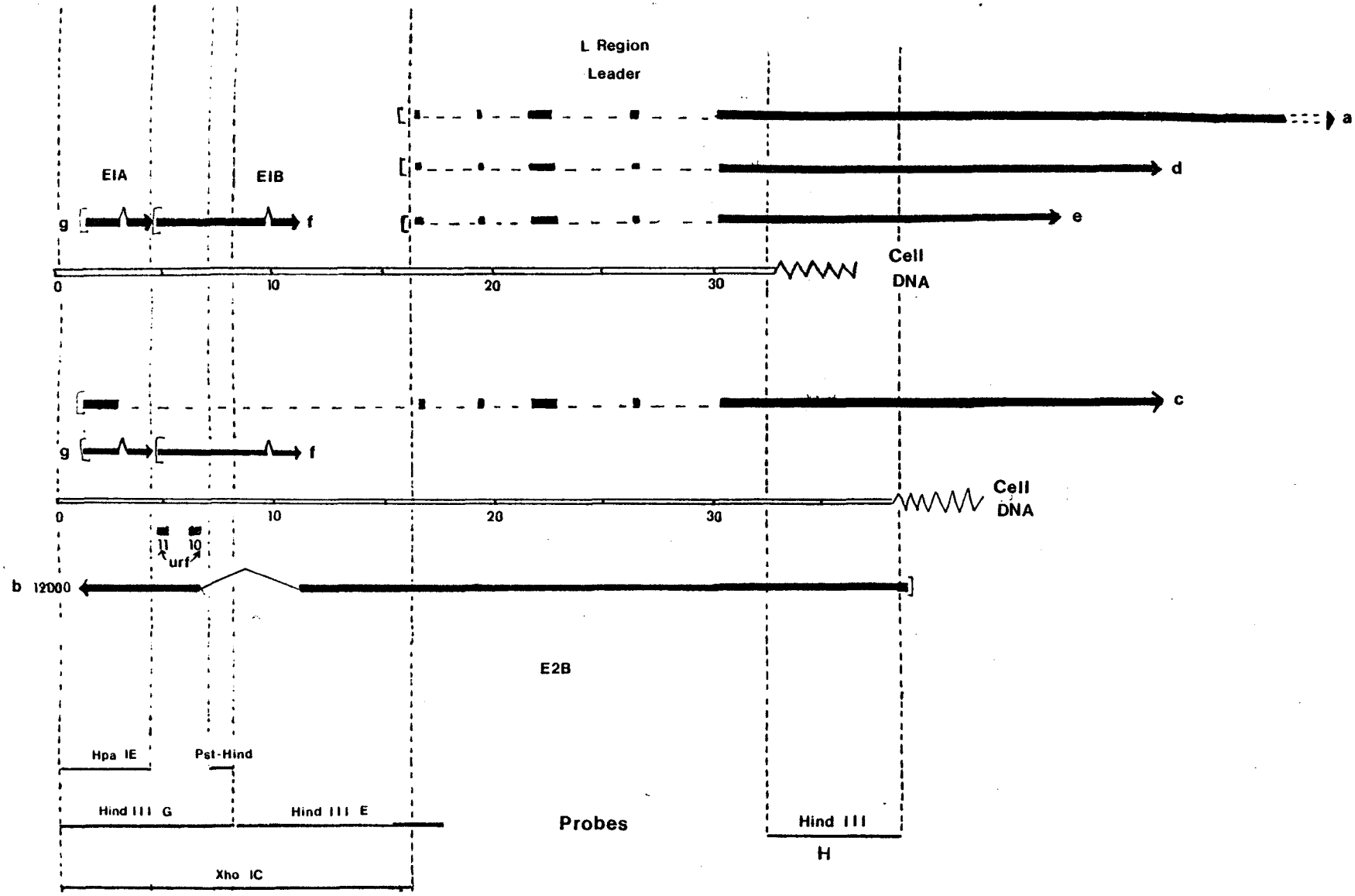
This line was included in this study because the line is highly oncogenic in newborn hamsters and contains a large insertion of Ad5 ts14 viral DNA (Table 1).

Aside from the "normal" E1A 13s and E1B 22s mRNAs a few unusually large mRNAs make this cell line interesting. About 5 large mRNAs contain Hind III H sequences and of these, two also hybridize to Hpa IE specific DNA. The mRNA maps in Figure 17 show possible arrangements of viral fragments to obtain mRNAs of this pattern, but with the available information these maps are very suggestive. As described earlier Yoshida and Fuginaga (1980) isolated mRNAs from

FIGURE 17: Possible transcription patterns for 14b cell line mRNA

The possible mRNA map for 14b is shown demonstrating the integrated Ad5 ts14 DNA regions and the mRNAs which DNA probes would hybridize. The promoters are depicted with square brackets; mRNAs correspond to the mRNAs listed in Table 3. Two integrated genomes are shown.

Viral/cell chimeric mRNAs may be responsible for the very large mRNAs hybridizing the Hind III H region, but unusual splicing must account for mRNAs with sequences from both the Hpa IE and Hind III H region.


L Region
Leader
a
d
e
EIA
EIB
g
f
Cell
DNA
0
10
20
30
c
g
f
Cell
DNA
0
10
20
30
11
10
urf
b 12000
E2B
Hpa IE
Pst-Hind
Hind III G
Hind III E
Xho IC
Probes
Hind III
H

tandem head to tail integrations creating mRNAs whose 5' ends are E1B sequences and 3' ends are E1A sequences. Other possibilities for the large Hind III H specific mRNAs include the viral/cell chimera promoted from the EL1 promoter which has not yet been shown to promote mRNAs in transformed cells.

Transformed cell line mRNAs and their protein products

Cell lines producing E1B 58k and 19k proteins all demonstrate a 22s like mRNA from E1B, which hybridizes to the Pst-Hind fragment (pHG-IPH). Cell line 945.C1 which does not have any detectable 58k does not appear to contain a 22s mRNA with the proper splicing pattern. Cell lines 1019.1 and 1019.2 do not produce 58k yet do contain E1B 22s-like mRNAs. The fact that the viral host range mutant used to transform these cells failed to produce 58k in a lytic infection explains the failure of transformed cells to produce this protein. The Group II transformed cell line 1019.3 which does not produce 58k or 19k proteins fails to demonstrate any E1B specific mRNA. This does not exclude the possibility that the mRNA is present in small quantities. Of course this raises interesting questions as to the requirement of E1B sequences in the transformed cell. Did the cell line have this information and lose it after induction of the transformed state, or does it contain a minimal undetectable level of E1B mRNAs and proteins?

Transformed cell lines and oncogenicity

The transformed cell lines selected for this study had a variety of oncogenic potentials, and were transformed with various regions of the adenovirus genome. Just as no correlation was found in these transformed cells for the presence of an adenoviral protein, and that cell line's oncogenic potential (see Tables 1,4), so has this study failed to relate oncogenicity and the adenoviral mRNAs from transformed cells. No clear trend can be detected with the presence or absence of any mRNA size class. All cell lines carried an E1A mRNA product, although the 972.2 E1A mRNA had to be inferred by the presence of an E1 13s mRNA, and the known ability of this line to make E1A proteins. The presence of an E1B mRNA may appear to be important since 14b, 983.2, and 954.1 all are highly oncogenic and also produce large quantities of the E1B mRNAs, but 945.C1 and 1019.3 which don't make large amounts of E1B mRNAs are also highly oncogenic.

Difficulties in sizing mRNAs from transformed cells

The mRNA values obtained in our calibrated Northern blot studies deviated somewhat from the reports in the literature for Adenoviral mRNAs. In Ad5 infected Kb cells typical sizes for Poly (A+) mRNAs are: E1A 13s = 1150 b, E1B 22s = 2450 b (Van den Elsen et al., 1983). These sizes are about 100 b smaller than those detected in our transformed cells: E1A 13s = 1250 b, E1B 22s = 2550 b (average of all results). Perhaps the apparently increased sizes are due to overloading of RNA samples. Adenovirus infected cells produce a greater amount of viral mRNAs compared to transformed cells;

TABLE 4: Summary of the transformed cell line products[1]

Transformed Cell line	# of E1 inserts	mRNA size class	Present in E1A	Present in E1B	follows[2] pattern	Tumorigenicity
1019.1	NT	2470		*	22 s	+
		1570	*			
1019.2	NT	3300	*			+
		2570		*	22 s	
1019.3	NT	11,000	*			+++
		7,000				
		1260	*			
945.C1	1	2480		*		+++
		1200	*			
954.1	1	17,000				+++
		3725		*	22 s	
		3050		*	22 s	
		3035	*			
		1160	*			
954.21	4	2500		*	22 s	-
		1200	*			
972.3	4	2860		*	22 s	-
		2515		*	22 s	
		1225	*			
972.2	2	2405		*	22 s	Not
		1280	*			tested
983.2	2	3800		*	22 s	++++
		3000		*	22 s	
		2700		*	22 s	
		1615		*	13 s	
		1250	*			
14b	5.5	15,500				++++
		12,025	*			
		9825	*			
		8100				
		6450				
		2450		*		
		1270	*			

1 Data on Ad5 DNA inserts and tumorigenicity from Rowe et al. (1983).
2 The E1B mRNA was determined to follow a transcription pattern similar to either the E1B 22s or 13s mRNAs (see figure 2).
NT Not tested

therefore, to achieve the same response of hybridizable viral mRNA in a Northern blot, more cytoplasmic RNA preparation from transformed cells must be used compared to infected cells. This difference in the amount of cytoplasmic RNA loaded in various lanes may cause a small effect in the migration of RNA products. Increased loading of cytoplasmic RNA creates a back-up effect in the migration of the rRNA present (data not shown).

A new E1 region mRNA from late infected cells?

The late infected KB cell mRNA preparation shown in Figure 5 demonstrated an unusual 1670 b mRNA hybridizing to the Pst-Hind fragment (pHG-1PH) (see Table 3). This might be an mRNA containing the 1 strand open reading frames URF 10 and 11 (Gingeras et al., 1982). It might also be an alternative E1B splicing mRNA previously undetected. It cannot be either the 13s or the postulated 1450 b E1B mRNA (Anderson et al., 1984) since it should have a splice in this region of the genome.

SUMMARY

The technique of Northern blot hybridization is convenient for determining the presence of adenoviral specific mRNA, provided adequate DNA probes are available for selecting different viral regions of hybridization. This study has shown that a small viral fragment of 300 bp is sufficient to detect specific complementary mRNAs.

The predominant transcription patterns observed in these transformed cell lines are not similar to those seen in lytically infected cells. The 22s mRNA appears to be the predominant E1B product, and is known to be capable of encoding both E1B 58 and 19 kd proteins (Bos et al., 1981). A preponderance of larger than normal viral mRNAs may indicate that preinitiation in viral sequences may be favoured in some transformed cells. Certainly the incidence of large E1B mRNAs (2950 and 3800 b) especially in 983.2 cells may indicate failure to terminate at viral signals and subsequent running on into cellular sequences. The cell line 954.1 with its viral sequence termination at the Hind III G site shows unusual mRNA sizes that definitely indicate viral/cell chimeric mRNA. A number of 7 kb and larger mRNAs may indicate either run through mRNAs or contamination of nuclear unprocessed RNAs.

Studies of transformed cells which examine gene expression at the protein level can overlook the complexity of mRNA expression. Chimeric mRNAs, either of the viral/cell or viral/viral type may still be in vitro translated to yield normal sized viral antigens. Indeed

as stated earlier Hashimoto et al. (1984) demonstrated that an E1A/E1B cotranscript can yield both the E1B 19 kd and the E1A cluster of antigens.

In conclusion, although this study failed to identify any relationship between oncogenicity and the mRNAs of Ad5 transformed cells, it is notable that these transformed cells do not express their integrated viral DNA in the same way that lytically infected cells do. Transformed cells may prove to be a good model for eukaryotic gene expression and regulation.

APPENDIX

Construction of a plasmid with a portion of the E1B coding sequence

We wanted to construct a probe for Northern blot hybridizations that would distinguish between the 22s and 13s mRNAs from region E1B. To do this our plan was to clone into pBR322 a fragment of viral DNA which complemented the 22s mRNA, but was in the intron splice region of the 13s mRNA (see Figure 18).

A 300 b fragment of viral sequences from the E1B region of the Ad5 genome which is bordered by a Pst 1 and a Hind III restriction endonuclease site was selected. Another Pst 1 site occurs about 1000 b upstream of the Hind III site, among sequences complementary to both mRNA transcripts. By analyzing the size of the fragment inserted into the final recombinant clone, it is possible to distinguish between these two fragments (see Figure 18).

One way of obtaining this fragment conveniently was to restriction endonuclease digest a recombinant plasmid, pHG-1 (donated by Dr. Graham) which contains the left 8% of Ad5 (Figure 18). DNA digestion at the Pst 1 and Eco R1 sites can generate fragments of different sizes (Figure 19A) if the digestion were not complete. One of the these fragments would be the selected E1B viral sequences adjoining a short 29 b sequence of pBR322. This fragment would easily be distinguishable from other pHG-1 fragments on the basis of its size alone.

To produce the desired recombinant plasmid, pBR322 and pHG-1 plasmid DNA were digested together with Pst 1 and Eco R1. The DNA fragments were ligated, and were then used to transfect the HB101 strain of E. coli. Figure 18 depicts the construction of the required plasmid. Briefly, the insertion of the 300 b fragment of pHG-1 into the pBR322 plasmid at the Pst 1 (-327 b) and the Eco R1 sites (-2 b) produces the required plasmid. The new plasmid recombinant confers resistance to tetracycline, but not to ampicillin ($tet^{R}amp^{S}$). E. coli containing recombinant plasmids of the $tet^{R}amp^{S}$ type can be distinguished from other recombinant plasmids, and the parental strains pBR322 ($tet^{R}\ amp^{R}$) and pHG-1 ($tet^{S}\ amp^{R}$), by drug resistance selection.

Obtaining plasmid recombinants of the tet amp phenotype we proceeded to screen for the size of inserted fragments as well as presence of an inverted repeat of pBR322 sequences about the Eco R1 site (see Figure 19B). By digesting pHG-1 and the recombinant plasmid with Eco R1 and Pst 1, we compared the insertion fragment size of the recombinant with the fragment sizes of the pHG-1 digest (see Figure 20, lanes C, E). As expected, the clone (lane E) and pHG-1 (lane C) contain a common Pst 1 and Eco R1 fragment of 327 b. To test for the inverted repeat of pBR322 about the Eco R1 site, the clone was digested with firstly Eco R1 and then either Pst 1 or Sal 1 (see Figure 19B for a map of the pHG-1PH). A further digest of this material with Hind III enzyme created a 29 b loss in the size of each of the smallest fragments, confirming the expected structure.

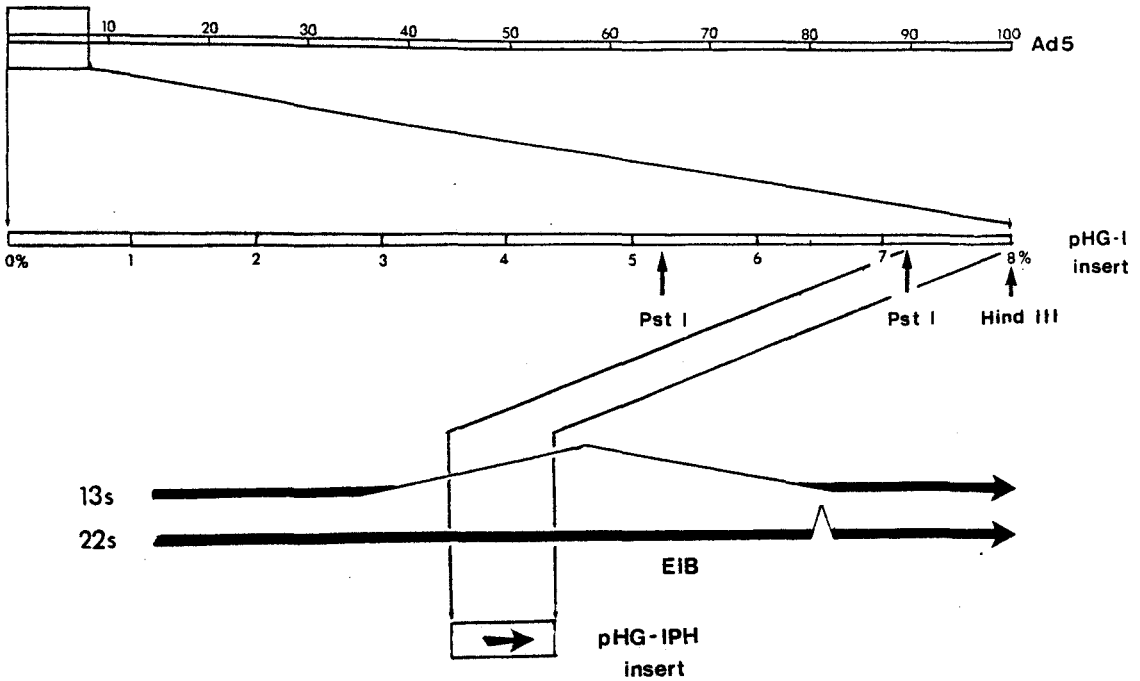
Figure 18: Construction of a Ad5 fragment recombinant plasmid specific for the E1B 22s mRNA

A Pst 1/Hind III restriction map of the Ad5 E1B region demonstrates the location of the Pst 1 - Hind III fragment with respect to the transcription map of the E1B region, and the 0-8% Ad5 insert in the pHG-1 plasmid.

A small fragment containing these Ad5 sequences and adjoining pBR322 sequences was released from pHG-1 by digestion of this DNA with Eco R1 and Pst 1 enzymes. In this same digestion mixture pBR322 DNA was also cut into 2 fragments, one of which contained DNA sequences with a gene for tetracycline resistance. The DNA fragments were allowed to ligate, E.coli were transfected and using drug resistance selection, E. coli with an amp tet phenotype were selected and amplified. Analysis of the size of the inserts from various isolates resulted in the final selection of E.coli carrying the plasmid pHG-1PH. Confirmation of the restriction map of this plasmid was done with the experiment shown in Figure 20.

are the 29 b of pBR322 sequences which are eventually present as an inverted repeat about the Eco R1 site.

the pHG-1 fragment which is inserted into the parental pBR322 plasmid to create the recombinant clone


10
20
30
40
50
60
70
80
90
100
Ad5
0%
1
2
3
4
5
6
7
8%
pHG-I
insert
Pst I
Pst I
Hind III
13s
22s
EIB
pHG-IPH
insert

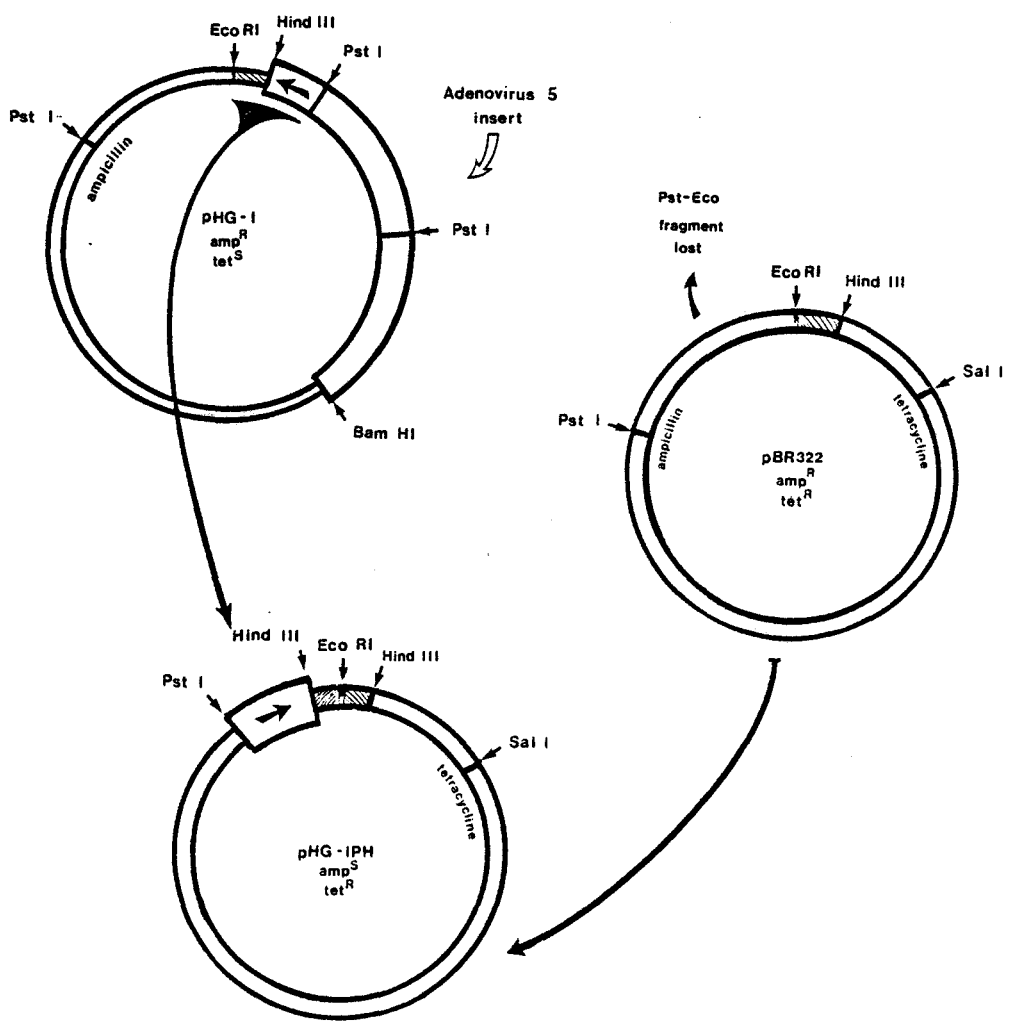

Eco RI
Hind III
Pst I
Adenovirus 5
insert
Pst I
ampicillin
pHG-I
amp^R
tet^S
Pst I
Bam HI
Pst-Eco
fragment
lost
Eco RI
Hind III
Sal I
Pst I
ampicillin
pBR322
amp^R
tet^R
tetracycline
Hind III
Eco RI
Hind III
Pst I
Sal I
tetracycline
pHG-IPH
amp^S
tet^R

FIGURE 19: Restriction endonuclease maps of pHG-1 and pHG-1PH

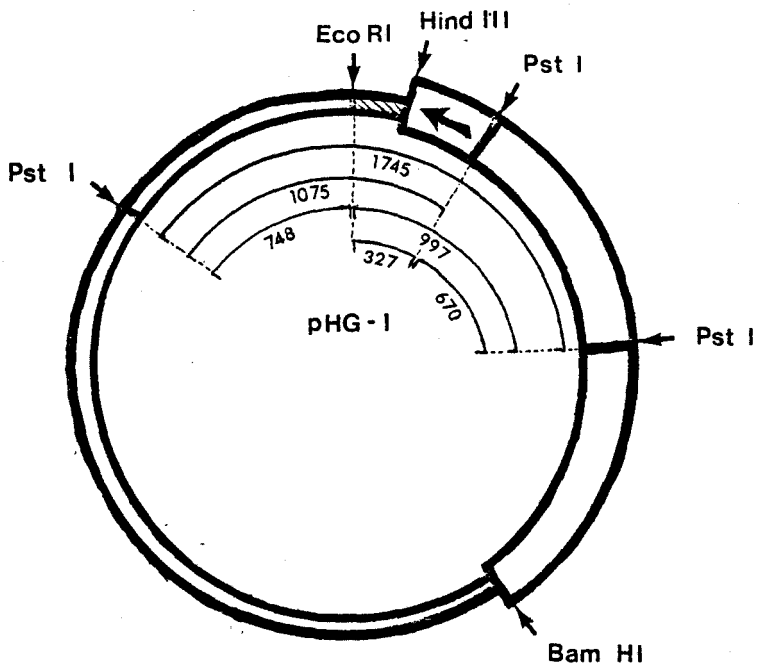
A. pHG-1

Digestion of plasmid pHG-1 with restriction endonucleases Pst 1 and Eco R1 can create fragments of various sizes, if digestion is incomplete. Any of the pairs of fragments listed could have been inserted into the recombinant clone. The smallest fragment with only 327 b contained the necessary sequences to make our recombinant clone, and is readily distinguishable by its small size from any other fragment.

B. pHG-1PH

This map depicts the fragments which would be detectable in a restriction endonuclease digestion of pHG-1PH DNA with Eco R1, Eco R1 and Pst 1, Eco R1 and Sal 1, Hind III and Pst 1, Hind III and Sal 1. By digesting first with Eco R1, and then with Hind III while codigesting with either Pst 1 or Sal 1, a 29 b loss in the size of the 327 b, and 650 b fragments can be discerned.

A


Eco RI
Hind III
Pst I
Pst I
1745
1075
748
997
327
670
pHG - I
Pst I
Bam HI

B

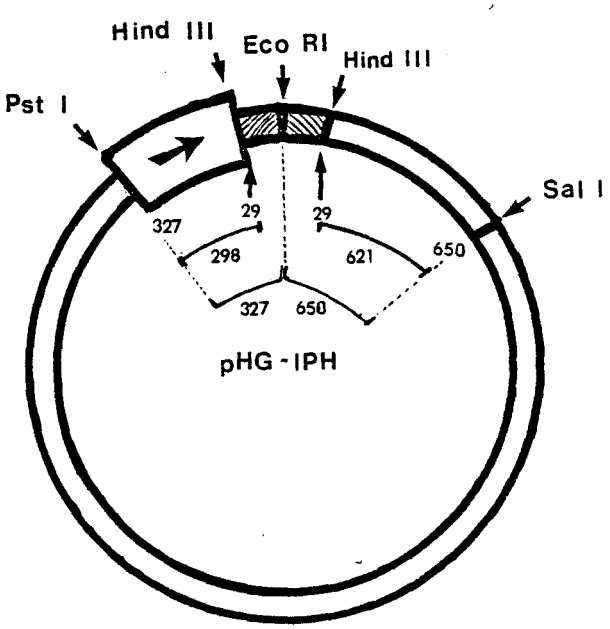

Hind III
Eco RI
Hind III
Pst I
Sal I
29
29
327
298
621
650
327
650
pHG - IPH

Figure 20: Gel electrophoresis of restriction endonuclease digested pHG-1 and pHG-1PH plasmid DNA

pHG-1 and recombinant clone pHG-1PH DNA were analyzed by restriction endonuclease digestion and polyacrylamide gel electrophoresis. 10 ul of a plasmid DNA preparation was digested with various enzymes, then electrophoresed through a 7% acrylamide gel for 3 hours at 150 volts. The DNA fragments were visualized by staining in an ethidium bromide solution, and illuminating with short wave UV light.

The size markers used were the pHG-1 fragments of known lengths. The bands of interest are the 327 and 298 b fragments in both pHG-1 and pHG-1PH DNA which contain the Adenoviral sequence of interest.

A. pHG-1PH DNA digested with Eco R1

B. pHG-1PH DNA digested with .....Pst 1

C. pHG-1 DNA digested with Eco R1...Pst 1

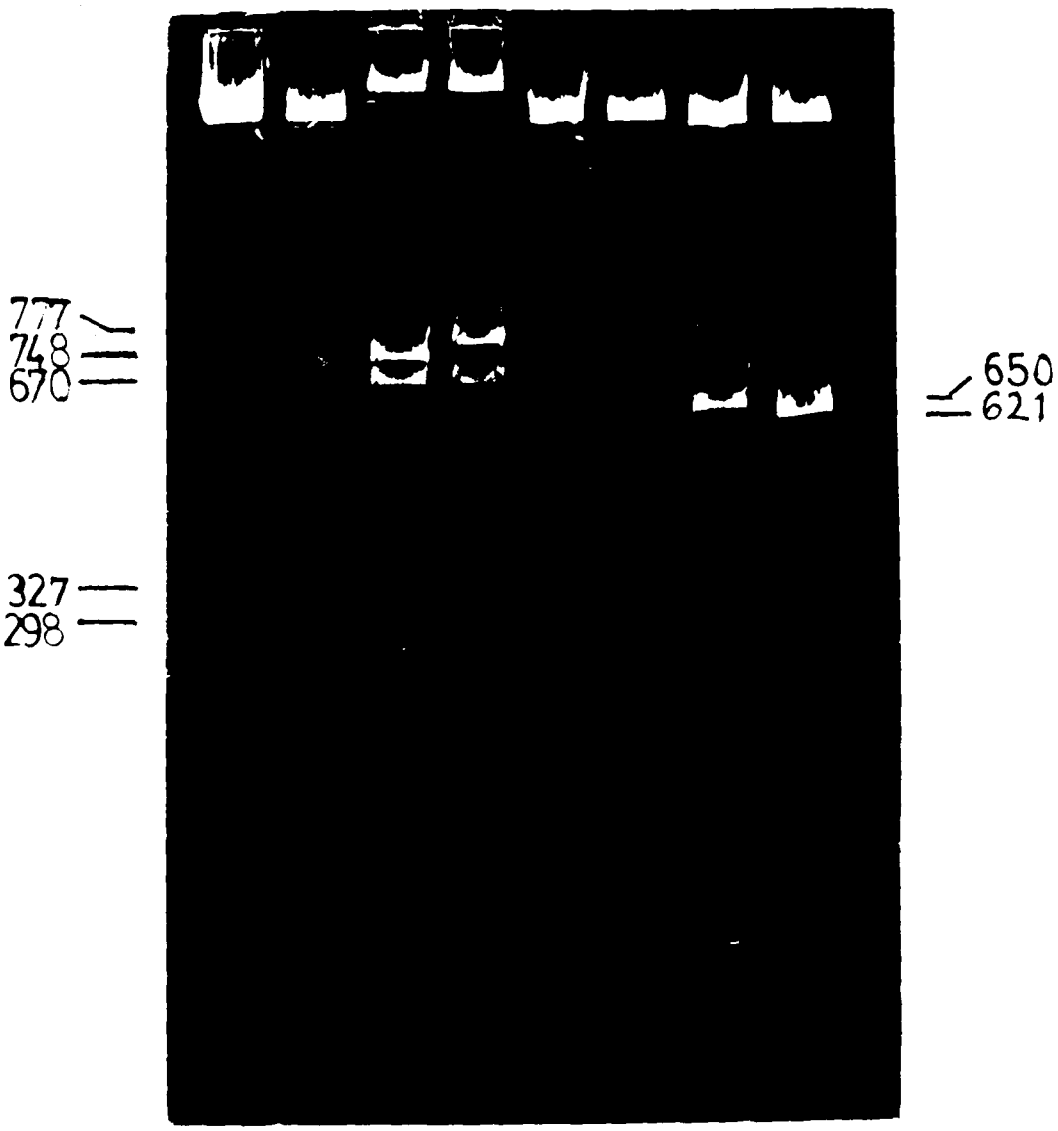
D. pHG-1 DNA digested with .....Pst 1...Hind III

E. pHG-1PH DNA digested with Eco R1...Pst 1

F. pHG-1PH DNA digested with Eco R1...Pst 1...Hind III

G. pHG-1PH DNA digested with Eco R1...Sal 1

H. pHG-1PH DNA digested with Eco R1...Sal 1...Hind III


777
748
670
650
621
327
298
A B C D E F G H

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