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Posttranscriptional regulation of adenovirus E1A and E1B genes during productive infection of human lymphoid cell lines

Lavery, Daniel Joseph, Ph.D.

City University of New York, 1991

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POSTTRANSCRIPTIONAL REGULATION OF ADENOVIRUS E1A AND E1B GENES DURING PRODUCTIVE INFECTION OF HUMAN LYMPHOID CELL LINES

by

Daniel J. Lavery

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, the City University of New York

1991

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

December 7, 1990
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The City University of New York

Abstract

POSTTRANSCRIPTIONAL REGULATION OF ADENOVIRUS E1A AND E1B GENE EXPRESSION DURING PRODUCTIVE INFECTION OF HUMAN LYMPHOID CELL LINES

by

Daniel J. Lavery

Advisor: Selina Chen-Kiang, Ph.D.

Expression of the group C adenovirus E1A and E1B genes was examined in cultured human B and T cells during productive infection. While human B and T cell lines are productively infectable by adenovirus types 2 and 5 with virus yields approaching those in HeLa cells, there was a characteristic and reproducible delay in the onset of the late infectious stage as compared with HeLa cells. To investigate this, early viral gene expression was analyzed during infection of a human B myeloma cell line, 2132. While mRNA accumulation from most viral genes was similar in 2132 and HeLa cells, E1A and E1B mRNA levels were reduced from 10- to 50-fold in 2132 cells throughout infection. Reduced E1A mRNA accumulation was reflected as well in reduced E1A polypeptide levels, as determined by immunoprecipitation. Reduced E1A and E1B mRNA accumulation was not due to reduced gene transcription, as determined by nascent RNA chain pulse labeling in isolated nuclei. Kinetic labeling of RNA to steady state levels indicated that, early after infection, nuclear events influencing the efficiency of E1A and E1B transcript utilization contributed to reduced accumulation of these mRNAs in 2132 cells. Similarly, in a human

T cell line, Jurkat, E1A and E1B mrNAs accumulated in the nucleus during the late infectious stage, indicating that the regulation of E1A and E1B gene expression by nuclear RNA processing events was not restricted to 2132 cells.

In addition, the influence of adenovirus infection on the expression of the immunoglobulin λ light chain gene of 2132 cells was examined. While λ gene transcription was slightly reduced early after infection, there was a three- to five-fold increase in transcription late after infection. However, despite increased λ gene transcription, post-transcriptional events presumably mediated by E1B gene products greatly reduced λ mRNA accumulation late after infection.

FORMAT OF THESIS

This thesis was prepared in accordance with guidelines of the City University of New York. Chapter II contains results published as Lavery et al., Journal of Virology 61:1466-1472 (1987). Chapter IIIA contains results published as Lavery and Chen-Kiang, Journal of Virology, 64:5349-5359 (1990). Chapters IIIB, IIIC, and IV contain unpublished results. Each section contains an introduction and discussion, with a general introduction and general discussion at the beginning and end of the thesis. To reduce redundancy, Materials and Methods sections have been consolidated, as have the Literature Cited sections.

ACKNOWLEDGMENTS

First and foremost, I would like to express my sincere gratification to Selina Chen-Kiang for serving as the motivating force behind my scientific training. Without her constant encouragement and faith in me even when my own faith was flagging, this thesis would never have been completed. Her constant good humor, prescient insight, and helpful fillips of Confucian philosophy taught us all that one need not be one-dimensional to be a top-notch scientist. I will always be in her debt.

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I. INTRODUCTION

Eukaryotic gene expression comprises many complex events, including the initiation of RNA transcription, the processing and cytoplasmic transport of mature messenger RNAs (mRNAs), and the translation and modification of their protein products. Each of these processes is a potential target for regulation. Thus, the combinatorial nature of gene regulation permits an exquisitely sensitive and rapid response to a broad range of biological signals. Understanding the mechanisms of this regulation is a common theme of molecular biology, whether in a developmental system, in the biology of disease, or in virus-host interactions.

Transcription initiation has been demonstrated to be the major level of gene regulation in many systems, and as such has been the focus of intense investigations. As a result, perhaps our most detailed knowledge of gene regulation mechanisms comes from the analysis of transcription factors and their ability to direct the initiation of RNA synthesis. However, post-initiation events can greatly influence gene expression as well. For example, premature transcription pausing/termination in a eukaryotic system was first described in adenovirus and SV40 systems. In HeLa cells early after infection by adenovirus, a block in polymerase elongation prevents RNA synthesis from the major late transcription unit with the exception of the L1 family of mRNAs (Fraser et al., 1979; Evans et al., 1979), while the appearance of 120- and 175-nucleotide prematurely terminated RNA transcripts from the major late promoter occurs temporally (Mok et al., 1984). Promoter-proximal attenuation events have also been described in the study of SV40 transcription termination (Hay et al.,

1982). Subsequent studies with cellular genes have demonstrated that premature transcription termination significantly alters expression of the c-myc (Bentley and Groudine, 1986) and c-myb (Bender et al., 1987) genes, as well as of the human immunodeficiency virus (HIV) genome (Kao et al., 1987). In the case of c-myc, this termination is enhanced upon retinoic acid-induced differentiation of the promonocytic cell line HL-60 (Bentley and Groudine, 1986). The alternative splicing of RNA transcripts into families of mRNAs, each encoding functionally distinct gene products, has proven to be an efficient means of expanding the genetic repertoire in both viral and cellular systems, and is also a potential site for regulation (Breitbart et al., 1987). During infection by HIV, RNA transport competes with RNA splicing events to allow production of viral structural proteins and packaging of virion RNA. This process is regulated by the activity of the viral rev gene product (Malim et al., 1989). Other nuclear events which have as yet been less well characterized, such as nuclear RNA stability and m⁶A-RNA methylation (Chen-Kiang et al., 1979), may also contribute to gene regulation.

Differential mRNA stability in the cytoplasm has also been demonstrated to be an efficient and sensitive means of gene regulation. In enucleated red blood cells, for example, β-globin mRNA is extremely stable, thus permitting the continued synthesis of β-globin in the absence of new mRNA production (Aviv et al., 1976). Conversely, rapid mRNA degradation contributes to the efficient down-regulation of proto-oncogene c-fos expression following its transient induction by serum in mouse fibroblasts. The RNA sequences regulating c-fos mRNA degradation have

been investigated, and include in part a motif in the 3' untranslated region, composed of a stretch of uridine residues bracketed by adenosine residues (Wilson and Treisman, 1988). This AU$_n$A motif was first identified in the 3' untranslated region of other rapidly degraded mRNAs, such as those encoding the c-*myc* proto-oncogene product, cytokines such as GM-CSF, interferons, and interleukins (Shaw and Kamen, 1986). However, while *cis*-sequences influencing mRNA stability from many different genes have been identified, characterization of possible *trans*-acting factors has only recently begun through the development of cell-free RNA degradation extracts (Brewer and Ross, 1989) and RNA band-shift assays (Malter, 1989; Weeks et al., 1990).

Regulated expression of the transforming genes E1A and E1B of the human adenovirus serotypes 2 and 5 has also been shown to involve post-transcriptional processes. Primary transcripts from both genes are alternatively spliced into mRNA species encoding functionally distinct gene products, and the ratios of the individual mRNAs vary with the stage of virus infection (Berk and Sharp, 1978). Furthermore, by analyzing mRNA half-lives by 3H-uridine labeling to steady state, Wilson and Darnell (1981) demonstrated that during the early stage of infection in HeLa cells, the half-lives of the E1A and E1B mRNAs were extremely short, but certain of these mRNAs became greatly stabilized during the late stage of infection. However, while much is known of E1A and E1B gene expression in HeLa cells, the highly complex posttranscriptional regulation of these genes has not yet been investigated in differentiated human cell types more closely approximating a natural host for infection, such as those of lymphoid cell origin.

Adenovirus as a model system in molecular biology. Since their discovery in 1953, the human adenoviruses, group C serotypes 2 and 5 (Ad2, Ad5) in particular, have proven effective as model systems for eukaryotic molecular biology. The relatively compact genome and well-defined virus infection cycle which relies extensively on host factors have provided opportunities to investigate mechanisms of gene expression, DNA replication, and virus-host interactions.

This icosahedral virus, containing a linear, double-stranded DNA genome of approximately 36 kilobasepairs, was first isolated from adenoid and tonsil explants taken from schoolchildren and military recruits suffering from acute respiratory disease (Rowe et al., 1953; Hilleman and Werner, 1954). The adenovirus infectious cycle has been extensively characterized in cultured human cell lines such as HeLa and KB. The virus attaches to the surface of these cells through interactions of the virion fiber protein with a specific surface receptor, and is internalized into a clathrin-coated endocytic vesicle (Helenius et al., 1986). Following endosome acidification, the virus undergoes a conformational change and is released to the cytoplasm, and from there migrates to the nuclear membrane, perhaps by attachment to cellular structural proteins (Luftig and Weihing, 1975). At the nuclear membrane, the viral nuclear core is removed from the virion and enters the nucleus to begin transcription (Chardonnet and Dales, 1972).

The first viral gene transcribed after entry into the nucleus is the E1A gene. Cellular factors including the AP-1 complex activate transcription of this regulatory gene, whose gene products in turn activate transcription of viral early genes E1B, E2, E3, and E4, as well

as E1A itself (Figure 1; Jones and Shenk, 1979; Berk et al., 1979; Müller et al., 1989). Expression of the viral early genes prepares the cell for later events in the infectious cycle. In addition to activating transcription of the viral early genes, E1A gene products also activate and repress certain cellular genes, as well as induce cellular DNA synthesis, which helps provide the cellular factors essential for viral DNA replication (for review, see Moran and Mathews, 1987, and Flint and Shenk, 1989). E1B gene products serve to protect viral and cellular DNA from degradation (White et al., 1987; Pilder et al., 1984), and recruit the cellular machinery for viral protein translation by preventing the accumulation of cellular mRNA in the cytoplasm (Babiss et al., 1985; Pilder et al., 1986).

The E2 gene encodes products essential for viral DNA replication, such as the viral DNA polymerase, single-stranded DNA binding protein (DBP), and the precursor to the terminal protein. In addition to functioning during DNA replication, the E2 DBP also serves as a transcriptional activator of the E1A, E1B, E2, E3, and major late transcription units, and as a repressor of E4 gene transcription (Chang and Shenk, 1990). The terminal protein, covalently attached to the 5' ends of the viral chromosome, acts as a primer in viral DNA replication, and mediates attachment of the viral chromosome to the nuclear matrix (Schaack et al., 1990).

The functions of gene products of the E3 transcription unit include protection from host immune defenses; consistent with this, E3 expression appears to be dispensible for productive infection of cells in culture. Expression of E3 gene products suppresses such cellular defense functions

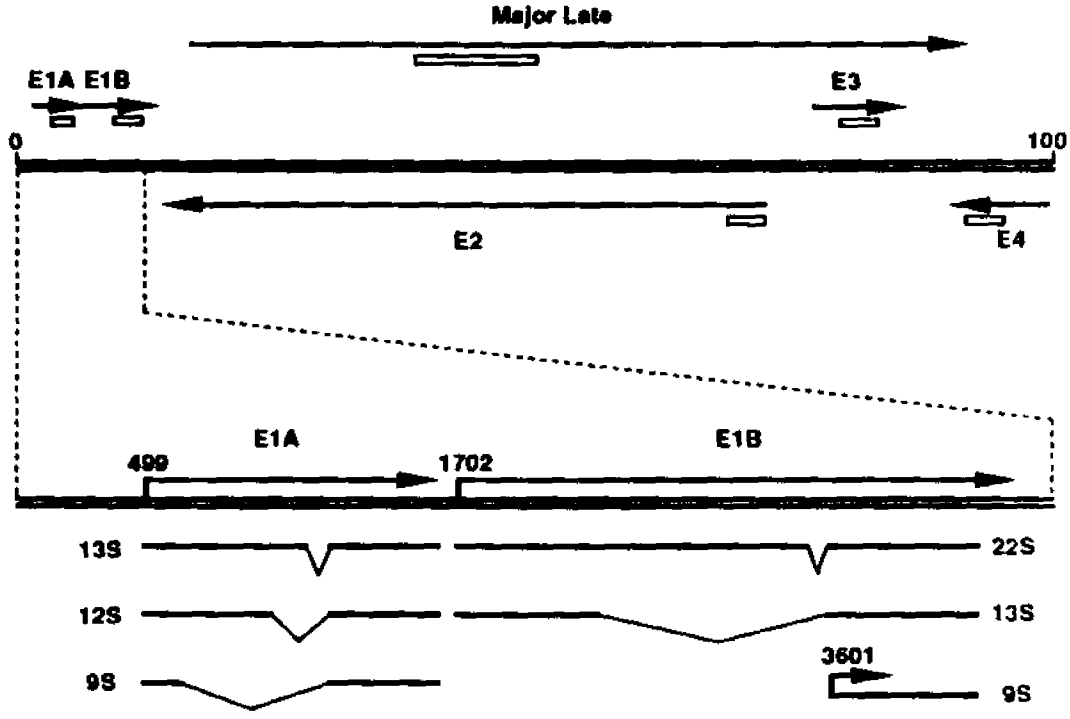


Figure 1: Map of adenovirus transcription units. Transcription units on the adenovirus chromosome are designated by arrows, indicating the orientation of transcription. Open boxes beneath each transcription unit represent the specific DNA probes used for Northern analysis (Fig. 2, 3, and 4). The lower portion of the diagram depicts an expanded map of early region 1, including the transcription initiation sites for the E1A gene (bp 499) and the E1B gene (bp 1702), and the RNA splicing patterns. Thick lines represent exons; thin lines represent introns. The unspliced 9S mRNA encoding protein IX is transcribed from a promoter internal to the E1B gene at bp 3601 of the adenovirus chromosome. The adenovirus chromosome is conventionally divided into 100 units with the left-hand terminus designated as 0. Each unit is approximately 360 bp (Flint, 1977).

as lysis of infected cells by tumor necrosis factor-alpha (Gooding et al., 1988) and expression of MHC class I antigen on the surface of infected cells (Burgert and Kvist, 1985). Finally, the functions of E4 gene products have not yet been completely defined, but include complexing with E1B gene products to cooperate in effecting the selective accumulation of viral mRNA in the cytoplasm (Halbert et al., 1985; Bridge and Ketner, 1989; Sandler and Ketner, 1989), and forming a stable complex with a cellular transcription factor, E2F, which binds within the E2 gene regulatory region (Hardy et al., 1989; Huang and Hearing, 1989).

With these early gene products preparing the cell to function as an efficient virus replication system, the virus enters the late phase of infection, characterized by replication of its genome and by synthesis of virion structural proteins in great abundance. The majority of structural proteins are encoded in a single transcription unit, the major late transcription unit (MLTU), whose promoter is one of the strongest yet characterized. Transcription of the MLTU initiates approximately 6 kb from the left-hand end of the viral chromosome and continues for greater than 26 kb, nearly reaching the right-hand end of the chromosome. This remarkably long transcript was the first to be characterized in its start and end points by UV crosslink mapping, nuclear run-on, and chemical analysis of mRNA cap structures, thus defining the first eukaryotic transcription unit (Darnell, 1982). Through an intricate combination of RNA splicing and alternate polyadenylation events, the primary transcript can be processed into one mRNA from one of five late gene families, defined by common polyadenylation sites, each of which contains multiple distinct members arising from RNA splicing events (Nevins and Chen-Kiang,

1981). Spliced to the 5' end of each mRNA derived from the MLTU is a three (and occasionally four [Chow et al., 1979]) exon leader, the "tripartite leader", encoded at the major late promoter. The addition of these segments was the first characterized to be the result of RNA splicing events (Chow et al., 1977; Berget et al., 1977).

In HeLa cells, viral DNA replication can be detected as early as 8 hours post-infection, and reaches a maximum rate by 18 hours post-infection. While cellular DNA may have replicated, cell division often will not occur, resulting in the formation of multinucleate host cells late in infection. During this time, the preferential translation of viral proteins, especially the structural proteins, is such that greater than 90% of polysome-bound mRNAs are virus-specific (Philipson et al., 1975), and by 24 hours post-infection the new synthesis of host proteins has been dramatically reduced (Ginsberg et al., 1967). These effects are due to several factors: the influence of E1B and E4 gene products on the preferential accumulation of viral mRNA in the cytoplasm; the presence of the "tripartite leader" sequence on the 5' end of most structural protein mRNAs which relieves the requirement of cap-binding protein for efficient translation initiation; and a host-induced inhibition of protein synthesis, presumably interferon-mediated, which adenovirus circumvents through the function of viral-encoded RNAs, the VA_1 and VA_2 RNAs. These polymerase III-transcribed RNAs appear to inhibit the block to protein synthesis by interfering with the activity of a protein kinase which can phosphorylate translation initiation factor eIF2-α and prevent recycling of the eIF2 (Kitajewski et al., 1986). However, the mechanism by which this permits only virus-specific translation is unknown.

Viral structural proteins are transported to the nucleus, where they are assembled with viral DNA into progeny virions. Approximately 10^4 progeny virions are released from the infected cell upon its disintegration, which appears to be a result of the inhibition of macromolecular synthesis as well as the toxic effects of the viral fiber protein.

The well-characterized infectious cycle of adenovirus has provided many opportunities to examine virus-host interactions. However, the dependency of the virus on many aspects of host metabolism also permits certain virus functions to serve as models for eukaryotic cell processes, thus contributing to important advances in molecular biology.

Adenovirus transforming genes E1A and E1B. The E1A and E1B genes are critical for productive infection of most cells. Expression of the E1A gene is required for efficient transcriptional activation of the viral early genes, and viruses containing mutations within the E1A gene show little or no virus replication in most cells (Shenk et al., 1979). E1B gene expression protects viral and cellular DNA from degradation and induces the preferential accumulation of viral mRNA in the cytoplasm, and E1B mutant viruses often display delayed infection kinetics with reduced yield of progeny virus (Babiss and Ginsberg, 1984; Pilder et al., 1984; White et al., 1984; Pilder et al., 1986).

During the early stage of infection, the E1A primary transcript is alternatively spliced into one of two mRNAs through the use of two 5' splice donor sites ligated to a common 3' splice acceptor (Figure 2). Use of a splice donor site at nucleotide 976 on the viral chromosome results in the formation of a 1.0 kb 12S mRNA, while the use of a splice donor

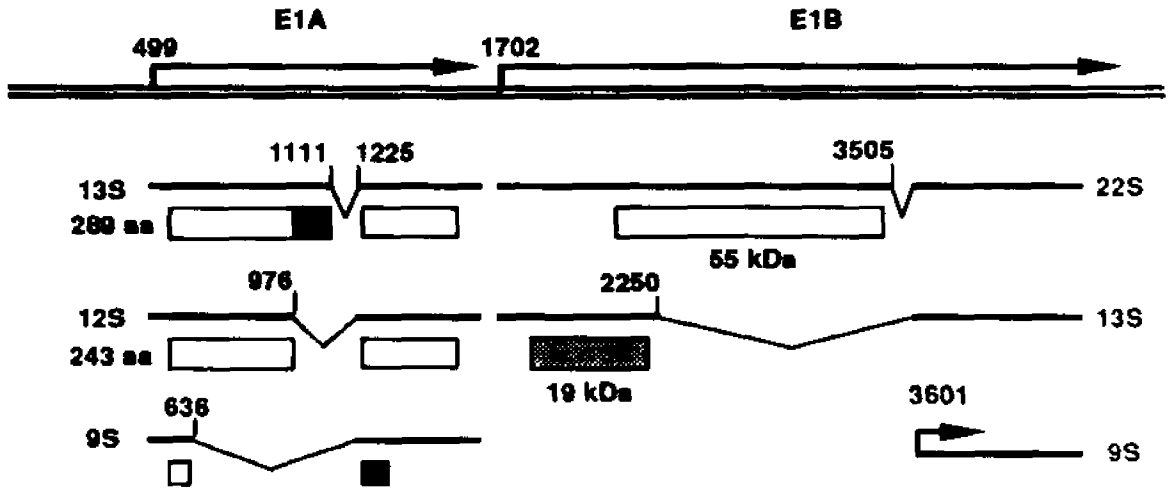


Figure 2: E1A and E1B transcription units and gene products. RNA splice acceptor and alternative splice donor sites are indicated, as well as transcription start sites. All positions are expressed as nucleotides from the left-hand viral terminus (Flint, 1977). Also indicated are the major translation products of the E1A and E1B genes. Differences in polypeptide translation reading frames within a transcription unit are expressed as different shading within the boxed coding region. In E1A, the black region identifies the transactivating domain specific to polypeptides translated from the 13S mRNA (for a review, see Moran and Mathews, 1987).

site at nucleotide 1111 results in the formation of a 1.2 kb 13S mRNA. Because the two splicing events occur within the same protein translation reading frame, the sole difference in the primary sequence of 13S and 12S gene products is the inclusion in the former of a 46-amino acid domain, encoded by sequences between 976 and 1111. Genetic and mutational experiments indicated that the activation of viral and cellular gene transcription can largely be attributed to the 13S-derived gene products, and to the 46-amino acid 13S-specific domain in particular, while the repression of certain enhancer-driven genes, the induction of cellular DNA synthesis, and the transformation phenotype can be predominantly attributed to 12S-derived gene products (for a review, see Moran and Mathews, 1987; Berk, 1986; Flint and Shenk, 1989). Late after infection, a third E1A mRNA of unknown function, termed the 9S mRNA, is also synthesized (Figure 2).

The ratio of 13S to 12S mRNA early in infection in HeLa cells is approximately 3:1, which may reflect the relative preference for the 13S-specific splice donor site. However, other posttranscriptional mechanisms influence E1A gene expression as well. By labeling mRNA to steady state levels with ^{3}H-uridine, Wilson and Darnell demonstrated that E1A mRNAs decayed very rapidly in the cytoplasm early in infection; at 5 hours p.i., the E1A cytoplasmic mRNA half-lives were less than 10 minutes. However, late in infection, the E1A mRNA appeared to be stabilized in the cytoplasm, with an average half-life of greater than 45 minutes. The reason for this stabilization is unknown, but perhaps relates to the observation that late in infection host protein synthesis is greatly reduced, which may reduce the activity of a labile host-encoded

ribonuclease.

As is the case with E1A, alternative splicing generates one of two major mRNAs from the E1B primary transcript early in infection (Figure 2). The use of a splice donor site at nucleotide 2250 produces the E1B 13S mRNA, encoding the 19 kDa polypeptide which protects viral and cellular DNA from degradation, while the use of a splice donor site at nucleotide 3505 produces the E1B 22S mRNA, which encodes the 55-58 kDa polypeptide influencing the selective accumulation of viral mRNA in the cytoplasm. Early in infection of HeLa cells, the 22S mRNA predominates, while late in infection the ratio is inverted, with more 13S mRNA accumulating.

E1B mRNAs also display short mRNA half-lives in the cytoplasm of HeLa cells early in infection, with half-lives of 10 to 15 minutes, but Wilson and Darnell reported that only the 13S mRNA became stabilized late in infection, while the 22S mRNA remained unstable (Wilson and Darnell, 1981). This was proposed to influence the change in the ratio of the two mRNAs during the infectious cycle of HeLa cells, though altered splicing preference for the 13S splice donor site may also contribute.

The E1A and E1B genes display distinct phenotypes when expressed outside of productive infection. When viral DNA fragments which are defective for productive infection are introduced into cells, the E1A and E1B genes can on rare occasion integrate into the cellular genome and induce cellular transformation, as determined by altered cell morphology, cell immortalization, loss of contact inhibition, and the ability to induce tumors in certain hosts (for a review, see Graham, 1984).

The E1A and E1B genes can also cooperate with certain other human oncogenes to induce transformation of primary rodent cultures in

cotransfection assays; in primary rat fibroblasts, E1A can induce foci in cooperation with the Harvey-ras oncogene, but not in cooperation with the myc oncogene, while for E1B the reverse is true (Ruley, 1983). Furthermore, gene products from both the E1A and E1B genes have been identified as complexing with cellular gene products which have been characterized as tumor-suppressors: E1A gene products can be coprecipitated with the 105 kDa product of the Rb retinoblastoma susceptibility gene when using antibodies directed against either E1A or Rb gene products (Whyte et al., 1988), and the 55 kDa E1B gene product can be coprecipitated with the tumor-suppressing cellular p53 gene product using an anti-55 kDa protein antibody (Sarnow et al., 1984). Both of these cellular proteins have been found to coprecipitate with other DNA tumor virus oncoproteins, such as the SV40 T antigen (DeCaprio et al., 1988) and the human papillomavirus type 16 E7 gene product (Dyson et al., 1989).

While cellular transformation may be viewed as a result of aborted viral infection, it can nonetheless provide insight into some functions which are essential for productive infection, as well as serving as a model system for transformation in general. For instance, Rb gene products are dephosphorylated in a cell-cycle dependent manner (Ludlow et al., 1990). If Rb gene products are involved in cell cycle control, the formation of an E1A-Rb complex may interfere with regulation of cellular DNA replication, which may permit greater viral DNA replication during productive infection, or unregulated cellular DNA replication in the transformed cell. Because the E1A and E1B genes play a central role in both productive viral infection and transformation, the regulation of

their own expression may provide clues to the underlying processes of these complex events.

Adenovirus and human lymphoid cell lines. Despite conventional wisdom to the contrary, interesting interactions between human lymphoid cells and human adenoviruses during productive infection were suggested by a curious finding uncovered upon the first isolation of adenovirus from adenoids and tonsils of patients with acute respiratory disease. Rowe et al. found that virus could be recovered following prolonged incubation of tissues from apparently "healthy" individuals (Rowe et al., 1953). Furthermore, low titers of virus could be derived from purified, activated human peripheral blood lymphocytes when co-cultured with fibroblasts (Van der Veen and Lambriex, 1973). Finally, the recurrence of adenovirus infection in humans, the persistent shedding of virus, and the maintenance of high titers of serum anti-adenovirus antibodies (Routes and Cook, 1989) suggest that productive infection in humans may not be as simple as represented by infection of cultured cells such as HeLa, in which the virus has subsequently been most frequently investigated. Because lymphoid cells comprise the majority of cells in adenoids and tonsils, their role in adenovirus infection of humans may be significant.

The interactions between adenovirus and human lymphoid cells present the opportunity to examine not only the influence of the differentiated cell background on viral gene expression, but also the influence of the virus on differentiated cell functions. Analysis of the expression of immunoglobulin genes and other lymphoid-specific functions during viral infection may uncover novel interactions between viral gene products and the expression of these lymphoid cell markers.

The investigations presented in this thesis analyze the regulated expression of adenovirus E1A and E1B genes during productive infection of with human cell lines of lymphoid origin. These investigations demonstrate that expression from the E1A and E1B genes in these cell lines is distinct from that in HeLa cells, predominantly as a result of posttranscriptional events. This regulation is cell type-specific and highly complex, involving nuclear and cytoplasmic processes which influence the efficiency with which primary RNA transcripts are processed into mature mRNAs in the cytoplasm. These findings may enhance our understanding of the functions of E1A gene products in a differentiated cell background.

MATERIALS AND METHODS

Cells and viruses. Suspension cultures of HeLa cells were cultured in Joklik modified minimal essential medium with 5% fetal calf serum. 293 cells (Graham et al., 1977) were grown as monolayer cultures in Dulbecco modified essential medium with 10% fetal calf serum. A549 cells were maintained as monolayer cultures in Dulbecco minimum essential medium with 5% heat-inactivated fetal calf serum. B and T lymphoid cell cultures were maintained in a humidified CO_2 incubator in RPMI 1640 medium with 10% heat-inactivated fetal calf serum. All media were supplemented with L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Ad2 and Ad5 stocks, which were phenotypically indistinguishable in all experiments performed, were propagated and used for infection as previously described (Chen-Kiang et al., 1982). Virus infections were performed essentially as described previously (Ruether et al., 1986), at a multiplicity of infection of 10 PFU per cell, except where noted. Representative early and late times after infection were 5 and 18 h post-infection (p.i.) for HeLa cells and 14 and 48 hours p.i. for human myeloma 2132 cells, respectively, unless otherwise indicated.

Southern blot analysis. After infection with Ad2 or Ad5 at 10 to 30 PFU per cell, 10^7 cells from each cell culture were withdrawn immediately after viral adsorption (time zero) and at various times post-infection (p.i.). Total DNAs were isolated, digested with restriction endonucleases, separated by agarose gel electrophoresis, and blotted onto nitrocellulose membranes as previously described (Mok et al., 1984). The blots were analyzed by hybridization to nick-translated (Rigby et al.,

1977) ^{32}P-labeled total Ad2 or Ad5 DNA in the presence of 50% formamide (Chen-Kiang et al., 1982). For detecting the presence of the EBV genome in B lymphoid cell lines, a cloned *Bam*HI W fragment of the EBV genome (kindly provided by W. Sugden) was used for Southern blot analyses.

RNA isolation and Northern analysis. Total RNAs were isolated by homogenization in 4 M guanidine thiocyanate (GTC; Chirgwin et al., 1979). For isolation of nuclear and cytoplasmic RNAs, cells were fractionated as described previously (Chen-Kiang and Lavery, 1989b). Cells were first treated with nonionic detergent NP-40, 0.5% in Iso-Hi buffer (NaCl, 0.14 M; Tris-base pH 8.4, 10 mM; $MgCl_2$, 1.5 mM), to disrupt the cytoplasmic membrane, and subjected to low-speed centrifugation. The supernatant was saved, and the pellet was again washed with a deoxycholate-Tween 40 solution to remove the outer nuclear membrane ["Magic Wash"; Tris-OH pH 8.3, 42.5 mM; NaCl 8.5 mM; $MgCl_2$, 2.6 mM; vanadyl adenosine 4 mM; phenylmethylsulfonyl-fluoride, 1.2 mM; Tween 40, 0.6% (v/v); sodium deoxycholate, 0.3% (w/v); Penman, 1966]. After low-speed centrifugation, the supernatant was combined with the previous supernatant; together, these cytoplasmic fractions were homogenized in GTC at a final concentration of 4 M. The nuclear pellet was also homogenized in 4 M GTC. RNA was separated from DNA and protein by overnight ultracentrifugation through cesium chloride (Chirgwin et al., 1979). Polyadenylated [poly(A)$^+$] and unpolyadenylated [less than 14 adenyl residues; poly(A)$^-$] RNAs were purified by poly(U)-agarose chromatography as described previously (Lavery and Chen-Kiang, 1989b).

Northern analysis of steady state mRNAs was performed as previously described (Lavery and Chen-Kiang, 1990). RNAs were size-fractionated on

denaturing formaldehyde-agarose gels in MOPS buffer (20 mM morpholino-propanesulfonic acid [Sigma Chemical Co.], 5 mM sodium acetate, 1 mM EDTA, pH 7.0), transferred to nitrocellulose membranes (Schleicher and Schuell), and baked at 80°C for 2-3 hours. DNA probes ^{32}P-labeled by random priming were hybridized to membranes at 42°C in the presence of 50% formamide, after which the membranes were washed four times with 2X SSC, 0.2% sodium dodecyl sulfate (SDS), for 10 minutes each at 42°C, and twice with 0.1X SSC, 0.2% SDS, for 15 minutes each at 55°C (1X SSC is 150 mM NaCl, 15 mM sodium citrate). Specific DNA probes used were a rat cDNA clone of the glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH; Fort et al., 1982), a genomic fragment of the human c-myc gene containing exon 1 and part of exon 2 (kindly provided by Drs. Andrew Bergemann and Ed Johnson), a cDNA clone specific for human class I major histocompatibility antigens HLA-B, pDP001 (Sood et al., 1980), c131, an E1A cDNA clone (Perricaudet et al., 1979), c244, an E1B cDNA clone (Perricaudet et al., 1980), restriction fragments of the Ad2 HindIII D for the L2 family (41.8 to 50.1 map units [m.u.] on the Ad2 genome), the Ad2 BalI K for E2 (63.2 to 66.3 m.u.), the Ad2 BglII F for E3 (78.3 to 84.7 m.u.), and the Ad2 Asp718 F for E4 (93.4 to 100 m.u.). (The Ad2 genome is 36 kb and conventionally divided into 100 units. The 0 at the left end is the 3' end of the rightward transcribed strand [15]. The transcription units are shown in Fig. 1.) For quantitation of RNA hybridization results, autoradiographic signals within the linear range of film sensitivity were analyzed using an LKB laser densitometer and were presented as arbitrary density units.

Inhibition of RNA synthesis by ActD. Dactinomycin (ActD), a potent inhibitor of RNA transcription (Sobell, 1985), was added to cell cultures

to a final concentration of 10 μg/ml at the hours after infection as indicated. Cell samples were removed at minutes after ActD addition as indicated by pouring cells over two volumes of crushed, frozen PBS, and washing two times with ice-cold PBS. Total RNAs were extracted and analyzed by Northern analysis as described above.

RNAse H assay. To assess whether shifts in electrophoretic mobility of certain mRNAs were due to differences in poly(A) tail length, the poly(A) sequences were removed by RNAse digestion in the presence of oligo-d(T)$_{12}$, as modified from Vournakis et al. (1975). Poly(A)$^+$ RNA from 10' cells (approximately 100 ng, with 1 μg yeast tRNA as carrier) was hybridized with 2 μg of oligo-d(T)$_{12}$ (Boehringer-Mannheim Biochemicals) in 90 μl of deionized water for 15 min at 37°C. RNAse H buffer (1X: KCl, 50 mM; $MgCl_2$, 10 mM; dithiothreitol, 1 mM; Hepes-OH, pH 7.9, 20 mM) was added, and samples were again incubated for 15 min at 37°C before addition of 10 U of RNAse H (Boehringer-Mannheim). Samples were incubated for 45 min at 37°C, after which the reaction was stopped with 0.3% NP-40, followed by ethanol precipitation. Control and RNAse H-digested RNAs were analyzed by Northern blotting as described above.

Nuclease S$_1$ protection assays. The 5' termini of E1A transcripts were assayed by using as a probe the Ad2 AvaI fragment (0 to 2.37 m.u.), radiolabeled at the 5' end with [γ-^{32}P]ATP (New England Nuclear) and polynucleotide kinase (Boehringer Mannheim Biochemicals). Hybridization of 10 ng of labeled probe to total RNA from 10' cells was performed in 20 μl of 80% formamide-40 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES; pH 6.4) (Sigma)-400 mM NaCl-1 mM EDTA for 3 h at 52°C, followed by

digestion with 1,000 to 2,000 U of nuclease S_1 (Sigma) at 42°C for 30 min (Favaloro et al., 1980). The reaction was stopped by phenol-chloroform extraction and isopropanol precipitation three times with 10 mM EDTA-2 M ammonium acetate. The protected fragments were separated on denaturing 8% acrylamide gels and exposed to X-ray film.

Nuclear run-on assays. Nuclei were isolated from 10^8 control or virus-infected cells by Dounce homogenization as described previously (Chen-Kiang and Lavery, 1989b), labeled for 10 min with 300 μCi of [^{32}P]UTP in 300 μl of transcription buffer at 30°C, and chased with 3 mM cold UTP. RNAs were extracted, subjected to limited alkaline hydrolysis, and hybridized to specific probes immobilized on nitrocellulose filters: M13 probes for E2 (Ad5 BglII L fragment, 60.2 to 63.6 m.u.), E3 (Ad5 EcoRI-KpnI fragment, 75.9 to 81.0 m.u.) and E4 (Ad5 HindIII-KpnI fragment, 89.1 to 93.5 m.u.), courtesy of P. Hearing; the major late promoter in both orientations (R17 and L17, 17.0 to 21.0 m.u.; Gingeras et al., 1982), double-stranded DNA probes for Ad5 E1A (FnuDII-HpaI fragment, 1.15 to 4.4 m.u.) and E1B (HindIII-BglII fragment, 7.8 to 9.2 m.u.) and cellular genes for glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Fort et al., 1985), the human λ light chain gene constant region (Raynal et al., 1989), and the human HLA class I gene, HLA-B7 (Sood et al., 1980). After hybridization in 50% formamide at 42° C, blots were washed as described previously (Chen-Kiang and Lavery, 1989b) and exposed to X-ray film. Autoradiographic signals were quantified by scanning of multiple exposures with an LKB laser densitometer.

Cumulative labeling of RNA with ^{3}H-uridine. To assess the kinetics of RNA biogenesis, RNAs were analyzed by cumulative labeling with [5,6-^{3}H]uridine as described by Chen-Kiang et al. (1979). At hours after infection by Ad2 as indicated, cells were pelleted, washed with serum-free medium, resuspended in prewarmed medium with 2% dialyzed, heat-inactivated fetal calf serum, and incubated for 30 minutes to deplete the endogenous uridine pool. Cells were then labeled with 0.2 mCi/ml of [5,6-^{3}H]uridine (37.5 Ci/mmol; New England Nuclear) in the presence of 20 μM adenosine, 20 μM guanosine, and 14 μM uridine to prevent uridine depletion during labeling. At intervals after labeling had begun, 2 X 10^7 cells were withdrawn and poured over two volumes of crushed, frozen phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, 1 mM $MgCl_2$, pH 7.0) containing 14 mM uridine, and washed twice with ice-cold PBS. Nuclear and cytoplasmic fractions were separated as described above, and RNAs were extracted from each by the GTC/CsCl method as described above. Poly(A)$^+$ and poly(A)$^-$ RNA fractions were isolated by poly(U)-agarose chromatography (Lavery and Chen-Kiang, 1989a). RNA samples were resuspended in a minimal (75-100 μl) volume of hybridization solution (750 mM NaCl, 75 mM sodium citrate, 50 mM Na_2PO_4, pH 7.4, 100 μg/ml yeast tRNA, 0.2% sodium dodecyl sulfate [SDS], 5X Denhardt's solution [ficoll, 1 mg/ml; polyvinylpyrrolidone, 1 mg/ml; bovine serum albumin, Fraction V, 1 mg/ml]), and hybridized to multiple small nitrocellulose filters, each bearing 1-2 μg of DNA probe specific for a viral or cellular gene. Probes used were: Ad5 E1A cDNA clone c131; Ad5 E1B gene BglII-HpaI fragment specific for the 22S mRNA; a rat cDNA clone

for the GAPDH gene; and plasmid pUC12, to serve as a background control. After hybridization for 24 hours at 65°C, the filters were washed four times with 2X SSC, 0.2% SDS, for 10 minutes each at 42°C, and twice with 0.1X SSC, 0.2% SDS, for 15 minutes each at 55°C. Filters were treated with RNAse A (Calbiochem), 5 μg/ml in 2X SSC, 0.2% SDS, for 5 minutes at 37°C. ^{32}P radioactivity hybridized to each filter was counted by liquid scintillation in a Beckman LS 5000TD scintillation counter. To facilitate comparison between cell lines, corrections for labeling efficiency were made by measurement of ^{32}P radioactivity incorporated into total nuclear RNA at 60 minutes after labeling, before the nuclear RNA pool of HeLa or 2132 cells had reached equilibrium. Similar results were obtained when comparing cytoplasmic poly(A)+ RNA pools at 240 minutes after labeling.

^{35}S-methionine labeling of cells and immunoprecipitation. A total of 5 X 10^7 cells at the indicated hours after infection were pelleted, washed in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, 1 mM $MgCl_2$, pH 7.0), and suspended in methionine-free medium supplemented with 2% (vol/vol) dialyzed fetal calf serum to deplete the endogenous methionine pool. After 1 h at 37°C, cells were pelleted and resuspended in the same medium to a concentration of 5 X 10^6 cells per ml and labeled with [^{35}S]methionine (150 μCi/ml, 1,245 Ci/mmol, New England Nuclear) for 2 or 3 hours at 37°C with occasional swirling. Cells were then pelleted, washed with PBS, and frozen as pellets for subsequent immunoprecipitation.

Immunoprecipitation was performed essentially according to Harlow et al. (1985), using an anti-E1A monoclonal antibody (Oncogene Sciences, Inc.). Cell pellets were thawed in weak RIPA buffer (10 mM Tris [pH 8.0],

150 mM NaCl, 1 mM EDTA, 1% [vol/vol] Nonidet P-40 [Sigma], 1% [wt/vol] sodium deoxycholate [Schwartz-Mann/ICN], 1 mM phenylmethylsulfonyl fluoride [Sigma]) and centrifuged at 15,000 x *g*. Total protein samples for analysis of protein synthesis were withdrawn from the supernatant, boiled, and separated on 10% polyacrylamide-SDS denaturing gels. After fixation, fluorography (En'Hance, New England Nuclear), and drying, gels were exposed to X-ray film.

For immunoprecipitation, nonspecific antibody interactions can often be reduced by preincubation of samples with preimmune serum and precipitation of the nonspecific complexes with protein A (Harlow and Lane, 1988). To accomplish this, supernatants from the weak RIPA lysis were incubated with 10% (wt/vol) fixed Staphylococcus aureus Cowan bacteria (Boehringer Mannheim Biochemicals), which had been previously complexed with goat preimmune serum (Harlow and Lane, 1988). After this preadsorption, the precleared supernatants were incubated with the anti-E1A monoclonal antibody as recommended by the supplier. Antigen-antibody complexes were recovered with protein A-Sepharose (Pharmacia), washed three times with weak RIPA buffer, and separated on a 10% SDS-polyacrylamide gel. After fluorography (En'Hance; New England Nuclear), gels were exposed to X-ray film.

Indirect immunofluorescence microscopy. Infected or control cells were pelleted by centrifugation, washed three times with ice-cold phosphate-buffered saline (PBS, pH 7, supplemented with 1 mM $MgCl_2$), and suspended in PBS. In the subsequent steps, PBS was not supplemented with $MgCl_2$. Samples of cell suspensions, 2 x 10^5 cells in 100 μl, were then

spun onto acetone-cleaned glass slides at 500 rpm (23 x g) for 2 min in a Cytospin centrifuge (Shandon-Southern). The attached cells were fixed in acetone at -20°C for 30 min, air dried, and quickly rinsed in distilled water. The fixed cells were then incubated with 100 μl of diluted (1:1,000 dilution in PBS, pH 7) rabbit antiserum to purified adenovirus virion (kindly provided by C. S. H. Young) for 45 min at 37°C in a humidified chamber. After incubation, the cells were washed three times, for 20 min each time, in PBS (pH 7) and then rinsed in distilled water, all at room temperature. Indirect immunofluorescent staining was carried out by blot drying the slides and incubating them with 100 μl of diluted (1:40 dilution in PBS, pH 8) fluorescein-conjugated goat antibody to rabbit immunoglobulin G (IgG; kindly provided by C. H. S. Young) for 45 min at room temperature in a humidified chamber. The stained cells were then washed three times in PBS, pH 8, for 20 min each time, rinsed in distilled water, and sealed in 90% glycerol under cover slips. We found that elevation of the pH to 8 greatly enhanced resolution and brightness of fluorescence. Photography under phase-contrast and UV microscopy was carried out with a Zeiss UV microscope (kindly provided by E. Wang).

Biological assay for production of infectious virus. Cells infected with Ad2 at 10 PFU per cell were collected at various times p.i. A portion was used for analysis of viral DNA replication, and lysates were prepared from the remainder. The lysates were then used to infect a second sample of uninfected cells from which line the lysates were prepared. DNAs isolated from the second round of infection were similarly analyzed by Southern blot analysis. Plaque assays of the lysates were

perfomed on A549 monolayer cells.

PART II: PRODUCTIVE INFECTION OF CULTURED HUMAN LYMPHOID CELLS BY ADENOVIRUS.

Results contained in Part II have been published (Lavery et al., Journal of Virology, 61:1466-1472).

Abstract: We investigated infection of cultures from established human B- and T-cell lines by adenoviruses. Infection by adenovirus type 2 or 5 was productive by the criteria of viral DNA replication, RNA synthesis, immunofluorescent staining of viral proteins, and assembly of biologically active virions. Whereas the kinetics of infection were reproducible and characteristic for each cell line, there appeared to be no correlation between the kinetics of infection and the origin from which the cell lines were established. In a myeloma and a T-cell line, the kinetics of infection approached those in HeLa cells. The presence of the Epstein-Barr virus genome in B lymphoid cells was not a prerequisite for adenoviral infection. Furthermore, expression of the E1A gene was repressed in myeloma cells in comparison with HeLa cells.

INTRODUCTION

Human adenoviruses were originally isolated from adenoids and tonsils of patients with acute respiratory disease (Enders et al., 1956; Hilleman and Werner, 1954; Rowe et al., 1953). Although 75% of adenoid tissue is composed of lymphoid cells, isolation of the viruses from fresh lymphocytes purified from adenoids and tonsils was unsuccessful. Even after lymphocyte culturing, only very low yields of infectious virus,

detected in approximately 1 in 10^7 cells infected, were observed (Strohl and Schlesinger, 1965; Van der Veen and Lambriex, 1973). Replication of exogenous adenovirus type 2 (Ad2) in cultured lymphocytes purified from adenoids was also poor; only 1 to 3% of cells were found to produce virus (Lambriex and Van der Veen, 1976). In their work superinfecting an Epstein-Barr virus (EBV)-transformed Burkitt's lymphoma B-cell line with adenovirus type 5 (Ad5), Faucon et al. (1974) showed by electron microscopy that approximately 3 to 6% of EBV-positive Jijoye cells contained viral particles, twice that of an EBV-negative cell line. This low level of infection of lymphocytes by Ad5 was also maintained in human cord blood lymphoblastoid cell lines transformed by EBV after several months of cultivation (Faucon and Desgranges, 1980).

The questions of whether and how lymphocytes are involved in the route of adenovirus infection remain unanswered. The viruses have been conventionally propagated in cell lines of nonlymphoid origin. Despite extensive studies of molecular interaction between adenoviruses and cells of nonlymphoid origin, little is known of the molecular biology of adenoviruses in a lymphoid cell background. Recent work by Chambon and colleagues pointed to the possibility of very interesting dialogues between adenoviral gene products and genes specifically expressed in B lymphoid cells. They showed (Hen et al., 1985; Borrelli et al., 1986) that, in mouse plasmacytoma cell line MPC-11, a nonpermissive host for adenoviral DNA replication, expression of immunoglobulin genes $\gamma 2b$ and κ was repressed after infection by Ad5.

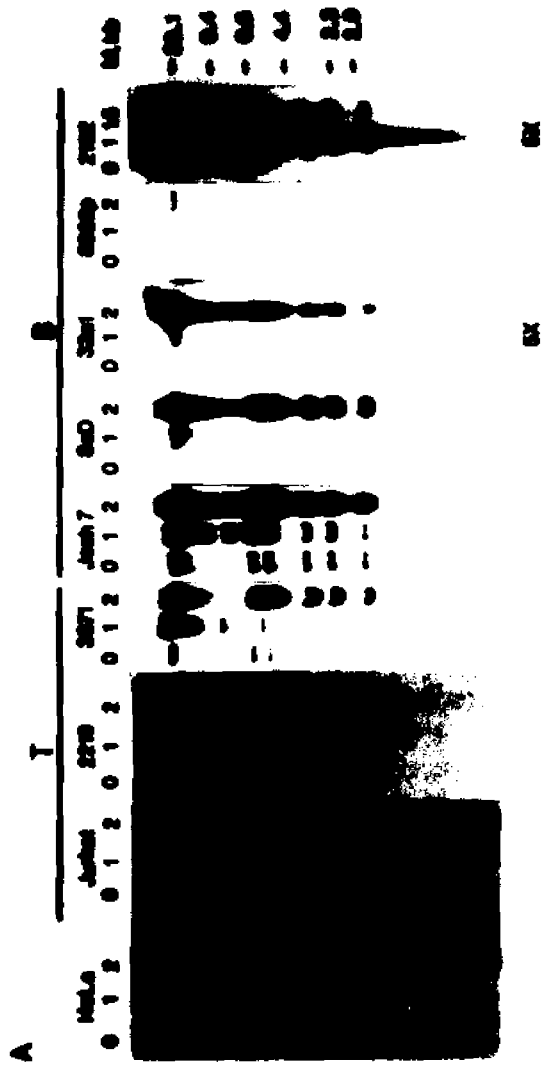
To understand the molecular interaction between adenoviruses and human lymphoid cells better, we undertook the present study. We showed that, with one exception, established human B- and T-cell lines supported viral replication, RNA synthesis, and assembly of infectious particles productively. In a B-cell myeloma line, 2132, and a T-cell line, Jurkat, the kinetics of infection by Ad5 approached those in HeLa cells. There appeared to be no correlation between the kinetics of infection on one hand and the stage of differentiation or the presence of EBV genome on the other. Expression of viral early region genes and use of adenovirus as a vector in human lymphoid cells are discussed.

RESULTS

Viral DNA replication in cultured lymphoid cells. Replication of adenoviral DNA in human lymphoid cells was assayed by Southern blot analysis with nick-translated total Ad2 DNA as a probe. Three T-cell lines, Jurkat (Gillis and Watson, 1980), GM2219 (Molt-4 [Minowada et al., 1972]) and BM3671 (CCRF-CEM [Foley et al., 1965]), were infected with Ad2 as were control HeLa cells (Fig. 3). Viral DNA replication was most efficient in Jurkat cells, in which the amount of Ad2 DNA synthesized at day 2 p.i. was comparable to that at day 1 p.i. in HeLa cells (Fig. 3). Replication of viral DNA in 2219 and 3671 cells followed slower kinetics. Repeated experiments showed, however, that the kinetics of DNA replication were reproducible and characteristic for each T-cell line.

Replication of Ad2 DNA was similarly analyzed in human B-cell lines with markers characteristic of various stages of B-cell differentiation (Fig. 3 and 4). Four of the cell lines studied here have been reported to contain EBV: Josh 7, a precursor B-cell line with Fc and C_3 receptors

Figure 3: Southern blot analysis of adenoviral DNA synthesis in cultured human B and T lymphoid cells. Cultured B and T cells were infected with Ad2 at 30 PFU per cell, as were HeLa cells. At the days p.i. indicated (numbers at the tops of lanes), 10^6 cells (except for lane 1.5 of 2132, in which only 2 X 10^6 cells were used) were withdrawn, and total DNAs were isolated as previously described (Mok et al., 1985). The DNAs were digested with restriction enzyme EcoRI, separated by agarose gel electrophoresis, and analyzed by the Southern method with ^{32}P-labeled nick-translated Ad2 DNA as a probe. M, kb indicates HindIII-digested λ DNA as size markers. The designation 0 represents 2 h from the beginning of viral adsorption, and X represents the time required for extended exposure of the autoradiograph to account for reduced loading of DNA in lane 1.5 of 2132, with the exposure time for HeLa cells being 1X. The numbers at the right indicate molecular size in kilobases.



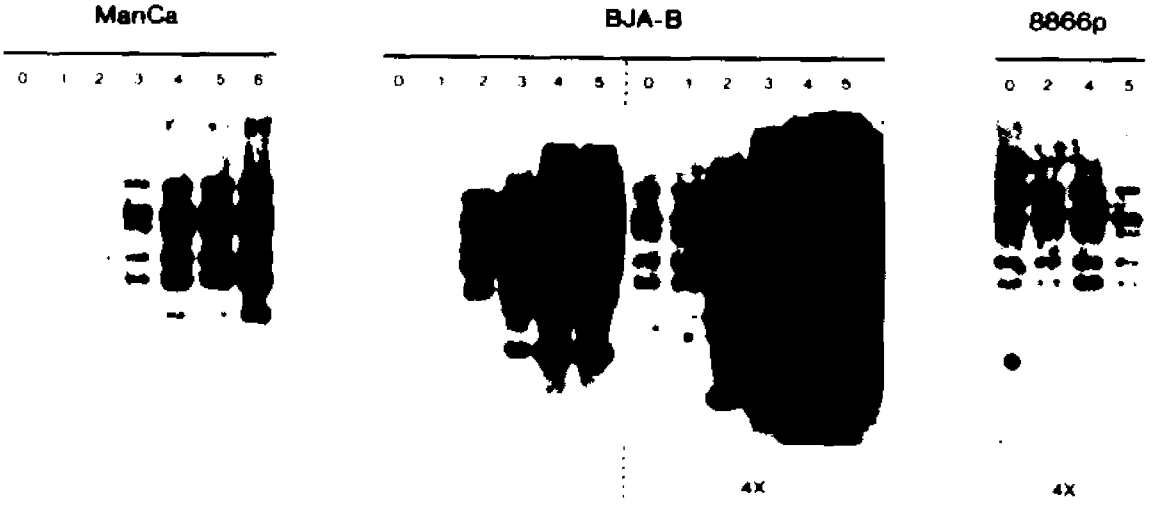


Figure 4: Southern analysis of adenoviral DNA synthesis during prolonged Ad5 infection. Manca, BJA-B, and 8866p cells were infected with Ad5 at 20 PFU per cell. Total DNAs were isolated after 1 h of adsorption (0) or the indicated number of days (1 to 6) p.i., digested with restriction enzyme HindIII, and analyzed by the Southern method with "-labeled nick-translated Ad5 DNA as a probe. A X symbol represents time required for extended exposure of the autoradiograph in order to detect adenovirus DNA in 8866p, with exposure time for Manca cells being 1X.

but no demonstrable immunoglobulin synthesis (Fu et al., 1980): SeD, a cell line producing predominantly membrane-bound and secreted IgM (McCune et al., 1980); 32a1, a cell line synthesizing both membrane-bound and secreted IgA (McCune et al., 1981); and RPMI 8866p, which produces predominantly secreted IgG (McCune and Fu, 1981). Three EBV-negative cell lines were used: GM 2132 (RPMI 8226), a λ light-chain-producing myeloma cell line (Matsuoka et al., 1967); Manca, a cell line derived from a patient with gastric lymphoma (S. M. Fu, unpublished data); and BJA-B, a Burkitt's lymphoma cell line (Menezes et al., 1975). Since EBV was used for establishment of most B lymphoid cell lines, we first confirmed the absence of the EBV genome in cell lines reported to be EBV negative by Southern blot analysis by using as a probe a cloned BamHIW fragment of the EBV genome (kindly provided by W. Sugden). Absence of the EBV genome in 2132, Manca, and BJA-B cells was confirmed as shown in Fig. 5 (lanes 2, M, and B, left panel), for which equal amounts of total cellular DNA of each line were analyzed (ethidium bromide-stained gel in the right panel), but only the DNA probe itself (lane E) and an EBV-positive reference cell line, Daudi (lane D)(Klein et al., 1968), were detectable by the EBV probe.

Adenovirus replicated in all of the B lymphoid cell lines studied, with the exception of cell line 8866p. Viral replication in the myeloma cell line 2132 was most efficient. The amount of adenoviral DNA present was comparable to that in HeLa cells at day one after infection at 30 pfu/cell (Fig. 3), or day 2 after infection at 10 pfu/cell (see section IIIA, Fig. 14). Viral replication in other B-cell cultures was

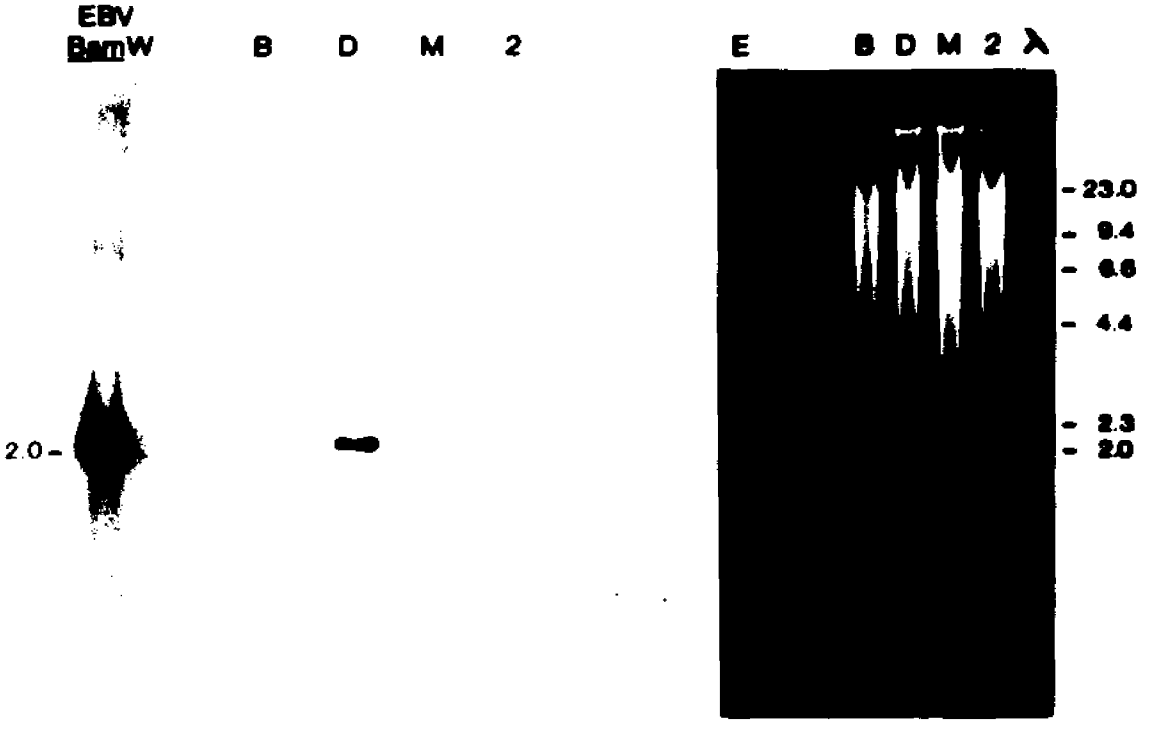


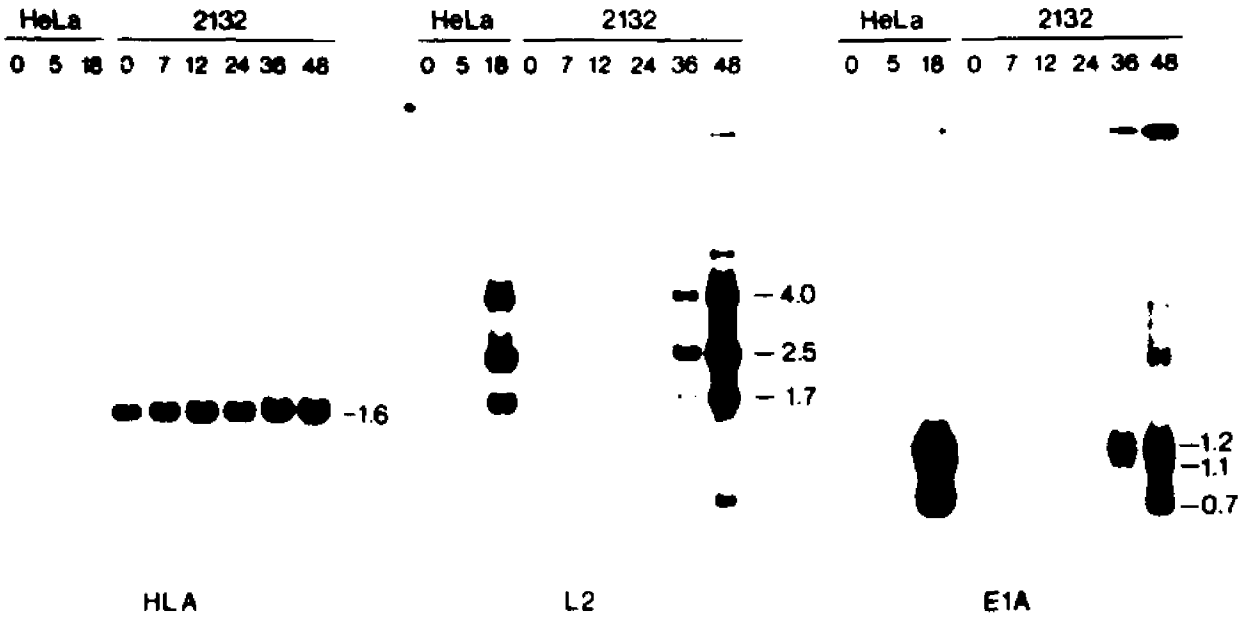
Figure 5: Southern blot analysis of the EBV genome in B cell lines. BamHI-digested DNAs (10 μg) from the four B cell lines (BJA-B [B], Manca [M], and 2132 [2] are EBV ; Daudi [D] is reported to be EBV [Klein et al., 1968]) were analyzed by the Southern method along with 100 pg of BamHI-W fragment (E; courtesy of W. Sugden) as a control. A nick-translated ^{32}P-labeled EBV BamHI-W DNA fragment was used as a probe for hybridization. The ethidium bromide-stained gel is shown on the right. λ represents HindIII-digested λ DNA as size markers. The numbers at the right indicate molecular size in kilobases.

considerably slower than in 2132 cells (Figs. 3, 4). Analysis of prolonged infection suggested a 10-fold increase of viral DNA in Manca cells at 6 days p.i. and that, after a delay of 1 day, viral replication in BJA-B cells was well supported for at least 5 days (Fig. 4). Viral DNA could be detected in 8866p cells immediately after adsorption (Fig. 4, lane 0, panel 8866p) but increased only negligibly at day 4. We found in repeated experiments that, with the same multiplicity of infection, replication of adenoviral DNA in B lymphoid cells, as in T cells, was reproducible and characteristic for each cell line. Furthermore, there appeared to be no apparent correlation between the kinetics of viral replication and the developmental stage from which the B lymphoid cell lines were isolated. Contrasting with earlier reports by Faucon et al. (1974), our data showed that adenoviral DNA replication was also not dependent on the presence of the EBV genome in B lymphoid cells.

Viral RNA synthesis. The presence of adenoviral RNA in lymphoid cells in the course of infection was assayed by Northern blot analysis with the following probes: c131 (Perricaudet, 1979), a cDNA clone for the E1A mRNAs; pHindIII-D, a genomic clone specific for the L-2 family of the Ad2 late mRNAs; and, as a control, a probe specific for human class I major histocompatibility antigen (Sood et al., 1980).

Myeloma cells 2132 and HeLa cells were infected with Ad2 at 10 PFU per cell, and total polyadenylated RNA from 10' cells was isolated at various times p.i. for assay. At this multiplicity of infection, viral DNA replication was detectable after 30 h p.i. in 2132 cells. The three major L-2 mRNA species, identical to those accumulated at 18 h p.i. in HeLa cells, were seen in 2132 cells at 36 h p.i. By 48 h p.i., the L-2

Figure 6: Synthesis of adenoviral mRNAs in human B lymphoid cells. Total poly(A)$^+$ RNAs were isolated (Chirgwin et al., 1979) from 10^7 2132 myeloma or HeLa cells at the indicated number of hours p.i. with Ad2 (10 PFU per cell). After formaldehyde denaturing agarose gel electrophoresis, RNAs on the same blot were analyzed by the Northern technique with the following probes: ^{32}P-labeled nick-translated inserts of clones pDP001 for HLA (Sood et al., 1980), c131 for E1A (Perricaudet et al., 1980), and pHindIII-D for Ad2 L2 mRNAs. The numbers at the right indicate molecular size in kilobases.


HeLa
2132
0 5 18 0 7 12 24 36 48
HeLa
2132
0 5 18 0 7 12 24 36 48
HeLa
2132
0 5 18 0 7 12 24 36 48
-1.6
-4.0
-2.5
-1.7
-1.2
-1.1
-0.7
HLA
L2
E1A

mRNA levels were equal to or slightly higher than those in HeLa cells at 18 h p.i. (Fig. 6). These results demonstrated that late viral mRNAs were synthesized appropriately after viral DNA replication. Similar results were obtained for other late adenoviral mRNAs and in other lymphoid cell lines (data not shown).

Late in infection, the steady-state levels of E1A mRNAs in 2132 cells at 48 h p.i., however, were reduced to 1/20th of those in HeLa cells at 18 h p.i., when late mRNAs were at comparable levels (Fig. 6). A longer exposure of the autoradiograph presented in Fig. 6 showed that the 13S (1.2-kilobase) E1A mRNA was detectable early in infection in 2132 cells, at 12 h p.i. The differential levels of E1A mRNAs in lymphoid and nonlymphoid cell backgrounds suggested that viral early genes are differentially regulated in lymphoid cells.

As a control, the levels of steady-state mRNAs for the host human class I histocompatibility antigen HLA-B were assayed. In the course of an 18-h adenoviral infection, HLA mRNA levels were maintained unchanged in HeLa cells. The slight increase in a 48-h infection period in 2132 cells may be attributed to the slower kinetics of infection, during which time cell mass also increased (Fig. 6).

Synthesis of viral proteins. Indirect immunofluorescent staining of adenoviral proteins with antisera directed toward purified Ad5 virion demonstrated that, in addition to synthesis of viral DNAs and RNAs, adenoviral proteins were translated in lymphoid cells. An example is shown in Fig. 7. Adsorption and internalization of infecting viral particles could be observed immediately after adsorption (O) and at early times p.i. (E) in myeloma 2132 cells as in HeLa cells. Late in

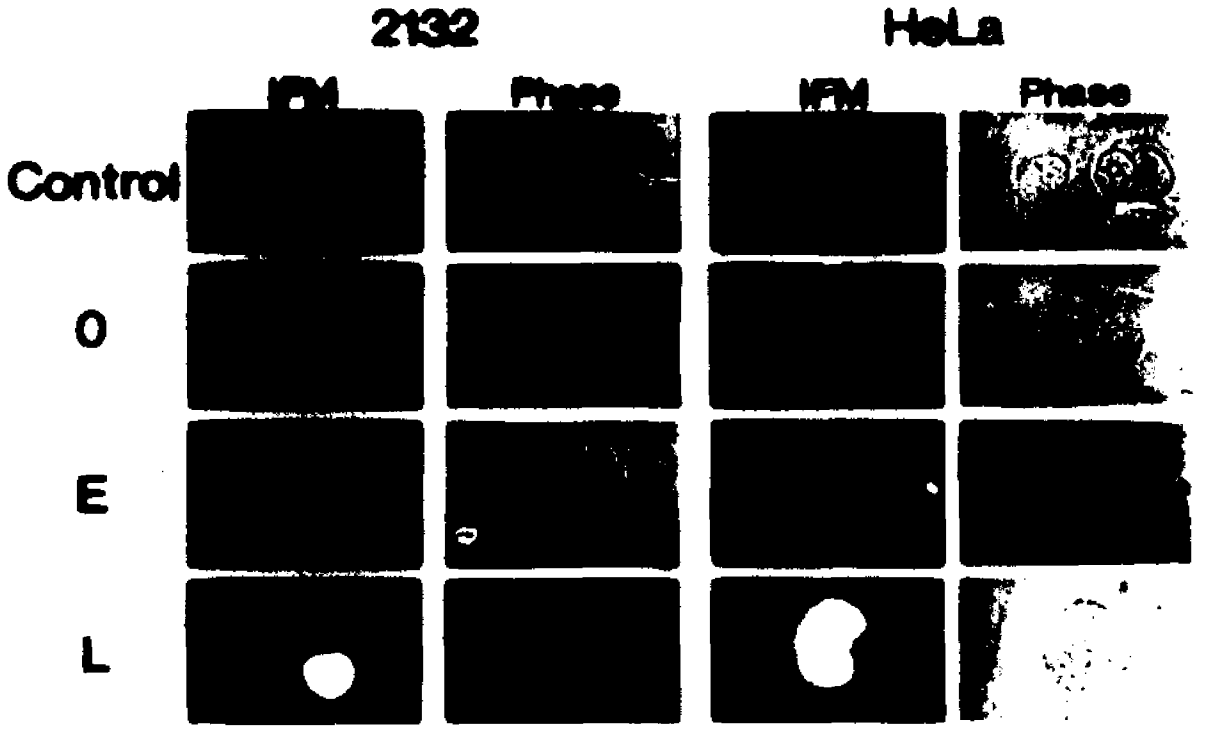


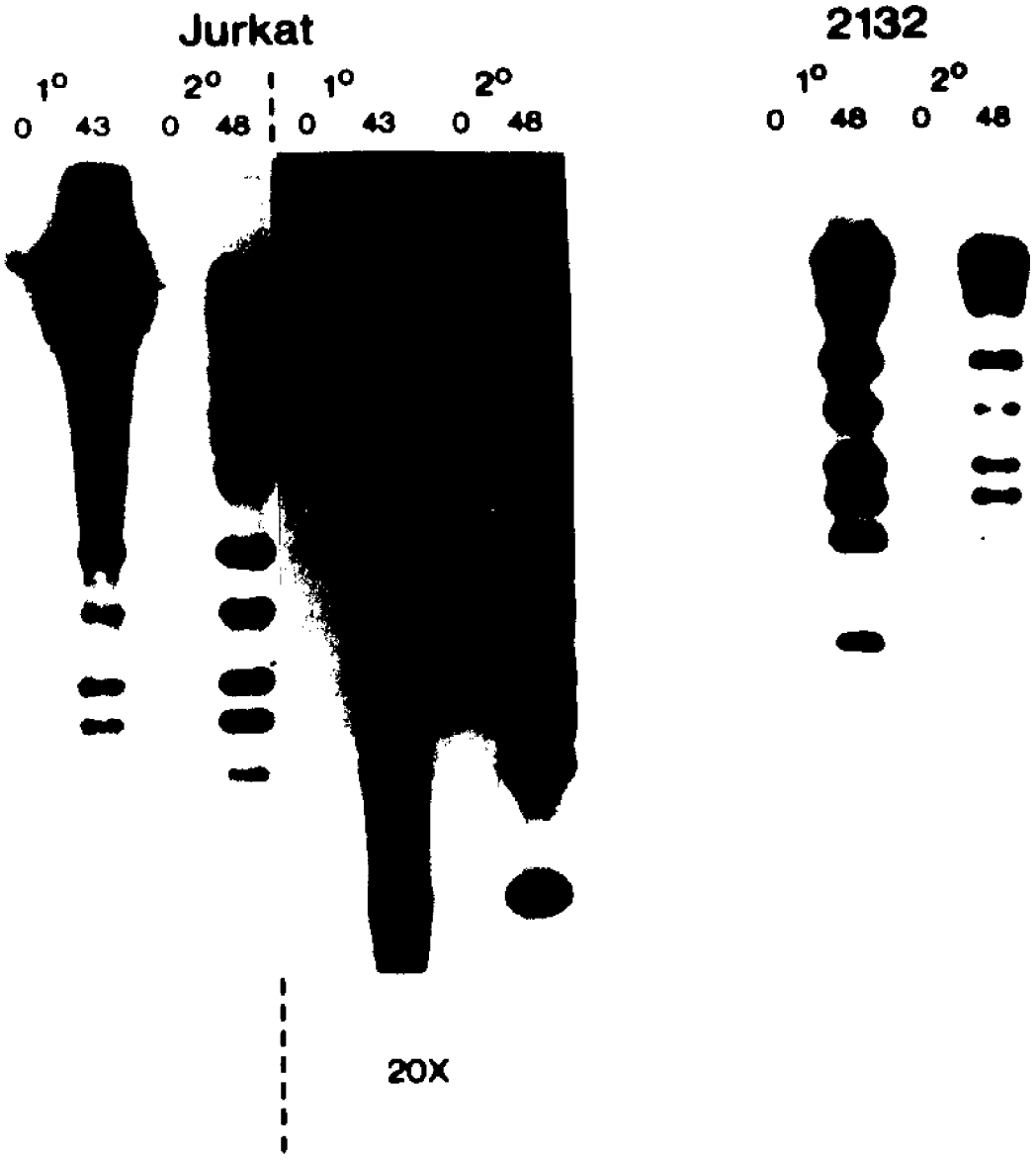
Figure 7: Indirect immunofluorescence and phase-contrast microscopy of Ad2-infected cells. Cells of an established myeloma cell line, 2132, and HeLa cells were infected with Ad2 at 20 PFU per cell and subjected to immunofluorescence as described in Materials and Methods. Samples were incubated first with rabbit antisera directed toward purified adenovirus virion and then incubated with fluorescein-conjugated goat antibody to rabbit IgG. Designations: IFM, immunofluorescent microscopy; Control, uninfected cells; 0, cells immediately after 30 min of adsorption at 37°C; E, cells at early times p.i. (5 h p.i. for HeLa and 7 h p.i. for 2132 cells); L, cells at late times p.i. (18 h p.i. for HeLa and 24 h p.i. for 2132 cells). All photographs were taken at X630 magnification.

infection, the virion proteins which reacted with the antisera were dramatically increased in 2132 cells as in HeLa cells. With our experimental protocol, at late times nearly all HeLa cells were immunofluorescence positive, and approximately 60% of 2132 cells stained positive for virion proteins. Similar positive results, albeit at lower percentages, were obtained in other B and T lymphoid cell lines.

Production of biologically active viruses. A two-cycle infection assay was used to determine whether active virions were produced in lymphoid cells. Ad5 virions prepared from HeLa cells and purified by cesium chloride gradients (Wall et al., 1972) were used to infect 2132 myeloma cells and Jurkat T cells at 20 PFU per cell. Total DNAs immediately after adsorption and at 43 (Jurkat) and 48 (2132) h p.i. were analyzed by Southern blot analysis with nick-translated total Ad5 DNA as a probe (Fig. 8, lanes 1°). Lysates were made from infected Jurkat and 2132 cells and used to infect an uninfected sample of the respective cell line from which the lysates were prepared (lanes 2°). Viral DNA synthesis in lymphoid cells during secondary infections was similarly assayed. Replication of adenoviral DNA during secondary infection demonstrated that biologically active virions were assembled in Jurkat and 2132 cells during primary infection and could be subsequently used to infect lymphoid cells.

A plaque assay was performed to quantify the yield of viruses from 2132 and Jurkat cells in the course of a 4-day infection with Ad2 at a multiplicity of infection of 10 PFU per cell. Taking into consideration the slight increase in cell number during the experimental period, 1.3-fold for 2132 cells and 2-fold for Jurkat cells, and the percentage of immunofluorescence-positive cells in the cultures, the virus produced

Figure 8: Synthesis of infectious viral particles in Ad2-infected human B and T cells. Human 2132 myeloma cells and Jurkat T cells were infected with Ad5 at 20 PFU per cell. At 48 h p.i. of 2132 and 43 h p.i. for Jurkat cells, DNAs from 4 X 10^6 cells were isolated as previously described (Mok et al., 1984). Lysates were made from the remainder of the infected 2132 or Jurkat cells and used for infecting 2132 and Jurkat cells, respectively. The multiplicity of the secondary infection was estimated to be 20 PFU per cell. DNAs were isolated from the secondary (2°), as well as the primary (1°) infection. Each DNA sample was digested with restriction enzyme KpnI and analyzed by the Southern blot method with ^{32}P-labeled nick-translated Ad2 DNA as a probe. X indicates the autoradiograph exposure time, that of 2132 cells being 1X.


Jurkat
1°
2°
0
43
0
48
1°
2°
0
43
0
48
20X
2132
1°
2°
0
48
0
48

per cell was 3,000 PFU for 2132 cells at 72 h p.i., 2,200 PFU for Jurkat cells at 96 h p.i., and 5,000 PFU for HeLa cells at 24 h p.i. (Fig. 9). Hence, the yield of viruses from lymphoid cells was comparable to that of nonlymphoid cells.

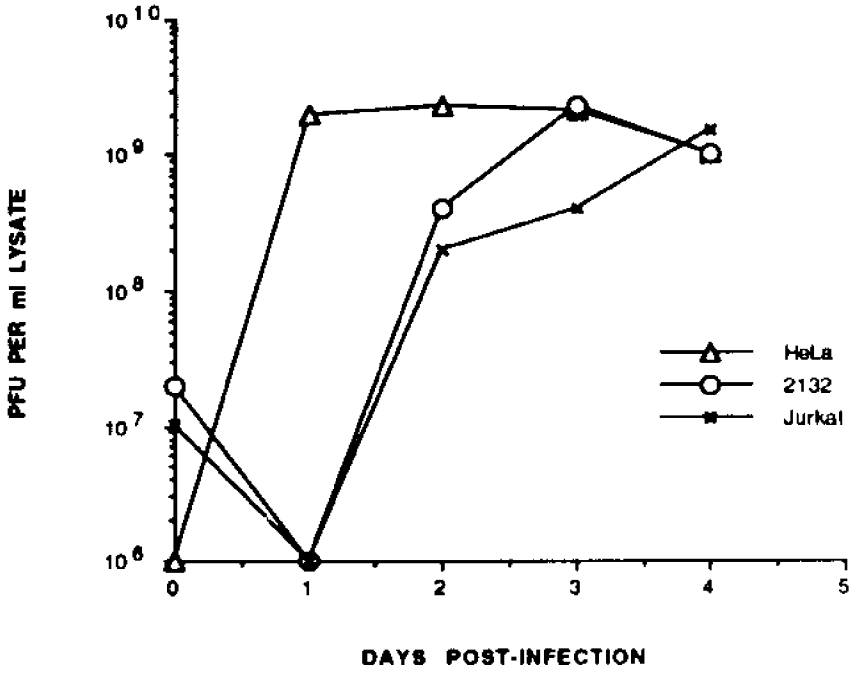


Figure 9: Plaque assay of viruses produced in 2132, Jurkat, and HeLa cells. Cultures at the log phase of growth were concentrated to 10^7 cells per ml, infected with Ad2 at 10 PFU per cell, and diluted to 2 X 10^6 cells per ml after a 30-min adsorption period. Lysates were prepared immediately after adsorption and on the days p.i. indicated. Plaque assay was performed on human A549 cells. Symbols: triangle, HeLa cells; circle, 2132 cells; X, Jurkat cells.

DISCUSSION

Our data demonstrated that infection of established B lymphoid cell lines by Ad2 or Ad5 was characteristic of each cell line regardless of its origin or the presence of the EBV genome. Results obtained from indirect immunofluorescent staining of infected cells and the cytopathic effect caused by infection with an Ad5 mutant (D. Lavery and S. Chen-Kiang, unpublished data), as well as the yields of viruses, suggested to us that the differences in infectivity among the cell lines were most likely due to the number of cells infected as opposed to the yield of infection by each cell in the culture. This has been pursued by other members of the laboratory. Replication of viral DNA in cell line 8866p was marginal. The basis for this exception is not understood at present. It appears, however, that the defect cannot be attributed to viral adsorption (Fig. 4).

With regard to natural infection of human lymphoid tissue by adenoviruses, previous investigators have found peripheral human lymphocytes to be largely refractory to infection by Ad2 or Ad5 (Strohl and Schlesinger, 1965; Van der Veen and Lambriex, 1973; Faucon and Desgranges, 1980). Our results with cultured lymphoid cell lines and peripheral blood lymphocytes (D. Lavery, S. M. Fu, and S. Chen-Kiang, unpublished data) suggest that whereas the entire peripheral lymphocyte population may not be productively infectable, an infectable subpopulation exists. Whether there is any functional relationship between the activation state of lymphocyte and its susceptibility to infection remains to be determined. This infectable subpopulation of lymphocytes may serve to carry adenoviral infection to other sites, where acute infection takes

place, a role previously suggested for both lymphocytes and fibroblasts (Strohl and Schlesinger, 1965; Faucon and Desgranges, 1980).

The presence of the EBV genome was postulated as enhancing the infectability of B lymphocytes by adenoviruses (Faucon et al., 1974; Faucon and Desgranges, 1980). Our results with more detailed investigations of many cell lines, however, indicated that the EBV genome was not detectable by Southern analysis in cell lines which permitted adenovirus replication. In particular, 2132, an EBV-negative myeloma cell line, showed the most rapid kinetics of infection of all of the lymphoid cells thus far studied. However, more sensitive analysis to detect the EBV genome, such as by polymerase chain reaction (PCR) analysis, has not been performed.

The levels of infection in other cell lines ranges from 6 to 60% of cells infected, much greater than those reported previously (Faucon et al., 1974). This must be in part due to the culture conditions we maintained, in which infected cells were not diluted by successive feeding, which would create a bias in favor of uninfected, dividing cells. Rapid and uniform infection of 2132 presents the intriguing possibility that regulation of the cellular genes specifically expressed in B lymphoid cells by adenoviral genes can by addressed. To this end, the expression of the immunoglobulin λ light chain gene was found to be significantly altered during adenovirus infection (see Section IV).

We demonstrated here for the first time that established T-cell lines could be infected by Ad2 with kinetics characteristic of each cell line as in lymphoid lines of B-cell origin. This would permit studies of

regulation of genes specifically expressed in host T cells by adenoviruses in cell lines such as Jurkat, which is highly infectable by the virus. Independently, H. Ginsberg and colleagues have also obtained results indicating that the Molt-4 (2219) cell line supports viral growth (H. Ginsberg, personal communication).

A recent report by Weber and colleagues showed that, by Southern blot analysis, several established cell lines contained very low levels of endogenous adenovirus genome, approximately one copy per cell (Horvath et al., 1986). However, Arrand et al. (1983), using stringent washing procedures after Southern hybridization, found that the endogenous viral genome bands were homologous but not identical to those of adenoviruses. Using stringent hybridization conditions (see Materials and Methods) and a 28S ribosomal clone to compete for nonspecific binding sites, we detected no discrete virus-specific DNA or RNA species in any lymphoid cell lines before infection by exogenous adenoviruses.

The well-characterized molecular genetics of adenovirus as well as its high copy number and episomal replication, have allowed it to be used by others as an effective vector system (Mansour et al., 1985). Successful infections of established human B- and T-cell lines, which are difficult to transfect, prompted us to modify adenovirus as an expression vector for lymphoid cells. This proved successful. Under control of the early region 1B promoter, the mouse immunoglobulin heavy-chain gene was found to be expressed as cell type specific mRNAs, independent of the immunoglobulin heavy-chain enhancer, and regulated posttranscriptionally (Ruether et al., 1986).

PART IIIA: ADENOVIRUS E1A AND E1B GENES ARE REGULATED POST-TRANSCRIPTIONALLY IN HUMAN LYMPHOID CELLS.

Results contained in Part III have been published (Lavery and Chen-Kiang, Journal of Virology, Volume 64, in press).

Abstract: The interactions of adenovirus with differentiated human cells have been investigated in human myeloma cells. Relative to HeLa cells, the E1A and E1B genes, but not other viral genes, were markedly repressed by differential RNA stabilization, resulting in 20- to 50-fold less E1A and E1B mRNAs at steady state late in infection. The reduced E1A level corresponded to an approximately 200-fold lower abundance of E1A polypeptides, which were nonetheless capable of efficient transactivation of E1A-dependent viral genes and were necessary for productive infection. The E1B gene was further regulated posttranscriptionally, yielding altered molar representation of alternatively spliced 22S and 13S mRNAs early in infection of myeloma cells. Taken together, these results suggested that repression and altered expression of E1A and E1B genes may provide a molecular basis of delayed kinetics of infection of lymphoid cells with adenovirus (D. Lavery, S. M. Fu, T. Lufkin, and S. Chen-Kiang, J. Virol. 61:1466-1472, 1987). The molecular mechanisms by which E1A and E1B are regulated and by which E1A transactivates viral genes in lymphoid cells are discussed.

INTRODUCTION

The E1A and E1B genes of adenovirus types 2 and 5 (Ad2 and Ad5) have been extensively studied as models of normal and oncogenic gene expression. The E1A gene is capable of efficient transcriptional activation of adenovirus early genes and many other viral and cellular genes, transcriptional repression of certain enhancer-driven genes, and induction of host DNA synthesis (for a review, see Moran and Mathews, 1987). The functions of the E1B gene products are less well defined. The E1B 55 kDa polypeptide binds to p53 (Sarnow et al., 1982) and has also been shown to be important for reducing the accumulation of host mRNAs after infection (Babiss et al., 1985; Pilder et al., 1986). The E1B 19 kDa polypeptide is suggested to be necessary for maintaining the integrity of viral DNA (Pilder et al., 1984; White et al., 1984). Furthermore, E1A and E1B genes together can induce transformation of primary rodent cells in culture (for a review, see Graham, 1984).

Although the functional significance of the E1A and E1B genes has been widely recognized and analyzed, little is known of the regulation of their own expression. By labeling of RNA to steady state, Wilson and Darnell (1981) showed that E1A and E1B transcripts were regulated posttranscriptionally in the viral infectious cycle in HeLa cells. The half-lives of E1A 13S and 12S mRNAs and the E1B 13S mRNA encoding the 19-kDa polypeptide increased from approximately 10 min early in infection to 45 min or greater late in infection.

We previously showed that late in productive infection of a human myeloma cell line, the steady state E1A mRNA level was 1/50 of that in HeLa cells late in infection (Lavery et al., 1987). The transcriptional

and post-transcriptional contributions to the differential accumulation of E1A mRNA have been further investigated. It is shown in this report that the reduced E1A mRNA accumulation in human myeloma cells can be attributed primarily to the instability of E1A mRNA in differentiated cells throughout infection. Likewise, the mRNAs of the other transforming gene, that encoding E1B, but not other adenoviral early mRNAs, are also less stable in myeloma cells late in infection. Furthermore, the two alternatively spliced E1B mRNAs are represented in different molar ratios in myeloma and HeLa cells early in infection. Despite reduced E1A gene expression, however, E1A-dependent genes are transcribed in myeloma cells as efficiently as in HeLa cells. These results imply that E1A and E1B genes are regulated by and interact with differentiated cells in a manner distinct from that with HeLa cells.

RESULTS

Accumulation of mRNA from E1A and E1A-dependent adenovirus transcription units is differential. Previously, we showed that synthesis of viral RNA, DNA, and biologically active particles after adenovirus infection proceeded with slower kinetics in lymphoid cells than in HeLa cells. After infection with Ad5 at 10 PFU per cell, viral DNA replication was not detectable until 24 h p.i. in the human myeloma cell line 2132, compared with 8 h p.i. in HeLa cells. Furthermore, accumulation of E1A mRNA late in infection at 48 h p.i. in 2132 cells was only 1/50 of that in HeLa cells at 18 h p.i., when DNA replication and the synthesis of mRNA from the L2 family of the major late transcription unit were comparable (Lavery et al., 1987).

The kinetics of accumulation of E1A 13S and 12S mRNA in 2132 cells and HeLa cells during viral infection was investigated by Northern blot analysis of steady-state RNA (Fig. 10). The E1A mRNA levels in 2132 cells were at least 20-fold less than those in HeLa cells throughout viral infection, including early times before the appearance of detectable viral DNA replication. Although the 13S and 12S mRNAs migrated very closely to each other in a denaturing agarose gel, RNase protection experiments showed that the two alternatively spliced E1A mRNAs were represented in similar molar ratios in the two cell lines (D. Lavery and S. Chen-Kiang, unpublished data). Thus, the 50-fold reduction of E1A mRNA in 2132 cells relative to HeLa cells late in infection is likely due to intrinsic differences in virus-host interactions and not restricted to late times after infection.

The E1A gene products are required for transactivation of viral transcription units E1B, E2, E3, and E4 as well as of E1A itself (Berk, 1986; Flint and Shenk, 1989). The possibility that reduced levels of E1A mRNA may correspond to reduced amounts of E1A polypeptides and lead to reduced expression of E1A-dependent genes was examined. Northern blot analysis showed that late in infection, when the levels of L2 mRNA were similar, the mRNA levels of E2, E3, and E4 were also comparable in these two cell lines (Fig. 1 and 11A). This result is dramatically different from the greater than 50-fold difference of E1A levels in the two cell types, suggesting that the lack of accumulation of viral mRNA in 2132 cells may be specific for the E1A gene.

However, before the onset of viral DNA replication, E3 and E4 mRNAs appeared to accumulate more slowly in 2132 cells than in HeLa cells (Fig.

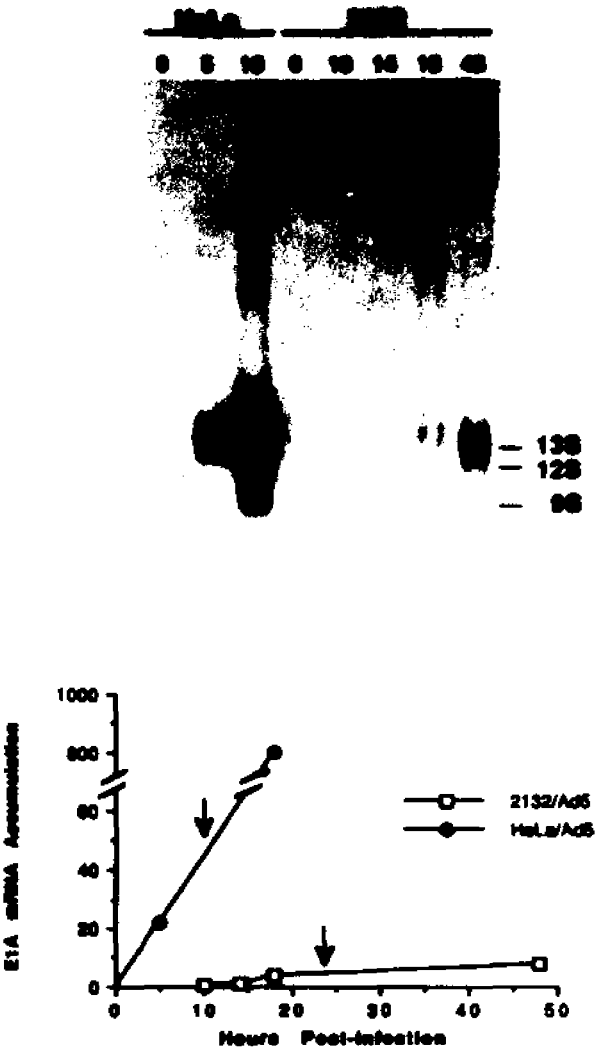
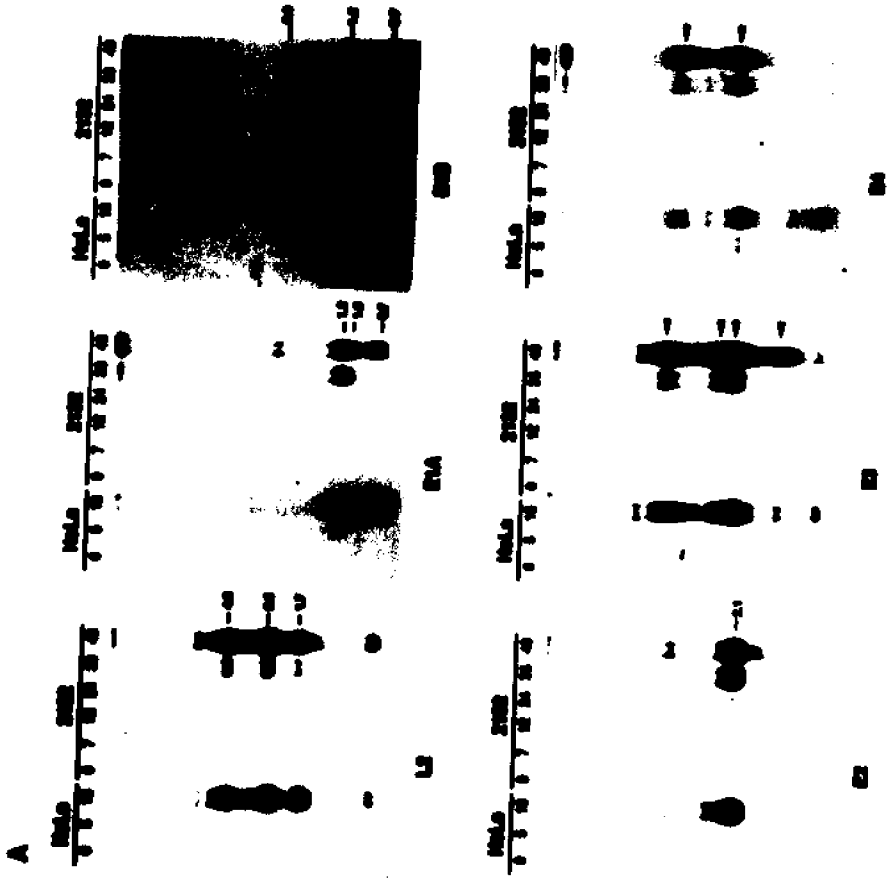
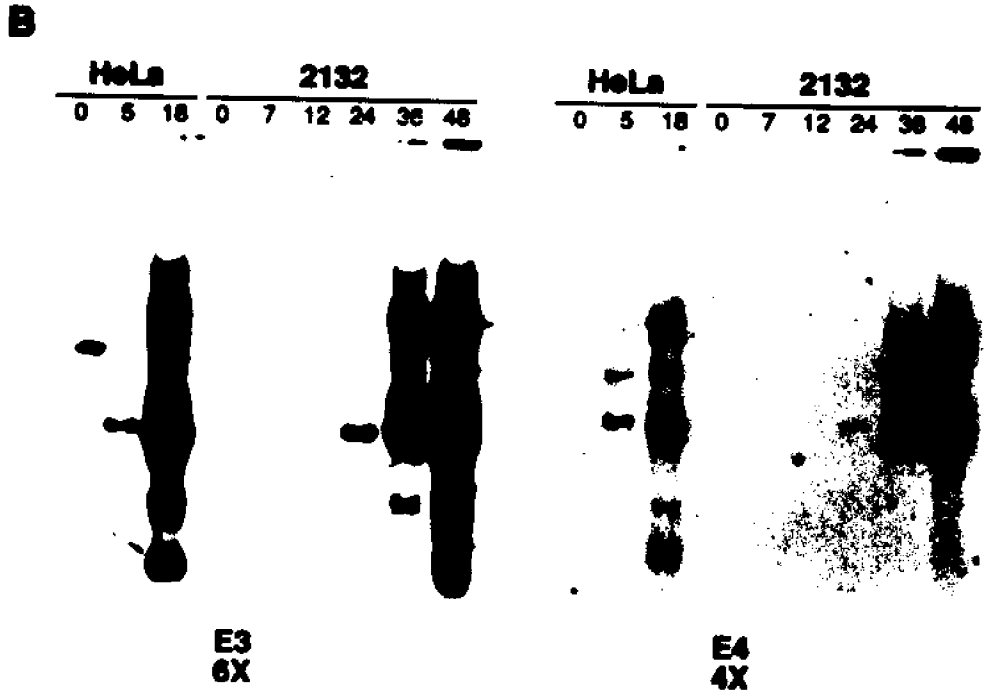


Figure 10: Northern blot analysis of E1A mRNA in Ad2-infected 2132 cells and HeLa cells. Poly(A)$^+$ RNAs from 2.5 X 10^7 HeLa and 2132 cells were isolated at the indicated hours after infection with Ad5 and analyzed for E1A mRNA as described in Materials and Methods. The autoradiographic signals are presented as a function of hours pi. Arrows indicate the earliest time after infection at which viral DNA replication was detected by Southern blot analysis (Lavery et al., 1987).

Figure 11: Northern blot analysis of viral mRNA accumulation in Ad-5-infected HeLa cells and 2132 cells. Poly(A)$^+$ RNAs were isolated from 10^7 cells at the indicated hours after infection with Ad5 and analyzed as for Fig. 2. (A) Viral mRNAs were detected by sequential probing of the same blot with nick-translated probes specific for each viral transcription unit as described in Materials and Methods. (To the right of each panel are the molecular sizes, in kilobases, of the major mRNA species detected.) The 1.2-, 1.0-, and 0.7-kb mRNA species of E1A correspond to the conventionally designated E1A 13S, 12S, and 9S mRNA species, respectively. The 2.3-, 1.2-, and 0.7-kb mRNA species of E1B correspond to the E1B 22S, 13S, and 9S mRNAs, respectively. The major E2, E3, and E4 mRNA species are represented by size in kilobases or by arrowheads according to Babich and Nevins (1981), Bhat et al. (1986), and Virtanen et al. (1984), respectively. (B) Extended exposure of blot probed for Ad-5 E3 and E4 mRNAs presented in panel A. X represents the exposure time relative to that in panel A.



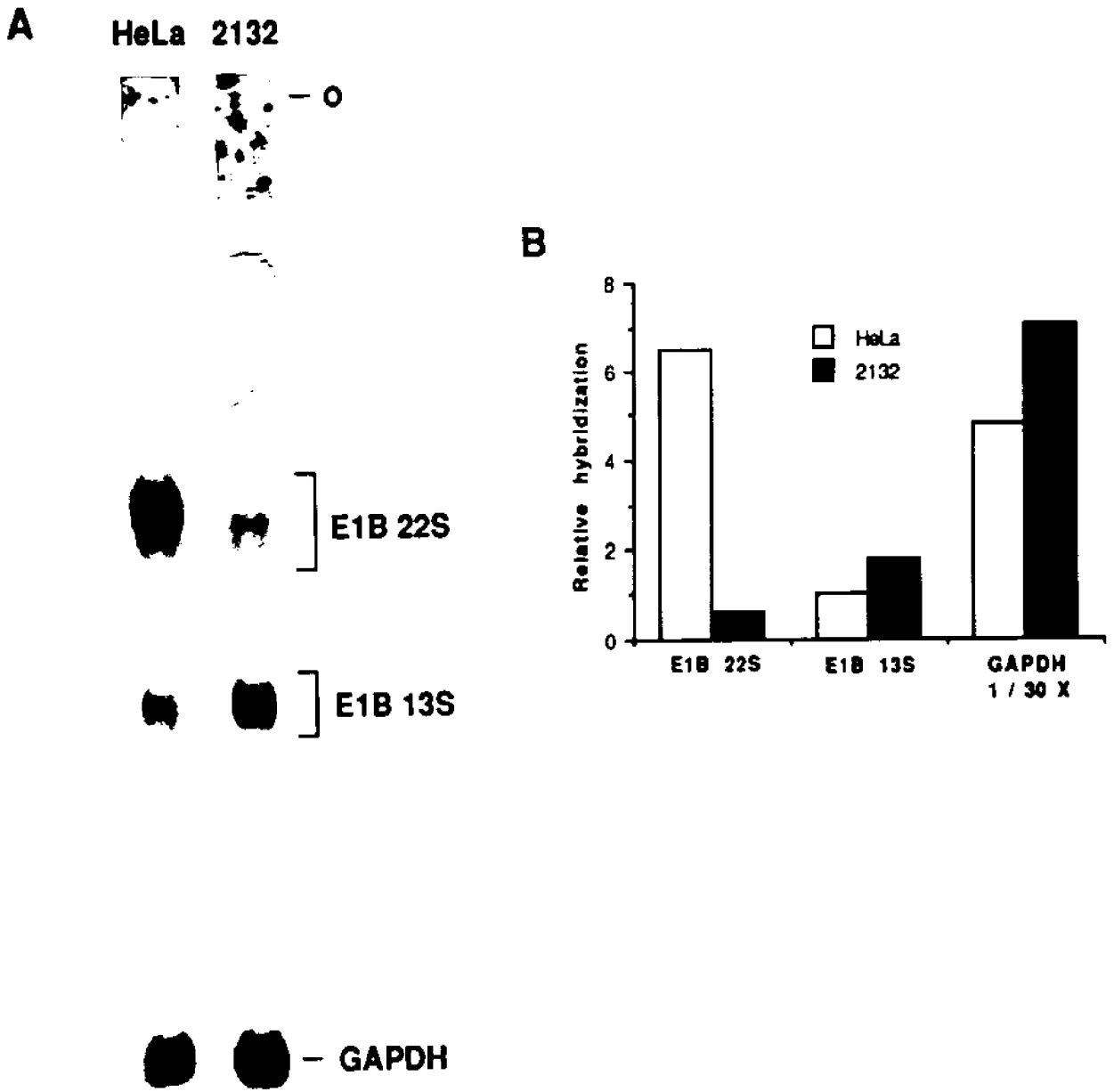

B
HeLa
2132
0 5 18 0 7 12 24 36 48
HeLa
2132
0 5 18 0 7 12 24 36 48
E3
6X
E4
4X

11B; compare lane 5 [HeLa] with lane 12 [2132]). Once viral DNA replication was detectable at 24 h p.i., there was a rapid increase in E3 and E4 mRNAs, such that by 48 h p.i. they reached levels comparable to those in HeLa cells at 18 h p.i. (Fig. 11B). This delay, followed by rapid mRNA accumulation, is consistent with the possibility that a threshold level of E1A gene expression must be reached before efficient activation of E1A-dependent genes can take place.

The two E1B mRNAs are represented in altered molar ratio early in infection and reduced levels late in infection in 2132 cells. The E1B transcripts are alternatively spliced into 22S and 13S mRNAs (Fig. 2). In contrast to the comparable levels of E2, E3, and E4 mRNAs in 2132 and HeLa cells, the E1B 22S and 13S mRNAs accumulated to a 20-fold reduced level late in infection in 2132 cells, as was the case with E1A mRNAs (Fig. 11A, E1B). Together with the E1A gene, the E1B gene is required for complete transformation of rodent cells (Graham, 1984). The reduced mRNA accumulation therefore appears to be specific to both transforming genes in 2132 cells. Consistent with this hypothesis, the steady-state level of the 9S E1B mRNA for a structural polypeptide protein IX, which is transcribed from a distinct, internal promoter (Alestrom et al., 1980; Fig. 1) and is not required for E1B transformation functions, was similar in the two cell lines (Fig. 11A).

Further analysis of polyadenylated RNAs isolated early after infection at 14 hr p.i. from 2132 cells revealed that the E1B mRNA levels were approximately one-third of those in HeLa cells early in infection at 5 h p.i. (Fig. 12). Since transcription of the E1B gene early in infection was twofold less in 2132 cells than in HeLa cells (see Fig. 15),

Figure 12: Northern blot analysis of E1B mRNAs in HeLa cells and 2132 cells early in infection. (A) Poly(A)$^+$ RNAs from 2 X 10^7 Ad2-infected HeLa and 2132 cells at 5 h and 14 h p.i., respectively, were isolated and analyzed by Northern blot analysis as for Fig. 2. A random-priming ^{32}P-labeled E1B cDNA probe, c244, was used to detect E1B 22S and E1B 13S mRNAs. As a control, the same blot was subsequently hybridized with a ^{32}P-labeled cDNA probe for the cellular GAPDH gene. (B) Relative abundances of E1B mRNAs after densitometric tracing of hybridization signals shown in panel A. The level of E1B 13S mRNA in HeLa cells is arbitrarily assigned 1 unit of density.

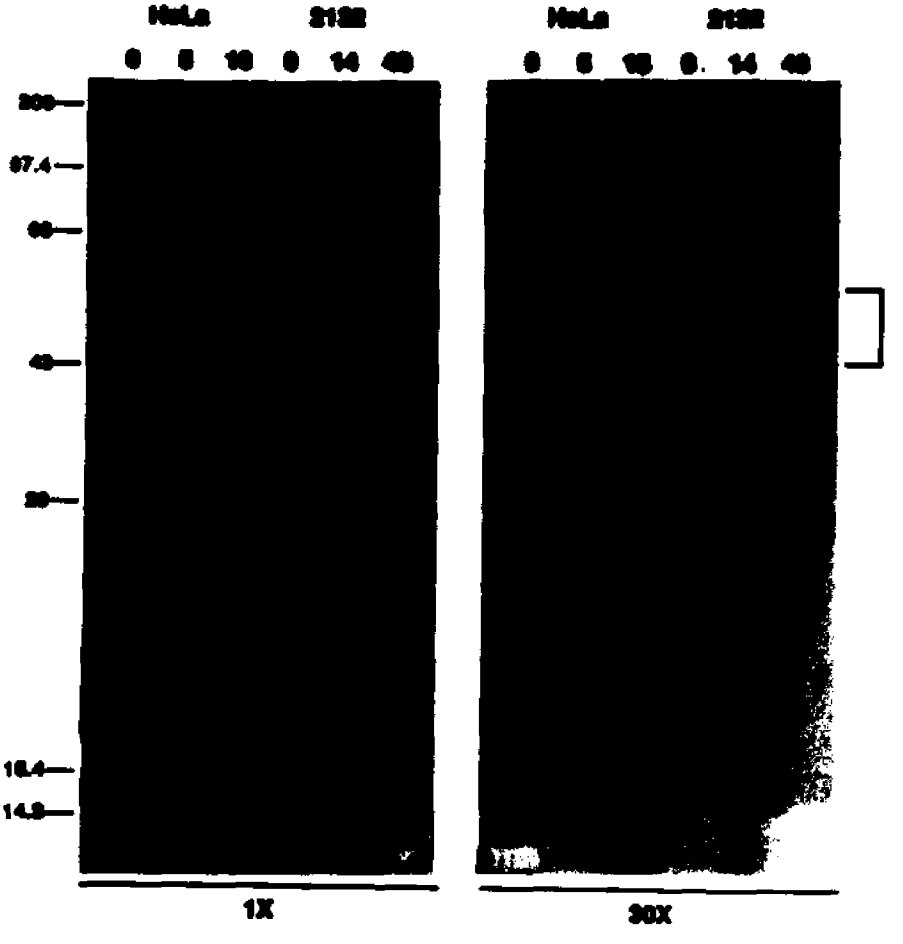

A
HeLa
2132
O
E1B 22S
E1B 13S
GAPDH
B
Relative hybridization
0
2
4
6
8
HeLa
2132
E1B 22S
E1B 13S
GAPDH
1 / 30 X

the difference in steady state E1B mRNAs is most likely reflective of differences in the rate of transcription in the two cell lines.

Surprisingly, whereas the 13S mRNA predominated late in infection in both cell lines (Figure 11A), early in infection the molar ratio of 22S mRNA to 13S mRNA, 6.5:1 in HeLa cells, was inverted (0.6:1.8) in 2132 cells (Fig. 12). The representation of these two mRNA species in HeLa cells (Fig. 11A and 12) is reminiscent of previous results reported by Wilson and Darnell (1981). The authors suggested that the increasing dominance of 13S mRNAs from early to late in infection in HeLa cells was due to stabilization of 13S mRNAs late in infection. In 2132 cells, the 13S mRNA was found to be dominant throughout infection (Fig. 11A and 12). The lack of accumulation throughout infection of E1B 22S mRNA, whose translation product is important for reducing host mRNA accumulation in the cytoplasm (Babiss et al., 1985; Pilder et al., 1986), may provide a molecular basis for the prolonged early phase after infection of 2132 cells (see Discussion). Furthermore, the 22S mRNA species from 2132 cells migrated consistently faster in denaturing gels than that from HeLa cells. This phenomenon may be associated with differences in poly(A) tail length (see Section IIIB).

E1A polypeptide levels are reduced in lymphoid cells. To determine whether reduced E1A mRNA accumulation in 2132 cells is effected at the protein level, E1A polypeptides in infected cells were immunoprecipitated with an E1A-specific mouse monoclonal antibody (Harlow et al., 1985; Harlow and Lane, 1988). Results of lysates prepared from cells labeled with [^{35}S]methionine for 3 h are presented in Figure 13; similar results are obtained from cells labeled for 2 h. E1A polypeptides, migrating at

Figure 13: Immunoprecipitation of E1A polypeptides in Ad2-infected HeLa and 2132 cells. [^{35}S]methionine-labeled lysates prepared from HeLa and 2132 cells at the indicated hours after infection with Ad2 were immunoprecipitated with a monoclonal antibody directed against an Ad5 E1A fusion polypeptide (21). Immunoprecipitates from 10' cells were denatured and separated on a 10% SDS-polyacrylamide gel. After fluorography using En^{3}Hance (New England Nuclear), the gel was dried and exposed to X-ray film. The molecular mass (in kilodaltons) of prestained protein standards (Bethesda Research Laboratories) are shown on the left. A 30-fold extended exposure is presented (30X) to reveal polypeptide in low abundance. The bracket at the right indicates the 48- to 52-kD region where E1A 13S and 12S mRNA-derived phosphoproteins are expected to migrate in an SDS-polyacrylamide gel (21).


HeLa
2122
0 6 10 0 14 40
HeLa
2122
0 6 10 0 14 40
200
97.4
68
43
29
18.4
14.3
1X
30X

approximately 48 to 52 kDa on a denaturing SDS-gel, were detectable at 5 h p.i. in HeLa cells and were increased by 40-fold late in infection at 18 h p.i. (Fig. 13). Early in infection in 2132 cells, the E1A polypeptides were estimated to be 1/10 of those in HeLa cells at a comparable stage of infection (compare lane 14 [2132] with lane 5 [HeLa] in Fig. 13). This difference was enhanced at comparable times late in infection: the E1A polypeptides in 2132 cells were less than 1/200 of those in HeLa cells (compare lane 48 [2132] with lane 18 [HeLa] in Fig. 13). These results confirm that the differences in the E1A mRNA levels are reflected at the protein level. They also suggest that in adenovirus infected myeloma cells, the E1A functions are derived from a very limited amount of E1A polypeptides available.

E1A gene is essential for viral replication in 2132 cells. Despite the differences in the amount of E1A polypeptides in 2132 and HeLa cells, adenovirus infection of 2132 cells is productive, yielding biologically active particles at levels comparable to those found in HeLa cells (Lavery et al., 1987). One possible explanation is that the E1A gene is dispensable for infection of 2132 cells as a result of complementation of its functions by cellular genes.

To test this hypothesis, infections were performed by using an Ad5 mutant, dl312, which carries a 902 bp deletion within the E1A gene, rendering it incapable of E1A expression (Jones and Shenk, 1979; Shenk et al., 1979; a kind gift of Dr. C. S. H. Young). Productive infection would occur if 2132 cells contained a factor(s) that complements E1A functions, as in the case of dl312 infection of 293 cells, a human embryonic kidney cell line that is transformed by and constitutively expresses the E1A and

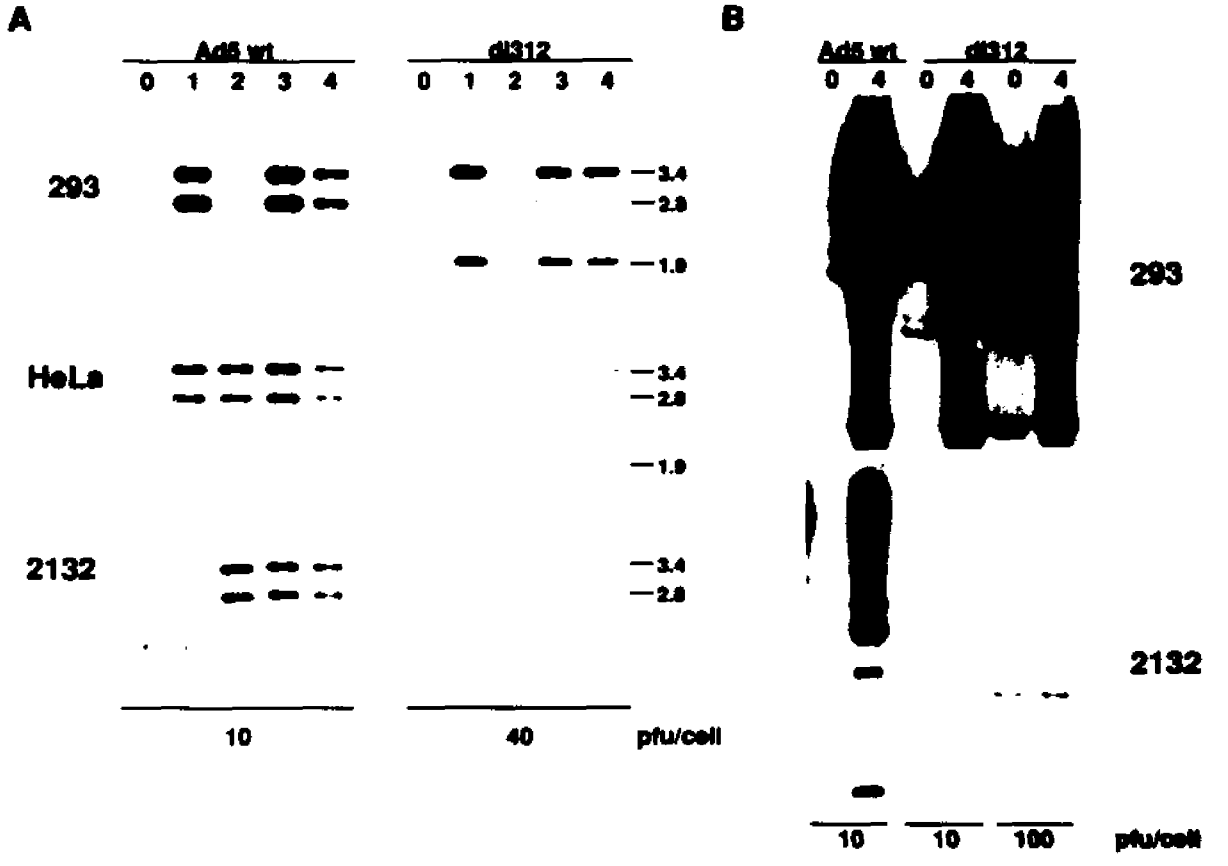
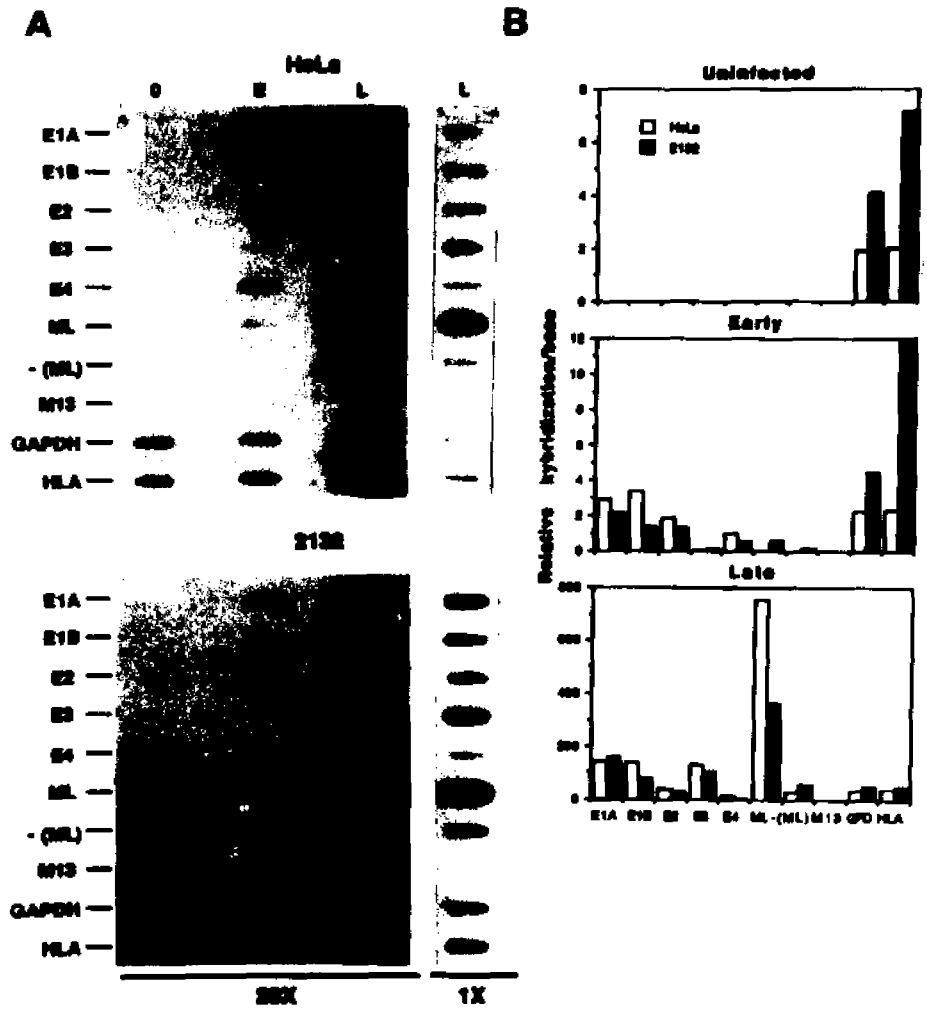


Figure 14: Southern analysis of Ad-5 and dl312 replication. (A) Total DNAs isolated from 10^4 HeLa, 293, and 2132 cells at the indicated days after infection with wild-type (wt) Ad5 (10 PFU per cell) or dl312 (40 PFU per cell) were digested with restriction enzyme HindIII and analyzed by Southern blotting. The ^{32}P-labeled probe used was the 5.4-kb XhoI-C DNA insert from plasmid p3wt18, which distinguishes the E1A region HindIII restriction fragments derived from the E1A region of wild-type virus (3.4- and 2.8-kb fragments) and dl312 mutant virus (3.4- and 1.9-kb fragments), as indicated. (B) Total DNAs isolated from 10^4 293 and 2132 cells at the indicated days after infection with Ad5 (10 PFU per cell) or dl312 (10 or 100 PFU per cell) were analyzed as in panel A except that the ^{32}P-labeled probe used was restriction-digested total Ad5 DNA.

E1B genes (Graham et al., 1977; Fig. 14). Viral replication, albeit with delayed kinetics, was detectable in HeLa cells infected with dl312 at 40 PFU per cell (Figure 14A; Shenk et al., 1979). However, no detectable viral replication was observed when 2132 cells were infected with dl312 at multiplicities of infection ranging from 10 to 100 PFU per cell (Fig. 14). Thus, in 2132 cells, the E1A gene is essential for productive infection by adenovirus. Although cellular factors must contribute to the viral infection, they cannot complement an E1A-deletion mutant, and the E1A gene, though expressed at 10- to 100-fold-reduced levels, is nonetheless required for productive infection of 2132 cells.

Transcription of E1A and E1A-dependent genes is comparable in 2132 cells and HeLa cells. Nuclear run-on experiments were performed to determine whether reduced accumulation of E1A and E1B mRNAs in 2132 cells was due to reduced transcription and to determine whether reduced levels of E1A polypeptides would influence transcription of E1A-inducible viral genes (Fig. 1). No significant difference was observed in the loading of polymerase on the E1A gene in 2132 and HeLa cells early and late in infection (Fig. 15). Therefore, relative to the level in HeLa cells, the reduced accumulation of E1A mRNA in 2132 cells was primarily due to instability of E1A transcripts. Furthermore, early in infection, the loading of polymerase on other E1A-dependent adenovirus genes ranged from 2-fold reduced for E1B to no appreciable difference for E2 and E4. E3 transcription appeared not to be reduced at all. Late in infection, when the abundance of E1A polypeptides differed by 200-fold in the two cell types, rates of transcription of E1A, E1B, E2, and E4 were comparable. The only consistent difference in rates of transcription was observed for

Figure 15: Nuclear run-on analysis of transcription in Ad5-infected 2132 cells and HeLa cells. Nuclei isolated from 10^6 HeLa and 2132 cells that were uninfected (0) or were early (E) or late (L) in infection by Ad2 were pulse-labeled with [^{32}P]UTP for 10 min, and nascent RNA transcripts were isolated and hybridized to DNA probes as described in Materials and Methods. Hours post-infection for early and late times were 5 and 18 in HeLa cells and 14 and 48 in 2132 cells, respectively. Probes for the viral E1A, E1B, E2, E3, and E4 genes, the major late promoter (ML) and, as a control, its opposite strand [-(ML)], are described in Materials and Methods; also used were probes for cellular GAPDH (GPD) and HLA genes. M13 represents single-stranded DNA from bacteriophage M13mp8, used as a nonspecific control. The total levels of [^{32}P]UTP incorporated into RNA in 10^6 HeLa cells were 1.4 X 10^7 cpm for uninfected cells, 2.1 X 10^7 cpm for early post-infection cells, and 4.9 X 10^6 cpm for late post-infection cells; values for 10^6 2132 cells were 2.7 X 10^7 cpm for uninfected cells, 1.6 X 10^7 cpm for early post-infection cells, and 2.1 X 10^7 cpm for late-post-infection cells. (A) Autoradiographic exposures of the first hybridization, with relative exposure times (1X and 20X), used to present data within the linear range of sensitivity of the film. (B) Densitometry scanning of autoradiographic signals from first (A) and second (not shown) hybridizations. Data were corrected for input counts per minute, length of the probe, G-C content and uridine content and are presented as histograms of relative hybridization per base.


A
B
HeLa
2102
E1A
E1B
E2
E3
E4
ML
-(ML)
M13
GAPDH
HLA
1X
Uninfected
Early
Late
Relative hybridization/Rate

the major late transcription unit, which was transcribed approximately twofold less in 2132 cells. (E3 transcription cannot be evaluated at late times because of the abundance of transcription initiated from the major late promoter in the same orientation and encompassing the E3 transcription unit [Figure 1].) Thus, despite the 10- to 200-fold differences in E1A protein levels, levels of transcription of E1A and E1A-dependent genes were comparable in 2132 and HeLa cells.

E1A mRNA 5′ termini are identical in 2132 and HeLa cells. Nuclear run-on experiments showed that levels of polymerase loading, and therefore the frequency of initiation in vivo, are similar in transcription of the E1A gene in 2132 and HeLa cells. However, inaccurate initiation of transcription, resulting in heterogeneous RNA 5′ termini, may influence RNA processing and stability. To address this possibility, S_1 nuclease protection assays were performed on total RNA from 2132 and HeLa cells uninfected or late in infection. The 5′ ends of E1A transcripts were assayed with a DNA probe spanning the E1A transcription start site at 499 bp from the left end of the virus chromosome. As presented in Fig. 16, the 5′ termini of E1A transcripts in 2132 cells are identical to those in HeLa cells, with the majority of transcripts initiated at nucleotide 499. Even the transcripts initiated at cryptic initiation sites within the E1A enhancer (Osborne and Berk, 1983; Hearing and Shenk, 1986) were represented at similar molar ratios in the two cell lines. Thus, there is no difference in the site of transcription initiation from the E1A gene in 2132 and HeLa cells.

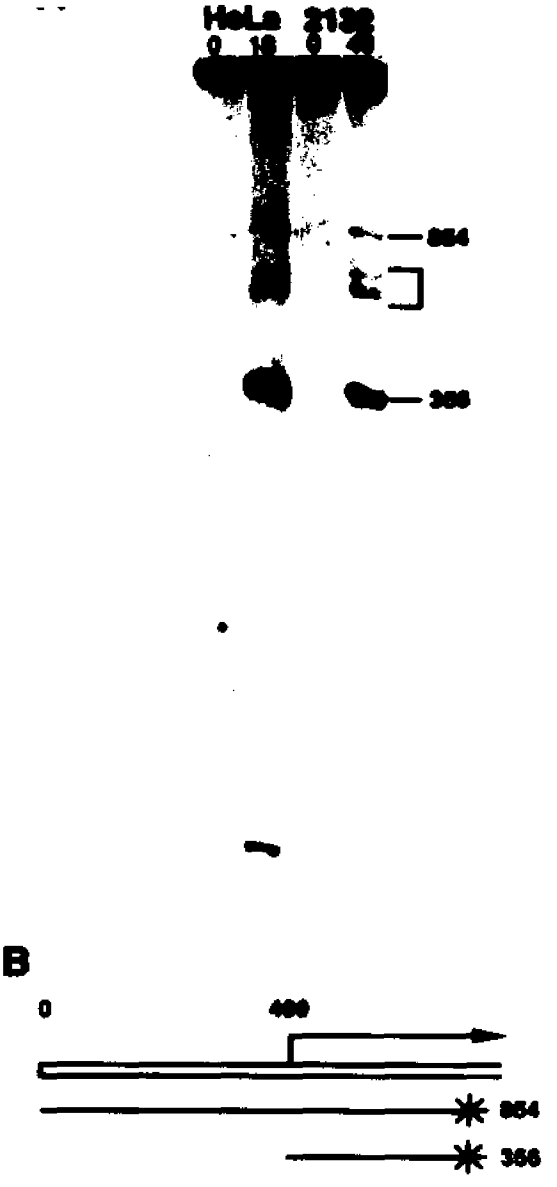


Figure 16: Nuclease S_1 protection assay for E1A RNA 5' termini. (A) RNAs from 10' HeLa or 2132 cells at the indicated hours after infection with Ad5 were analyzed by the nuclease S_1 protection assay as in Materials and Methods. (B) Diagram of the E1A transcription start site (arrow) on the adenovirus chromosome (double line), the end-labeled DNA fragment used for nuclease protection experiment (long line with "*"), and the expected size of the protected fragment (short line with "*").

DISCUSSION

At the molecular level, the most pronounced difference in the interactions of adenovirus with myeloma cells and HeLa cells lies in the steady state mRNA levels of the transforming E1A and E1B genes. The 20- to 50-fold relative reduction of E1A and E1B mRNAs in myeloma cells is specific, since mRNA levels of other viral genes are nearly identical at comparable times post-infection. It is not the result of general breakdown of cellular functions late in infection, since E1A mRNAs are reduced by 20-fold relative to those in HeLa cells early in infection as well. Nuclear run-on results showed that loading of polymerase on the viral genes is within a twofold range in the two cell types. Thus, the marked reductions of E1A mRNAs throughout infection and the E1B mRNAs late in infection are due to posttranscriptional mechanisms that are specific for the two genes and intrinsic to their interactions with myeloma cells.

In HeLa cells, the increase of E1A and E1B mRNAs at steady state from early to late in infection was largely attributed to mRNA stabilization (Wilson and Darnell, 1981). Results from infection of HeLa cells with a viral E2 gene mutant suggested that the E2 gene product might be involved in regulating the stability of E1A and E1B mRNAs in these cells (Babich and Nevins, 1981). It would be of interest to investigate the generality of this finding. Attempts to assess the turn-over time of E1A and E1B mRNAs by the conventionally used dactinomycin chase method have been hampered by specific stabilization of E1A and E1B mRNAs induced by this inhibitor in HeLa cells (see Section IIIB, Fig. 19). However, preliminary results suggest that the reduced accumulation of E1A and E1B

mRNAs in myeloma cells is not the consequence of retention of correctly processed mRNA in the nucleus. Rather, the polyadenylated E1A and E1B mRNAs appear to be very unstable, with half-lives of approximately 20 min. They also fail to be significantly stabilized late in infection in myeloma cells (see Part IIIB).

The stabilization of E1A and E1B mRNAs from early to late in infection of HeLa cells had prompted Wilson and Darnell (1981) to propose the existence of a nuclease(s) that is responsible for the rapid turnover of E1A and E1B mRNAs early in infection and is shut off late in infection. It is enticing to speculate that in myeloma cells the E1A and E1B mRNAs fail to accumulate to significant levels because the specific nuclease(s) persists throughout infection. This may in turn be due to lack of general shutoff of host protein synthesis (Lavery and Chen-Kiang, unpublished data) or the possibility that the nuclease(s) is cell type specific.

There is no obvious similarity in the primary sequences of the adenovirus E1A and E1B genes. This lack of an apparent common target might argue against the possibility that the same nuclease(s) recognizes transcripts from both genes but does not exclude the possibility that a similar secondary or tertiary structure, or perhaps a similar complex in a ribonucleoprotein particle, might be a requirement for substrate. Whether one or a family of nucleases is involved, it is of interest that human myeloma cells might possess a mechanism capable of selectively recognizing and degrading viral oncogene mRNAs. This could imply the existence of cellular genes similar to E1A and E1B in structure or regulation. Answers to this and other questions are dependent on identification of factors regulating E1A and E1B mRNA instability in an

in vitro system. Progress has recently been made toward this end in the analysis of mRNAs from the c-myc gene (Brewer and Ross, 1988; Pei and Calame, 1988), histone H4 gene (Peltz and Ross, 1987), and mRNAs containing the A/U-rich destabilizing element (Malter, 1989). It would be interesting to adapt these systems to recapitulate the regulated degradation of E1A and E1B mRNAs in human myeloma cell extracts.

Analysis of E1B mRNAs early in infection revealed that the alternatively spliced 22S and 13S mRNAs are represented in different molar ratios in the two cell types. Genetic evidence has strongly suggested that the 55 kDa polypeptide translated from the E1B 22S mRNA is important for preventing host mRNAs from accumulating in the cytoplasm of HeLa cells as viral infection progresses (Babiss et al., 1985; Pilder et al., 1986). The lack of E1B 22S mRNA early in infection of myeloma cells offers a plausible explanation for the observation that host protein synthesis is not severely inhibited in the course of infection of these cells (Part IIIB). In this regard, it is noted that E1A polypeptides, albeit present in small amount, are required for productive infection of myeloma cells. It is therefore entirely possible that the products of the E1A and E1B genes, which are expressed at reduced levels, have to accumulate to threshold levels before viral replication commences, hence the prolonged early phase in infection of many lymphoid cells, including myeloma cells (Lavery et al., 1987).

With respect to the molecular mechanism by which the alternatively spliced E1B 22S and 13S mRNAs are represented at steady state, the 22S mRNA from myeloma cells migrated considerably faster in denaturing gels (Fig. 12). Whether the different molar ratios of 22S to 13S mRNA in the

two cell types can be attributed to differential mRNA stability, poly(A) length, or preference of 5' splice site remains to be investigated. These results may imply a very complex and intricate regulation of E1A and E1B genes at the posttranscriptional level in a cell type-specific manner.

It is noteworthy that comparable transcription of all E1A-dependent genes is achieved in the two cell lines despite a 20- to 200-fold difference in levels of E1A polypeptides. This is in contrast to results reported by Brunet and Berk (1988), who demonstrated that transcriptional activation of E1A-dependent promoters of E2, E3, and E4 genes responded in a linear fashion to increasing concentrations of E1A protein in HeLa cells. Consequently, a 10-fold reduction in E1A mRNA led to an 8- to 10-fold reduction in E1A-dependent gene transcription. We do not yet understand why in myeloma cells a reduced amount of E1A proteins activates transcription nearly as well as a greater amount of E1A in HeLa cells. Whether the E1A polypeptides are modified identically in myeloma and HeLa cells remains to be determined. It is certain that E1A is essential for productive infection of myeloma cells, since some but not all E1A-dependent viral genes fail to be transcribed (Lavery and Chen-Kiang, unpublished data) and viral DNA is not replicated during infection with dl312 (Fig. 14). Perhaps the differentiated B cell background allows the transcriptional machinery to compensate for reduced levels of E1A. This may be accomplished through greater abundance of certain general transcription factors. Indeed, 2132 cells display greater sequence-specific binding activity of transcription factor Sp1 (Kadonaga et al., 1986; Pugh and Tjian, 1990) than do HeLa cells (Lavery and Chen-Kiang, unpublished data). The Sp1 binding site within the E1B promoter has been

shown to be necessary for efficient E1A transactivation (Wu et al., 1987). Consistent with this possibility, the cellular GAPDH and HLA genes were more actively transcribed in myeloma cells than in HeLa cells. The activities were enhanced late in infection, implying an elevation of general transcriptional activity in adenovirus-infected cells (Fig. 15). Alternatively, E1A responsiveness may be enhanced via constitutively high levels of second messengers, such as cyclic AMP, which has been shown to act in synergy with E1A for transcription activation of certain genes (Engel et al., 1988).

Although the ability of the E1A gene products to regulate the expression of other viral and cellular genes has been extensively studied, much less is known about the regulation of E1A gene expression itself. Dissection of the E1A enhancer and characterization of binding factors have identified participants in transcriptional activation, including E1A itself (Hearing and Shenk, 1986; Kovesdi et al., 1986; Hardy et al., 1989), yet other aspects of regulation, such as post-transcriptional mechanisms, have only begun to be addressed, and no aspect of E1A gene regulation has been studied in differentiated human cells. The investigation of interactions of adenovirus with human lymphoid cells offers a point of view different from those obtained from studies conducted in HeLa cells. Indeed, relative to what is found for HeLa cells, reduction of E1A and E1B mRNA levels prevails in many human cell lines of various tissues of origin, including liver, lung fibroblasts, and B and T lymphocytes (J. Spergel, D. Lavery, and S. Chen-Kiang, unpublished data), suggesting that repression of these transforming genes in differentiated cells may be the rule rather than the exception.

PART IIIB: ACCUMULATION OF E1A AND E1B TRANSCRIPTS IN THE NUCLEUS OF 2132 CELLS.

INTRODUCTION

The processing of eukaryotic RNA to mature mRNA is a complex series of events resulting in the accumulation of mature mRNA in the cytoplasm. For most mRNAs, nuclear events include cleavage and polyadenylation of the RNA at the 3' end, and RNAs generated from complex transcription units may be alternatively spliced or polyadenylated. While the basic mechanisms of polyadenylation and splicing have been biochemically dissected, regulated polyadenylation and splicing have not been completely recapitulated in vitro (Takagaki et al., 1988, and references therein). Other nuclear events that might have an equally important influence on mRNA biogenesis have been less well characterized. For example, the HIV rev gene product has been suggested to regulate the transport of RNA from the nucleus to the cytoplasm (Malim et al., 1989), and products of the adenovirus E1B and E4 genes are thought to induce the selective accumulation of viral mRNA in the cytoplasm late after infection by selective mRNA transport (Babiss et al., 1985; Pilder et al., 1986; Halbert et al., 1985; Bridge and Ketner, 1989; Sandler and Ketner, 1989). Furthermore, methylation of adenosine residues on nascent RNAs is conserved in mature, cytoplasmic RNAs from the adenovirus major late transcription unit (Chen-Kiang et al., 1979).

To properly address the regulation of RNA processing, all aspects of nuclear events should be considered; that is, how much do the

degradation or aberrant processing of RNA in the nucleus and the nuclear-cytoplasmic transport influence gene expression? Recently, considerable emphasis has been placed on the analysis of cytoplasmic mRNA stability, yet remarkably little effort has been directed toward RNA turnover in the nucleus.

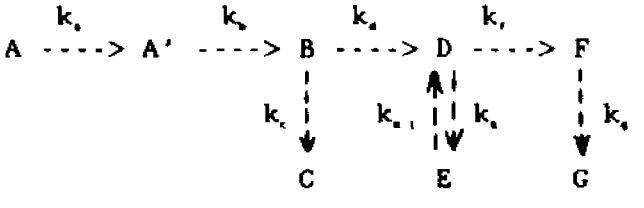
We have examined the cell type-specific expression of the adenovirus E1A and E1B genes as models for posttranscriptional gene regulation. During infection of the human myeloma cell line 2132, E1A mRNA accumulation is reduced by 20- to 50-fold relative to that in HeLa cells, and this reduced accumulation is the result of posttranscriptional mechanisms (Part IIIA). To further investigate the mechanism by which E1A and E1B mRNA levels are reduced at steady state, we assayed the accumulation of E1A RNA in the nuclear unpolyadenylated (poly(A)$^-$) and polyadenylated (poly(A)$^+$) RNA fractions, as well as in the cytoplasmic poly(A)$^+$ RNA fraction, in both 2132 and HeLa cells by cumulative labeling of RNA with ^{3}H-uridine to steady state levels. These analyses showed that reduced E1A mRNA accumulation in 2132 cells early after infection was due to greater accumulation of E1A RNA in the nuclear poly(A)$^-$ fraction, as unpolyadenylated pre-mRNA and/or as RNA degradation intermediates, whereas E1A poly(A)$^+$ RNA sequences accumulated to a much reduced level in the nucleus of HeLa cells. These results may suggest that the E1A primary transcripts were more efficiently processed into poly(A)$^+$ RNAs, or were more rapidly degraded, in the nucleus of HeLa cells than in 2132 cells. However, although similar results were found for the cellular gene GAPDH, the adenovirus E1B 22S RNA appeared to be less efficiently processed into the nuclear poly(A)$^+$ fraction in both HeLa and 2132 cells early in

infection, but was efficiently processed into the nuclear poly(A)$^+$ in HeLa cells during the late stage of infection. An estimate of cytoplasmic half-life by the kinetics of ^{3}H-uridine accumulation indicated that E1A and E1B 22S cytoplasmic mRNAs were unstable in both cell lines early after infection. Late after infection, the E1A and 22S E1B cytoplasmic RNAs in HeLa cells were significantly more stable than early after infection, while in 2132 cells they remained relatively unstable throughout infection. Finally, the measurements of mRNA half-life by cumulative labeling to approach steady state appear to be more reliable than those derived by the dactinomycin chase method, which were found to stabilize of the E1A and E1B mRNAs in HeLa cells, thus altering their apparent mRNA half-lives.

Taken together, these results suggested that cell type- and gene-specific nuclear events leading to retention of RNA sequences in the nucleus can significantly influence E1A and E1B gene expression in both 2132 and HeLa cells.

RESULTS

1. Rationale for the analysis of RNA kinetics by cumulative labeling. We have previously demonstrated (Lavery et al., 1987; Lavery and Chen-Kiang, 1990) that E1A mRNA accumulation was reduced throughout infection by 10- to 50-fold in the B myeloma cell line 2132 when compared with E1A mRNA accumulation in HeLa cells. Furthermore, nuclear run-on analyses indicated that this reduction did not result from a reduction in transcription initiation, and therefore was the result of post-transcriptional mechanisms. To further investigate these mechanisms, E1A RNA biogenesis was analyzed by assessing the kinetics of RNA accumulation during the labeling of RNA to steady-state levels. ^{3}H-radiolabeled uridine is quickly taken up by HeLa cells and incorporated into nascent RNA with a UTP pool equilibration time of less than 20 minutes (Latham and Darnell, 1965; Nevins and Darnell, 1978). After this time, with the specific activity of the UTP pool assumed to be constant, the approach to equilibrium of a given RNA species is the function of the rates of the RNA synthesis and decay (Chen-Kiang et al., 1979). The kinetics of RNA accumulation in the nucleus and cytoplasm can be represented in equation form as follows:



(1)

where A represents precursor nucleosides, A' represents nucleoside triphosphates, B represents unpolyadenylated nascent transcripts, D represents polyadenylated nuclear RNAs, and F represents polyadenylated cytoplasmic RNAs. C represents nuclear RNA degradation products of unpolyadenylated nascent transcripts. E represents deadenylated nuclear RNA, including degradation products of polyadenylated nuclear RNA molecules, and G represents cytoplasmic RNA degradation products. Each reaction can be assigned an individual rate constant, k_n, which is used to quantitate reaction kinetics. Thus, k_a through k_g represent the rate constants for nucleoside triphosphate pool equilibration, synthesis of nascent RNA transcripts, decay of nascent RNA transcripts, polyadenylation of nascent transcripts, decay of nuclear polyadenylated RNA (and its reverse reaction, the polyadenylation of deadenylated RNA), transport of RNA from the nucleus to the cytoplasm, and decay of mRNA in the cytoplasm, respectively.

In comparing E1A mRNA accumulation in 2132 and HeLa cells, we have previously established two components of the processing equation: B, or nascent RNA transcripts, is equivalent in 2132 and HeLa cells, while F is reduced significantly in 2132 cells relative to HeLa cells. We sought to determine which reaction(s) in the processing equation might account for the change between B and F.

The kinetics of cytoplasmic mRNA accumulation has been conventionally assessed with two major assumptions: first, that nuclear kinetics of accumulation are constant, and thus do not influence cytoplasmic mRNA kinetics of accumulation; second, that cytoplasmic mRNA decay proceeds with first order kinetics. With these assumptions, the

equation for cytoplasmic mRNA concentration, n, as a function of time, t, is:

$$n = n_o e^{(-k_o)t}$$

or

$$n/n_o = e^{(-k_o)t} \qquad (2)$$

where n_o is the maximal RNA concentration, and k_o is the cytoplasmic mRNA degradation constant (equation [1]). The average mRNA half-life, $t_{1/2}$, can be calculated from k_o, where

$$k_o = (\ln 2)[(1/t_{1/2}) + (1/t_c)]$$

where t_c is the cell doubling time. When $t_c >> t_{1/2}$ for a particular mRNA, the term $(1/t_c)$ can be assumed to approach zero, and thus the equation for k_o can be simplified:

$$k_o = (\ln 2)/t_{1/2} \ ,$$

or,

$$t_{1/2} = (\ln 2)/k_o \ .$$

If the specific activity of the RNA pool remains constant during labeling, the proportion of labeled RNA in the cytoplasmic pool will increase as the unlabeled RNA decays, until the labeled RNA concentration, a, approaches a maximal concentration, a_o, as the unlabeled RNA concentration approaches zero (Greenberg, 1972):

$$a/a_o = 1 - n/n_o$$

Substituting from equation (2):

$$a/a_o = 1 - e^{(-k_o)t}$$

and for $t_c >> t_{1/2}$ for a particular RNA:

$$a/a_o = 1 - e^{(-\ln 2/(t_{1/2}))t} \qquad (3)$$

By plotting values for the fractional amount of labeled RNA, a/a_o,

over a labeling period sufficiently long to reach a plateau value a_∞, regression analysis or semilogarithmic plotting will generate an approximate value for the $t_{1/2}$. However, the kinetics of cytoplasmic mRNA accumulation is much more complex than a first-order kinetics, as it is a downstream event of nuclear-cytoplasmic RNA transport.

The analysis of nuclear RNA kinetics is even more complicated. Because of the multiple reactions influencing accumulation of RNA transcripts in nuclear poly(A)$^+$ and poly(A)$^-$ fractions, their accumulation can rarely be viewed as following first-order kinetics. Furthermore, even given the ability to derive higher order equations (Chen-Kiang et al., 1979), the experimental design in many cases may not permit sufficient data points to permit statistically significant modelling. As a result, a qualitative comparison, rather than a strictly quantitative one, will be relied on for the nuclear analyses. A quantitative analysis of nuclear and cytoplasmic RNA accumulation kinetics has been presented (Chen-Kiang et al., 1979) to analyze the kinetics of accumulation of 6-methyl-adenosine (m6A) in nuclear and cytoplasmic RNA fractions. The high degree of statistical confidence in the large number of data points permitted the prediction of values for rate constants in the metabolism of m6A, including their incorporation into methylated RNA. When such values were determined, the mathematical model could predict the changes in accumulation kinetics resulting in variation of several criteria: precursor pool equilibration time, mean nuclear dwell time, mean cytoplasmic lifetime, and conservation of precursor molecules in cytoplasmic RNA (Chen-Kiang et al., 1979; Figure 17). Variation in pool equilibration time had very little influence on the kinetics of

accumulation for nuclear or cytoplasmic RNA. Therefore, while UTP pool equilibration time in 2132 cells was not directly assessed, its variation from HeLa pool equilibration time by as much as 2-fold will not greatly influence the kinetics of RNA accumulation (Figure 17). However, changes in nuclear dwell time and cytoplasmic half-life can have significant influence on RNA accumulation kinetics (Figure 17).

Poly(A)$^+$ E1A transcripts accumulate in the nucleus of 2132 cells. When the accumulation kinetics of E1A transcripts was analyzed in HeLa cells early after infection, cytoplasmic polyadenylated (poly(A)$^+$) RNA was found to accumulate to a maximal value by 90 min after labeling, displaying a cytoplasmic half-life of approximately 20 min (Figure 18). This is consistent with previously published E1A cytoplasmic half-life values in early-infected HeLa cells (Wilson and Darnell, 1981; Babich and Nevins, 1981). In the nucleus, poly(A) E1A RNA reaches a maximum level by 120 minutes after labeling, and actually decreases later in labeling, as the nuclear poly(A)$^+$ E1A RNA population increases. By comparison with predicted kinetics of accumulation from the mathematical model (Figure 17), these data are consistent with a relatively short nuclear dwell time for E1A transcripts.

Early after infection in 2132 cells (Figure 18A), E1A cytoplasmic poly(A)$^+$ RNA reached a maximal level between 90 and 120 min after labeling. By best-fit curve and graphic plotting analysis, these data indicated that the cytoplasmic half-life of E1A mRNA in 2132 cells early in infection was approximately 30 min, slightly longer than the 20 min E1A cytoplasmic mRNA half-life in HeLa cells. Therefore the reduced accumulation of E1A mRNA in 2132 cells early after infection, ten-fold reduced compared with that

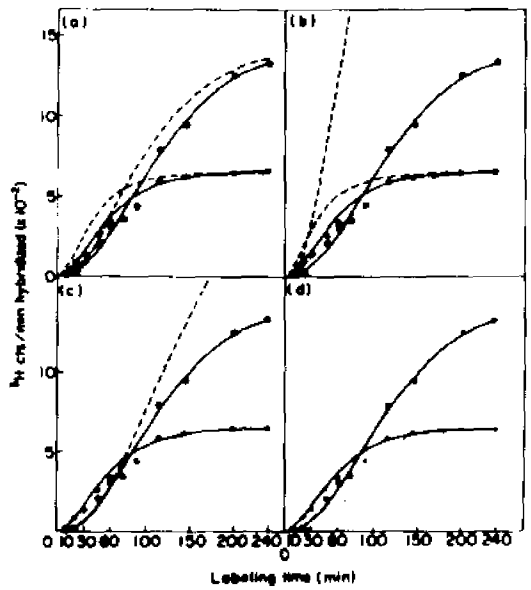


FIG. 5. Effect on the kinetics of accumulation of nuclear and cytoplasmic RNA by varying pool equilibration, mean nuclear dwell time, mean cytoplasmic life time or extent of conservation of nuclear sequences.

Data from Fig. 2(b) were taken as a model for testing the effect on the kinetics of accumulation when the Ado-Met pool equilibration (a), mean nuclear dwell time (b), mean cytoplasmic life time (c) or both mean cytoplasmic life-time and extent of conservation (d) were varied. (a) to (d), —●—●—, [^{3}H]methionine in nuclear RNA hybridized to 40·5–52·6 of the genome; —○—○—, [^{3}H]methionine in cytoplasmic RNA hybridized. In (a) curves were calculated with a half-time of pool equilibration of 10 min (– – – –) or 40 min (·····) as compared with 22 min (—●—●—) estimated from experimental results in Fig. 2(b) and Fig. 3. In (b) accumulation curves were calculated with mean nuclear dwell time of 20 min (—●—●—, nuclear, and —○—○—, cytoplasmic) based on data of Fig. 2(b) and compared to 10 min (– – – –) or 50 min (·····). In (c) accumulation curves were calculated with a mean cytoplasmic life-time of 70 min (—●—●—, nuclear; —○—○—, cytoplasmic) compared to 140 min (– – – –) or 35 min (·····). The change in the mean cytoplasmic life-time does not affect the nuclear curve. In (d) theoretical curves were plotted (·····) when only 50% of conservation and a mean cytoplasmic life-time of 140 min were assumed, as compared with the plot when complete conservation and a mean cytoplasmic life-time of 70 min were assumed (—○—○—), using the data from Fig. 2(b) for pool equilibration (22 min) and mean nuclear dwell time (20 min). All the theoretical curves were calculated using the mathematical model detailed in the Appendix.

Figure 17: Kinetics of RNA accumulation under various conditions as predicted by a mathematical model. Reprinted from Chen-Kiang et al., 1979, with permission of the publisher, Academic Press.

in HeLa cells, cannot be attributed to decreased cytoplasmic half-life, and is most likely the result of nuclear events.

Indeed, unlike kinetics of E1A RNA accumulation in HeLa cells, poly(A)$^+$ E1A transcripts accumulated to a much higher level in the nucleus of 2132 cells early after infection. The nuclear poly(A)$^+$ E1A RNA pool in 2132 cells continued to accumulate throughout the labeling period, to levels greater than in the poly(A)$^+$ cytoplasmic RNA fraction after 180 minutes (Figure 18A). These data are consistent with an increase in the nuclear dwell time (Figure 17) for the E1A RNA in 2132 cells as compared to HeLa cells, and suggest that nuclear processing of E1A RNA is less efficient in 2132 cells, resulting in nuclear accumulation of poly(A)$^+$ E1A transcripts and reduced E1A mRNA accumulation in the cytoplasm.

During the late stage of infection in HeLa cells (Fig. 18B), cytoplasmic E1A RNA appears to be more stable than at early times; cytoplasmic accumulation of E1A RNA does not reach a maximum level during the 240-minute labeling period, and thus appears to have a half-life of greater than 60 minutes, much longer than the 20-minute half-life early after infection. The stabilization of E1A RNA in HeLa cells from early to late after viral infection is consistent with a previous report by Wilson and Darnell (1981). In contrast, E1A mRNA is not substantially stabilized in the cytoplasm of 2132 cells late in infection, displaying kinetics of accumulation similar to that during the early stage of infection, with an apparent cytoplasmic half-life of approximately 35 minutes. Thus, in the late stage of infection, the reduced accumulation of E1A mRNA in 2132 cells is influenced by differential cytoplasmic half-lives in 2132 and HeLa cells. Furthermore, similar to early after

Figure 18: Kinetics of RNA accumulation during adenovirus infection of 2132 and HeLa cells. At early and late times after infection with Ad2, 2132 and HeLa cells were labeled with [^{3}H]uridine as described in Materials and Methods. At minutes after labeling as indicated, 2 X 10^7 cells were withdrawn, and nuclear poly(A)$^+$ and poly(A)$^-$ RNAs, as well as poly(A)$^+$ cytoplasmic RNA, were isolated. RNAs were hybridized to DNA probes bound to nitrocellulose filters. Probes used were an E1A cDNA clone, an E1B 22S exon-specific DNA fragment, and a rat cDNA clone for cellular gene GAPDH (detailed in Materials and Methods). Plasmid pUC12 DNA was included as a non-specific DNA control for hybridization. Following hybridization and washing of filters as described in Materials and Methods, hybridized ^{3}H radioactivity was quantitated by liquid scintillation spectroscopy. To facilitate comparisons between cell lines, values were corrected for total radioactivity incorporated into nuclear RNA by 60 minutes after labeling, when the accumulation of nuclear ^{3}H radioactivity was increasing linearly in HeLa and 2132 cells.

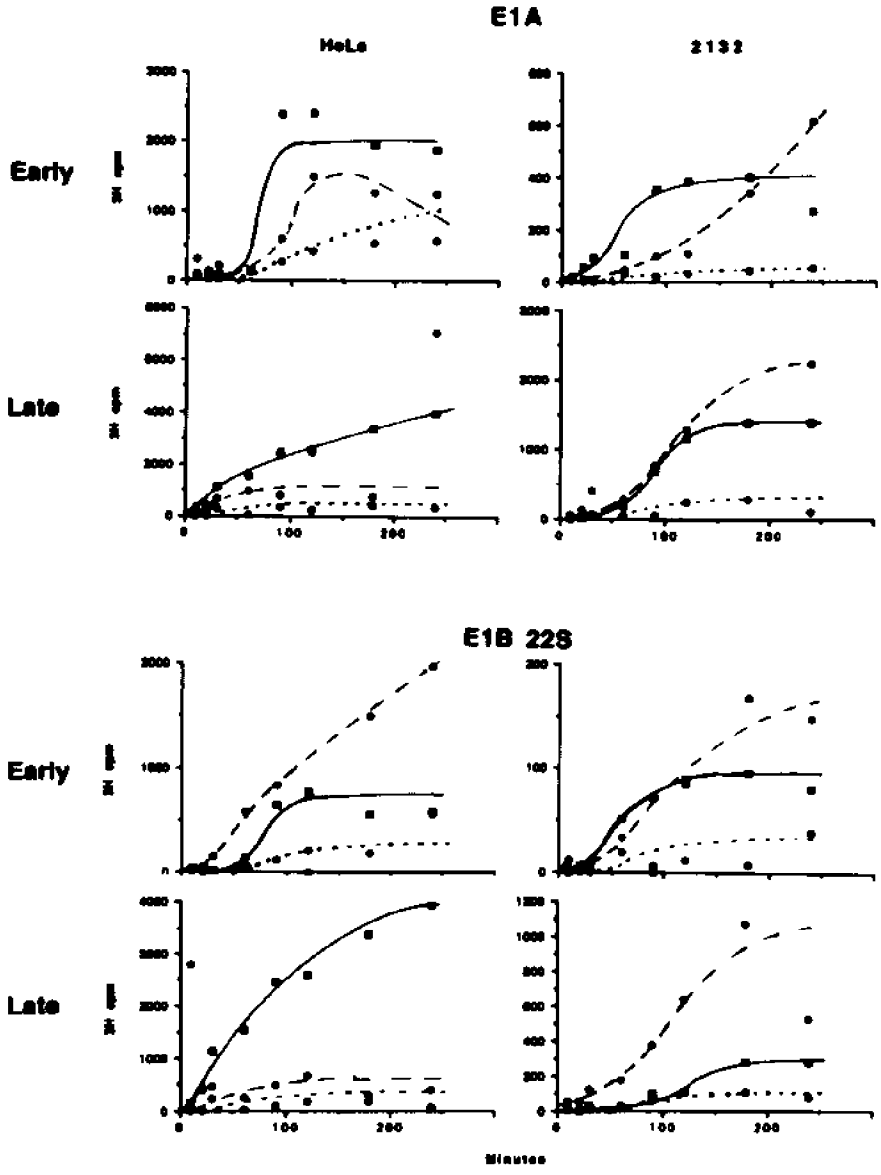


Figure 18A: E1A and E1B

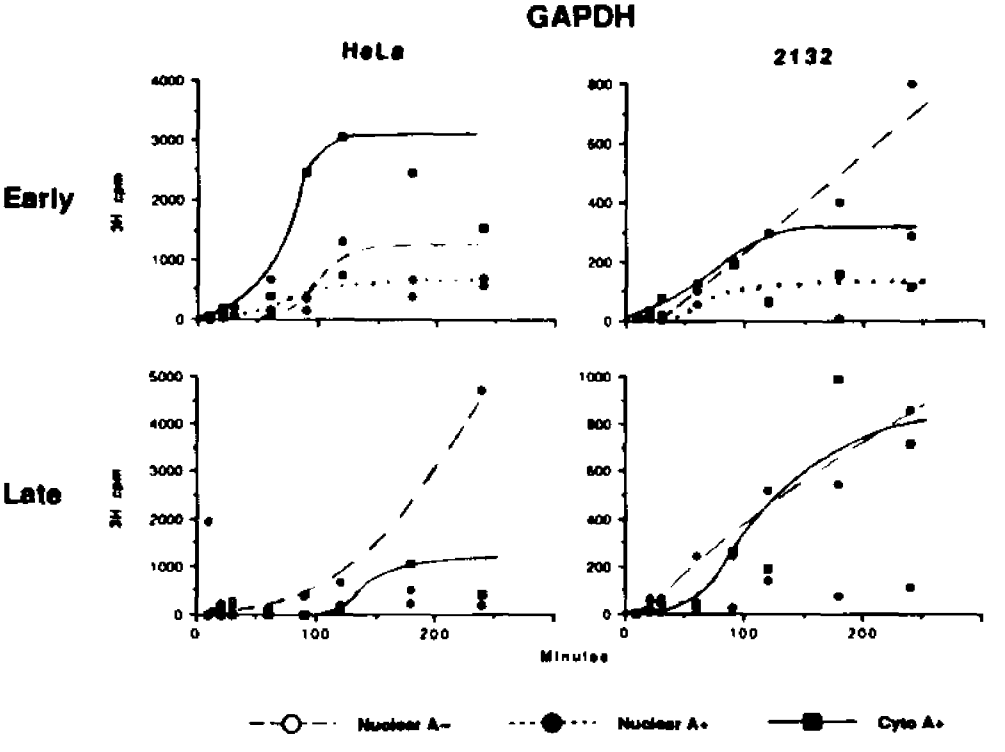


Figure 18B: GAPDH

infection, E1A RNA sequences accumulate in the poly(A)$^+$ nuclear RNA pool, suggesting that E1A transcripts are inefficiently processed in the nucleus late after infection as well.

Nuclear poly(A)$^+$ E1B 22S transcripts accumulate in HeLa cells early after infection and in 2132 cells throughout infection.

The adenovirus E1B gene primary transcript can be alternatively spliced into two major E1B mRNAs through the use of alternative splice donor sites. Use of the splice donor site at nucleotide 2550 generates the 13S mRNA, while use of the splice donor site at nucleotide 3505 generates the 22S mRNA (Figure 2).

The study of the half-lives of each of these two species and their precursors presents technical difficulties. For example, as a result of overlapping sequences, any DNA probe which will detect the 13S mRNA will also detect the 22S mRNA; even the 22S exon-specific probe would detect intron sequences removed during splicing of the 13S mRNA in the nucleus. We therefore chose to focus on metabolism of the 22S mRNA, with the caveat that 13S intron sequences may influence our analysis of nuclear accumulation data.

In HeLa cells early after infection, there is more E1B 22S mRNA than 13S mRNA in the cytoplasm. Since the 22S and 13S mRNAs have been shown to have similar cytoplasmic half-lives (Wilson and Darnell, 1981), this selective accumulation of 22S mRNA may result from nuclear events such as RNA splicing. The ratio of 22S to 13S mRNA changes in HeLa cells late after infection, with 13S predominating over 22S. In contrast, in 2132 cells the ratio of 13S to 22S mRNAs favors the 13S mRNA throughout infection (Part IIIA, Figures 11 and 12).

Analysis of 22S RNA biogenesis by cumulative RNA labeling analysis with a 22S exon-specific probe indicated that early after infection in both HeLa and 2132 cells, a substantial proportion of E1B 22S-specific transcripts accumulated in the poly(A)⁺ nuclear RNA pool (Figure 18A). As predicted by the mathematical model (Figure 17), this suggests that the E1B 22S-specific sequences have a nuclear dwell time relatively longer than that for E1A transcripts in HeLa cells early after infection. Furthermore, the retention of the 22S sequences in the nucleus of HeLa cells suggests that inefficient processing is not restricted to 2132 cells, but may also be cell-specific and sequence-specific (i.e., E1A but not E1B 22S RNAs are efficiently processed in HeLa cells). It is possible that the large accumulation of 22S-specific sequences as nuclear poly(A)⁺ sequences in HeLa cells represents intron sequences removed from E1B transcripts during efficient 13S mRNA splicing reactions. This could be resolved through analysis with a probe detecting only exon sequences, or through infection with mutant adenovirus deficient in 13S mRNA production.

Late after infection of HeLa cells, the majority of E1B 22S sequences accumulated in the cytoplasm, with very little accumulation in the nucleus (Figure 18B). This dramatic change may be due to functions of the translation product of the 22S mRNA itself: genetic evidence suggests that the 22S-derived E1B 55 kDa polypeptide functions in concert with the 34 kDa E4 gene product to enhance viral mRNA accumulation in the cytoplasm, presumably by enhancing the transport of the viral mRNA from the nucleus (Babiss et al., 1985; Pilder et al., 1986; Leppard and Shenk, 1989). The kinetics of E1B transcript accumulation in the nucleus of HeLa cells, suggesting that nuclear dwell time and the levels of 22S RNA

accumulation in the nucleus are reduced from early to late after infection, may be interpreted to arise as a result of the expression of these viral gene products. In addition, cytoplasmic 22S mRNA is stabilized late in infection in HeLa cells, with a half-life of greater than 60 minutes. Thus, nuclear and cytoplasmic events contribute to the increased E1B 22S accumulation in HeLa cells late in infection.

In 2132 cells, however, the partitioning of E1B 22S transcripts in the nucleus appeared not to change dramatically from early to late after infection (Figures 18A, 18B). While late after infection cytoplasmic mRNAs appear to be slightly more stable than early after infection, with a half-life of approximately 37 minutes, accumulation of nuclear poly(A) E1B 22S transcripts remained high, again presumably reflecting increased RNA degradation or inefficient RNA processing. The inability of 2132 cells to efficiently process E1B 22S mRNAs, even during later times of infection, may reflect the reduced expression of the E1B 55 kDa polypeptide.

Transcripts from cellular gene GAPDH are processed and accumulate differentially between HeLa and 2132 cells. Early after infection, accumulation of RNA transcripts from the cellular GAPDH gene was similar to that for E1A RNA in HeLa and 2132 cells (Fig. 18A). In HeLa cells, GAPDH RNA appeared rapidly in the cytoplasm, and accumulation kinetics of nuclear poly(A) transcripts was consistent with a relatively short nuclear dwell time. In 2132 cells a significant amount of GAPDH RNA accumulated as nuclear poly(A) RNA, as was seen for E1A and E1B transcripts in these cells. The cytoplasmic half-lives were approximately 20 minutes in HeLa cells and 45 minutes in 2132 cells.

Late after infection in HeLa cells (Fig. 18B), the cytoplasmic half-life of GAPDH mRNA, approximately 30 minutes, was not significantly changed from that early after infection. However, the appearance of GAPDH RNA in the cytoplasm was delayed until after 90 minutes and its cytoplasmic steady state accumulation was greatly reduced when compared to early after infection. Together with the data for E1B 22S RNA accumulation in HeLa cells early and late after infection, these data are consistent with the E1B 55 kDa protein functioning to induce the preferential appearance of viral mRNA in the cytoplasm, at the expense of cellular RNA transport out of the nucleus. In late-infected 2132 cells, GAPDH RNA did not display accumulation kinetics dramatically different from those early after infection. This is further support for the lack of E1B function during infection of 2132 cells.

Dactinomycin stabilizes E1A and E1B mRNAs in HeLa cells. The kinetics of RNA accumulation analyses presented above demonstrated that early after infection in both HeLa and 2132 cells, E1A and E1B mRNAs displayed similar cytoplasmic half-lives of between 20 and 35 minutes. This is in agreement with previously published values for E1A and E1B cytoplasmic mRNA half-life, as determined by the same method (Wilson and Darnell, 1981; Babich and Nevins, 1981). However, as a substitute for the more laborious and expensive cumulative labeling experiments, dactinomycin (ActD) chase experiments are often used to assess mRNA half-lives. The rationale is that ActD rapidly intercalates into DNA by binding to deoxyguanosine residues, and blocks RNA transcription (Sobell, 1985). With no new RNA synthesis, the decay of mRNA over a period of time is directly assessed as a first-order decay reaction by monitoring steady

state mRNA levels by Northern blotting or nuclease protection analyses. The rapidity of ActD action is an advantage over cumulative RNA labeling with ^{3}H-uridine labeling, which may be hampered by UTP pool equilibration times longer than the half-life of the mRNA being studied.

However, treatment of cells with ActD appears to influence more than nascent RNA transcription; several investigators have reported apparent preferential mRNA stabilization or destabilization after ActD treatment (Singer and Penman, 1973; Steinberg et al., 1975; Casey et al., 1983). Therefore, the use of ActD to assess the half-life of a certain mRNA may in fact itself alter the mRNA half-life. To date, no studies have compared the discrepancies in predicted half-life of a particular mRNA by the ActD method with that by kinetics of accumulation methods.

To accomplish this, HeLa and 2132 cells were treated with ActD early after infection by adenovirus. Total RNAs were isolated, and poly(A)$^+$ RNAs were analyzed by Northern analysis (Figure 19). While E1A mRNA appears to decay exponentially up to 60 minutes after ActD treatment, with an apparent half-life of 20 to 30 minutes in both HeLa and 2132 cells, approximately 30% of the E1A mRNA detected in untreated cells is detectable even after 100 minutes of ActD treatment, yielding an overall mRNA half-life of greater than 45 minutes. This is not due to the ineffectiveness of ActD in inhibiting RNA transcription, as c-myc mRNA sequences decayed to less than 10% of the untreated c-myc mRNA concentration within 20 minutes (Figure 19), comparable to its half-life assessed by both cumulative labeling and ActD chase (Dani et al., 1984). It is noted, however, that the c-myc mRNA concentration was not reduced to background levels with extended ActD treatment.

Figure 19: ActD chase analysis of mRNA half-life. Early after Ad2 infection of HeLa and 2132 cells, ActD was added to cultures to a final concentration of 10 μg/ml. At minutes after ActD addition as indicated, total poly(A)$^+$ RNAs were isolated and analyzed by Northern blotting. (A) Viral E1A and E1B mRNAs and cellular c-myc mRNA were detected using specific radiolabeled probes described in Materials and Methods. (B) Quantitation of E1A and E1B mRNA accumulation in HeLa and 2132 cells, and c-myc mRNA accumulation in HeLa cells. Quantitation of c-myc mRNA accumulation in 2132 cells was not possible due to high background radioactivity in the Northern blotting. Densitometric signals from autoradiograms in (A) were quantitated with an LKB laser densitometer, and values are presented on a semi-log scale as percentage of maximum mRNA accumulation versus time.

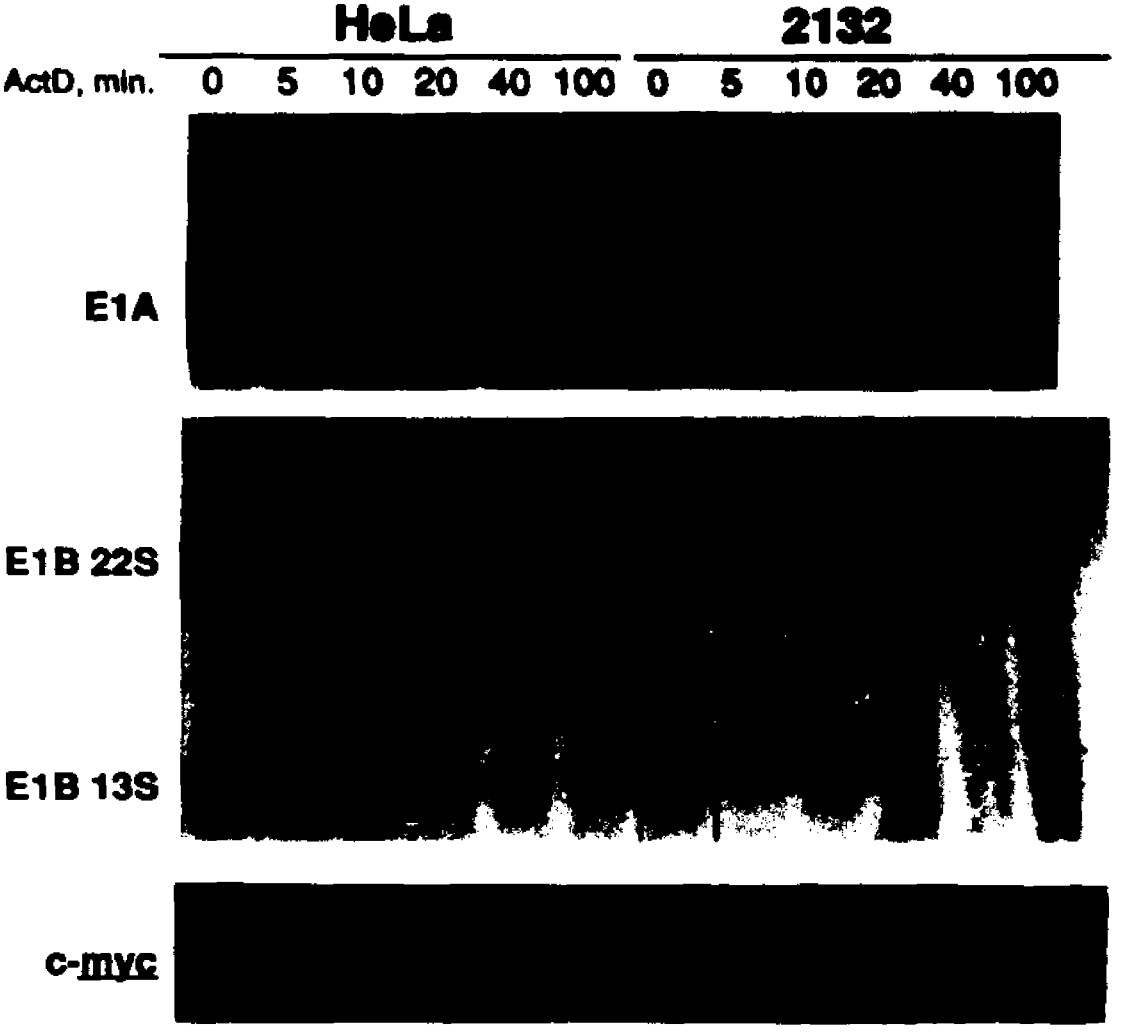

HeLa
2132
ActD, min.
0 5 10 20 40 100 0 5 10 20 40 100
E1A
E1B 22S
E1B 13S
c-myc

Figure 19A

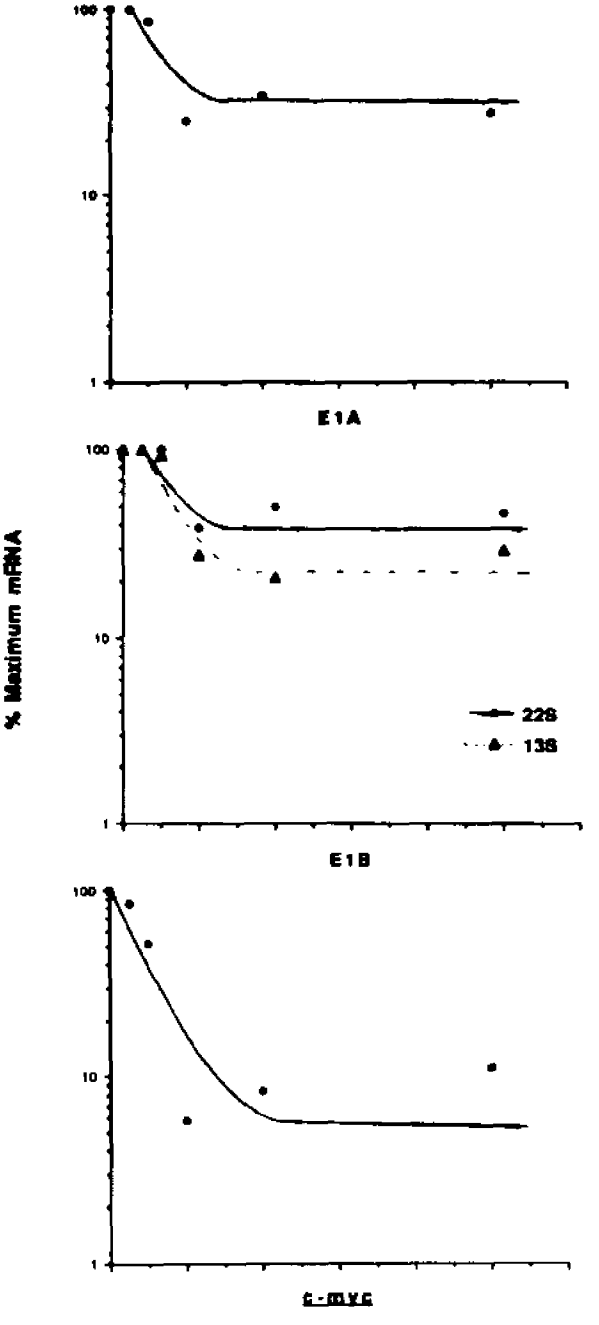
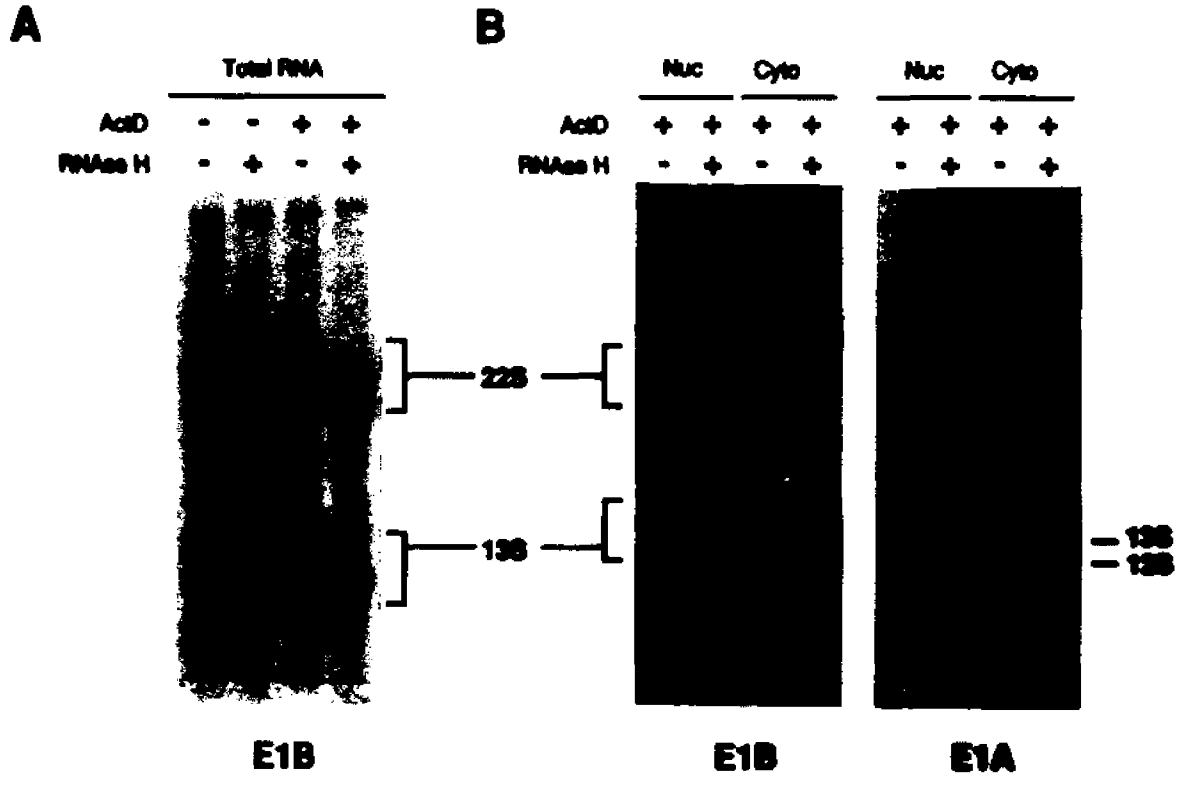


Figure 19B

During ActD treatment, E1A mRNAs accumulated predominantly in the cytoplasm, with poly(A) tail length similar to that of E1A mRNA before ActD treatment (Figure 20B). In 2132 cells, assessment of the ActD influence on E1A mRNA accumulation is more difficult, because of the low abundance of E1A mRNA in 2132 cells which presents difficulties in detecting the reduced E1A population above background after 100 minutes of ActD treatment. Northern analysis of E1A mRNA accumulation after ActD treatment does suggest the accumulation of ActD-stable E1A mRNA; however, the significance of this population is unclear due to the high background from the Northern probing. The improved quantitation and increased sensitivity of nuclease protection assays may afford better evaluation of E1A mRNA in ActD-treated 2132 cells.

ActD treatment induces poly(A) lengthening of nuclear E1B 22S mRNAs in HeLa cells. Similar to E1A mRNAs, significant amounts of E1B 22S and 13S mRNA species were detected in HeLa cells during prolonged ActD treatment. Unexpectedly, the E1B 22S mRNA population shows a pronounced and reproducible separation into two distinct species: one population which is larger than the predominant 22S mRNA from untreated cells, and one which is smaller. Digestion of poly(A) RNA with RNAse H after hybridization to oligo-d(T)$_{12}$ demonstrates that the altered mobilities of these two species is due to lengthening of the poly(A) tail in the slower migrating species, and shortening of the poly(A) tail in the faster migrating species (Figure 20). Furthermore, the long poly(A)-containing 22S mRNA species is detected almost exclusively in the nucleus, while the shorter poly(A)-containing species is detected predominantly in the cytoplasm (Figure 20B). Similar lengthening of E1B 22S mRNA poly(A) tail

Figure 20: Act D-stabilized E1A and E1B mRNAs in HeLa cells. (A) Early after infection by Ad2, HeLa cells were treated with ActD (10 μg/ml) for 0 minutes (-) or 100 minutes (+). Total poly(A)$^+$ RNA was isolated from 2 X 10^7 cells. Half of each RNA sample was digested with 10 units of RNAse H in the presence of molar excess oligo-d(T)$_{12}$ as described in Materials and Methods. Control (-) and RNAse H-treated (+) RNAs were analyzed by Northern blotting, and E1B mRNAs were detected with a DNA probe specific for the E1B 3' end. (B) Nuclear and cytoplasmic poly(A)$^+$ RNAs were isolated from HeLa cells early after Ad2 infection and 100 minutes after treatment with ActD (10 μg/ml; ActD "+"). Following RNAse H digestion and Northern blotting as above, E1B mRNAs were detected in control (-) or RNAse H-treated (+) RNA samples with an E1B 3' specific probe, and E1A mRNAs were detected with an E1A cDNA probe.


A
Total RNA
ActD
RNAse H
22S
13S
E1B
B
Nuc
Cyto
Nuc
Cyto
ActD
RNAse H
E1B
E1A

was not detectable by Northern blot analysis during ActD chase periods; this may have been impaired by the lack of accumulation over background after 20 minutes of ActD treatment (Figure 19).

In HeLa cells, the alternatively processed E1B 13S mRNA shows neither nuclear accumulation nor poly(A) tail lengthening; however, as for E1A and E1B 22S mRNAs, the 13S mRNA is significantly represented even after 100 minutes of ActD treatment (Figure 20). Therefore, ActD stabilizes E1A as well as E1B 13S and 22S E1B mRNAs in HeLa cells. However, the mechanism of stabilization is not clear, as mRNAs in both the nucleus and cytoplasm are stabilized, and stabilization is independent of the poly(A) elongation detected in the E1B 22S mRNA species.

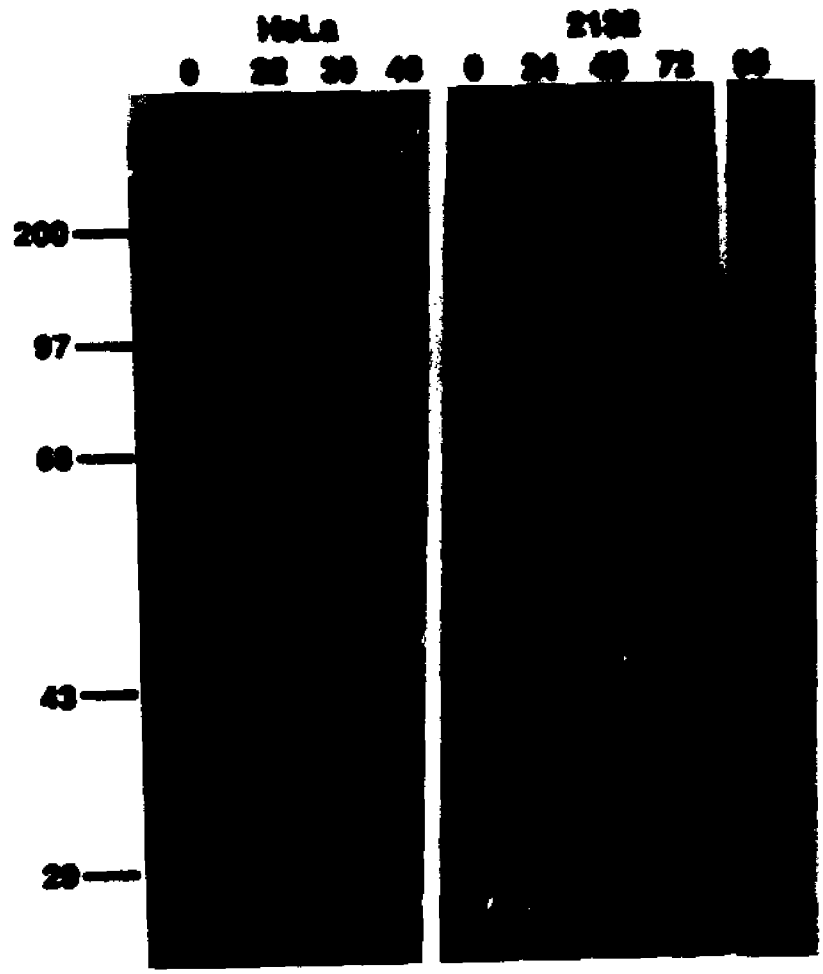
Host protein synthesis is not significantly impaired in adenovirus-infected 2132 cells. Adenovirus infection of HeLa cells induces a significant inhibition of host protein synthesis during the late stage of infection (Ginsberg et al., 1967). As adenovirus infection progresses from the early to the late stage in HeLa cells, E1A mRNAs become stabilized in the cytoplasm, with an increase in the average cytoplasmic half-life from 20 minutes early after infection to greater than 60 minutes late after infection. It is possible that these two events are related; Wilson and Darnell suggested that the stabilization of E1A mRNA resulted from the inactivation of a host-encoded nuclease by the virus-induced inhibition of host protein synthesis (Wilson and Darnell, 1981).

E1A mRNA is not substantially stabilized in the cytoplasm of 2132 cells late after infection; the kinetics of E1A cytoplasmic mRNA accumulation was similar to that during the early stage of infection (Figure 18). If E1A mRNA stabilization in the cytoplasm of HeLa cells

results from the effects of the virus-induced inhibition of new host protein synthesis, then the lack of stabilization of E1A mRNA in 2132 cells late after infection may result from the lack of such an inhibition of host protein synthesis. To analyze this, the rate of new protein synthesis was evaluated by labeling of cells with 35S-methionine at different hours post-infection, and comparing the new synthesis of host proteins to that in uninfected cells. As presented in Figure 21, synthesis of host proteins in HeLa cells was significantly inhibited by 30 hours post-infection, approximately coincident with the accumulation of viral virion proteins. This is consistent with earlier reports (Ginsberg et al., 1967; Babiss and Ginsberg, 1984). However, in 2132 cells, little inhibition of host protein synthesis is detected, even after 4 days of infection, after the peak of virus production in 2132 cells as judged by plaque assay (Lavery et al., 1987; Figure 9). This does not merely reflect a reduced population of infected cells, as greater than 70% of cells were found to be infectable by indirect immunofluorescence and cytopathic effect analyses (see Section II).

This suggests that the lack of E1A mRNA stabilization in 2132 cells may correlate with the lack of host protein synthesis shut-off. However, preliminary experiments indicate that treatment of 2132 cells with the translation inhibitor anisomycin early after adenovirus infection did not increase E1A mRNA accumulation, even after three hours of anisomycin treatment. Similar treatment of HeLa cells with anisomycin resulted in a 5-fold increase in E1A mRNA accumulation. This suggests that while a labile nuclease can be inhibited in HeLa cells by blocking protein synthesis, a similar factor in 2132 cells is sufficiently stable to be

Figure 21: Analysis of new protein synthesis by ^{35}S-methionine metabolic labeling. At hours after infection by Ad2 as indicated, HeLa and 2132 cells were labeled for 2 hours with ^{35}S-methionine as described in Materials and Methods. Samples equivalent to 1 X 10^4 cells were withdrawn from cytoplasmic extracts of labeled cells, boiled, and separated on a 10% polyacrylamide-SDS gel. After fixation and fluorography (En^{3}Hance, New England Nuclear), gels were dried and exposed to X-ray film. Numbers to the left refer to the molecular mass in kilodaltons of prestained high-range molecular weight protein standards (Bethesda Research Laboratories).


HeLa
2102
0
24
48
72
96
200
97
68
43
29

unaffected by a three-hour treatment with anisomycin. We cannot exclude the possibility that the increase in E1A mRNA accumulation in HeLa cells is not the result of greater transcription activity after treatment with protein synthesis inhibitors, as has been demonstrated following cycloheximide treatment of early-infected HeLa cells (Cross and Darnell, 1983). This must be confirmed by direct assessment of transcription rates by nuclear run-on analyses of infected cells with and without anisomycin.

Thus the putative E1A-specific nuclease, which may be inactivated in HeLa cells by virus infection, appears not to be influenced in the same way in 2132 cells. This may provide an explanation for the significant stabilization of E1A mRNA in HeLa cells following ActD treatment: if the E1A-specific nuclease were labile, treatment with ActD to inhibit transcription of its RNA would reduce the nuclease activity during prolonged treatment periods.

DISCUSSION

While *cis*-acting sequences and *trans*-acting factors influencing cytoplasmic mRNA turnover have been widely investigated, little is known of possible mechanisms leading to the degradation or retention of RNA in the nucleus, and their contribution to gene regulation. This bias is in part due to the relative simplicity of cytoplasmic RNA kinetic analysis, which is assumed to follow first-order decay kinetics. The multivariable nature of nuclear RNA processing greatly increases the complexity of its analysis. While much has been learned about nuclear processes through the dissection of individual processes such as splicing and polyadenylation *in vitro*, the regulation of these processes and their contribution to the

kinetics of nuclear RNA processing in vivo are still not fully understood. The development of a mathematical model by Chen-Kiang et al. (1979) which accurately fits nuclear and cytoplasmic RNA kinetics data permits a quantitative analysis of the efficiency of nuclear RNA processing events in vivo. By varying parameters such as nuclear dwell time, the model can be used to predict kinetics of accumulation under a variety of cellular conditions. Comparison of these predicted accumulation kinetic values with actual data provides a first approximation of events within the cell.

In the kinetic analysis of E1A and E1B RNA metabolism in HeLa cells and 2132 cells, the E1A cytoplasmic mRNA half-life was found to be similar between 2132 and HeLa cells early after infection, when transcription of the E1A gene was similar in the two cell lines. Therefore, nuclear processing events most likely accounted for the 10-fold relative decrease in E1A mRNA accumulation early after infection. When nuclear poly(A) and poly(A)$^-$ E1A RNAs were analyzed, HeLa cells displayed a modest nuclear (A) pool. In 2132 cells, the nuclear poly(A) pool was much larger than in HeLa cells, and actually exceeded the size of the E1A cytoplasmic mRNA pool. These data from HeLa and 2132 cells are consistent with predictions of the mathematical model for a difference in nuclear dwell times between the two cell lines, with a shorter E1A nuclear dwell time in HeLa cells. Thus, from kinetic analysis, E1A gene expression appears to be reduced in 2132 cells early after adenovirus infection due to increased accumulation of E1A RNA in the nucleus.

This experiment does not permit the distinction between accumulation in the nuclear poly(A) population as degraded RNA or as unadenylated RNA; experiments to distinguish these two possibilities by sizing of nuclear

poly(A)⁻ E1A species are in progress. In preliminary investigations, poly(A)⁻ E1A RNA was not detected as full-length species in steady state RNA from 2132 cells by Northern blotting, suggesting that in these cells the predominant poly(A)⁻ E1A RNAs are partially degraded. However, this evaluation may not have been sensitive enough, owing to the large quantity of ribosomal RNA and other RNA species which often contribute high background to Northern analysis of poly(A)⁻ RNA.

It is not known whether deadenylated RNAs would have arisen due to the lack of polyadenylation, or due to the degradation of the poly(A) tail from the properly polyadenylated RNAs. This could be distinguished through analysis of polyadenylation efficiency on newly transcribed RNAs during brief pulse labeling of cells. However, the appearance of E1B 22S mRNAs containing long poly(A) tails in the nucleus of ActD-treated HeLa cells suggests that unpolyadenylated RNA can exist as whole molecules in the nucleus, and can serve as substrates for a slow poly(A) addition activity (Diez and Brawerman, 1974; Sawicki et al., 1977).

The significant change in E1B 22S RNA accumulation kinetics from early to late times after infection in HeLa cells (as well as the equally remarkable lack of such a change in 2132 cells) is consistent with an increase in the efficiency of RNA metabolism, resulting in decreased nuclear dwell time and rapid appearance in the cytoplasm. This indicates that efficient RNA processing in HeLa cells may depend on specific cis-elements. In addition, this is consistent with the proposed mode of action of an adenovirus gene product encoded by the 22S mRNA itself, the E1B 55 kDa protein. Mutant viruses containing deletions within the 55 kDa coding region show a defect in the progression of viral RNA through

biochemically defined nuclear compartments at late times after infection, resulting in reduced viral mRNA accumulation in the cytoplasm (Leppard and Shenk, 1989). This is in agreement with our findings in HeLa cells early in infection, and in 2132 cells throughout infection. Perhaps in HeLa cells the E1B 22S RNA requires its own translation product, the 55 kDa protein, for efficient RNA processing, in contrast to the E1A RNA, which is efficiently processed early after infection. Thus, expression of the 22S mRNA would be regulated by a positive feedback mechanism, whereby increased accumulation of 22S mRNA in the cytoplasm leads to translation of the 55 kDa protein, which in turn increases 22S RNA transport out of the nucleus.

The inefficient processing of E1B 22S mRNA in 2132 cells throughout infection may reflect reduced expression of the E1B 55 kDa protein, resulting in its inability to efficiently activate this positive feedback mechanism. E1A and host gene GAPDH RNA sequences are also processed inefficiently in 2132 cells. If these cells are generally inefficient in the processing of RNA, this would impede the upregulation of 22S mRNA expression by the 55 kDa protein, and thus prevent the transformation to a more efficient RNA processing mechanism.

The ActD chase method for the assessment of half-life for the E1A and E1B mRNAs was found to artificially stabilize these mRNAs, thus altering their apparent mRNA half-lives. A significant proportion of E1A and E1B mRNAs are detected in poly(A)$^+$ RNA of HeLa cells, even after 100 minutes of ActD treatment. This sub-population probably arose as a result of ActD treatment, because the presence of such a stable RNA population *in vivo* would have distinctly altered the cytoplasmic accumulation

kinetics in the cumulative labeling experiments; no such alteration in accumulation kinetics was observed. We do not know the mechanism of ActD-induced mRNA stabilization; it does not stabilize all mRNAs, but appears to stabilize certain RNA species in both the nucleus and cytoplasm. Possible mechanisms by which ActD might stabilize mRNA include intercalation into RNA directly, and thus disruption of a degradation signal, or the inactivation of a labile nuclease through the shut-off of transcription of its own RNA.

While ActD chase experiments may not permit the accurate evaluation of E1A or E1B mRNA half-lives, they may offer some insights into RNA processing events in HeLa and 2132 cells. For instance, E1A mRNAs become stabilized in the cytoplasm of HeLa cells late in infection. This may be the result of host protein synthesis inhibition, because treatment of HeLa cells with anisomycin to block protein synthesis also appears to increase E1A mRNA accumulation in HeLa cells. The anisomycin experiment does not distinguish whether increased E1A mRNA accumulation results from greater mRNA stabilization through inactivation of a labile nuclease in HeLa cells, or from increased transcription activation (Cross and Darnell, 1983), perhaps due to the inactivation of a transcription repressor. However, if the stabilization of E1A mRNA by ActD also results from inactivation of a host factor through a block in its RNA transcription, then the inactivated host factor in this case cannot be a transcription repressor, since new RNA synthesis is blocked. This does not prove, however, that ActD and anisomycin increase E1A mRNA accumulation by the same mechanism; it merely lends support to the proposed inactivation of a labile nuclease.

ActD treatment of HeLa cells also increased the accumulation of poly(A)$^+$ E1B 22S mRNA in the nucleus. These mRNAs contained poly(A) tails longer than those on the majority of E1B mRNAs in untreated HeLa cells, and the poly(A) tail appears to grow longer with time of ActD treatment. These surprising results are consistent with the data from the cumulative labeling experiments which suggested that E1B 22S RNA is inefficiently processed in HeLa cells early after infection, and accumulated in the nucleus as poly(A)$^+$ RNA. If these poly(A)$^+$ RNAs are stable in HeLa cells, or become stabilized after ActD treatment, they may remain in the nucleus long enough to become polyadenylated by a slow poly(A) addition activity (Diez and Brawerman, 1974; Sawicki et al., 1977). Because they appear not to be transported from the nucleus, and because they are not degraded while in the nucleus, these RNAs will eventually become polyadenylated sufficiently to be retained in the poly(A)$^+$ RNA fraction, and their poly(A) tail will become progressively longer during the ActD chase. While after 100 minutes of ActD chase E1B 22S mRNAs in 2132 cells are not readily detectable above background by Northern analysis, perhaps more sensitive methods may determine whether a similar process occurs in 2132 cells. If so, it would indicate that E1B 22S RNA sequences can accumulate in the nucleus of 2132 cells as whole, unpolyadenylated RNA molecules, as in HeLa cells.

IIIC: RETENTION OF PROCESSED E1A AND E1B mRNA IN THE NUCLEUS OF T CELLS.

PRELIMINARY RESULTS

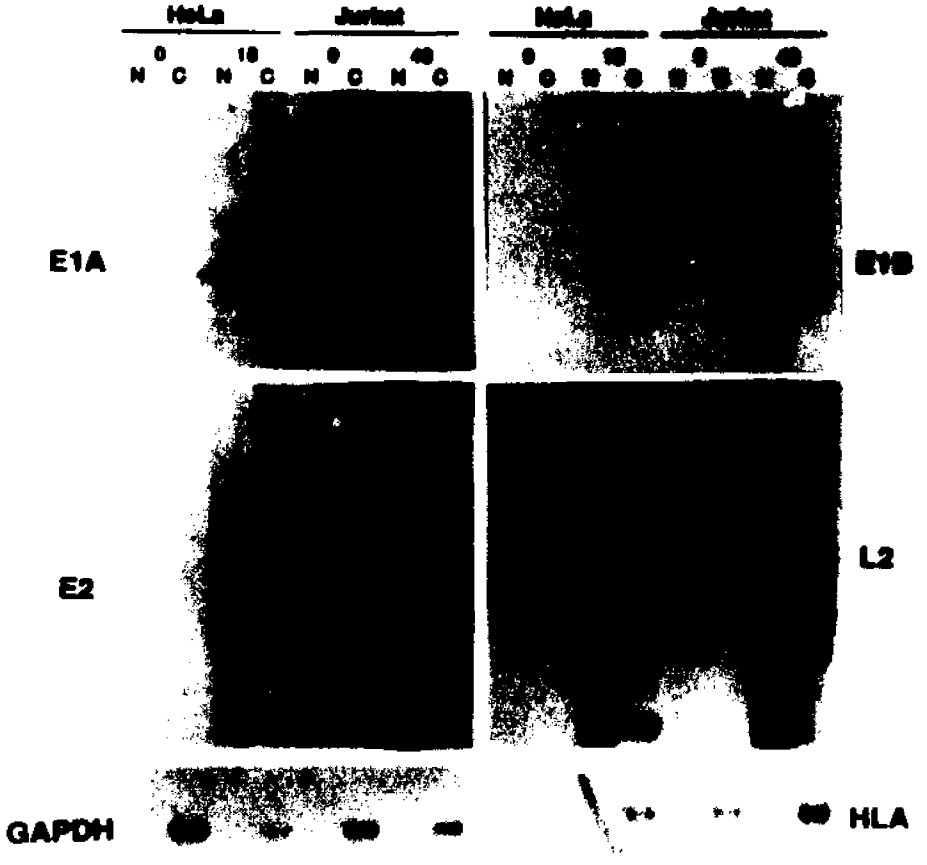
In the human myeloma cell line 2132, adenovirus E1A and E1B gene expression is reduced due to nuclear events leading to increased retention of RNA transcripts in the nucleus when compared to HeLa cells. We sought to examine whether posttranscriptional influences on E1A and E1B gene expression were particular to this cell line, or were detected in other cell lines as well. The human T cell line, Jurkat, was used as a model to examine expression of the E1A and E1B genes late after adenovirus infection. Similar to 2132 cells, Jurkat cells showed an extended lag phase in the onset of viral DNA replication and late gene expression, and in the appearance of progeny virions. This suggested a defect in early gene functions delaying the progression to the late stages of viral infection. Preliminary results indicate that E1A and E1B RNAs accumulate in the nucleus of Jurkat cells late after virus infection. However, unlike the case in 2132 cells, E1A and E1B nuclear RNAs were detectable as spliced and polyadenylated RNAs at steady state in the nucleus of Jurkat cells. Other mRNAs, including viral mRNAs from the viral E2 gene and L2 late mRNA family, accumulated primarily in the cytoplasm, suggesting that in Jurkat cells, as in 2132 cells, retention of E1A and E1B RNA in the nucleus significantly influences expression of these viral genes. However, unlike 2132 cells, Jurkat cells displayed accumulation of these RNAs as spliced and polyadenylated RNA molecules.

RESULTS AND DISCUSSION

Nuclear and cytoplasmic RNA was isolated from Jurkat cells uninfected or 48 hours after infection by adenovirus, when viral DNA replication and late gene mRNA accumulation are detected at levels comparable to those in HeLa cells late after infection (Lavery et al., 1987; D. Lavery and S. Chen-Kiang, unpublished data). Northern analysis of polyadenylated RNA indicated that greater than 50% of E1A mRNAs and greater than 90% of E1B mRNAs accumulated in the nucleus of Jurkat cells late in infection (Figure 22). While these mRNAs were detected in the poly(A)$^+$ fraction of RNA, they appeared slightly smaller than their cytoplasmic species, indicating accumulation of degradation intermediates or RNA species with short poly(A) tails in the nucleus. Greater than 50% of mRNA from the L2 gene family and greater than 70% E2 gene mRNA accumulated in the cytoplasm. However, steady state mRNA of host genes for GAPDH and HLA class I antigen HLA-B7 (HLA) were detected exclusively in the cytoplasm.

While these results are preliminary, they do suggest several interesting possibilities. The first is that Jurkat cells may be similar to 2132 cells in repressing E1A and E1B gene expression by retention of their RNAs in the nucleus. However, Jurkat cells differ from 2132 cells in that their nuclear RNA species accumulate as properly processed and polyadenylated molecules, while in 2132 cells these transcripts are detected as unpolyadenylated RNA. We cannot exclude that a large proportion of E1A and E1B RNAs do indeed accumulate as unpolyadenylated RNA in Jurkat cells, since only poly(A)$^+$ RNA has been analyzed by Northern analysis. This would be testable by kinetic labeling experiments,

Figure 22: Retention of E1A and E1B mRNAs in the nucleus of Jurkat T cells. Nuclear and cytoplasmic RNAs from Jurkat and HeLa cells which were uninfected or in the late infectious stage (48 hours for Jurkat cells, 18 hours for HeLa cells) were isolated as described in Materials and Methods. Poly(A)$^+$ RNA from 10^7 cells were analyzed by Northern blotting, using as probes an E1A cDNA clone, an E1B cDNA clone, and Ad2 DNA restriction fragments specific for the E2 transcription unit and the L2 family of mRNA, as well as cDNA clones for cellular genes GAPDH and HLA class I antigen. Left and right panels represent probings of duplicate blots. We cannot explain the appearance of a band detected by the E1A cDNA probe in uninfected Jurkat cytoplasmic RNA. However, it does not migrate the same as the conventional 13S, 12S, or 9S E1A gene products, and is not reproducible. Furthermore, no other viral mRNAs were detected in this sample.


HeLa
Jurkat
HeLa
Jurkat
N C N C N C N C
E1A
E1B
E2
L2
GAPDH
HLA

as have been performed with 2132 cells (Part IIIB), or by analysis of steady state RNA with methods of greater sensitivity, such as ribonuclease protection. However, E1A transcription appears similar to that in HeLa cells, as judged by nuclear run-on experiments (D. Lavery and S. Chen-Kiang, unpublished data), and total E1A mRNA accumulation is similar in Jurkat and HeLa cells. Since the same proportion of E1A RNA transcripts arrive as spliced and polyadenylated RNA in HeLa and Jurkat cells, nuclear poly(A)$^+$ accumulation is probably similar in the two cell lines. Therefore the greater accumulation of properly processed mRNAs in Jurkat cells than in 2132 cells may be due to the increased activity of a nuclear-localized nuclease activity in 2132 cells, which acts efficiently to degrade E1A and E1B nuclear RNAs.

A second interesting possibility arising from these observations is the possible existence of a cell-specific hierarchy for RNA transport out of the nucleus. While kinetic analysis rather than steady state Northern analysis is required to properly evaluate this proposal, these data suggest that E1B mRNA is least efficiently transported from the nucleus, followed by E1A and L2 mRNAs, while E2 mRNA appears more efficiently transported to the cytoplasm, and cellular gene GAPDH and HLA mRNAs appear very efficiently transported. These data suggest <u>cis</u>-elements can influence nuclear RNA transport. It is not clear what influence the location of the RNA within the nucleus exerts on this <u>cis</u> effect. However, de la Pena and Zasloff presented evidence that a gene promoter can influence export of RNA from the nucleus, perhaps by determining its orientation with the cellular transport machinery (de la Pena and Zasloff, 1987). It is interesting that selective RNA transport from the nucleus,

which is utilized by adenovirus to enhance its own gene expression, may also repress expression of certain adenovirus genes, including the E1B gene, whose 55 kDa gene product aids in the selective transport of viral mRNA. The resulting deficiency in E1B 55 kDa protein does not permit the alteration of the cellular transport machinery to one which favors viral mRNA transport, thus further impeding the progression of viral infection.

Previous studies on tRNA transport from the nucleus of Xenopus oocytes demonstrated that it is a carrier-mediated, saturable process (Zasloff, 1983). It is also sensitive to subtle changes in the tRNA molecule; a single point mutation in $tRNA^{Met}$ results in its accumulation in the nucleus (Zasloff et al., 1982). If mRNA transport is also a carrier-mediated process, the nuclear accumulation of E1B in Jurkat cells may provide the opportunity to identify not only *cis*-sequences regulating transport, but also the cellular factors interacting with these *cis*-sequences.

IV. REGULATION OF IMMUNOGLOBULIN GENE EXPRESSION DURING PRODUCTIVE ADENOVIRUS INFECTION

Previously Chambon and colleagues described an interesting interaction between mouse immunoglobulin genes and the human adenovirus E1A gene products: E1A expression by infection or transfection could repress expression of endogenous immunoglobulin κ and γ2b genes in the mouse plasmacytoma cell line MPC11, but could enhance transcription directed by the immunoglobulin heavy chain enhancer within an integrated plasmid in the mouse fibroblast L cell line (Hen et al., 1985; Borrelli et al., 1986). This suggested that the lymphoid cell background could significantly influence the ability of E1A gene products to effect changes in immunoglobulin gene expression.

Productive infection of human cell lines of lymphoid origin by adenovirus presents an opportunity to examine the influence of E1A gene products on endogenous human immunoglobulin gene expression during productive virus infection. Through the use of cell lines which are nearly uniformly infectable by adenovirus, E1A interactions with immunoglobulin genes can be examined in cell populations in which the majority of cells will express the E1A gene, in contrast to transient E1A gene transfection studies, where only a small percentage of cells may express the E1A gene. The human myeloma cell line 2132 expresses the λ light chain gene at high levels (Matsuoka et al., 1967), and is well characterized in its infectability by adenovirus (Lavery et al., 1987). We examined the transcription and mRNA accumulation from the λ light chain

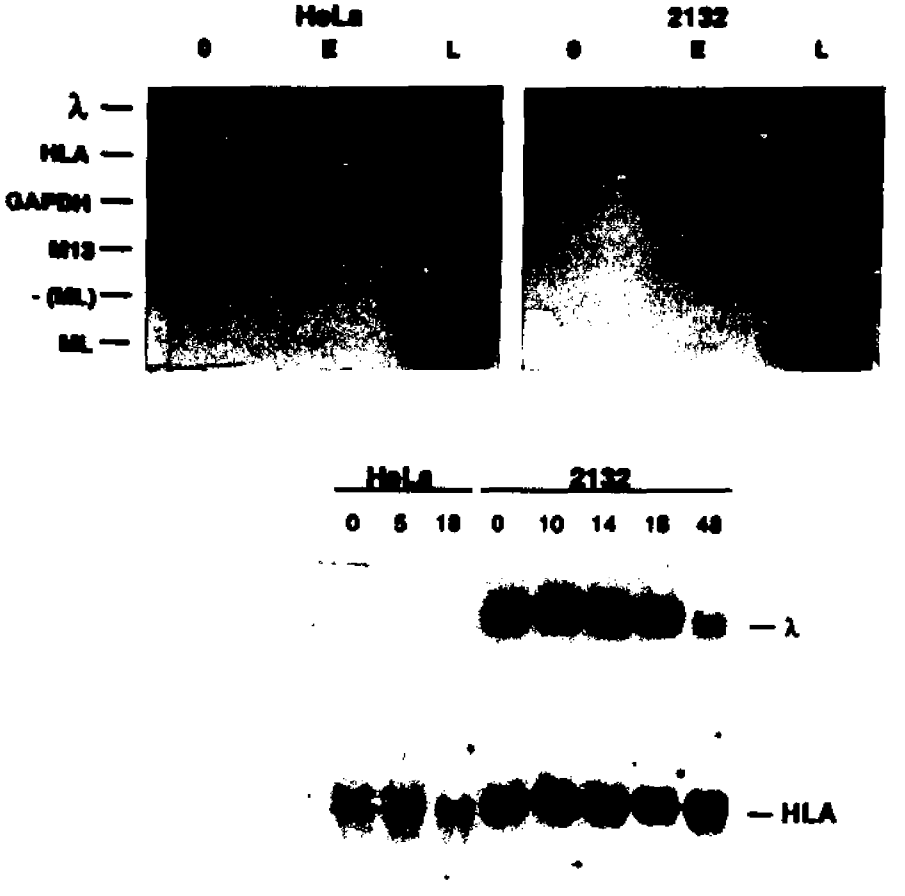
gene during productive adenovirus infection of 2132 cells and, as a control, HeLa cells. Our results indicate that λ light chain gene transcription in isolated nuclei is enhanced late after infection, in 2132 cells as well as in HeLa cells, which do not normally transcribe the λ gene. Despite increased λ gene transcription in 2132 cells late after infection, λ mRNA was greatly reduced at steady state as compared to levels in uninfected or early-infected cells. Therefore, while λ gene transcription was significantly increased, accumulation of its mRNA was reduced as a result of posttranscriptional, presumably virus-mediated, mechanisms.

PRELIMINARY RESULTS

Transcription of the λ light chain gene is increased during adenovirus infection of 2132 cells. E1A mRNA and protein expression were detectable by 14 hours after infection of 2132 cells, although at slightly lower levels than those detected in HeLa cells at 5 hours after infection. At this time, there was no detectable late gene mRNA accumulation or viral DNA replication (Lavery and Chen-Kiang, 1990; Figure 23). During this early stage of infection, polymerase loading on the λ gene was not significantly changed from that in uninfected cells, as assayed by nuclear run-on analysis (Figure 23). Thus, this level of E1A gene expression, sufficient to efficiently activate transcription of viral early genes, does not appear to significantly alter λ gene transcription.

By 48 hours after infection, adenovirus infection in 2132 cells had progressed to the late stage of infection, as characterized by the

Figure 23: Transcription and mRNA accumulation from the immunoglobulin λ light chain gene in adenovirus-infected 2132 and HeLa cells. Nuclei isolated from 10^8 HeLa and 2132 cells that were uninfected (0) or were early (E) or late (L) after infection by Ad2 were used for nuclear run-on experiments as described in Materials and Methods and the legend to Figure 15. Probes used were single-stranded DNA probes specific for transcription from the major late promoter (ML), and, as a control, its opposite DNA strand (-[ML]), and double-stranded DNA probes for the cellular genes for human λ light chain constant region (λ), human HLA class I antigen (HLA), and GAPDH.


HeLa
2132
0
E
L
0
E
L
λ
HLA
GAPDH
M13
(RSL)
RSL
HeLa
2132
0 5 18 0 10 14 16 48
λ
HLA

abundant transcription from the major late promoter (ML, Figure 23), and replication of viral DNA (Lavery et al., 1987). At this time, λ gene transcription is increased by three-fold. However, transcription of cellular genes GAPDH and HLA were also increased, by nine-fold and four-fold, respectively. Thus, the increase in λ transcription in 2132 cells was not λ gene specific, but rather due to a general increase in the transcription of some cellular genes.

Induction of "λ-like" gene transcription in HeLa cells by adenovirus. Surprisingly, late after adenovirus infection of HeLa cells, hybridization of labeled RNA to the λ gene DNA probe is reproducibly detected at significant levels, even following stringent washing and RNAse A treatment. Weak transcription of the λ gene can also be detected early after infection (Figure 23). Further characterization of this observation to ensure that the hybridized RNA is indeed transcribed from the λ gene is required; however, no such hybridization to the λ DNA was detected by nascent transcripts from uninfected HeLa cells, and no hybridization was detected to the control DNA, from an M13 bacteriophage. It is possible that this hybridization represents RNA from a gene sharing significant homology to λ gene sequences, though the homology would have to be extremely high in order to be resistant to RNAse A and stringent blot washing conditions. In light of the increase in GAPDH and HLA transcription in both HeLa and 2132 cells, perhaps the detection of "λ-like" RNA transcripts in HeLa cells suggests a general de-repression in transcription following adenovirus infection, resulting in augmented transcription from previously active genes, and new transcription from

previously silent genes.

Accumulation of λ gene mRNA is reduced in 2132 cells late after infection. Steady state levels of λ mRNA were assessed to determine whether posttranscriptional mechanisms might alter λ gene expression. Indeed, Northern analysis indicated that late after infection, at 48 hours p.i., λ mRNA accumulation was significantly reduced over levels in uninfected or early-infected 2132 cells (Figure 23). Because λ gene transcription was increased late after infection in 2132 cells, this was the result of posttranscriptional processes, presumably the virus-induced reduction in host mRNA accumulation induced by adenovirus E1B and E4 gene products. No polyadenylated RNA species were detected in HeLa cells, despite the detection of nascent RNA transcripts hybridizing to the λ constant region DNA probe in nuclear run-on experiments (Figure 23).

DISCUSSION

In addition to its biological significance, the strict cell type-specificity and multiple levels of regulation, including DNA rearrangement, transcriptional activation, and RNA processing, have made immunoglobulin gene expression an excellent system for molecular studies. Recent advances in protein purification and assay methods have made immunoglobulin gene transcriptional activation more amenable to analysis. As a result, several transcription factors influencing transcription of these genes have been characterized, purified, and cloned (see, for example, Roman et al., 1990; Beckmann et al., 1990).

The interplay of these factors in effecting the high levels of immunoglobulin gene transcription remains unknown. The alteration of normal expression patterns may provide insight into these protein interactions. While Chambon and colleagues have reported that during high multiplicity of infection of the mouse plasmacytoma cell line by human adenovirus type 2, transcription of endogenous γ2b and κ genes were reduced (Hen et al., 1985; Borrelli et al., 1986). However, no decrease in λ gene transcription was detected in human myeloma 2132 cells during productive infection by Ad2. Perhaps E1A polypeptide levels sufficient to efficiently activate transcription of viral early genes in 2132 cells are too low to alter λ gene transcription. Alternatively, the λ gene may be more refractory to E1A influences than the κ gene. Although certain sequence motifs are conserved between λ and κ genes (Falkner and Zachau, 1982), the human and murine λ genes have not been demonstrated to possess a κ- or heavy chain enhancer-like element (Blackwell and Alt, 1988). Perhaps E1A-mediated transcription repression requires the presence of enhancer-binding transcription factor sites. A further possibility is that during productive infection, other viral products may counterbalance the repression of λ transcription. Thus, while E1A gene products might reduce transcription, other viral or viral factors might serve as activators, thereby negating the influence of E1A gene products on λ transcription.

Surprising results were obtained in nuclear run-on experiments when nascent RNA transcripts from nuclei of late-infected, but not uninfected, HeLa cells were found to hybridize to the λ gene-specific probe. No λ

mRNA accumulation is detectable in uninfected or infected HeLa cells, and presumably the λ gene locus is not rearranged in HeLa cells. However, the possibility exists that sterile λ RNA transcripts (Calame, 1985) are synthesized from the λ gene late after infection in HeLa cells, presumably due to virus-induced changes in the nuclear environment. Kadesch and colleagues (Beckmann et al., 1990) noted that the DNA binding site for the cellular transcription factor USF/MLTF in the adenovirus major late promoter is nearly identical to the μE3 motif of the immunoglobulin heavy chain enhancer and the κE3 motif of the κ chain enhancer. We have noted that within the λ light chain J-C intron there is a sequence with significant homology (9 of 11 identical bases) to the USF/MLTF binding site. Perhaps USF/MLTF is a common factor in the activation of both immunoglobulin and adenovirus genes; factors present in non-lymphoid cells contribute to the basal transcription of transfected immunoglobulin genes (Wasylyk and Wasylyk, 1986). Whether USF/MLTF DNA binding activity is regulated after adenovirus infection is being investigated in our laboratory. If USF/MLTF-induced transcriptional activation is increased in infected cells, transcription of previously active immunoglobulin genes, such as the λ gene in 2132 cells, might be increased. Indeed, we have found λ gene transcription to be increased four-fold late after infection of 2132 cells. Furthermore, previously inactive genes, such as the λ gene in HeLa cells, might also be transcribed at a significant level, resulting in the production of sterile λ transcripts. Adenovirus infection has also been found to induce reorganization of the cellular chromatin (Zhai et al., 1987). It is possible that this may alter the

immunoglobulin gene locus to make it more accessible to transcription factors or nuclear structures which previously had not been able to interact with immunoglobulin gene regulatory regions.

Inhibition of host protein synthesis by adenovirus may also influence the ectopic transcription of the λ gene in HeLa cells. Recent studies in our laboratory indicated that the DNA binding activity of a nuclear factor which has been shown critical for immunoglobulin gene expression in transfection (Araki et al., 1988; Liberman et al., 1990; Nelsen et al., 1990) is increased in nuclear extracts from HeLa cells late after infection. Conversely, the DNA binding activity of NF-IL6, a member of the C/EBP nuclear factor family (Akira et al., 1990) whose DNA binding sequence is present within the heavy chain promoter (S. Chen-Kiang, unpublished), decreases in HeLa cells late after infection. Similar results are obtained in HeLa cells treated with the protein synthesis inhibitor anisomycin (C. Smith and S. Chen-Kiang, unpublished data), though it is not yet known whether anisomycin treatment induced λ gene transcription. If λ gene transcription in HeLa cells is repressed by a labile factor, protein synthesis inhibition induced either by adenovirus or anisomycin may repress the repressor and allow activation of λ transcription.

The slight induction of transcription from the λ gene in HeLa cells early after infection, however, suggests activation independent of protein synthesis inhibition and possibly independent of nuclear reorganization, both of which are predominantly late infection events. Therefore, the possibility exists that virus early gene products, presumably E1A gene

products, can activate λ gene transcription in HeLa cells. In this respect, HeLa cells may be similar to mouse L cells, in which E1A gene expression activated transcription from integrated plasmid sequences containing the heavy chain enhancer (Borrelli et al., 1986). It remains to be determined why activation of transcription from the endogenous λ gene is possible in HeLa cells, while it was not detected in L cells (Borrelli et al., 1986).

Genetic studies have indicated that gene products of the adenovirus E1B and E4 genes cooperate to enhance viral mRNA accumulation, while leading to reduced cellular mRNA accumulation. Mutations reducing expression of either the E1B 55 kDa protein or the E4 34 kDa protein severely reduced expression of this phenotype (Babiss et al., 1985; Pilder et al., 1986). Analysis of steady state RNA and results from the biochemical fractionation of nuclei (Babiss et al., 1985; Pilder et al., 1986; Leppard and Shenk, 1989) suggested that this phenotype is the result of preferential mRNA transport from the nucleus, associated with preferential flow of RNA through defined nuclear compartments. Apparently, mRNAs which are impeded in their flow through these compartments are degraded. The greater reduction in mRNA accumulation from the λ gene than from the HLA gene may reflect relative half-lives of their cytoplasmic mRNAs; if HLA mRNA were more stable in the cytoplasm, its accumulation might be less influenced by reductions in nuclear RNA transport.

V: DISCUSSION

The E1A and E1B genes of group C adenoviruses encode multifunctional polypeptides with dramatic influences on cellular gene transcription, DNA replication, and other aspects of macromolecular synthesis. The E1A and E1B genes can also cooperate to induce the transformed phenotype in cultured rodent cells, which often serves as a model for malignant cell growth in vivo. Thus, the understanding of the functions of these gene products as well as the regulation of their expression may provide insights into processes of great interest in molecular biology.

The regulation of expression from the E1A and E1B genes has been conventionally studied in such cell lines as HeLa and KB, in which complex regulation of these genes at the levels of transcriptional activation and alternative RNA splicing were characterized. The examination of E1A and E1B gene expression in a differentiated background such as human lymphoid cells may uncover regulatory aspects not previously observed in HeLa or KB cells. While the investigations presented here bear out this fact, it also reveals interesting new information, such as ActD-induced poly(A) tail lengthening of E1B 22S mRNA and ectopic transcription of the λ light chain gene, in HeLa cells, which were supposedly serving as controls.

Adenovirus and human lymphocytes during natural infection. In studying adenovirus gene expression in human cell lines of lymphoid origin, we have undertaken to determine whether E1A and E1B gene expression, as well as virus replication, displays distinctions in this differentiated cell background. While much has been learned of adenovirus

functions in more conventional cell lines such as HeLa, the possibility exists that the adenovirus life cycle may be distinct in the background of lymphoid cells. The isolation of virus from apparently "normal" adenoid and tonsil explants, the recurrence of viral infection and shedding, and the high antibody titer against adenovirus in many individuals suggested that natural infection by adenovirus was more complex than that suggested by the 18-hour infectious cycle in HeLa cells. In addition, the expression of human papillomavirus E6 and E7 genes in HeLa cells may also exert an as yet unrecognized influence on virus replication.

Because lymphocytes comprise the majority of cells in adenoids and tonsils, these cells are very likely a primary cell type for virus infection. However, the majority of peripheral blood lymphocytes do not appear to be highly infectable, and productive infection of established human cell lines was not reported prior to investigations from our laboratory in 1987. The reason for this may be that only a subpopulation of peripheral blood lymphocytes are infectable at a detectable level, and cultured lymphocytes are representative of these lymphocyte subpopulations. While only a fraction of peripheral B and T cells appeared infectable by adenovirus (Horvath et al., 1986; D. Lavery and S. Chen-Kiang, unpublished), preliminary results indicate that the proportion of infectable T cells from peripheral blood increased upon stimulation of T cells with phytohemagglutinin (D. Lavery and S. Chen-Kiang, unpublished data). Also, certain established B cell lines are only weakly infectable by adenovirus, while some cell lines (notably 2132) display infection kinetics approaching that in HeLa cells. The distinction between cells

which are highly infectable versus those that are not is unknown; it does not correlate with cellular growth kinetics or the stage of B cell development. One observation, however, is that the B cell lines which were the poorest in supporting viral DNA replication correlate with those which have undergone class switching to immunoglobulin heavy chain isotypes other than IgM. However, since only three such lines have been examined, this remains only a preliminary observation.

The observation that λ light chain gene mRNA accumulation is reduced late after infection indicates that B cell-specific functions, like other cellular functions, can be inhibited by adenovirus infection. Work by Kvist and colleagues (Burgert and Kvist, 1985; Burgert et al., 1987) indicated that E3 gene products could suppress MHC class I-restricted antigen presentation to cytotoxic T cells. If MHC class II-restricted presentation of virus antigens is also inhibited, perhaps through the inhibition in transport of the MHC class II antigen mRNA, antigen presentation by virus-infected B cells to helper T cells might be impaired. The result might be the establishment of prolonged or latent viral infection. Our experiments present the first opportunity to examine these interactions and their relevance to natural infection.

E1A and E1B gene expression during infection of 2132 cells. The high infectability of 2132 cells, as judged by immunofluorescence, and kinetics of infection similar, but not identical, to that in HeLa cells prompted the further investigation of adenovirus infection of this cell line. Adenovirus infection in these cells demonstrated a prolonged eclipse phase: the onset of viral DNA replication, early and late gene

mRNA accumulation, and progeny virus production were delayed by one to two days. After this delay, however, viral DNA replication, mRNA accumulation and progeny virus titer increased, with kinetics and productive yield similar to those in HeLa cells. These observations suggested the defect in infection of 2132 cells was not the result of the cells' inability to support virus replication. Rather, these data were consistent with a defect in early gene function, which delayed, but did not reduce, the ability of the virus to efficiently switch to the late stage of infection. The logical early gene to examine was the E1A gene, the master control gene of adenovirus gene expression, required for efficient activation of transcription from the viral early genes. Indeed, E1A mRNA accumulation was found to be greatly reduced throughout infection, compared with its accumulation in HeLa cells. It appeared that the progression of adenovirus infection was delayed in 2132 cells until the amount of E1A gene expression reached a critical threshold level. Once that level was achieved, infection progressed as efficiently as in HeLa cells.

Expression of the E1B gene remained altered throughout infection. Early after infection, E1B mRNAs accumulated in 2132 cells with a ratio of its alternatively spliced 22S and 13S mRNAs inverted relative to their accumulation in HeLa cells, and late after infection E1B mRNA accumulation was reduced by 10- to 20-fold in 2132 cells. E1B gene expression, therefore, was distinct from that of the other E1A-dependent early genes, and shared similarity to E1A gene expression.

With slightly reduced E1B gene transcription rate compared to HeLa cells, and with the 13S mRNA accumulating preferentially over 22S mRNA, the E1B 22S mRNA was reduced by 8-fold in 2132 cells relative to HeLa

cells early after infection. The 22S mRNA encodes the E1B 55 kDa polypeptide which influences viral mRNA accumulation. Since 2132 cells seem to process both host and viral RNA transcripts inefficiently, the reduced accumulation of 22S mRNA can only serve to make a bad situation worse: with E1B transcripts inefficiently processed, the E1B 55 kDa polypeptide would be more urgently needed to increase viral mRNA accumulation in the cytoplasm. Such a reduction in 22S mRNA might contribute to a vicious cycle of inefficient viral RNA metabolism further delaying the virus infectious cycle.

While E1A mRNA and polypeptide expression were greatly reduced in 2132 cells, in some respects its functions were not: a 20- to 50-fold reduced amount of E1A polypeptides was nearly as efficient at activating transcription of E1A-dependent genes in 2132 cells as were greater amounts of E1A polypeptides in HeLa cells. E1A functions in 2132 cells appeared not to be sensitive to E1A concentrations in a linear fashion, as appears to be the case in HeLa cells (Brunet and Berk, 1988). The B cell background of 2132 cells may provide a transcription apparatus more sensitive to E1A concentrations than that in HeLa cells. An alternative possibility is distinction in the post-translational modifications, such as protein phosphorylation, which might not have been apparent from one-dimensional electrophoresis analysis of E1A polypeptides. Nonetheless, these data show that E1A functions are not only regulated differentially in the lymphoid cell background than in HeLa cells; E1A functions themselves are distinct in the background of HeLa cells.

Posttranscriptional mechanisms influencing E1A and E1B gene expression. What, then, is the mechanism by which E1A mRNA accumulation

is reduced in 2132 cells? Analysis of nascent transcripts by nuclear run-on assays indicated that at early or late times after virus infection, E1A gene transcription initiation was nearly identical between 2132 and HeLa cells. Therefore, reduced E1A mRNA accumulation arose as the result of posttranscriptional mechanisms. Transcription termination appeared not to influence E1A gene expression; the use of probes comprising the entire gene or a 3′ portion of E1A produced similar results in nuclear run-on assays.

Cytoplasmic mRNA stability has been demonstrated to influence E1A mRNA accumulation in HeLa cells, with the mRNA half-life in the cytoplasm increasing as adenovirus infection progresses (Wilson and Darnell, 1981). We sought to determine whether reduced E1A mRNA accumulation in 2132 cells reflected a difference from HeLa cells in cytoplasmic mRNA stability. First attempts to assess the E1A cytoplasmic mRNA half-life were complicated by the method itself. Treatment of HeLa and 2132 cells with the transcription inhibitor dactinomycin (ActD) not only stopped transcription, as judged by c-myc mRNA decay, but also stabilized E1A mRNA such that nearly one-third of E1A mRNA detected before ActD addition was still detectable 100 minutes after ActD addition. Furthermore, ActD induced the appearance of an E1B 22S mRNA population with an inordinately long poly(A) tail. This novel species of 22S mRNA accumulated almost exclusively in the nucleus, while a 22S mRNA population with a shorter poly(A) tail length was detected in both the nucleus and the cytoplasm, even after 100 minutes of treatment with ActD. While this made proper evaluation of E1A and E1B mRNA stability impossible, it supported previous observations by others on the unreliability of ActD chase experiments in

the analysis of certain mRNAs.

Proper evaluation of E1A and E1B RNA kinetics was undertaken using analysis of the kinetics of RNA accumulation. By assessing the time required for a radiolabeled RNA pool to reach steady state labeling, the kinetics of pool synthesis and decay can be evaluated. The assessment of cytoplasmic mRNA accumulation kinetics is rather straightforward; if one assumes that both RNA flow from the nucleus and the pool specific activity are constant, then the cytoplasmic labeling kinetics are inversely related to the mRNA decay kinetics. By analyzing accumulation data over a period sufficiently long to reach a plateau value, a cytoplasmic mRNA half-life value can be derived from the RNA cumulative labeling curve. When HeLa and 2132 cells were analyzed early and late after adenovirus infection, the cytoplasmic half-lives of E1A and E1B mRNAs were actually slightly longer in 2132 cells, between 30 and 35 minutes, than in HeLa cells, approximately 20 minutes. Therefore, a difference in cytoplasmic mRNA half-life did not account for reduced E1A mRNA accumulation in 2132 cells early after infection.

Because the analysis of nuclear RNA kinetics is more complex than cytoplasmic RNA kinetics, the paucity of data points from the cumulative labeling experiments precluded a quantitative approach to their analysis. Rather, we relied on the mathematical model developed by Chen-Kiang et al. (1979) which quantitated nuclear and cytoplasmic RNA kinetics in the conservation of 6-methyl-adenosine residues in the formation of mRNA from the adenovirus major late promoter. By using many more reliable data points to fit curves predicted for multiple-variable accumulation kinetics, Chen-Kiang et al. were able to demonstrate the changes in RNA

accumulation kinetics resulting from variation of such factors as equilibration of the labeled precursor pool to a constant specific activity, conservation of precursors in the formation of mRNA, or the nuclear dwell time for an RNA (Chen-Kiang et al., 1979).

By comparing our data with the curves predicted for alterations of these variables, an interesting contrast was apparent. While E1A RNA sequences in the nucleus of HeLa cells displayed accumulation kinetics predicted for an RNA with a short nuclear dwell time, E1A nuclear RNA sequences in 2132 cells displayed kinetics of accumulation identical to those for an RNA with longer nuclear dwell time. With E1A gene transcription being the same in HeLa and 2132 cells, and cytoplasmic mRNA half-life being approximately the same, the increased retention of E1A RNA sequences in the nucleus appears to lead to decreased E1A mRNA accumulation in 2132 cells as compared with HeLa cells early after infection. This is the first demonstration of the regulation of E1A mRNA accumulation through the nuclear accumulation of transcripts.

E1B 22S RNA sequences display kinetics of nuclear RNA accumulation consistent with inefficient processing early after infection, in both HeLa cells and 2132 cells. This may explain why there is little difference in the steady state E1B mRNA accumulation between HeLa and 2132 cells early after infection (3-fold; Figure 12). This would also explain the appearance of polyadenylated E1B 22S RNA in the nucleus of HeLa cells treated with ActD. Nuclear poly(A) 22S mRNA produced by inefficient RNA metabolism which is either inherently stable or stabilized by ActD will accumulate sufficiently long to serve as templates for poly(A) addition. These would then appear in poly(A)$^+$ RNA fractions, and their poly(A) tails

would continue to grow throughout the ActD chase period.

Striking differences arise in E1B 22S RNA kinetics late after infection, when the 22S RNA sequences appear to be efficiently exported from the nucleus of HeLa cells, while in 2132 cells this is not the case. It would be of interest to examine whether this switch in 22S RNA processing efficiency is induced by E1B 55 kDa or E4 34 kDa viral proteins. If this were the case, mutant viruses defective for expression of these proteins would be expected to display inefficient nuclear processing of 22S RNA throughout infection.

The exact mechanism leading to increased accumulation of poly(A) RNA sequences in the nucleus remains unknown. More insight would be gained by sizing of the poly(A) nuclear RNAs to determine whether they are deadenylated, intact pre-mRNA molecules or RNA degradation products, and by pulse labeling experiments to assess efficiency of polyadenylation. It is interesting to note that the intron of the E1A gene contains multiple repeats of the AU$_n$A motif, which was identified by Shaw and Kamen to be capable of conferring instability on the GM-CSF cytoplasmic mRNA (Shaw and Kamen, 1986). The identification of these sequences as affecting stability of E1A RNA in the nucleus would be an interesting variation of this regulatory mechanism.

Late after viral infection, E1A cytoplasmic mRNAs are significantly more stable in HeLa cells than in 2132 cells; this contributes to the greater accumulation of E1A mRNA in HeLa cells over 2132 cells late after infection. When Wilson and Darnell (1981) first reported the stabilization of E1A mRNA during the late stage of infection, they suggested that the inhibition of host protein synthesis late after

infection might inactivate a labile nuclease responsible for E1A mRNA degradation. In support of this, E1A mRNA accumulation increased when HeLa cells were treated with inhibitors of protein synthesis, and E1A mRNA does not completely decay in Ad2-infected HeLa cells treated with the transcription inhibitor ActD.

The situation in 2132 cells is more complicated, however. Though host protein synthesis is not efficiently inhibited in 2132 cells, this is does not explain the lack of E1A mRNA stabilization in these cells. Preliminary experiments indicate that E1A mRNA levels did not increase in 2132 cells treated with anisomycin for three hours, which was sufficient to increase E1A mRNA accumulation in HeLa cells. Therefore, the putative E1A-specific nuclease would presumably be more stable at the protein level in 2132 cells than in HeLa cells. However, it is interesting to speculate on the nature of an E1A-specific nuclease. Presumably, it does not exist in 2132 cells and HeLa cells to protect against viral infection. A plausible explanation is that the true target of the nuclease is not E1A, but rather a cellular E1A analog, which may perform a function similar to E1A, acting as a transcriptional activator, or perhaps as an activator of cellular DNA synthesis. By inactivating this nuclease through protein synthesis inhibition, adenovirus may enhance the ability of E1A gene products to enhance viral replication.

The continuous culture of HeLa cells for nearly 40 years may have imposed selective pressure in favor of a labile nuclease regulating transcriptional activators, DNA synthesis induction, or maintenance of differentiated cell functions. The less robust growth characteristics of 2132 cells may be a result of a more stable nuclease regulating cell

growth and function. The characterization of this nuclease may be of interest, either in understanding E1A mRNA stability or in the investigation of a cellular E1A analog. Assays such as RNA mobility-shift gels (Malter, 1989; Weeks et al., 1990) and *in vitro* RNA degradation systems (Brewer and Ross, 1989) would aid in the characterization of this nuclease.

The investigation of adenovirus gene regulation in human lymphoid cell lines presents new opportunities to examine long-standing questions, such as nuclear events regulating gene expression, the role of E1A gene products in transcription induction, and the multiple action of ActD. It is hoped that this thesis provides the initiative for further studies to expand our understanding of these molecular biology phenomena.

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