Adenovirus E1A and E1B Genes Are Regulated Posttranscriptionally in Human Lymphoid Cells†‡

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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.

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 reference 30). The functions of the E1B gene products are less well defined. The E1B 55-kDa polypeptide binds to p53 (39) and has also been shown to be important for reducing the accumulation of host mRNAs after infection (3, 36). The E1B 19-kDa polypeptide is suggested to be necessary for maintaining the integrity of viral DNA (43). Furthermore, E1A and E1B genes together can induce transformation of primary rodent cells in culture (for a review, see reference 18).

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 (44) 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 (27). The transcriptional and posttranscriptional 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.

MATERIALS AND METHODS

Cells and viruses. 2132 cells, from an immunoglobulin λ light-chain-secreting human myeloma B-cell line (29), were cultured in RPMI 1640 medium with 10% heat-inactivated fetal calf serum (Hyclone). Suspension cultures of HeLa cells were cultured in Joklik modified minimal essential medium with 5% fetal calf serum. 293 cells (19) were grown as monolayer cultures in Dulbecco modified essential medium with 10% fetal calf serum. All media were supplemented with L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μ g/ml). Ad2 and Ad5 stocks, which were phenotypically indistinguishable in all experiments performed, were propagated as described previously (10) and plaqued on A549 cells. Virus infections were performed essentially as described previously (27), 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 postinfection (p.i.) for HeLa cells and 14 and 48 h p.i. for 2132 cells, respectively, unless otherwise indicated.

DNA analysis. Total DNA from 10^4 uninfected or adenovirus-infected cultures were isolated and analyzed by Southern blotting as described previously (27). Adenovirus DNA was detected using a 32 P-labeled probe of total adenovirus DNA or plasmid p3wt18 (38), which contains the left-hand 5.4 kb of Ad5.

RNA isolation and Northern (RNA) analysis. Total RNAs were isolated by the guanidinium thiocyanate method (11), and polyadenylated RNAs were selected by poly(U)-agarose chromatography (8). Steady-state RNAs were fractionated on 1.4% agarose-formaldehyde gels in MOPS buffer (20 mM

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FIG. 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 to 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 1,702), 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 (15).

morpholinopropanesulfonic acid [Sigma Chemical Co.], 5 mM sodium acetate, 1 mM EDTA, pH 7.0) and analyzed by Northern blotting. The specific probes were c131, an E1A cDNA clone (34), c244, an E1B cDNA clone (35), 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.) Hybridization was performed in the presence of 50% formamide at 42°C overnight as described previously (27); blots were washed four times with 2× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% sodium dodecyl sulfate (SDS) for 10 min at 42°C and two times with 0.1× SSC–0.2% SDS for 30 min at 55°C. Probes were ³²P labeled by nick translation or random priming.

Nuclear run-on assays. Nuclei were isolated from 10⁸ control or virus-infected cells by Dounce homogenization as described previously (9), labeled for 10 min with 300 μCi of [³²P]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.; 17); 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 (GADPH; 16) and human class I major histocompatibility antigen HLA-B (41). After hybridization in 50% for-

amide at 42°C, blots were washed as described previously (9) and exposed to X-ray film. Autoradiographic signals were quantified by scanning of multiple exposures with an LKB laser densitometer.

Nuclease 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 [³²P]ATP (New England Nuclear) and polynucleotide kinase (Boehringer Mannheim Biochemicals). Hybridization of 10 ng of labeled probe to total RNA from 10⁷ 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 S1 (Sigma) at 42°C for 30 min (13). 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.

Immunoprecipitation. A total of 5 × 10⁷ cells at the indicated hours after infection were pelleted, washed in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄ · 7H₂O, 1.4 mM KH₂PO₄, 1 mM MgCl₂ [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 × 10⁶ cells per ml and labeled with [³⁵S]methionine (150 μCi/ml; 1,245 Ci/mmol; New England Nuclear) for 2 or 3 h 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. (21), 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 × g. Nonspecific antibody interactions can often be reduced by preincubation of samples with preimmune serum and precipitation of the nonspecific complexes with protein A (22). 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 (22). After this preadsorption, the pre-cleared 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 (En³Hance; New England Nuclear), gels were exposed to X-ray film.

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

FIG. 2. Northern blot analysis of E1A mRNA in Ad2-infected 2132 and HeLa cells. Poly(A)⁺ RNAs from 2.5×10^7 HeLa and 2132 cells were isolated at the indicated hours after infection with Ad5. After RNAs were fractionated on formaldehyde-agarose gels and transferred to nitrocellulose, E1A mRNAs were detected by hybridization with a randomly primed ³²P-labeled E1A cDNA clone as described in Materials and Methods. The autoradiographic signals were quantitated by densitometric scanning and are presented in graphic form as E1A mRNA units as a function of hours postinfection. Arrows indicate the earliest time after infection at which DNA replication was detected by Southern blot analysis (27).

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 (27).

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. 2). 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. J. 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 (4, 14). 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 3A). 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. 3B; 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. 3). 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. 1). 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. 3A, E1B). Together with the E1A gene, the E1B gene is required for complete transformation of rodent cells (18). 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 (1; Fig. 1) and is not required for E1B transformation functions, was similar in the two cell lines (Fig. 3A).

Further analysis of polyadenylated RNAs isolated early after infection at 14 h 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. 4). Since transcription of the E1B gene early in infection was twofold less in 2132 cells than in HeLa cells (see Fig. 7), 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 (Fig. 3A), 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. 4). The representation of these two mRNA species in HeLa cells (Fig. 3A and 4) is reminiscent of previous results reported by Wilson and Darnell (44). 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

FIG. 3. Northern blot analysis of viral mRNA accumulation in Ad5-infected HeLa and 2132 cells. Poly(A)⁺ RNAs were isolated from 10⁷ 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 (2), Bhat et al. (5), and Virtanen et al. (42), respectively. (B) Extended exposure of blot probed for Ad5 E3 and E4 mRNAs presented in panel A. × represents the exposure time relative to that in panel A.

dominant throughout infection (Fig. 3A and 4). The lack of accumulation throughout infection of E1B 22S mRNA, whose translation product is important for reducing host mRNA accumulation in the cytoplasm (3, 36), may provide a molecular basis for the prolonged early phase after infection of 2132 cells (see Discussion).

E1A polypeptides 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 (21, 22). Results of lysates prepared from cells labeled with [³⁵S]methionine for 3 h are presented in Fig. 5; similar results were obtained from cells labeled for 2 h. E1A polypeptides, migrating at 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. 5). 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. 5). 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. 5). 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 (27). 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*, that carries a 902-bp deletion within the E1A gene, rendering it incapable of E1A expression (24, 40; a kind gift of 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

FIG. 4. (A) Northern blot analysis of E1B mRNAs in early-infected HeLa and 2132 cells early in infection. Poly(A)⁺ RNAs from 2 × 10⁷ 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 ³²P-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 ³²P-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.

FIG. 5. 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 10^7 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 masses (in kilodaltons) of prestained protein standards (Bethesda Research Laboratories) are shown on the left. A 30-fold-extended exposure is presented (30 $\times$) to reveal polypeptide in low abundance. The bracket at the right indicates the 48- to 52-kDa region where E1A 13S and 12S mRNA-derived phosphoproteins are expected to migrate in an SDS-polyacrylamide gel (21).

transformed by and constitutively expresses the E1A and E1B genes (19; Fig. 6). Viral replication, albeit with delayed kinetics, was detectable in HeLa cells infected with *d*/312 at 40 PFU per cell (Fig. 6A; 40). However, no detectable viral replication was observed when 2132 cells were infected with *d*/312 at multiplicities of infection ranging from 10 to 100 PFU per cell (Fig. 6). 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. 7). 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 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 initiation from the major late promoter in the same orientation and encompassing the E3 transcription unit [Fig. 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

FIG. 6. Southern analysis of Ad5 and *dl312* replication. (A) Total DNAs isolated from 10⁴ 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 *Hind*III and analyzed by Southern blotting. The ³²P-labeled probe used was the 5.4-kb *Xho*I-C DNA insert from plasmid p3wt18, which distinguishes the E1A region *Hind*III 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. Note that for 293 cells, DNA samples from 0, 1, 3, and 4 days p.i. are presented. (B) Total DNAs isolated from 10⁴ 293 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 ³²P-labeled probe used was restriction-digested total Ad5 DNA.

processing and stability. To address this possibility, S1 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. 8, 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 (23, 31) 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.

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 postinfection. 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 (44). 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 (2). It would be of interest to investigate the generality of this finding. Attempts to assess the turnover 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 (Lavery and Chen-Kiang, unpublished data). However, preliminary results suggest that the reduced accumulation of E1A and E1B mRNAs in myeloma cells is not the consequence of retention of transcripts 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 stabilized late in infection in myeloma cells (Lavery and Chen-Kiang, unpublished data).

The stabilization of E1A and E1B mRNAs from early to late in infection of HeLa cells had prompted Wilson and Darnell (44) 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

FIG. 7. Nuclear run-on analyses of transcription in Ad5-infected 2132 cells and HeLa cells. Nuclei isolated from 10^8 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 postinfection 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 the 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 10^8 HeLa cells were 1.4×10^7 cpm for uninfected cells, 2.1×10^7 cpm for early-postinfection cells, and 4.9×10^6 cpm for late-postinfection cells; values for 10^8 2132 cells were 2.7×10^7 cpm for uninfected cells, 1.6×10^7 cpm for early-postinfection cells, and 2.1×10^7 cpm for late-postinfection cells. (A) Autoradiographic exposures of the first hybridization, with relative exposure times (1 $\times$ and 20 $\times$) 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.

FIG. 8. Nuclease protection assay for E1A RNA 5' termini. (A) RNAs from 10^7 HeLa or 2132 cells at the indicated hours after infection with Ad5 were hybridized to a 5'-end-radiolabeled Ad5 *Ava*I DNA fragment spanning nucleotides 0 to 854 on the Ad5 genome (B). After digestion with nuclease S1, the nuclease-resistant products were separated on a denaturing 8% polyacrylamide gel, which was dried and exposed to X-ray film. Numbers on the right indicate the sizes in bases of nuclease-resistant products. (B) Diagram of the E1A transcription start site (—►) on the adenovirus chromosome (—) the end-labeled DNA fragment used for nuclease protection experiment (—*), and the expected size of the protected fragment (—*).

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 ribonucleoprotein complex, is 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 (6, 32), histone H4 gene (33), and mRNAs containing the A/U-rich destabilizing element (28). Experiments are in progress 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 (3, 36). The lack of E1B 22S mRNA early in infection of myeloma cells offers a plausible explanation for the observation that host protein synthesis is never completely shut off in the course of infection of these cells (Lavery and Chen-Kiang, unpublished data). 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 (27).

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. 3A). 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 (7), 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 *dl*312 (Fig. 6). 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 (25, 37) 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 (45). 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. 7). 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 (12).

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 (20, 23, 26), yet other aspects of regulation, such as posttranscriptional 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. Spengel, 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.

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