JOURNAL OF VIROLOGY, June 1993, p. 3226–3231
0022-538X/93/063226-06$02.00/0

Vol. 67, No. 6

Regulated Splicing of Adenovirus Type 5 E4 Transcripts and Regulated Cytoplasmic Accumulation of E4 mRNA

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Received 12 November 1992/Accepted 10 March 1993

The E4 gene of human type C adenoviruses has been shown previously to give rise to an array of mRNAs via differential splicing. In this study, the pattern of expression of these mRNAs during lytic infection was examined, and two distinct temporal classes were defined. mRNAs of the early class were distinguished from those of the late class by the presence, in the early class, of a sequence in the 3′ half of the mRNA that was removed as an intron in the late class. A single mRNA of the late class was found to show a strong dependence on the presence of the 55-kDa protein from region E1b and the open reading frame 6 protein from region E4 for its normal cytoplasmic accumulation. One feature of this mRNA that distinguishes it from other E4 mRNAs expressed at late times is the retention within it of an intron from the 5′ half of E4; it may therefore be recognized as incompletely spliced by the host cell and retained in the nucleus. It is proposed that the E1b 55-kDa/E4 open reading frame 6 protein complex facilitates accumulation of this mRNA by overcoming this retention mechanism.

The lytic infectious cycle of human adenovirus type 5 (Ad5) is divided by convention into early and late phases, separated by the initiation of viral DNA replication. The products of genes expressed during the early phase of infection are generally involved in the regulation of gene expression and viral DNA replication, whereas the products of genes expressed after DNA replication function in the structure or assembly of the particle (reviewed in reference 14). However, expression of most of the viral genes is not strictly confined to only one phase of the infection: early genes are still transcribed well into the late phase of infection, and restricted expression from the major late promoter occurs at early times (reviewed in reference 9). Most Ad5 primary transcripts are differentially spliced to allow expression of the virus's many open reading frames (ORFs) from a limited number of promoters, and the pattern of differential splicing observed changes during the course of infection. This is exemplified by expression of the L1 segment of the major late transcription unit, during which only large mRNAs are produced at early times, specifying the 52- and 55-kDa proteins, and predominantly a distinct smaller species at late times, specifying protein IIIa (1, 19).

The E4 region of Ad5 is located between 91.3 and 96.8 map units on the viral genome and is transcribed from right to left. E4 transcription initiates at a unique site, beginning in the early phase and continuing into the late phase of infection. RNA transcribed from the equivalent region of Ad2 is subject to a complex pattern of differential splicing, producing a set of mRNAs that share a common poly(A) site (Fig. 1) (10, 13, 32, 33). These mRNAs potentially encode at least seven different polypeptides, products of ORF1, ORF2, ORF3, ORF3/4, ORF4, ORF6, and ORF6/7, of which three, ORF3, ORF6, and ORF6/7, have been identified during the course of infection of HeLa cells (5, 7, 29, 30). Studies of appropriate mutants have implicated these proteins in regulating late protein synthesis, nuclear and cytoplasmic accumulation of late messages, viral protein synthesis at late times, and the activation of the E2a promoter (3, 11, 15, 28, 34). Recently, the ORF4 product has been shown to regulate the phosphorylation of certain viral and cellular proteins (24).

Previous studies of E4 mRNAs indicated some degree of temporal regulation of the pattern of splicing (27, 32), although individual mRNA species were not identified. To characterize the pattern of expression of the known and putative E4 products in more detail, a systematic study of the levels of individual mRNAs during the infectious cycle was undertaken, revealing distinct early and late classes of E4 mRNA. Given the established role of the E1b 55-kDa and E4 ORF6 proteins in regulating cytoplasmic levels of the major late mRNAs (2, 26, 35), the effect on E4 mRNA levels of deleting these functions was examined. Accumulation of one E4 mRNA species was strongly dependent on both E1b 55-kDa and E4 ORF6 protein functions. The structural features and pattern of expression of this mRNA support the idea that the presence of intact splice donor and/or acceptor signals within an mRNA is a determinant of E1b dependence for efficient mRNA efflux from the late adenovirus-infected cell nucleus.

MATERIALS AND METHODS

The origins of Ad5 *dl*309, *dl*338, *dl*355, and *dl*367 and methods for their growth in cell culture have been described previously (5, 11, 26). Infections were at a multiplicity of 10 PFU per cell except as noted. Cytoplasmic and nuclear fractions of infected HeLa cells were prepared by Nonidet P-40 lysis and subsequent low-speed centrifugation, and total RNA was prepared from each fraction by phenol-chloroform extraction in the presence of sodium dodecyl sulfate and EDTA, all as previously described (18).

Quantitative RNase protection assays were carried out as described by Melton et al. (21), using unselected RNA and an excess of [α-^{32}P]UTP-labeled antisense probe RNA transcribed in vitro from cloned fragments of Ad5 genomic DNA in pGEM vectors (Promega). The positions on the Ad5 genome of the E4 probes generated for this study are shown in Fig. 1. The E1a probe has been described previously (18) and was the generous gift of J. Schaack. Protected fragments

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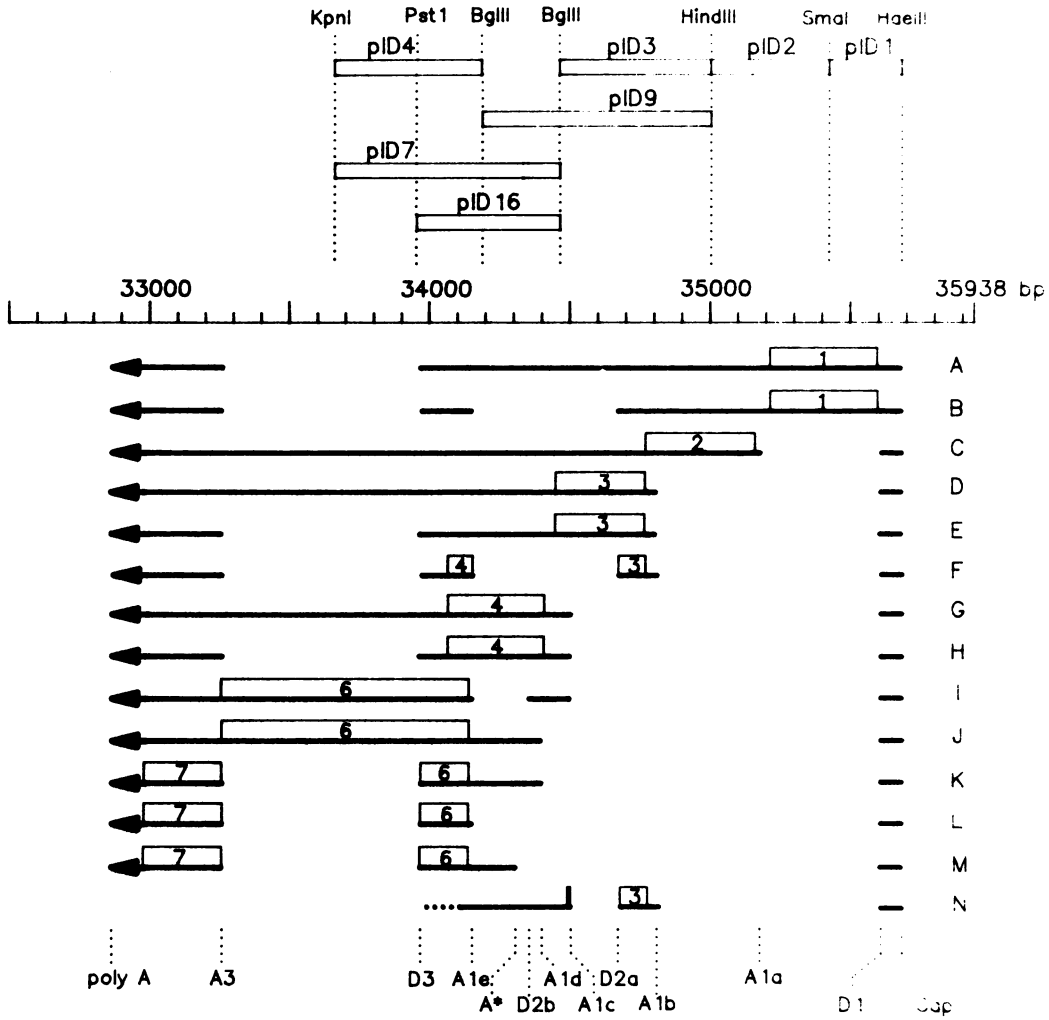

FIG. 1. Constructs used. The right terminal portion of the Ad5 genome (bp 32500 to 35938) is shown as a line scale. Below the line is a transcription map inferred from studies of the closely related virus, Ad2. mRNAs A to L were described by Virtanen et al. (33), and mRNAs M and N are additional species described by Freyer et al. (10). Splice sites are indicated below the transcription map in the notation of Virtanen et al. (33). The additional splice acceptor site inferred from the detection of mRNA M is labeled A*. Minor splice acceptor sites in the 3′ portion of E4 and the mRNAs deriving from their use (10, 32) are not shown; the study reported here did not distinguish these mRNA species. The positions on the genome map of the cloned fragments from which RNA probes were derived are shown at the top (pID1, -2, -3, etc).

were separated on 5% polyacrylamide–7 M urea gels and detected by exposure to preflashed Fuji RX film at −70°C. Protected fragments were quantified by laser scanning densitometry (Molecular Dynamics Corp.), using suitable film exposures. To estimate relative levels of different RNA species, these data were corrected to take account of the number of uridine residues in the protected fragment, using information from the Ad5 sequence (4, 6).

RESULTS

Temporal classes of E4 mRNA. The E4 region primary transcript is spliced to produce at least 14 distinct mRNAs, as indicated in Fig. 1 (10, 31, 32). To examine further the temporal control of E4 RNA splicing, the cytoplasmic level of each E4 mRNA was determined at various times after infection with wild-type Ad5. Each RNA probe used gave protected fragments of characteristic length, allowing identification and quantitation of individual mRNAs, or in some cases of pairs of mRNAs predicted to encode the same translation product. mRNA-probe RNA duplexes covering the region around position 34350 consistently showed sensitivity to RNase that resulted in 50 to 80% cleavage of the probe. Fragment lengths were in each case consistent with an mRNA-probe RNA discontinuity at this position, although no splice donor or acceptor sequence was expected. Inspection of the sequence revealed a stretch of 11 A residues in the E4 mRNA. Stuttering of RNA polymerase on this sequence, either in the cell or, in the antisense, during probe synthesis in vitro, could explain this observation.

Results of representative assays are shown in Fig. 2. The probe used to detect mRNA A did not distinguish mRNAs A and B. However, other probes showed that mRNA B was not present in these experiments. Similarly, the probe for mRNA D did not distinguish mRNAs D and E. However, from results with other probes detecting mRNAs G and H, I

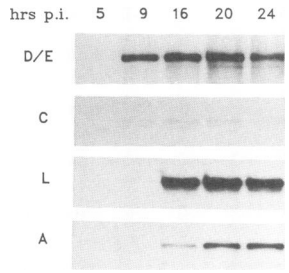

FIG. 2. Analysis of cytoplasmic levels of E4 mRNAs during infection of HeLa cells by wild-type virus, *dl*309, by RNase protection assay. Total cytoplasmic RNA was isolated at the time p.i. indicated above each lane. Ten micrograms of RNA was probed for specific E4 mRNAs, indicated at the left. Fragments shown: D/E, pID3 (345 nucleotides); C, pID2 (174 nucleotides); L, pID7 (178 nucleotides); A, pID1 (258 nucleotides).

and J, and E and H, it could be deduced that mRNA E was significantly less abundant than mRNA D and was expressed with different kinetics (see below). The major component of the protection shown for probe D+E is therefore due to mRNA D. A similar conclusion could be reached for levels of mRNA G in relation to mRNA H (data not shown).

Two distinct classes of E4 mRNAs were identified by this analysis. mRNAs belonging to the early class (mRNA D; Fig. 2) were first detected in cytoplasmic RNA 5 h postinfection (p.i.). Amounts of these mRNAs rose rapidly to 9 h p.i. and then remained constant or declined slightly during the late phase of infection. The small increase in the level of mRNAs D and E that was observed between 9 and 16 h p.i. is consistent with estimates of mRNA E synthesis from other experiments. mRNAs of the late class were only barely detectable at 9 h p.i., and levels increased dramatically thereafter. mRNA A was observed in low amounts at 16 h p.i., but its levels did not peak until 20 h p.i. Other RNAs with late kinetics (mRNA L; Fig. 2) reached maximum or near-maximum levels at 16 h p.i. The temporal classification of each E4 mRNA detected is shown in Table 1.

Relative abundance of E4 mRNAs. Accurate determination of the relative amounts of the different mRNAs is complicated by the need to assume that different probe RNAs were labeled to the same specific activity. This assumption is reasonable only for probes prepared in parallel from the same mixture of α-^{32}P-labeled and unlabeled UTP. For such probe sets, protected fragments were quantified, and length-adjusted data were taken as a measure of relative mRNA levels. A full data set was obtained by comparing the results for groups of mRNAs having one or more members in common. These estimates of relative E4 mRNA levels, which are in reasonable agreement with previous data for Ad2 (32), are shown in Table 1. mRNAs E and H have not been identified individually; however, neither is found in significant amounts at 9 h p.i., and their collective abundance is significantly less than that of mRNA D. mRNAs B, F, I, and N were not detected in any experiment.

TABLE 1. Classification of E4 mRNAs

E4 RNA	Relative amt		Kinetic class
	9 h p.i.	20 h p.i.	
A	−	++	Late
B	−	−	
C	+	+	Early
D	+++	+++	Early
E[a]	−	++	Late
F	−	−	
G	++	++	Early
H[a]	−	++	Late
I	−	−	
J	+	+	Early
K	−	++	Late
L	(+)	++++	Late
M	−	+	Late
N	−	−	

[a] Amounts shown are for mRNAs E and H collectively.

Late E4 mRNAs depend on DNA replication for accumulation. Transcription of Ad5 late genes is severely restricted prior to the onset of viral DNA replication. To determine whether expression of the late class of E4 mRNAs was similarly dependent on viral DNA synthesis, their levels in cells infected in the presence of the DNA synthesis inhibitor hydroxyurea were assayed. As shown in Fig. 4, expression of mRNA D (early class) in a wild-type infection was unaffected by the inhibitor, whereas accumulation of the late E4 mRNAs L and A was largely and completely prevented, respectively. Thus, these late E4 mRNAs, like other viral late mRNAs, depend on DNA replication for their expression.

Dependence of late E4 expression on E1b 55-kDa protein function. Previous work has shown that cytoplasmic mRNA accumulation for genes expressed from the Ad5 genome at late times is dependent on a function provided by the E1b 55-kDa protein product (2, 18, 26, 34). The possible dependence for proper accumulation of the late class of E4 mRNAs, as defined above, on this E1b function was therefore examined by using a mutant virus, *dl*338 (26), which is unable to express the E1b 55-kDa protein.

E4 mRNAs present during a *dl*338 infection were quantified in a comparative analysis with wild-type virus (*dl*309). Representative analyses are shown in Fig. 3. Results with the control E1a probe confirmed that the multiplicities of infection of the two viruses were similar. Because E1a products are involved in the activation of the E4 promoter, E1a mRNA appears in the cytoplasm before E4 mRNA. The low levels of E1a mRNA detected at 5 h p.i. in this experiment may explain why little or no E4 mRNA was detected at this time point. Other experiments have shown the presence of early E4 mRNAs at 5 h p.i.

As expected, the early class of E4 mRNA (mRNA D; Fig. 3) accumulated in the cytoplasm of wild-type- and *dl*338-infected cells with identical kinetics. More surprisingly, most of the late class of E4 mRNAs showed only minimal dependence on E1b 55-kDa protein function for normal accumulation (mRNA L; Fig. 3). The one exception was mRNA A, which had shown the latest kinetic profile in a wild-type infection. Accumulation of this mRNA was found to be strongly dependent on E1b function, with cytoplasmic levels in *dl*338-infected cells only 5 to 10% of the wild-type level at 20 to 24 h p.i.

E4 ORF6 is necessary for E4 mRNA A accumulation. E1b 55-kDa protein exists as a molecular complex with the E4 ORF6 protein in infected cells (28), and in previous studies, virus *dl*355 (lacking E4 ORF6) and *dl*367 (lacking E4 ORF6 and E1b 55-kDa protein) showed phenotypes very similar to that of E1b 55-kDa mutant *dl*338 in respect of late gene expression (5, 11). To confirm that the effect of the E1b mutation on levels of E4 mRNA A was due directly to absence of the E1b mRNA transport regulatory function, the levels of mRNA A in E4 ORF6 mutant infections were

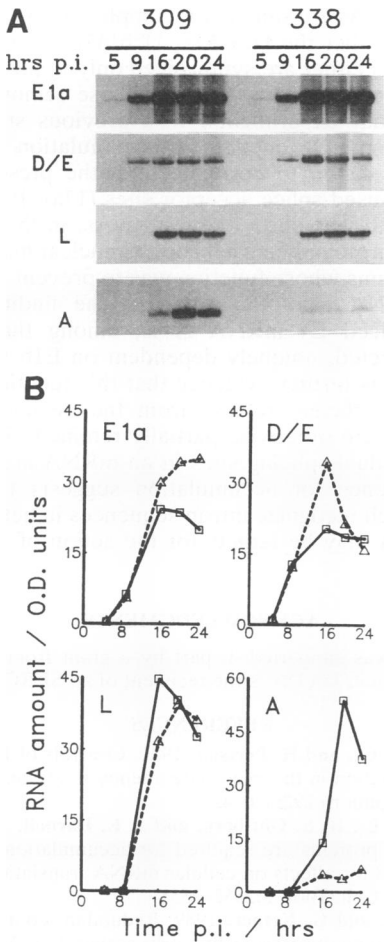

FIG. 3. (A) Analysis of cytoplasmic levels of E1a and E4 mRNAs during infection of HeLa cells by wild-type virus *dl*309 or E1b 55-kDa mutant virus *dl*338, as indicated. The E1a fragment shown is protected by the 3′ common exon (404 nucleotides). Other details are as for Fig. 2. (B) Quantification of data shown in panel A. Results of laser scanning densitometry are shown in arbitrary units plotted against time p.i. for *dl*309 (□) and *dl*338 (△). These data are not corrected for protected fragment length and specific activity differences; therefore, amounts are not comparable between panels. O.D., optical density.

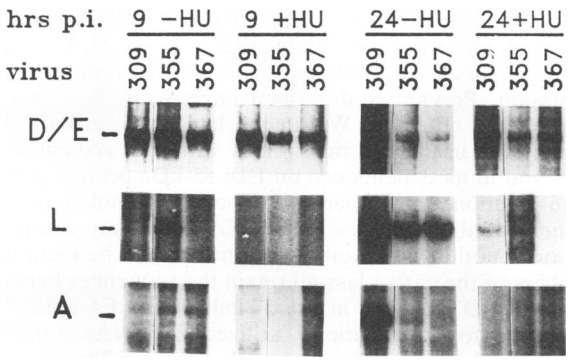

FIG. 4. Analysis of cytoplasmic levels of E4 mRNAs in HeLa cells infected by wild-type *dl*309, E4 ORF6 mutant *dl*355, or E1b 55-kDa/E4 ORF6 mutant *dl*367 at the times p.i. indicated, in either the absence (−HU) or presence (+HU) of hydroxyurea, added at 10 mM to the culture medium. Virus stocks were CsCl gradient-purified particle preparations and used at 500 particles per cell. Five micrograms of unselected RNA was probed for specific mRNAs indicated at the left; other details are as for Fig. 2.

examined (Fig. 4). Levels of mRNA D (early class) were unaffected at 9 h p.i. in these infections and only marginally affected at 24 h p.i.; mRNA L showed a similar pattern at 24 h p.i. These marginal reductions at late times may be due to the slight reduction in the rate of DNA replication previously noted for these mutants (5, 11). In contrast, levels of mRNA A were severely depressed in both E4 mutant infections, as they were in *dl*338 infections.

E4 mRNA A dependence on E1b is at a postprocessing level. Since all E4 mRNAs derive from the same primary transcript, the selective depression of mRNA A levels seen in *dl*338 infection must be due to a posttranscriptional effect. mRNA A results from nonusage of the E4 5′ splice donor site D1 (Fig. 1). To determine whether the absence of E1b 55-kDa protein resulted in increased usage of this site, the relative abundance of RNA spliced and unspliced at this site in nuclear RNA was assessed for cells infected with either wild-type or *dl*338 virus. The results of this analysis are shown in Fig. 5.

Levels of a control E4 RNA (mRNA L) in both nucleus and cytoplasm were unaffected by the absence of E1b 55-kDa protein. Levels of mRNA A in the cytoplasm were, as before, greatly reduced in the mutant infection. However, no similar reduction in nuclear levels of mRNA A was seen. This result shows that, as for other mRNAs which depend on E1b 55-kDa protein function for accumulation, E4 mRNA A requires this function for its efficient movement out of the nucleus or for stabilization rather than to modulate its splicing. No probe fragment protected by mRNAs spliced at site D1 could be detected in either wild-type- or *dl*338-infected cell nuclear RNA. Also, the cytoplasmic/nuclear ratio for mRNA L was very much greater than that for mRNA A. These findings suggest that, once spliced at the D1 site, E4 mRNA is rapidly exported to the cytoplasm. A similar conclusion was reached previously regarding E2 mRNA expression (18).

DISCUSSION

The various Ad5 E4 mRNAs have been divided into two temporal classes based on a detailed analysis of RNA levels

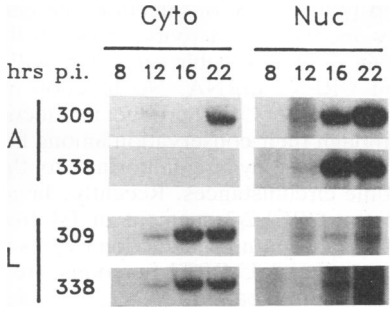

FIG. 5. Analysis of cytoplasmic and nuclear levels of E4 mRNAs during infection of HeLa cells by wild-type virus *dl*309 or E1b 55-kDa mutant virus *dl*338. Total cytoplasmic (Cyto) and total nuclear (Nuc) RNAs were isolated at the times p.i. indicated, and 5-μg samples were analyzed. The virus used and E4 RNA detected are indicated at the left. Other details are as for Fig. 2.

over the time course of wild-type infection. mRNAs C, D, G, and J form an early class, while mRNAs A, E, H, K, L, and M form a late class, dependent on viral DNA replication for expression. Previously described mRNAs B, F, I, and N could not be detected. Within the late class, mRNA A is unique, both in the late time p.i. at which its accumulation peaks and in its dependence on E1b 55-kDa protein and E4 ORF6 functions for proper cytoplasmic accumulation.

When members of these E4 mRNA classes are compared with one another, structural relationships can be identified. Members of the early class all retain the sequences between splice sites D3 and A3 in the 3′ half of the E4 unit. This donor-acceptor combination is utilized in mRNAs of the late class to produce a family of shorter mRNAs. The usage of these sites is therefore temporally regulated during the course of Ad5 infection; recent studies of E4 expression in abortively infected monkey cells suggested that the E2a DNA-binding protein was important for this regulation (27). As with other examples of regulated adenovirus splicing, in expression of the E1a, E1b, and L1 genes (1, 8, 19, 22, 31), processing in E4 moves toward more heavily spliced, shorter mRNAs in the late phase of infection.

The undetected E4 mRNAs are also related, being formed by the use of donor site D2a or D2b. The lack of usage of these sites may be sequence related, as they are more divergent from the consensus sequence (23) than are sites D1 and D3, where splicing was observed. Given the detection of mRNAs spliced at these sites by other workers, their absence in our experiments may indicate that usage of D2a or D2b is regulated and apparent only under certain circumstances.

The observed temporal pattern of RNA expression from the E4 region suggests that ORF2, ORF3, ORF4, and ORF6 products are synthesized initially during the early phase of infection, while the products of ORF1 and ORF6/7 are synthesized later. The early expression of ORF3 and ORF6 is in agreement with the role of their products in facilitating viral gene expression from the onset of the late phase (3, 11, 15, 28, 34). ORF4-mediated down-regulation of E1a phosphorylation in HeLa cells (24) was observed from early in the infection, in agreement with our data. The delay in the expression of ORF6/7 is surprising given its role in the activation of the E2 early promoter (16, 20, 25). Formation of an infection-specific, E4-dependent complex on an E2 promoter fragment has been observed in extracts prepared from HeLa cells from 6 h p.i. (12). However, amounts of this complex increased dramatically between 6 and 12 h p.i. in these experiments. Given the differences in experimental protocol used (multiplicity of infection and cell type), this delayed-early increase in activity, now attributed to E4 ORF6/7 protein, is in reasonable agreement with our data on expression of ORF6/7 mRNA. No function has yet been assigned to the ORF1 or ORF2 product in infections of HeLa cells (11), although their conservation among human adenoviruses implies that they are important to the infectious process in some circumstances. Recently, Javier et al. (17) showed that the ORF1/2/3 segment of E4 from Ad9 was essential for mammary tumor induction by this virus in rats. Our data suggest that Ad5 ORF1 functions late in infection, while ORF2 is required earlier.

Among the E4 mRNAs detected in this study, mRNA A is the only one to retain splice donor site D1 unused. Furthermore, this mRNA also retains a number of acceptor sites (A1a to A1e) that are used, with D1, to form other detectable E4 mRNAs. Thus, mRNA A is an incompletely spliced mRNA which contains a readily removable intron. Although other E4 mRNAs are similarly incompletely spliced, through retention of either the D2a/A1e, D2b/A1e or D3/A3 intron, these mRNAs either are synthesized only in the early phase of infection or else carry introns whose removal was not detected in our experiments. In a previous study, strong dependence on E1b function for accumulation of late viral mRNA was shown to correlate with the presence in the mRNA of unused splice acceptor sites (17a). It was argued that this result was due to viral mRNAs, in the absence of E1b 55-kDa protein, being held on the nuclear matrix by host cell mechanisms whose function was to prevent the efflux of immature RNA from the nucleus. The finding that the partially spliced E4 mRNA A is, among the many E4 mRNAs detected, uniquely dependent on E1b 55-kDa protein function is further evidence that this function is necessary for the efficient release from the nuclear matrix of mRNAs that are otherwise partially retained. The implication that residual splicing sites in an mRNA are a cause of E1b dependence for accumulation suggests that nuclear proteins which recognize intron sequences in heterogeneous nuclear RNA may be targets for the action of E1b 55-kDa protein.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Medical Research Council. Ian Dix is the recipient of an SERC studentship.

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