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# Genetic Analysis of mRNA Synthesis in Adenovirus Region E3 at Different Stages of Productive Infection by RNA-Processing Mutants

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**Region E3 of adenovirus encodes about nine overlapping mRNAs (*a* to *i*) with different spliced structures and with two major RNA 3′ end sites termed E3A and E3B. Synthesis of E3 mRNAs was examined by the nuclease-gel procedure at early and late stages of infection by wild-type virus (*rec*700) and by several E3 deletion mutants. Our results, together with those obtained by electron microscopy (L. T. Chow, T. R. Broker, and J. B. Lewis, J. Mol. Biol. 134:265–303, 1979), suggest that E3 may be differentially regulated at early and late stages at both the promoter and RNA-processing levels. Early after infection, the E3 promoter is used to make mainly mRNAs *a* and *h*. Late after infection, the E3 promoter appears to be shut off and the major late promoter is used to make mainly mRNAs *d* and *e*. The late L4 mRNA 3′ end site is not used early even though early E3 pre-mRNAs transcribe through the L4 RNA 3′ end site. The nucleotide 768–951 exon, which is the y leader on many L5 mRNAs, is very abundant late. (Nucleotide +1 is the major E3 transcription initiation site.) Early after infection, the 951 5′ splice site is enhanced 5- to 10-fold in the *dl*712 (Δ1691 to 2122) group of mutants; late after infection, these mutants resemble the wild type. We speculate that the activity of the 951 5′ splice site is regulated at early and late stages; it is suppressed early to permit synthesis of mRNA *a*, and it is activated late to permit synthesis of mRNAs *d*, *e*, and L5. With *dl*719 (Δ2173 to 2237), the 951→2157 splice is enhanced both early and late; we suggest that this deletion enhances the 2157 3′ splice site.**

We are interested in adenovirus region E3 (12, 13, 35) as a model to understand gene organization and regulation, and in particular differential RNA processing, in a "complex" transcription unit (14, 25). E3 is located at genomic map position 76 to 86 and is expressed off *r* strand. E3 encodes about nine overlapping mRNAs (*a* to *i*) with different exon structures and with two major 3′ ends termed E3A and E3B (Fig. 1). These mRNAs are formed by differential processing of pre-mRNA (14). *rec*700 (Fig. 1) is the wild-type version of the mutants that we are analyzing; it has adenovirus type 5 (Ad5) sequences from map position 0 to 76, adenovirus type 2 (Ad2) sequences from map position 76 to 83, and Ad5 sequences from map position 83 to 100.

Few details are known about the regulation of E3 mRNAs. In common with other classical early regions, E3 is transcribed during early stages, before DNA replication. E3 transcription is induced by the product(s) of E1A (4, 19). There is evidence that E3 transcription is reduced at late stages of infection (9, 27, 30, 34). It is not known whether the RNA processing pattern for E3 mRNAs changes as a function of time during early stages of infection. An interesting aspect of E3 is that it is embedded within the major late transcription unit. The major late transcription unit is initiated from the major late promoter at map position 16 and is transcribed off *r* strand between map positions 16 and 92 to produce five families (L1 to L5) of late mRNAs. The RNA 3′ end site (the polyadenylation site) for the L4 family of mRNAs is located in E3 (Fig. 1). Also, L5 (fiber) pre-mRNAs must transverse through E3. These features provide a unique opportunity to examine how RNA processing signals function at different stages of infection. That is, is the L4 RNA 3′ end site used early, are the E3A and E3B sites used late, and are the E3 5′ and 3′ splice sites used late? The available information indicates that the L4 site is not used early, the E3A and E3B sites are used at low efficiency late, and some of the splice sites are used late (5, 11, 18).

We have isolated a number of viable virus mutants with deletions of E3 (6–8, 16, 37; B. M. Bhat, H. A. Brady, M. H. Pursly, and W. S. M. Wold, J. Mol. Biol., in press). Many of these mutants are defective in E3 pre-mRNA processing, sometimes in a surprising manner (Bhat et al., in press) (the phenotypes are described in Results). In this communication we report the E3 mRNA patterns in wild-type- and mutant-infected cells at different stages of infection.

## MATERIALS AND METHODS

**Isolation of virus mutants.** Isolation of the virus mutants used has been previously described (37). Mutants were constructed in such a way that they have Ad5 sequences from map position 0 to 76, Ad2 sequences from 76 to 86, and Ad5 sequences from 86 to 100. Ad5 and Ad2 are closely related in region E3 (13). *rec*700 is the wild-type version of the mutants (Fig. 1).

**Extraction and nuclease-gel analysis of mRNA.** Procedures for infection of KB cells and preparation of cytoplasmic and poly(A)$^+$ RNA have been previously described (12). RNA was extracted at different times postinfection (p.i.) in the absence of drugs that inhibit DNA or protein synthesis. Early RNA isolated at 6 h p.i. produced the same results as RNA from cycloheximide-treated cells (data not shown). Chow et al. (11) also found that the relative distribution of E3 mRNAs was not significantly affected by drugs.

Nuclease-gel analyses (5) were done with $^{32}$P-labeled RNA probes (23) and 6% sequencing gels as described elsewhere (6; Bhat et al., in press). The probes are described in the figure legends. Cytoplasmic RNA (30 μg) or poly(A)$^+$ RNA (3 μg) was used. RNAs from each infection at the equivalent time point contained similar quantities of total E3 and L4 mRNAs. E3 mRNAs were measured by the abundance of the 1 to 372 exon, and L4 mRNAs were measured by the abundance of their 3′-proximal exon. The abundances of

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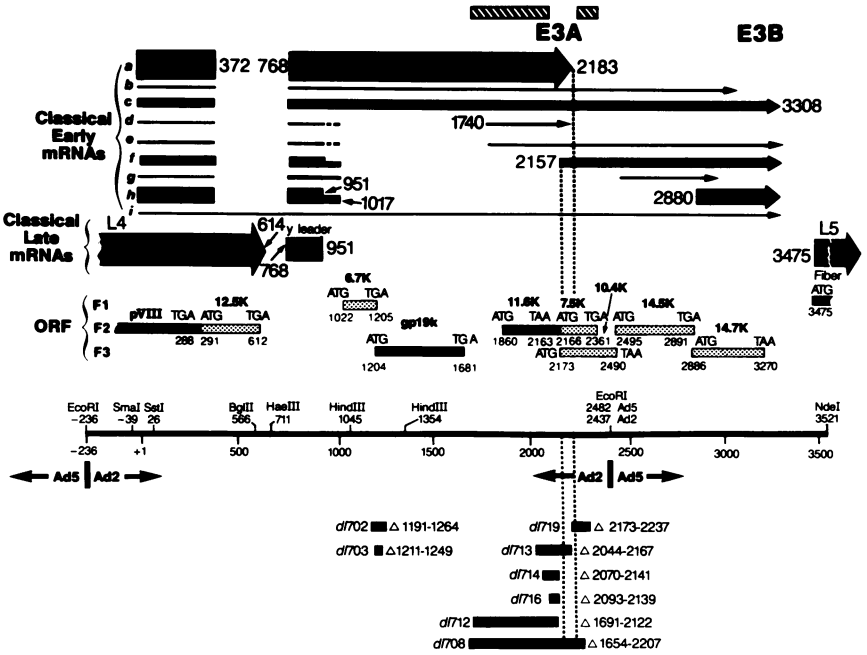



FIG. 1. Schematic representation of the E3 transcription unit in *rec*700 and of deletion mutants. E3 in *rec*700 has Ad2 sequences between the *Eco*RI sites at nts −236 and 2437 and Ad5 sequences elsewhere. Ad2 numbers are used from nts −236 to 2437, and Ad5 numbers are used downstream from the *Eco*RI site at nt 2482 in the Ad5 sequence. Nt +1 is the transcription initiation site. Restriction sites are numbered at the 5′ base in the recognition sequence, not at the actual cleavage site. Arrows *a* to *i* indicate the exon structures of the mRNAs (11). The same letters are used for early and late mRNAs, as well as those that have the 768–951 or 768–1017 exon. Open reading frames (ORFs) are shown; solid bars indicate proven proteins and hatched bars indicate proteins that we propose to exist (13). Evidence in support of this schematic is discussed in references 12 and 13.

these exons were determined by tracing of gel bands with a Joyce-Loebl microdensitometer. mRNAs from region E1B were also at similar concentrations among mutants (data not shown). Therefore, the infections were equally efficient and the deletions did not significantly affect accumulation of total E3 or L4 mRNAs at early or late stages of infection.

## RESULTS

Synthesis of E3 and L4 mRNAs by *rec*700 and by the deletion mutants was analyzed by the quantitative nuclease-gel method (5) with $^{32}$P-labeled RNA probes. With this method, hybrids between the probe and exons in the mRNA are resistant to RNase digestion, and the resistant fragments are identified by gel electrophoresis. Since the probe is in excess, the abundance of the exon is reflected by the intensity of the gel band. The schematics at the bottom of the gel figures show the exons from the various overlapping mRNAs that should anneal to the probe; the numbers above each exon indicate the sizes of the fragments that were observed in the analysis.

**Expression of region E3 mRNAs and the L4 family of mRNAs at different stages after infection by *rec*700.** *rec*700 RNA extracted at different times p.i. was analyzed for exons encoded by the left about one-third of E3. The 1–372 exon (Fig. 2, 372 nucleotide [nt] band) was apparent at 3 h (lane c), peaked at 6 h (lane d), declined at 9 h (lane e), and disappeared at 12 h (lane f). Clearly, the classical early E3 mRNAs with the 1–372 exon are very scarce at intermediate (12 h) times p.i.

The 283-nt band in Fig. 2A corresponds to the second exon in mRNAs *a* to *c*, extending from the 3′ splice at nt 768 to the 5′ end of the probe at nt 1050. We will refer to this as the "768–E3A/B" exon. The 250-nt band corresponds to the 768–1017 exon in mRNAs *d* to *h*. The precise mapping of this exon will be described elsewhere (B. Bhat and W. Wold, manuscript in preparation). The temporal synthesis of these two exons was similar to that of the 1–372 exon except that low amounts of the 768–E3A/B exon remained from 12 to 31 h (lanes f to h) p.i. The 184-nt band corresponds to the 768–951 exon in mRNAs *d* to *h*. This exon was relatively scarce from 3 to 9 h p.i., increased dramatically at 12 h, and continued to increase until 31 h p.i. The 768–951 exon is the y leader found on ~25 to 50% of L5 mRNAs (3, 10, 17, 35, 38). The increase in this exon at 12 h presumably mainly reflects synthesis of L5 mRNAs. These results indicate that the 768 3′ splice site is used actively at early and late times p.i. In addition, the 951 5′ splice is very active late such that most pre-mRNAs that use the 768 3′ splice also use the 951 5′ splice. This is different from the situation early, in which only ~20% of pre-mRNAs that use the 768 3′ splice site also use the 951 5′ splice site (the remaining ~80% of the pre-mRNAs either are not spliced again or use the 1017 5′ splice site) (11). We propose that the 951 5′ splice site somehow becomes activated late. The 768–951 exon is spliced to the E3 1–372 exon early and presumably to the major late tripartite leader late.

The ~650-nt band in Fig. 2A corresponds to the last exon in the L4 family of mRNAs. The 3′ ends of these mRNAs are formed near nt 615 downstream of the AATAAA at nt 597 (18, 21). This exon was absent at 3 and 6 h p.i., appeared at 9 h p.i., and persisted until 31 h p.i. (the decreased abundance of this exon at 31 h was not observed in other experiments).

The temporal synthesis of the E3 1–372 exon and the last L4 exon was confirmed by using a shorter specific probe

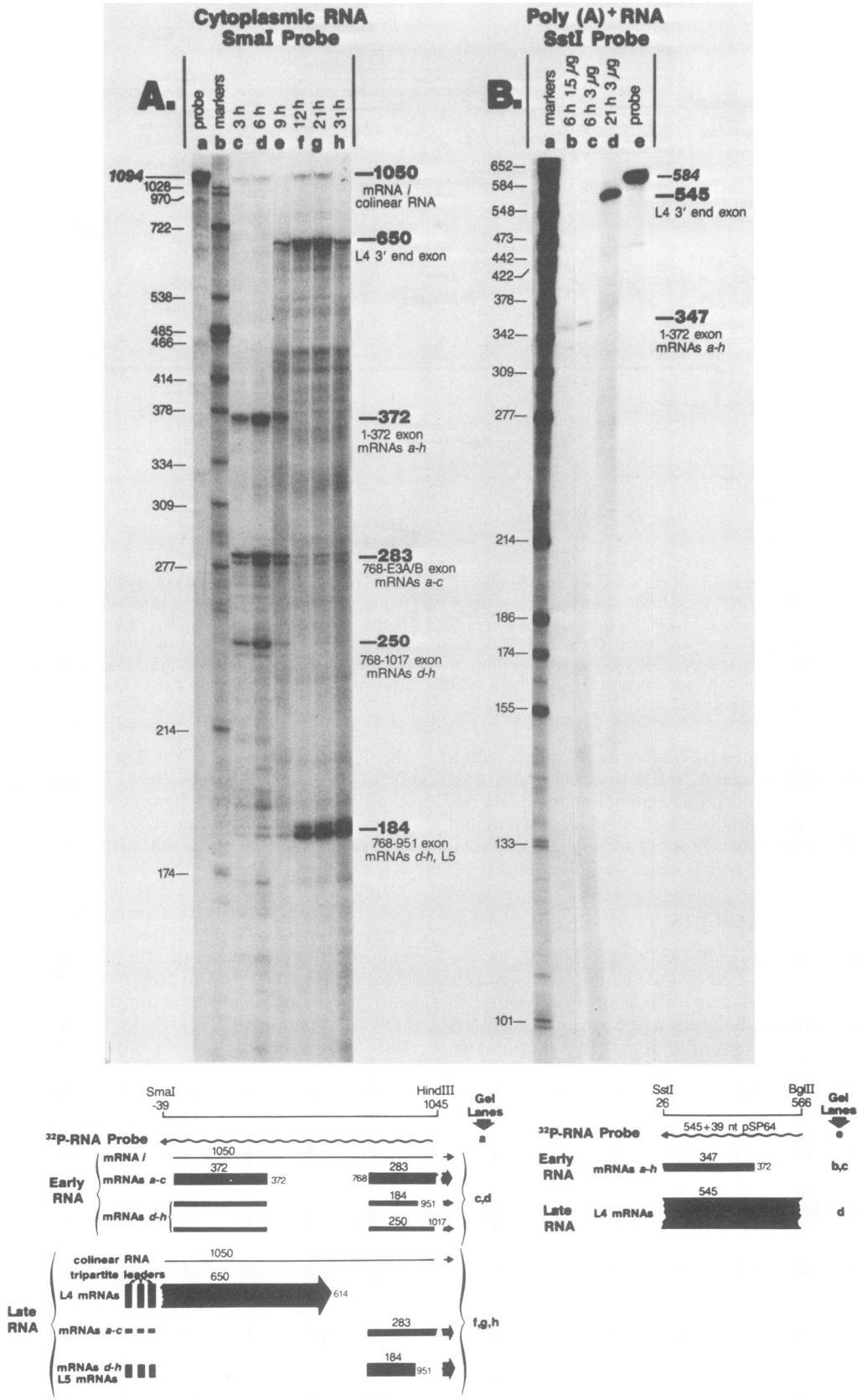



FIG. 2. Nuclease-gel assay of exons from the left one-third of region E3 in RNA extracted at different stages of infection by *rec*700. The schematic at the bottom indicates the probes used as well as the RNase-resistant fragments observed in the analysis. The *Hin*dIII→*Sma*I and

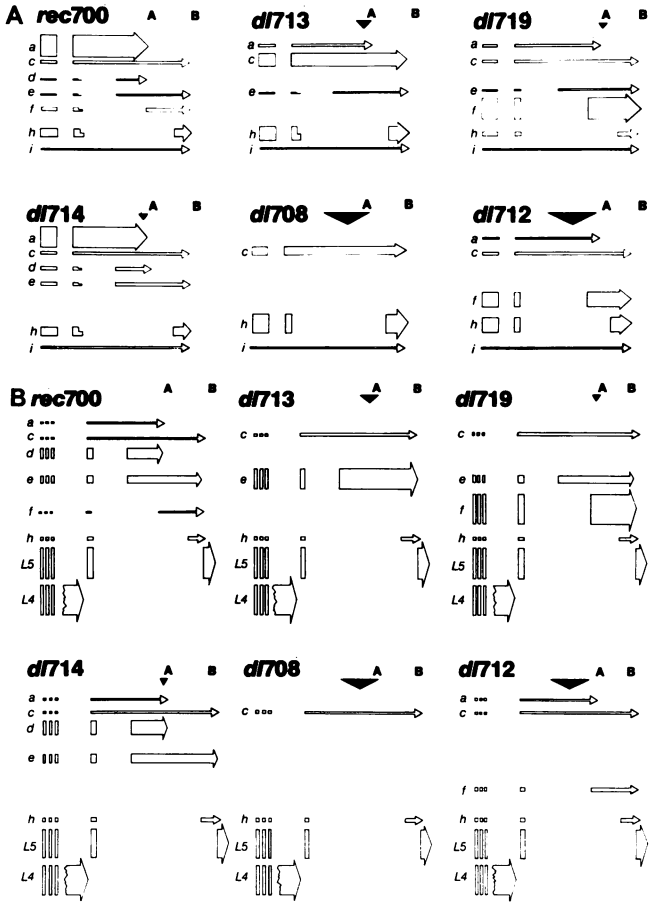



FIG. 3. Schematic representation of the major mRNAs synthesized by *rec*700 and by deletion mutants at early (A) and late (B) stages of infection. *dl*703 (Δ1211 to 1249), *dl*702 (Δ1191 to 2164), and *dl*716 (Δ2093 to 2139) are similar to *rec*700.

(584-nt *Bgl*II→*Sst*I probe) and poly(A) mRNA (Fig. 2B). That is, the 1–372 exon was detected at 6 h but not at 21 h, and the L4 exon was detected at 21 h but not at 6 h. The L4 exon was much more abundant than the 1–372 exon (Fig. 2B, lanes c and d); this is the expected result because late mRNAs are much more abundant than early mRNAs.

An x leader has been observed by electron microscopy on some L5 mRNAs (10); this leader should use the 372 5′ splice site and a 3′ splice site ~100 to 300 nt upstream. We have never detected the x leader in late RNA (Fig. 2A and B).

Use of the other splice sites and E3A and E3B RNA 3′ end sites by *rec*700 at early and late stages of infection will be discussed below, together with the mutants. The results are summarized in Fig. 3.

**Analysis of mutants at early and late stages of infection for synthesis of mRNAs with the 768–951, 768–1017, and 768–E3A/B exons.** We have described two groups of splice-enhancing deletion mutants (17; Bhat et al., in press). The effects that these deletions have on the accumulation of E3 early mRNAs are depicted in Fig. 3A. *dl*712 (Δ1691 to 2122) is a prototype member of one group; its deletion enhances the 951 5′ splice site such that most pre-mRNAs are processed into mRNAs *f* (951→2157 splice) and *h* (951→2880 splice). *dl*708 (Δ1654 to 2207) resembles *dl*712 except that the 2157 3′ splice and E3A RNA 3′ end sites are deleted; with *dl*708, most pre-mRNAs are processed into mRNA *h* and lesser amounts of mRNA *c*. *dl*719 (Δ2173 to 2237) is the prototype of a second group; its deletion enhances the 951→2157 splice such that almost all pre-mRNAs are processed into mRNA *f*. Because of enhanced splicing at the 951 5′ splice site, the 768–951 exon is 5- to 10-fold more abundant in early mRNA from the *dl*712-*dl*708 and *dl*719 groups than from *rec*700. *dl*702 (Δ1191 to 1264), *dl*703 (Δ1211 to 1249), *dl*713 (Δ2044 to 2167), *dl*714 (Δ2070 to 2141), and *dl*716 (Δ2093 to 2139) do not have enhanced splicing activity at the 951 site. *dl*713 has the 2157 3′ splice site deleted, so mRNA *f* is not formed. *dl*713 also has an ATTAAA sequence near the E3A RNA 3′ end site deleted; this destroys most of the E3A RNA 3′ end signal so that mRNAs *a* and *d* are very scarce (7). *dl*714 has the 2157 3′ splice signal deleted so that mRNA *f* is not formed (6).

Figure 3B depicts the RNA processing phenotypes of these mutants at late stages of infection as described in this report. Figure 4A shows nuclease-gel analysis for the 768–951, 768–1017, and 768–E3A/B exons in wild-type Ad2, in *rec*700, and in mutants with the wild-type phenotype with respect to splicing activity at the 951 5′ splice site. RNA extracted at 6, 12, and 21 h p.i. was analyzed. No significant differences were observed in the abundances of these three exons from any virus at any time point. Also, the same results were obtained with all viruses as those described earlier for Fig. 2. That is, the 768–951 exon (184-nt band) increased and the 768–1017 exon (250-nt band) and 768–E3A/B exon (283-nt band) decreased from 6 to 12 h p.i.

Figure 4B shows results with three representative splice-enhancing mutants. The 768–951 exon was elevated 5- to 10-fold in 6-h RNA from *dl*719 (lane a), *dl*712 (lane d), and *dl*708 (lane g) compared with wild-type *rec*700 and the non-splice-enhancing mutants in Fig. 4A (lanes c, f, i, l, o, and r). Thus, use of the 951 5′ splice site by *dl*719, *dl*712, and *dl*708 is markedly enhanced relative to the wild type during early stages of infection; this finding has been reported previously (17; Bhat et al., in press). This enhancement relative to the wild type is not so apparent during late stages of infection. In 12- or 21-h RNA from *dl*719 (Fig. 4B, lanes b and c), the 768–951 exon was elevated about two- to three-fold relative to *rec*700 and the non-splice-enhancing mutants in Fig. 4A. In 12- or 21-h RNA from *dl*712 (Fig. 4B, lanes e and f) and *dl*708 (Fig. 4B, lanes h and i), the 768–951 exon was at about the same abundance as *rec*700 and the non-splice-enhancing mutants (Fig. 4A). Thus, the *dl*712-*dl*708 deletions do not enhance the 951 5′ splice site late after infection, relative to the wild type, as they do early. The two- to three-fold enhancement of the 768–951 exon in *dl*719 relative to the wild type late after infection probably reflects the two- to three-fold enhancement of the 2157 3′ splice site late after infection (see below).

With all three splice-enhancing mutants, the 768–1017 and 768–E3A/B exons (250- and 283-nt bands) were very scarce, not only late after infection as with *rec*700 and the non-splice-enhancing mutants but also early after infection (Fig. 4B). This is because the 951 5′ splice is enhanced both early

*Bgl*II→*Sst*I probes were used in A and B, respectively. These probes have 6 and 39 nt of vector sequences, respectively, at their 5′ termini. In the gel, heavy roman type indicates RNase-resistant fragments, medium italics indicate probes, and light roman type indicates $^{32}$P-labeled RNA size markers.

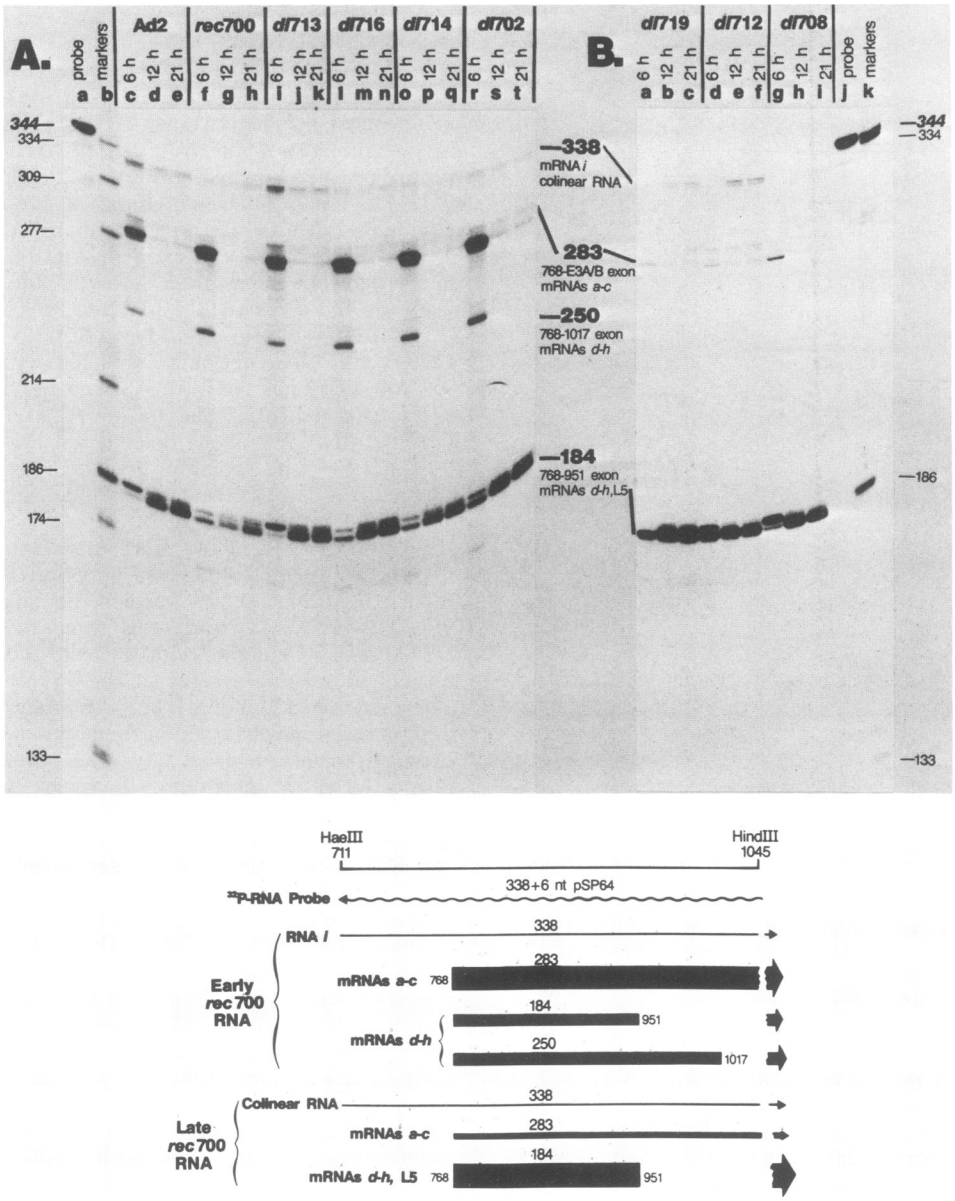
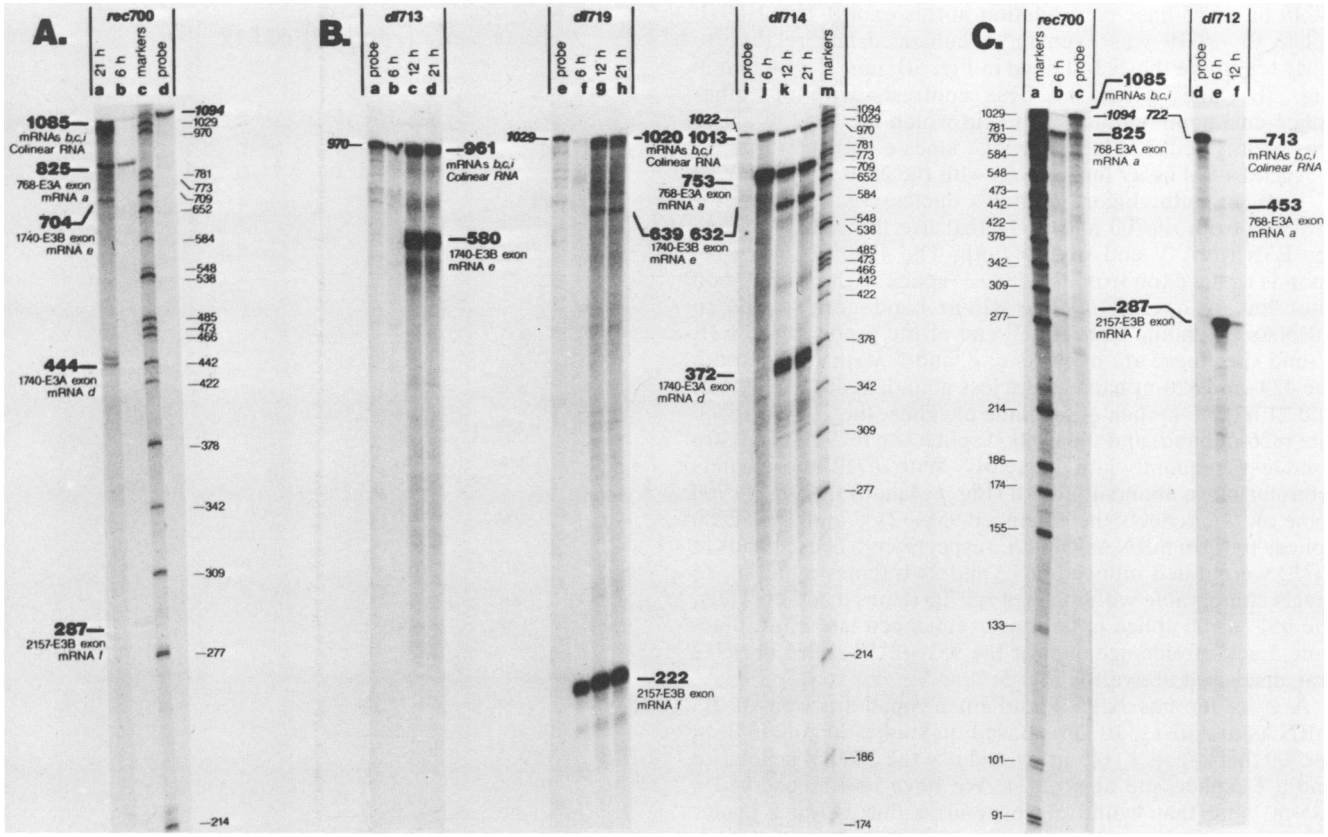



FIG. 4. Nuclease-gel assay for the 768–E3A/B, 768–951, and 768–1017 exons extracted at 6, 12, and 21 h p.i. from cells infected by *rec*700 or deletion mutants. The *Hin*dIII→*Hae*III probe was used. (A) The wild type and mutants with the wild-type phenotype. (B) Mutants with enhanced splicing at the 951 5′ splice site.

and late, and this causes the 768–1017 and 768–E3A/B exons to be spliced away.

**Analysis of mutants at early and late stages of infection for synthesis of mRNAs with the 1740 3′ splice, E3A 3′ ends, and the 2157 3′ splice.** Figure 5 shows the analysis of *rec*700 and representative mutants for mRNAs *d* and *e* with the 1740 3′ splice, mRNAs *a* and *d* with E3A RNA 3′ ends, and mRNA *f* with the 2157 3′ splice. In part A, the gel was run longer to resolve the bands. Parts A and B show cytoplasmic RNA, and part C shows poly(A)$^+$ RNA for comparison. The schematic at the bottom shows the sizes of the RNase-protected fragments that were generated for *rec*700 from the exons in the various mRNAs. The top of the schematic indicates the deletions in the mutants. The deletions alter the sizes of some of the RNase-protected fragments. The gel bands in the figure are labeled to indicate the correspondence between the fragments and exons. For example, the 1740–E3B exon for mRNA *e* is represented by a 704-nt band for *rec*700 (Fig. 5A, lane a) and a 580-nt band for *dl*713 (Fig. 5B, lanes c and d). The major points from this analysis are as follows.

First, the 1740 3′ splice was very infrequent (undetectable in these gels) early but was greatly increased late. This splice is indicated in *rec*700 by the 444-nt (mRNA *d*) and 704-nt (mRNA *e*) bands (Fig. 5A, lane a), in *dl*713 by the 580-nt (mRNA *e*) band (Fig. 5B, lanes c and d), and in *dl*714 by the 372-nt (mRNA *d*) and 632-nt (mRNA *e*) bands (Fig. 5B, lanes k and l). The 1740 3′ splice was also elevated late in *dl*719 but not to the same extent; this splice is indicated by the 639-nt (mRNA *e*) band (Fig. 5B, lanes g and h). No bands from the



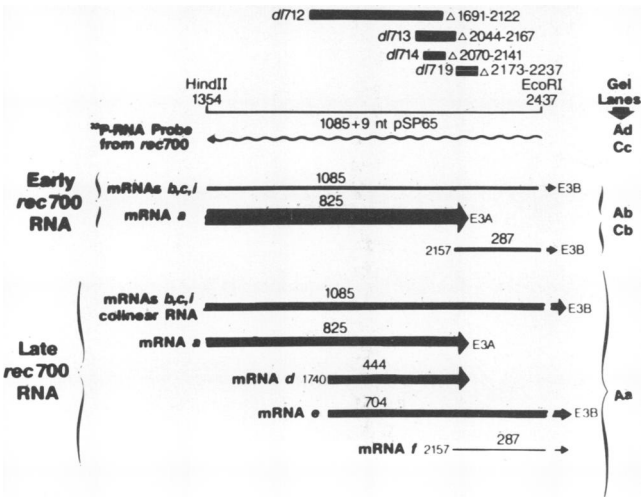




FIG. 5. Nuclease-gel assay for exons with E3A RNA 3′ ends and for exons with the 1740 and 2157 3′ splices. RNAs were extracted at 6, 12, or 21 h p.i. Each *Eco*RI→*Hin*dIII probe used was prepared from the same mutant DNA corresponding to the virus preparation. The probes differ in size because of the deletions. Cytoplasmic RNA (30 μg) was used in A and B, and poly(A)$^+$ RNA (3 μg) was used in C. All lanes in B were from the same gel exposure, as were all lanes in C.

1740 3′ splice were obtained from *dl*712 (Fig. 5C, lanes e and f) or *dl*708 (data not shown) because the 1740 3′ splice site is deleted.

Second, the bands corresponding to mRNA *d* (see above) indicate that the E3A RNA 3′-end signal functions both early and late after infection. Use of the E3A RNA 3′-end signal by mRNA *a* is indicated in *rec*700 by the 825-nt band (Fig. 5A, lanes a and b) and in *dl*714 by the 753-nt band (Fig. 5B, lanes j to l). No bands for exons from mRNAs *a* and *d* with E3A 3′ ends were obtained from *dl*713 (lanes b to d) or *dl*719 (lanes f to h) because these mutants are defective in E3A RNA 3′-end formation (7). The faint 453-nt band in *dl*712 6-h RNA (Fig. 5C, lane e) corresponds to mRNA *a* with E3A RNA 3′ ends. We have described earlier that *dl*712 makes very little mRNA *a* with E3A RNA 3′ ends early after infection (8). This also appears to be true late (Fig. 5C, lane f).

Third, in contrast to the 1740 3′ splice, the 2157 3′ splice (287-nt band) was reduced late in *rec*700 (Fig. 5A, lane a) compared with the early stage in *rec*700 (Fig. 5A, lane b). (The 287-nt band representing the 2157 3′ splice is clearly visible on the original gel in Fig. 5A, as well as in other gels. The 2157 3′ splice for mRNA *f* was quite minor early although more abundant than the 1740 3′ splice). With *dl*712, the 2157 3′ splice was enhanced early because the 951→2157 splice is enhanced relative to *rec*700 (6, 15; Bhat et al., in press) (Fig. 5C; compare the 287-nt bands in lanes b and e). However, late after infection this splice was much reduced (Fig. 5C; compare the 287-nt bands in lanes b and f), down to roughly wild-type levels. Thus, as with the 951 5′ splice, the deletion in *dl*712 enhances the 2157 3′ splice early but not late. With *dl*719, the 2157 3′ splice was greatly enhanced early relative to *rec*700 (compare the 222-nt band from *dl*719 in Fig. 5B, lane f, with the 287-nt band for *rec*700 in Fig. 5A, lane b, or Fig. 5C, lane b). (The band representing the 2157–E3B exon is 222 nt for *dl*719 versus 287 nt for *rec*700 because

*dl*719 has a 65-base-pair deletion in this exon.) The 2157 3′ splice for *dl*719 was even more enhanced late relative to early (compare the 222-nt band in Fig. 5B, lane f, with that in Fig. 5B, lanes g and h). This contrasts with the other splice-enhancing mutant, *dl*712, in which the 2157 3′ splice was greatly reduced late (Fig. 5C, lanes e and f).

**Nuclease-gel assay for mRNAs with the 2880 3′ splice, E3B 3′ ends, or both.** Figure 6 shows nuclease-gel analysis for mRNAs from *rec*700 and *dl*712 that use the 2880 3′ splice, the E3B RNA 3′ end site, or both. The 429-nt band corresponds to the exon from the 2880 3′ splice to the E3B 3′-end site; this is mRNA *h*. The 826-nt band corresponds to mRNAs extending from the 3′ end of the probe to the E3B 3′-end site; these are mRNAs *c*, *e*, and *f*. With *rec*700, both the 429- and 826-nt bands were less abundant at 12 h (lane d) and 21 h (lane e) than at 6 h (lane c). Thus, the 2157 3′ splice site (826-nt band) and the 2880 3′ splice site (429-nt band) are used less frequently late than early. With *dl*712, these bands were far more abundant at 6 h (Fig. 6, lane f) than in *rec*700 (lane c); this reflects the enhanced 951→2157 and 951→2880 splices to form mRNAs *f* and *h*, respectively. These bands in *dl*712 were much reduced at 12 and 21 h (lanes g and h), to levels comparable with those of *rec*700 (lanes d and e). Thus, the 951→2880 splice is no longer enhanced late after infection. Lack of enhancement of the 951→2157 splice in *dl*712 was discussed above for Fig. 5.

A z leader has been found on a small fraction of L5 mRNAs in Ad2 (3, 10, 35). Based on studies in Ad2 (35), in *rec*700 this z leader (145 nt) would use the 2880 3′ splice site and a 5′ splice site at nt 3024. We have never observed a 145-nt band that would correspond to this 145-nt z leader (Fig. 6).

The region between the 2157 and 2880 3′ splice sites was analyzed in *rec*700 at early and late stages for other 5′ or 3′ splice sites. No sites were detected (data not shown).

## DISCUSSION

We studied how use of various splice and RNA 3′-end sites in E3 varies in response to a number of deletions and to the infection stage (Fig. 3). The following discussion is based on our data with *rec*700, as well as the quantitative electron microscopy data of Chow et al. (11) with Ad2.

**E3 expression is temporally regulated at the promoter and RNA processing levels.** Synthesis of classical E3 mRNAs initiated from the E3 promoter peaked at 6 h and was virtually undetectable by 12 h p.i. The decline probably results from a decline in transcription rate (27, 30, 34) effected by repression, or lack of activation, of the E3 promoter. Transport and stability of E3 mRNAs could also be reduced. Early E3 mRNAs are not very stable as judged by their decline at 6 to 9 and 9 to 12 h p.i.

Early E3 pre-mRNAs are processed with different efficiencies into the individual E3 mRNAs. Studies on other transcription units indicate that the first RNA processing event is RNA 3′-end formation rather than splicing (25). This may not necessarily be so in E3 because spliced pre-mRNAs have been detected in both poly(A)$^+$ and poly(A)$^-$ nuclear RNA (31). Half of mRNAs end up with E3A 3′ ends, and half end up with E3B 3′ ends (11, 20). It is not known how the E3A site versus the E3B site is selected. Almost all pre-mRNAs use the 372→768 splice, and roughly 60% are not spliced again (5, 11, 20, 29). About half of E3 mRNAs are mRNA *a* with E3A 3′ ends, and ~10% are mRNA *c* with E3B 3′ ends. The remaining pre-mRNAs are spliced a second time (5, 11, 20, 29) at either the 951 (768–951 exon) or

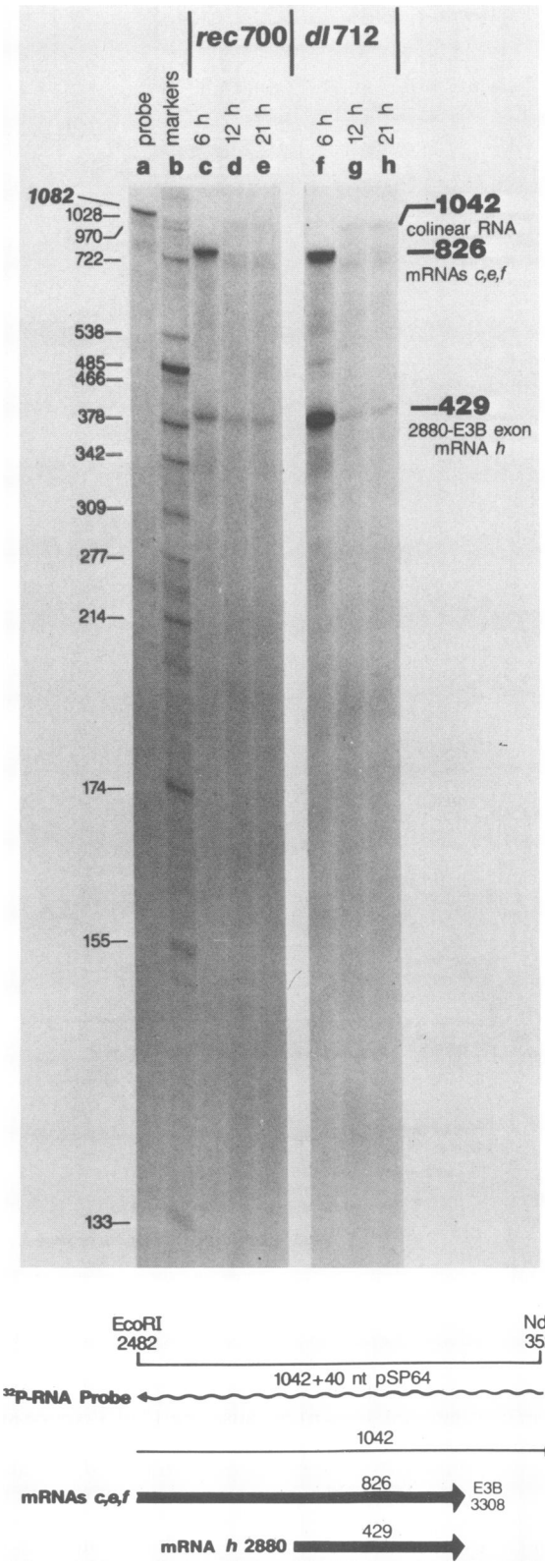



FIG. 6. Nuclease-gel assay for exons with the 2880 3′ splice or E3B 3′ ends in RNAs from *rec*700 and *dl*712 or both. RNAs were extracted at 6, 12, or 21 h p.i. The probe plasmid contained the *Nde*I-*Eco*RI fragment (nt 3521 to 2482) of Ad5 cloned between the *Sma*I and *Eco*RI sites of pSP64. The *Nde*I site was blunt ended with the Klenow fragment so that it would clone into *Sma*I.

1017 (768–1017 exon) 5′ splice sites. These two 5′ splice sites are used with about equal efficiency (Fig. 4). The 768–951 or 768–1017 exon is spliced to one of three 3′ splice sites at nt 2880, 2157, or 1740. The splice to nt 2880 predominantes so that mRNA *h* represents one-third of total E3 mRNAs. The splice to nt 2157 is much less frequent. According to Chow et al. (11), the 1740 3′ splice is used about 11 to 25% of the time. However, in our hands the 1740 3′ splice is very infrequent because we can barely detect it in early RNA from *rec*700. In Ad5, however, the equivalent of the 1740 3′ splice occurs more frequently (12). We have never detected mRNAs *b* and *g*; these mRNAs were included in Fig. 1 only because they were reported to exist as minor species by Chow et al. (11).

E3 mRNAs continue to be made as the infection proceeds from early to late stages. However, they are made from the major late promoter at map position 16, and they contain the major late tripartite leader coded at map positions 17, 20, and 27 (11). They also contain the 768–951 exon (11). The mRNAs contain E3A or E3B 3′ ends so that these RNA 3′-end sites continue to function late after infection. Significantly, the splicing pattern changes from early to late stages. The 768 3′ splice site remains very active, but the 951 5′ splice site is dramatically activated so that very little of mRNAs *a* and *c* is formed. This 768–951 exon is the y leader found on 25% (11) to 50% (17) of L5 mRNAs. The 1017 5′ splice site is used very infrequently late, as indicated by the absence of the 250-nt band in Fig. 4. The 1740 3′ splice site is activated late so that mRNAs *d* and *e* become the prominent E3 mRNAs (Fig. 5). The 2880 and 2157 3′ splice sites continue to be used late but at lower frequency than early. Our results differ from those of Chow et al. (11), who found by electron microscopy that mRNAs *a* and *h* predominated late after infection. We don't know whether this is due to adenovirus strain differences or to the different techniques used.

In summary, the E3 transcription unit appears to be differentially expressed at early and late stages. At early times, the early promoter is used to make mainly mRNA *a*, moderate amounts of mRNA *h*, and low amounts of mRNAs *b* to *g*. At late times, the major late promoter is used to make mainly mRNAs *d* and *e* and much lesser amounts of other mRNAs.

Regulation of E3 expression at the promoter and RNA processing levels is similar to that of some other adenovirus transcription units. The major late promoter is active early (11, 30), but the 3′ ends of most transcripts are formed at the L1 site, and specific splices are carried out such that the 52,000- and 55,000-molecular-weight proteins are made (1, 2, 22). Late after infection, the L1 to L5 RNA 3′ end sites are used and the L1 mRNAs are spliced differently. The region E2 and major late transcription units are similar to E3 in that which of the RNA 3′-end and splice sites are selected determines which mRNA will be made. The E1A and E1B transcription units each have a single promoter and RNA 3′ end site early and late, but the splicing pattern changes (11, 24, 32).

**Why does the frequency of use of E3 RNA processing sites differ between early and late stages of infection?** The E3A and E3B RNA 3′-end sites continue to be used late after infection, although not nearly as frequently as the classical late 3′-end sites, e.g., L4. Although pre-mRNAs are transcribed through the L4 site early after infection, mRNAs with L4 3′ ends are not detectable early. As discussed above, the frequencies of specific splices are different at early and late stages. However, perhaps excepting the 768 site, none of the E3 3′ splice sites are used with efficiencies comparable to those of classical late 3′ splice sites. What accounts for these observations? There are four general models to explain this differential RNA processing. First, in the case of the E3A and E3B RNA 3′-end sites and the 3′ splice sites, the signals may simply be not as strong as the late signals. However, signal strength does not explain why the L4 RNA 3′-end site is not used early. Second, virus-coded or cell-coded and virus-induced RNA or protein factors could be required for efficient use of the early- and late-specific signals (33). Third, pre-mRNA secondary and tertiary structure could determine the efficiency of RNA processing sites; early and late pre-mRNAs would have different structures and therefore could be processed with different efficiencies. The fourth model invokes a dynamic interaction between the rate of RNA 3′-end formation and the rate of specific splices. For example, if the 372→768 splice occurs in early pre-mRNA before cleavage and polyadenylation occurs at the L4 site, then the L4 site would be spliced out of the pre-mRNA. Similarily, late after infection the active tripartite leader→L5 splice and the 951→L5 splice would excise the E3A and E3B RNA 3′-end sites from the pre-mRNA. This model can also explain why *dl*712, in which the 951→2157 and 951→2880 slices are enhanced early, makes almost exclusively mRNAs with E3B 3′-ends early (8). Arguing against this model are the observations that polyadenylation precedes splicing in adenovirus late pre-mRNA (26) and in at least some early E3 pre-mRNA (14, 31). However, polyadenylation is not obligatory for splicing (39) and it is possible that in many pre-mRNAs splicing precedes polyadenylation.

**Possible *cis*-acting sequences that regulate the activity of the 951 5′ splice site.** Early after infection, the 951 and 1017 5′ splice sites are each used in ~20% of E3 mRNAs as judged by the ratio of mRNAs with these splices to total E3 mRNAs (Fig. 2 and 4). We have suggested that sequences somewhere within nts 1691 to 2044 function in *cis* specifically to suppress the 951 5′ splice site (16; Bhat et al., in press). Deletion of these sequences in *dl*712 and *dl*708 relieves this suppression, and consequently the 951→2157 or 951→2880 splice or both are enhanced. As we have shown here, late after infection the 951 site is activated in the wild type such that almost all pre-mRNAs that use the 768 3′ splice site also use the 951 site. *dl*712 and *dl*708 have essentially a wild-type RNA-processing phenotype late instead of the splice-enhancing phenotype manifested early; i.e., neither the 951 5′ splice site nor the 2157 or 2880 3′ splice site, is enhanced late compared with the wild type. Since the 951 site is normally highly activated late, the lack of enhancement of this site by the deletion in *dl*712 and *dl*708 is not surprising. In any case, it is apparent that the 951 suppressor function of the nt 1691 to 2044 region which operates early no longer operates at late stages. To visualize how this could occur, suppose that the nt 1691 to 2044 region affects the early pre-mRNA higher-order structure such that the 951 site is shielded from the splicing apparatus. Deletion of this region, as has occurred in *dl*712 and *dl*708, could alter the structure and expose the 951 sites. In contrast, late pre-mRNAs could have a different higher-order structure such that the 951 site is normally exposed to the splicing apparatus and deletion of the nt 1691 to 2044 region could have no effect on the exposure of the 951 site. Of course, other explanations are possible. For example, a factor could be present early that binds to the nt 1691 to 2044 region in pre-mRNA and suppresses the 951 5′ splice site. Perhaps this factor cannot bind in *dl*712 or *dl*708 so that the 951 site is not suppressed. Late after infection, this factor could be somehow removed so that the 951 site is not suppressed.

Why would it be beneficial for the virus to regulate the 951 5′ splice site? Early after infection, suppression of this site would permit mRNAs *a* and *c* and gp19K, which is encoded by these mRNAs (29), to predominate. Late after infection, relief of suppression of the 951 site would result in the observed enhanced synthesis of mRNAs *d* and *e* with the 951→1740 3′ splice. It may not be coincidental that the 1740 3′ splice site is located in the nt 1691 to 2044 region which we have postulated to control the 951 5′ splice site. That is, whatever relieves the 951 suppression may also activate the 1740 3′ splice site. Relief of 951 suppression would also enhance synthesis of those L5 mRNAs that contain the y leader. It has been postulated that the y leader may be important for efficient translation of L5 mRNA (3), although such an effect was not observed in a cell-free translation system (17). Finally, if the kinetic model for RNA 3′-end selection is correct (see the preceding section), then activation of the 951→L5 splice would excise the E3A and E3B 3′-end sites from L5 pre-mRNA.

Our suggestions regarding the regulation of the 951 5′ splice site and its biological benefit are speculative. However, we noted that the 1017 5′ splice was not enhanced late nor was it enhanced early by any of our splice-enhancing mutants. This was true even though the 1017 site is closer to the E3 and L5 3′ splice sites than is the 951 site. Therefore, whether by coincidence or by evolutionary selection, there is something special about the 951 5′ splice site.

**Similar *cis*-acting sequences are required at early and late stages of infection for E3A 3′-end formation and for splicing at the 2157 3′ splice site.** *dl*713, *dl*719, and *dl*708 are defective in E3A RNA 3′-end formation both early and late after infection (7; Fig. 5). Thus, perhaps not surprisingly, similar *cis*-acting sequences are required for E3A RNA 3′-end formation at early and late stages. These *cis*-acting sequences include the ATTAAA sequence, as well as sequences immediately downstream from the ATTAAA.

The deletion in *dl*714 leaves 15 nt of the pyrimidine-rich region upstream from the 2157 3′ splice site. This deletion prevents splicing at the 2157 site both early (6) and late (Fig. 5B). Therefore, the sequences deleted in *dl*714 are required for the 2157 3′ splice to function both early and late.

**The deletion in *dl*719 enhances the 951→2157 splice both early and late after infection.** The deletion in *dl*719 leaves 17 nt in the exon downstream from the 2157 3′ splice site. Early after infection, this deletion activates the 951→2157 splice such that it is used by almost all pre-mRNAs (6; Bhat et al., in press). This splice continues to be enhanced late after infection (Fig. 5). Considering that the 951 site is already enhanced late, this result suggests that the deletion in *dl*719 activates the 2157 3′ splice site. If so, then *dl*719 is the first mutant described in which an exon deletion enhances an upstream 3′ splice site.

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