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Map of *cis*-Acting Sequences That Determine Alternative Pre-mRNA Processing in the E3 Complex Transcription Unit of Adenovirus

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The E3 complex transcription unit of adenovirus encodes four major mRNAs (*a*, *c*, *f*, and *h*) and two minor (*d* and *e*) mRNAs with overlapping exons, alternative splice sites, and two polyadenylation sites, termed E3A (upstream) and E3B (downstream). mRNAs *a* and *d* use the E3A polyadenylation site, and mRNAs *c*, *e*, *f*, and *h* use the E3B site. We have analyzed virus mutants with deletions throughout the E3 region in order to identify *cis*-acting sequences that function in E3 pre-mRNA processing. The results presented in this report as well as previous results are summarized as follows. (i) Deletions in the first (5′) intron at nucleotides (nt) 372 to 768 in E3 had no effect unless they removed the consensus sequence for the nt 372 5′ splice site; however, the overall pattern of E3 mRNAs did not change significantly. (ii) Deletions in region I (nt 1441 to 2044) eliminated mRNAs *a* and *c* and resulted in corresponding increases in mRNAs *f* and *h*; we propose that region I contains sequences that suppress splicing. (iii) Mutations in region II (nt 2161 to 2243) resulted in nearly exclusive synthesis of mRNA *f*; this phenotype is understood and is discussed. (iv) Changing the AUUAAA component of the E3A poly(A) addition signal to AAUAAA resulted in increased mRNA *a* levels, suggesting that the E3A poly(A) addition signal is intrinsically inefficient. (v) Deletions in region III (nt 2488 to 3002) decreased mRNA *a* levels about two- to threefold and specifically increased mRNA *f* levels; we suggest that region III facilitates use of the E3A polyadenylation site. (vi) Deletions in region IV (nt 2904 to 3251) increased mRNA *a* levels about two- to threefold; we suggest that region IV may contain sequences that facilitate use of the E3B polyadenylation site. A map of sequences that determine alternative pre-mRNA processing in region E3 is now nearly complete.

Complex transcription units encode two or more different mRNAs derived from a single pre-mRNA precursor. Although there is considerable information available on the mechanisms of splicing and on RNA 3′-end formation, much less is known about the determinants of alternative pre-mRNA processing (reviewed in references 30 and 42). In *Drosophila melanogaster*, sex is determined by a cascade of alternative splicing events, in which RNA-binding proteins bind specifically to 3′ splice sites, thereby blocking splicing to that site and allowing splicing to an alternative 3′ splice site (34, 35). With the *Drosophila P*-transposase pre-mRNA, alternative splicing is regulated by a factor that binds to the exon adjacent to a 5′ splice site (50). In humans, the protein factor ASF, also called SF2, enhances the use of a proximal 5′ splice site at the expense of a distal 5′ splice site (25, 38, 39). ASF (SF2) has homology to RNA-binding proteins and to the *Drosophila* splicing regulators (26, 40). A different human protein, termed DSF, promotes the use of distal 5′ splice sites at the expense of proximal sites (32).

The retrovirus genome is a complex transcription unit which is alternatively expressed as unspliced genomic RNA and mRNAs or as spliced mRNAs. Some mechanism must exist to maintain the proper balance between these RNAs. In Rous sarcoma virus, a *cis*-acting negative regulator of splicing element has been identified which appears to reduce splicing of the *env* and *src* mRNAs, allowing increased synthesis of the genomic RNA and *gag-pol* mRNA (2, 44, 45, 51). Also, the 3′ splice site of the *env* mRNA may be intrinsically weak (23). In human immunodeficiency virus type 1, the virally coded Rev protein inhibits splicing by binding to the *cis*-acting Rev-responsive element (37).

We are studying the E3 complex transcription unit of human adenovirus as a model to understand alternative pre-mRNA processing (4–11, 18). During early stages of infection (prior to viral DNA replication), a variety of overlapping E3 mRNAs are synthesized from a single promoter and by the alternative use of splice sites as well as the alternative use of two polyadenylation sites, termed E3A and E3B (Fig. 1). The major mRNAs are mRNA *a* (approximately 40% of the total), mRNA *c* (approximately 15%), mRNA *f* (approximately 15%), and mRNA *h* (approximately 25%). mRNAs *d* and *e* are made in small amounts. Synthesis of the E3 mRNAs appears to be constitutive under these conditions, i.e., the pattern of synthesis remains constant provided that the early stage of infection is maintained. As is the case with retroviruses, specific sequences or mechanisms must operate to determine which splice and polyadenylation sites are used and at what frequency; this in turn will determine which E3 proteins are synthesized and in what abundance (see Discussion). One approach to identifying these "determination" sequences or mechanisms is to create mutations throughout the transcription unit and analyze how the mutations affect the accumulation of E3 mRNAs. E3 is a particularly useful model for such studies because mutations in E3 do not affect the replication of adenovirus in cultured cells. Thus, mutations can be analyzed under conditions that are as "natural" as possible.

We have previously shown that deletions in a specific region, denoted region I (Fig. 1), eliminate synthesis of

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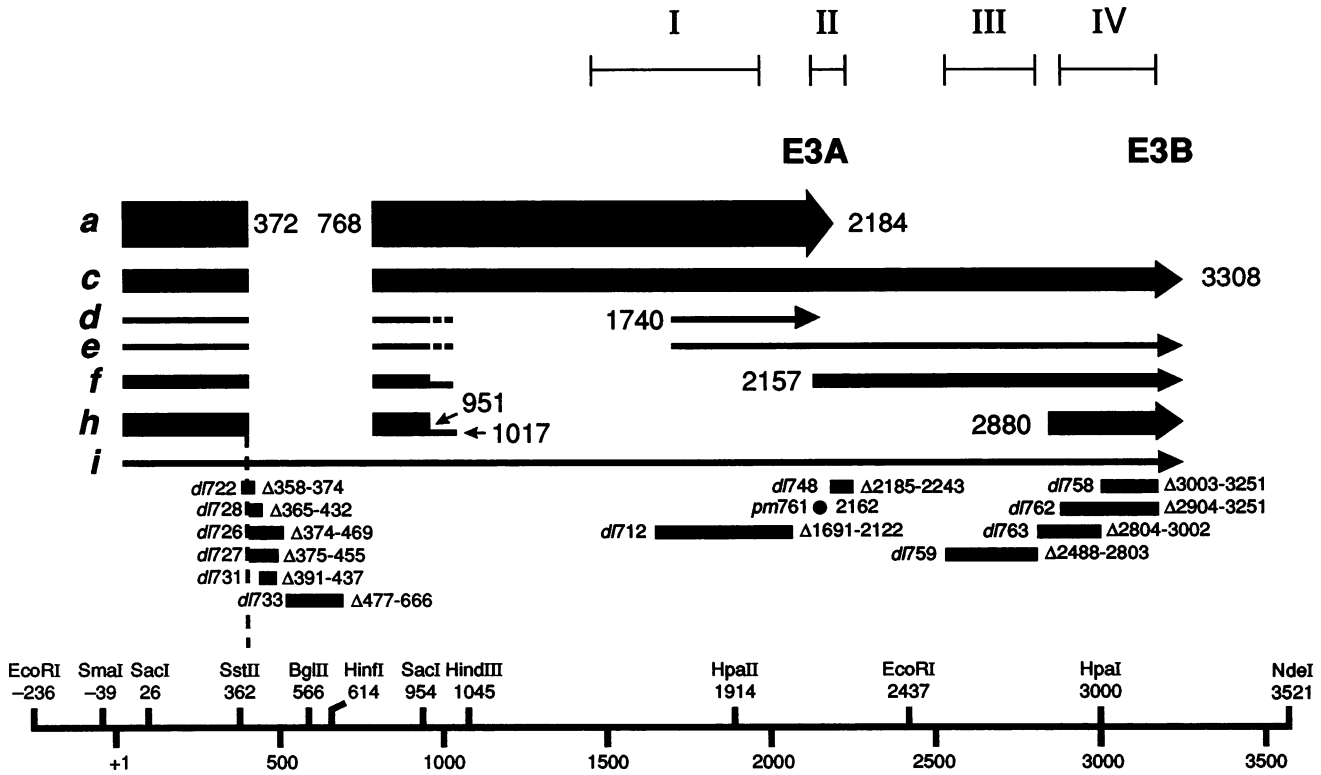

FIG. 1. Schematic representation of the E3 transcription unit. The arrows indicate the exons of mRNAs *a* through *i*, with the thickness of the arrow indicating the abundance of the mRNA. Nucleotide (nt) +1 is the transcription initiation site. E3A and E3B refer to polyadenylation sites. The regions designated I, II, III, and IV are regions where deletions affect E3 pre-mRNA processing (see text). The sequences deleted in the virus mutants are indicated. *pm*761 is a point mutant with the T at nt 2162 converted to an A. The restriction sites at the bottom are numbered at the 5′ base pair in the recognition sequence. The mRNAs shown are from H5/2*rec*700, which has Ad2 sequences between the *Eco*RI sites at nt −236 and nt 2437 and Ad5 sequences elsewhere.

mRNAs *a* and *c* and result in the nearly exclusive synthesis of mRNAs *f* and *h* (4, 7–9, 18). Mutations in region II (Fig. 1) result in the nearly exclusive synthesis of mRNA *f* at the expense of the other E3 mRNAs (10, 11). We have hypothesized how and why regions I and II control E3 mRNA synthesis (see Discussion). In this communication, we report the mRNA phenotypes of mutants with deletions in most of the remaining portions of E3 that have not been analyzed previously. The mapping of *cis*-acting sequences that affect E3 pre-mRNA processing is now nearly complete.

MATERIALS AND METHODS

Isolation of virus mutants. Viruses were grown in suspension cultures of human KB cells, banded in CsCl, and counted in monolayers of human A549 cells as described before (31). A549 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum.

The parental virus for all the mutants used in this study is H5/2*rec*700, whose genome consists of the adenovirus type 5 (Ad5) *Eco*RI-A (genomic map positions 0 to 76), Ad2 *Eco*RI-D (76 to 83), and Ad5 *Eco*RI-B (83 to 100) fragments (62). Mutants *dl*712 (18), *dl*748 (10), and *dl*759 (53) have been described.

For mutations near the nucleotide (nt) 372 5′ splice site, deletions were generated in the cloned Ad2 *Eco*RI-D fragment by Bal31 exonuclease digestion at either the *Sst*II site at nt 362 (*dl*722) or the *Apa*I site at nt 413 (*dl*726, *dl*727, *dl*728, and *dl*731). (The sequence of the E3 transcription unit is presented in an earlier report by Cladaras and Wold [15]). A *Bam*HI linker (CGGGATCCCG; New England Biolabs, Inc., Beverly, Mass.) was inserted at the deletion. The sequences deleted are presented in Fig. 2. The deletions were built into the virus genome as described before (18, 62). Briefly, the Ad2 *Eco*RI-D fragment was ligated between the Ad5 *Eco*RI-A and *Eco*RI-B fragments. This DNA was transfected into A549 cells, and the resulting plaques were screened for the mutation by restriction enzyme analysis of the viral DNA in the Hirt supernatant (62).

The sequence ATTAAA, located at nt 2161 to 2166 in region E3 of H5/2*rec*700, is a component of the E3A polyadenylation signal (11). This sequence was converted to AATAAA by oligonucleotide mutagenesis and then built into the H5/2*rec*700 genome as described by Brady and Wold (11). The mutant was named *pm*761.

Mutants *dl*758, *dl*762, and *dl*763 were constructed starting with the *Kpn*I-A fragment (map positions 71 to 94) of H5/2*rec*700 cloned at a *Kpn*I site that had been introduced at the original *Eco*RI site in pBR322; the plasmid was called pKA. For *dl*763, pKA was digested with *Hpa*I and partially digested with *Nae*I; *Bam*HI linkers were ligated, and the DNA was digested with *Bam*HI, gel purified, circularized, and cloned. For *dl*758, pKA was digested with *Hpa*I, *Bam*HI linkers were ligated, and the DNA was partially digested with *Bgl*II, digested with *Bam*HI, gel purified, circularized, and cloned. For *dl*762, pKA was partially digested with *Bgl*II to excise the fragment from nt 2900 to 3251, gel purified, circularized, and cloned. The deletions in these pKA-de-

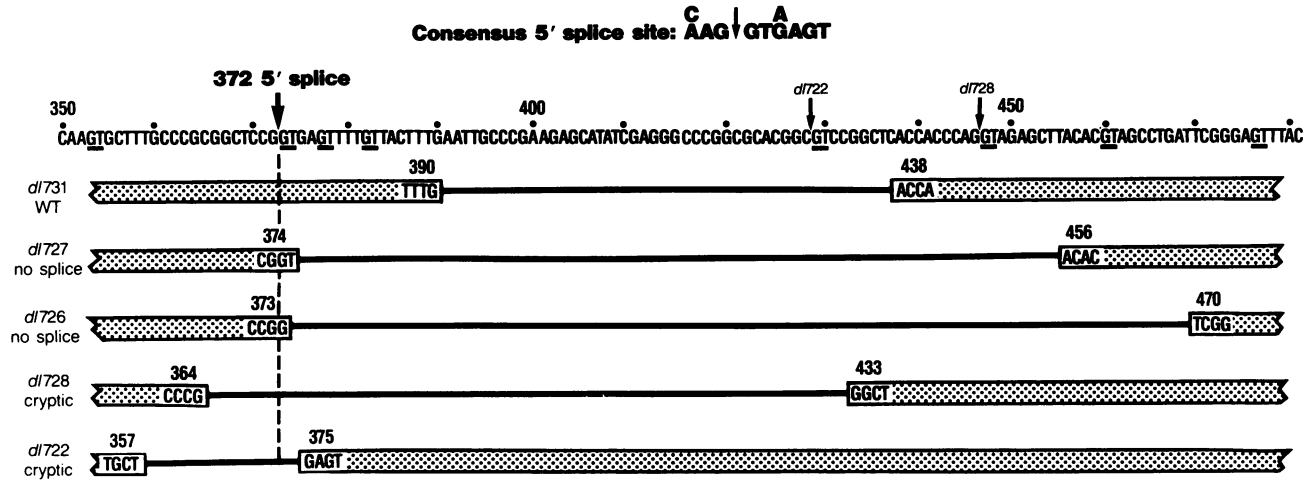

FIG. 2. DNA sequence near the nt 372 5′ splice site, and sequences deleted in the mutants. The 5′ splice consensus sequence is taken from reference 30. *dl*731 has a 10-bp *Bam*HI linker (CGGGATCCCG) in the deletion. *dl*727 has two such linkers (20 bp). *dl*726 has only 7 bp of linker sequences (GATCCCG), because the 3 bp at the 5′ end of the linker are identical to the Ad2 sequences. *dl*728 has 8 bp of linker (GGATCCCG). *dl*722 has 9 bp of linker (CGGGATCCC).

rived plasmids were built into the adenovirus genome by the double-overlap recombination method (36).

For all mutants, the mutations in the plasmids were sequenced before they were built into the H5/2*rec*700 genome. All virus mutants were plaque purified at least once before expansion into high-titer CsCl-banded stocks.

Nuclease protection and RNA blot analysis. KB cells were infected with mutants and maintained in cycloheximide (25 μg/ml) from 3 to 9 h postinfection. Cytoplasmic and polyadenylated RNAs were prepared (14). S1 nuclease analysis (3) was carried out with ^{32}P-labeled RNA probes (see figure legends) prepared with SP6 polymerase, 30 μg of cytoplasmic RNA, and S1 nuclease, as described before (6, 18). ^{32}P-labeled RNA size markers were prepared with SP6 polymerase. All gels are 6% polyacrylamide sequencing gels. In all experiments, probes were in excess, so the results are quantitative. RNA blots were done (4) with the ^{32}P-labeled RNA probes described in Fig. 6.

For the mutants with deletions near the nt 372 5′ splice site, the relative abundance of adenovirus-specific mRNA in each RNA preparation was standardized by S1 nuclease analysis of mRNAs from region E1B (the E3 deletions do not affect the synthesis of E1B mRNAs). This analysis indicated that E1B mRNAs were similar in abundance in all mutants, indicating that the infections and expression of early genes were equally efficient.

RESULTS

Analysis of deletions near the nt 372 5′ splice site. Figures 1 and 2 illustrate the mutants with deletions near the nt 372 5′ splice site. Figure 3 shows the analysis of mRNAs from wild-type virus (H5/2*rec*700) and from the mutants. The probes extend from the *Bgl*II to the *Sma*I site, nt 566 to −39. H5/2*rec*700 and *dl*731 were analyzed with the H5/2*rec*700 probe. The other mutants were analyzed with probes prepared from the corresponding mutant plasmid; thus, these probes and the nuclease-protected fragments vary in size according to the deletion. H5/2*rec*700 yielded a strong band of 372 nt and a very weak band of 566 nt (Fig. 3, lane c). The 372-nt band corresponds to the abundant nt 1 to 372 exon in mRNAs *a* through *h*. The 566-nt band is derived from the minor mRNA *i*, which lacks the nt 372 to 768 splice and which is colinear with the probe.

*dl*731 has a deletion of 47 bp in the "first" intron of the nt 372 to 768 splice, leaving 18 bp downstream from the nt 372 5′ splice site. This deletion has no effect on use of the nt 372 site, as indicated by the abundance of the 372-nt band (Fig. 3, lane d). *dl*733 has a deletion of 190 bp in the intron, 106 bp downstream from the nt 372 site. This deletion also has no effect on the nt 372 to 768 splice (9).

*dl*722 and *dl*728 have deletions of the nt 372 site. Both these deletions activate cryptic 5′ splice sites. With *dl*722, the 420-nt band corresponds to the exon extending from nt 1 to a cryptic 5′ splice site at nt 428 (Fig. 3, lane f). The 558-nt band is derived from colinear mRNAs that are unspliced in this region. With *dl*728, the 388-nt band represents the exon from nt 1 to a cryptic site at nt 446 (Fig. 3, lane h). The 506-nt band is derived from unspliced colinear mRNA. The sizes of the 420- and 388-nt bands were estimated to within ±3 nt from data obtained from other gels. Based on this, we deduce that the cryptic splices occur immediately prior to the GT's at nt 429 and 447 (Fig. 2). The increased intensity of the unspliced colinear bands in *dl*722 (558 nt) and *dl*728 (506 nt) compared with that of the band in H5/2*rec*700 (566 nt) indicates that the cryptic sites are not used as efficiently in the mutants as is the nt 372 site in H5/2*rec*700. The cryptic nt 446 site in *dl*728 is used more efficiently than is the cryptic nt 428 site in *dl*722.

*dl*726 and *dl*727 have deletions of intron sequences to within 1 or 2 bp, respectively, of the nt 372 site (Fig. 2). These deletions essentially abolish splicing at the nt 372 site, as indicated by the absence of the 372-nt band (Fig. 3, lanes j and l). These mutants make mainly unspliced mRNA in this region, as indicated by the 480-nt and 504-nt bands (Fig. 3, lanes j and l). The abundance of transcripts in *dl*727 was reduced compared with that in the other mutants, suggesting that this deletion affects the cytoplasmic accumulation of total E3 mRNAs. The deletions in *dl*726 and *dl*727 do not activate a single prominent cryptic 5′ splice site. The cryptic sites used by *dl*722 and *dl*728 are deleted in *dl*726 and *dl*727, but there are other nearby GT's (nt 353, 460, and 476) that could have been used as cryptic sites.

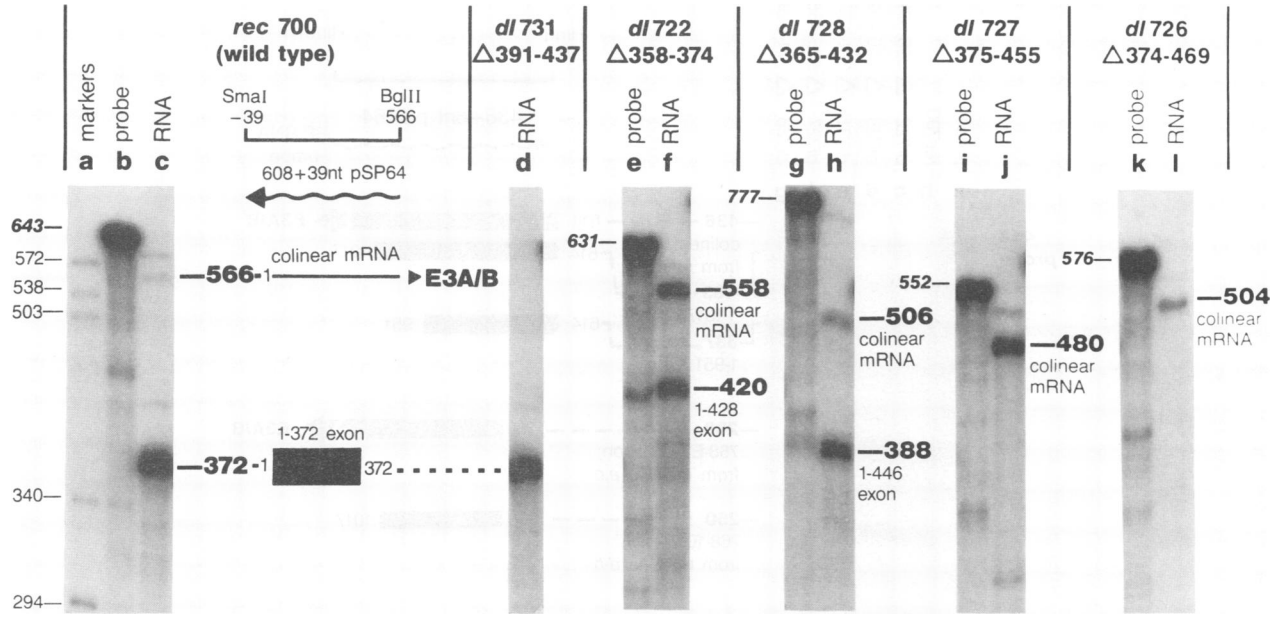

FIG. 3. S1 nuclease analysis for use of the nt 372 5′ splice site. The ^{32}P-labeled RNA probes were prepared in vitro with SP6 polymerase. The pSP64 plasmid clones were cleaved at the *Sma*I site (nt −39) prior to transcription. H5/2*rec*700 and *dl*731 cytoplasmic mRNAs were analyzed with probes prepared from the H5/2*rec*700 plasmid. The other mutants were analyzed with probes prepared from the corresponding mutant plasmids. The probes and the nuclease-protected fragments are indicated in the schematic. In the gel, boldface roman type indicates nuclease-protected fragments, italics indicate probes, and lightface roman type indicates ^{32}P-labeled RNA size markers (in nucleotides). All lanes are from the same exposure of the same gel.

We conclude that the intron sequences required for use of the nt 372 5′ splice site map somewhere between 18 bp (deleted in *dl*731, which uses this 5′ splice site) and 2 bp (deleted in *dl*727) downstream from the nt 372 site. No doubt the 5′ splice consensus sequences which are deleted in *dl*726 and *dl*727 are important. Sequences within the region from nt 391 to 666 can be deleted with no significant effect on E3 pre-mRNA processing.

Figure 4 presents the S1 analysis of the nt 768 3′ splice site and the nt 951 and nt 1017 5′ splice sites. The probe will detect four overlapping exons: (i) the colinear mRNA *i*; (ii) the nt 768 to E3A/B exon from mRNAs *a* and *c*, which extends from the nt 768 3′ splice site to either the E3A or E3B polyadenylation site; and (iii) the nt 768 to 951 and nt 768 to 1017 exons in mRNAs *f* and *h*, which extend from the nt 768 3′ splice site to either the nt 951 or nt 1017 5′ splice site. The optional nt 951 and nt 1017 5′ splice sites are spliced to either the nt 1740 3′ splice site (the scarce mRNAs *d* and *e*), the nt 2157 3′ splice site (mRNA *f*), or the nt 2880 3′ splice site (mRNA *h*).

H5/2*rec*700 produced three detectable nuclease-protected fragments of 283, 250, and 184 nt (Fig. 4, lane b). The strong 283-nt band corresponds to the nt 768 to E3A/B exon in the abundant mRNAs *a* and *c*. The 250- and 184-nt bands are derived from the nt 768 to 951 and nt 768 to 1017 exons, respectively, in mRNAs *d* through *h*. (A very weak 436-nt band representing the scarce colinear mRNA *i* is not visible in this gel.) *dl*731 (lane e) gave results similar to those with H5/2*rec*700, consistent with the conclusion from Fig. 3 that the deletion does not affect the nt 372 to 768 splice. We further conclude that the deletion does not affect the frequency of use of the nt 951 and nt 1017 5′ splice sites.

*dl*726 and *dl*727 are defective in excision of the first intron (Fig. 3). This is confirmed by the results in Fig. 4, lanes f and g. Neither mutant produced the 283-, 250-, or 184-nt bands indicative of the nt 768 to E3A/B exon, the nt 768 to 951 exon, and the nt 768 to 1017 exon, respectively. Instead, these mutants yielded bands of 436, 403, and 337 nt. The 436-nt band is derived from unspliced versions of mRNAs *a* and *c* as well as from mRNA *i* (here, mRNAs *c* and *i* have the same structure). The 403- and 337-nt bands represent exons extending from nt 1 to the 5′ splice site at nt 1017 and nt 951, respectively. It is of interest that the alternative nt 951 and nt 1017 sites continue to be used despite the fact that the first intron is not excised.

*dl*722 and *dl*728 have the nt 372 site deleted and use cryptic 5′ splice sites instead (Fig. 2 and 3). These cryptic sites are spliced to the nt 768 3′ splice site, as indicated by the 283-, 250-, and 184-nt bands (Fig. 4, lanes c and d). These two deletions, and the use of cryptic 5′ splice sites, have little effect on the use of the alternative 5′ splice sites at nt 951 (184-nt band) and nt 1017 (250-nt band). The cryptic sites in the mutants are not used as efficiently as the nt 372 site in H5/2*rec*700 (Fig. 3); this is reflected by the 436-, 403-, and 337-nt bands which result when the first intron is not excised (Fig. 4).

The deletions in *dl*731, *dl*722, and *dl*728 and the use of cryptic 5′ splice sites in *dl*722 and *dl*728 did not affect the frequency of use of the other pre-mRNA processing sites downstream in E3. However, with *dl*726 and *dl*727, the inability to excise the first intron reduced the use of the downstream processing sites by a factor of about 2 or 3, so that mRNA *c* (unspliced) and mRNA *i* became the most prominent mRNAs. The results are shown in Fig. 5 for the E3A polyadenylation site used by mRNA *a* and the scarce mRNA *d* and for the nt 2157 3′ splice site used by mRNA *f*. The probe extends from the *Eco*RI to the *Hpa*II sites, nt 2437 to 1914. H5/2*rec*700 and all the mutants yielded three

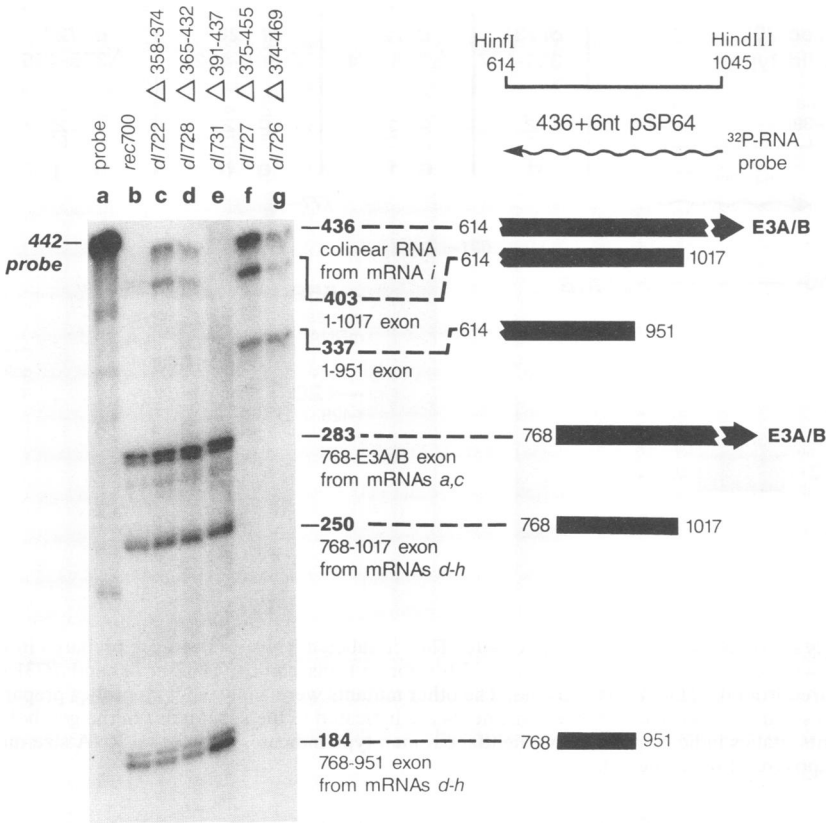

FIG. 4. S1 nuclease analysis for use of the 3′ splice site at nt 768 and the 5′ splice sites at nt 951 and nt 1017. The probe was prepared by cleaving at the *Hin*fI site (nt 614) prior to transcription. Sizes are shown in nucleotides.

sets of bands; the 529-nt band is derived from the colinear mRNAs *c* and *i*, the 287-nt band corresponds to the nt 2157 E3B exon in mRNA *f*, and the 270- and 277-nt bands represent the 3′ portion of the nt 768 to E3A exon in mRNA *a*. For H5/2*rec*700, *dl*731, *dl*722, and *dl*728 (Fig. 5, lanes c to f), the mRNA *a* bands were the most abundant, and the mRNA *f* and *c* bands were equally less abundant. In these mutants, the first intron is excised with high (H5/2*rec*700 and *dl*731) or reasonable (*dl*722 and *dl*728) efficiency. For *dl*726 and *dl*727 (Fig. 5, lanes g and h), in which the first intron is not excised, mRNA *c* plus *i* is increased relative to mRNAs *f* and *a*; i.e., the mRNA *c* plus *i* bands are similar to those for H5/2*rec*700, but the mRNA *a* and *f* bands are much reduced. (mRNAs *a*, *c*, and *f* in *dl*726 and *dl*727 do not have the first intron excised.)

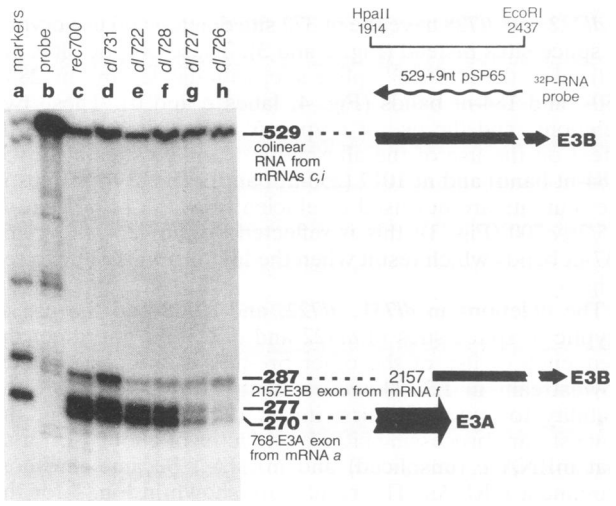

FIG. 5. S1 nuclease analysis for use of the E3A cleavage and polyadenylation site and of the 3′ splice site at nt 2157. The probe was prepared by cleaving at the *Hpa*II site (nt 1914) prior to transcription. Sizes are shown in nucleotides.

RNA blot analysis was used to confirm the S1 nuclease data for one of the nonsplicing mutants, *dl*727. Three different ^{32}P-labeled RNA probes were used to distinguish the mRNAs (Fig. 6). Probe I will detect all E3 mRNAs. Hybridization of probe I to H5/2*rec*700 revealed mRNA *a* in high abundance and mRNAs *c* and *f* in lesser abundance (Fig. 6A, lane a). mRNA *h* in this blot appears as a weak diffuse band, but in other blots it is more abundant (10, 11). These same mRNAs were detected in *dl*727 (Fig. 6A, lane b), except they are larger than in H5/2*rec*700 because the first intron (327 bp in *dl*727) has not been excised. With probe II, mRNAs *a* and *c* plus *i* were detected, as expected (Fig. 6B). With probe III, mRNAs *c* plus *i*, *f*, and *h* were detected but no mRNA *a*, as expected (Fig. 6C). With all three probes, mRNA *c* plus *i* was relatively more abundant in *dl*727 than in H5/2*rec*700; this is consistent with the data in Fig. 5.

These results establish that nt 391 to 666 in the nt 372 to 768 intron are not required for the synthesis of wild-type levels of E3 mRNAs. The results also show that the nt 372 to 768 splice is not obligatory for the other downstream splicing

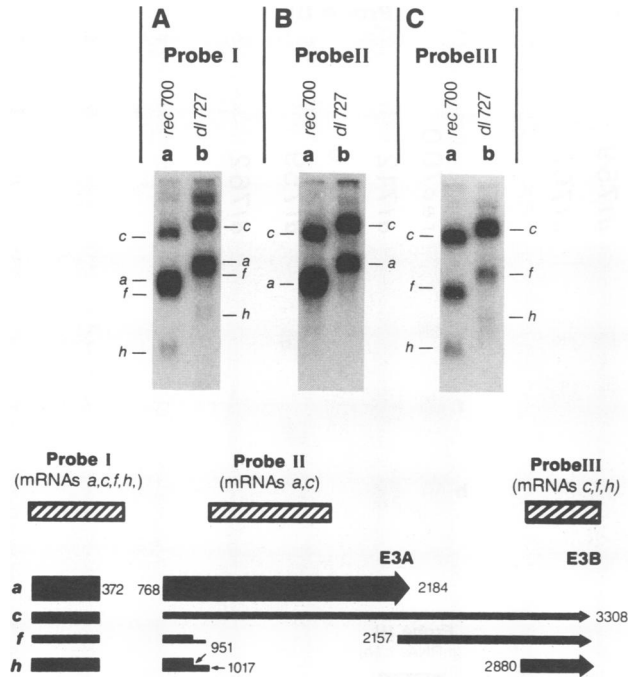

FIG. 6. RNA blot analysis for synthesis of E3 mRNAs of H5/2*rec*700 and *dl*727. Probes were ^{32}P-labeled SP6 RNAs. Probe I consists of H5/2*rec*700 sequences from nt 27 to 560, probe II of nt 1297 to 1825, and probe III of nt 3003 to 3521. The minor mRNAs *d* and *e* are omitted from the schematic (see Fig. 1).

events or for the use of the E3A or E3B polyadenylation site. However, the lack of the nt 372 to 768 splice in *dl*727 significantly decreases the efficiency with which the E3A polyadenylation site and the nt 2157 3′ splice site are used.

Mutation of the E3A polyadenylation signal from AUUAAA to AAUAAA increases the efficiency of the signal. AAUAAA is found about 20 nt upstream of the site of 3′-end formation in the vast majority of mammalian mRNAs, and it is an essential component of the polyadenylation signal (reviewed in references 47 and 58). However, AUUAAA is found at some polyadenylation sites, including the E3A site, where genetic studies have shown that AUUAAA constitutes part of the E3A polyadenylation signal (11). Why is AUUAAA rather than AAUAAA present at the E3A site? We considered that if AUUAAA were a less efficient signal than AAUAAA, then the use of the former at the E3A site would be advantageous because this would allow increased synthesis of mRNAs *c*, *e*, *f*, and *h*, which require use of the E3B polyadenylation site (Fig. 1). To address this possibility, a virus mutant (*pm*761) was constructed which has AUUAAA converted to AAUAAA, and synthesis of E3 mRNAs by *pm*761 was analyzed.

First, a nuclease protection experiment was carried out which indicated that the abundance of the nt 1 to 372 exon in cytoplasmic RNA from H5/2*rec*700 was equal to that in *pm*761 (data not shown). Since this exon is present in nearly all E3 mRNAs (Fig. 1), this control allowed us to conclude that the total E3 RNA concentration in the two RNA preparations was equal. Accordingly, any differences observed in the relative abundance of individual E3 mRNAs must be due to differences in pre-mRNA processing. As shown in the RNA blot in Fig. 7, mRNA *a*, which uses the E3A site, was increased about twofold in *pm*761 compared

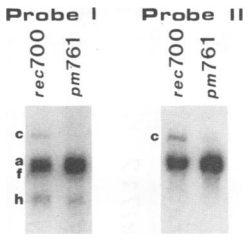

FIG. 7. RNA blot analysis for synthesis of E3 mRNAs by H5/2*rec*700 and *pm*761. Probes I and II are the same as used in Fig. 6.

with H5/2*rec*700, and mRNA *c*, which uses the E3B site, was almost undetectable in *pm*761. A nuclease protection assay with the same probe that was used in Fig. 5 also indicated that *pm*761 synthesized more mRNA *a* than did H5/2*rec*700 (see Fig. 9B). These results indicate that in the context of the E3A site, AAUAAA is a more efficient polyadenylation signal than is AUUAAA.

Deletions in region III result in decreased mRNA *a* and increased mRNA *f*, and deletions in region IV result in increased mRNAs *a*, *d*, and *e* and decreased mRNA *f*. In our previous studies (4–11, 18), *dl*753 (deletion of nt 2229 to 2436) carried the deletion that was closest to the 3′ end of the E3 transcription unit to be analyzed, and the E3 mRNA phenotype was wild type (10). Here we present the analysis of *dl*759 (deletion of nt 2488 to 2803), *dl*763 (nt 2804 to 3002), *dl*758 (nt 3003 to 3251), and *dl*762 (nt 2904 to 3251), whose deletions extend from the *dl*753 deletion to near the E3B polyadenylation site. The E3-specific cytoplasmic mRNAs were at an equivalent concentration in all RNA preparations from these mutants, as indicated by the abundance of the nt 1 to 372 exon (data not shown).

The RNA blot in Fig. 8 shows that *dl*759 synthesized less mRNA *a* and more mRNAs *c* and *f* than did H5/2*rec*700. *dl*762, on the other hand, synthesized relatively more mRNA *a* and less mRNA *f* than H5/2*rec*700, as indicated from the results with probes I and II; no *dl*762 RNA was detected with probe III because the probe III sequences are deleted in *dl*762. *dl*762 also synthesized more of the minor mRNAs *d* and *e* than did H5/2*rec*700. The results with *dl*758 were similar to those with *dl*762, and those with *dl*763 were similar to those with H5/2*rec*700 (data not shown). The results from the RNA blots were confirmed by nuclease protection experiments with the same probe as was used in Fig. 5 (Fig. 9A).

The deletions in *dl*759, *dl*758, and *dl*762 caused about a two- to threefold alteration in the levels of some of the E3 mRNAs, and the data were reproducible. Accordingly, we have designated the region deleted in *dl*759 region III and that deleted in *dl*758 and *dl*762 region IV. Regions III and IV have opposite effects on the synthesis of mRNAs *a* and *f*, as discussed above. The deletion in *dl*763 lies between that of *dl*759 and *dl*758; since *dl*763 resembles H5/2*rec*700, the deleted sequences either do not play a role in E3 pre-mRNA processing or perhaps include the 3′ boundary of region III and the 5′ boundary of region IV, so that the net phenotype resembles that of the wild type.

For comparison purposes, Fig. 8 also shows the phenotype of a region I mutant, *dl*712, and a region II mutant, *dl*748. As described previously (4, 7–9, 18), *dl*712 essentially synthesizes only mRNAs *f* and *h* at the expense of mRNAs *a* and *c*. Also as described previously (10), *dl*748 essentially synthesizes only mRNA *f* at the expense of the other mRNAs.

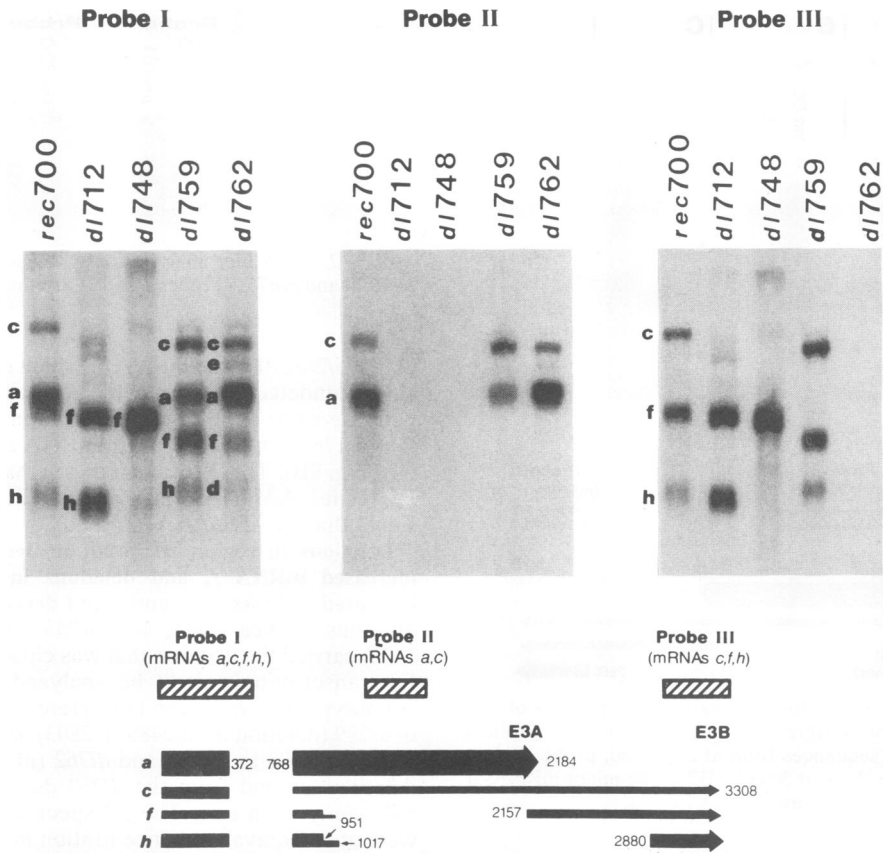

FIG. 8. RNA blot analysis for synthesis of E3 mRNAs by mutants with deletions in region I (*dl*712), region II (*dl*748), region III (*dl*759), and region IV (*dl*762). The probes are the same as in Fig. 6. The schematic does not show the minor mRNAs *d* and *e* (see Fig. 1); probe I will detect both mRNAs *d* and *e*, probe II will detet mRNAs *d* and *e*, and probe III will detect mRNA *e*. For H5/2*rec*700 and *dl*759, mRNA *d* comigrates with mRNA *h*, and mRNA *e* cannot be detected. The sequences for mRNA *h* are deleted in *dl*762, so mRNA *d* can be visualized.

DISCUSSION

Region E3 encodes about nine overlapping mRNAs which are translated into seven known proteins. The singly spliced mRNAs *a* and *c* encode gp19K, whose function is to bind to major histocompatibility complex class I antigens and cause them to be retained in the endoplasmic reticulum, thereby preventing cytolysis by cytotoxic T lymphocytes (reviewed in reference 63). mRNAs *a* and *c* also encode a 6.7-kDa protein whose function is unknown (59). The doubly spliced mRNA *h* encodes a 14.7-kDa protein (56), which prevents cytolysis by tumor necrosis factor (27, 29). The doubly spliced mRNA *f* encodes the 10.4-kDa (53) and 14.5-kDa (52) proteins, which function as a complex to prevent cytolysis by tumor necrosis factor (28) and to downregulate the epidermal growth factor receptor (12, 55). The minor doubly spliced mRNAs *d* and *e* encode an 11.6-kDa protein (61) which is synthesized much more abundantly at late stages of infection (54), and the unspliced mRNA *i* encodes a 12.5-kDa protein (33); the functions of these proteins are unknown.

As mentioned earlier, E3 must have a mechanism which ensures that the proper mRNAs are synthesized so that the E3 proteins are made in the required amounts. We have previously shown that deletions in a specific region, denoted region I (Fig. 1), eliminate synthesis of mRNAs *a* and *c* and result in the nearly exclusive synthesis of mRNAs *f* and *h* (4, 7–9, 18). We have hypothesized that region I contains splice-suppressing sequences that function under normal

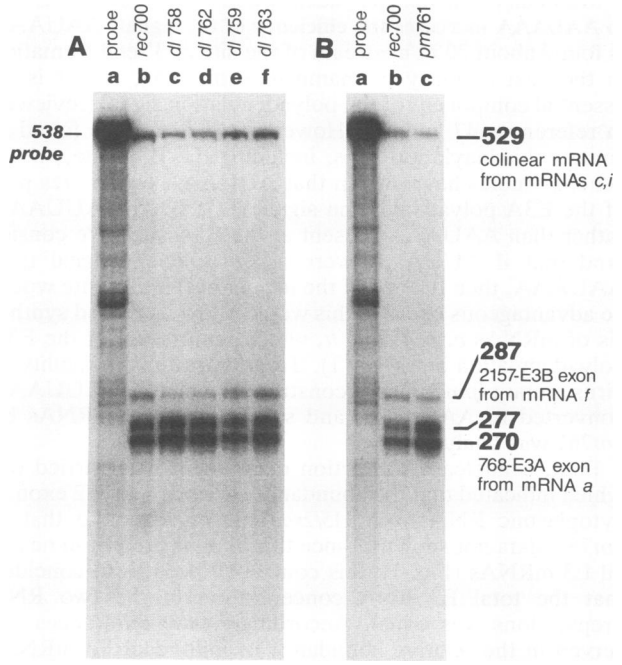

FIG. 9. S1 nuclease analysis for use of the E3A cleavage and polyadenylation site and of the 3′ splice site at nt 2157. The same probe was used as in Fig. 5.

conditions to suppress splicing, allowing mRNAs *a* and *c* to accumulate in quantity (18). Such suppression of splicing would allow gp19K to be synthesized in large amounts, which is important because large amounts of gp19K are required to bind stoichiometrically to class I antigens. Region I may be analogous to the negative regulator of splicing element of Rous sarcoma virus, which inhibits splicing (2, 44, 45, 51). However, there is no obvious sequence similarity between region I and the negative regulator of splicing element.

We have also shown that mutations in region II (Fig. 1) result in the nearly exclusive synthesis of mRNA *f* at the expense of the other E3 mRNAs (10, 11). This occurs because mutations in region II destroy the E3A polyadenylation signal, relieving the competition between polyadenylation factors and splicing factors for binding to overlapping sequences in the E3 pre-mRNA. As a result, nearly all E3 pre-mRNAs become spliced to form mRNA *f*. This very close proximity of the E3A polyadenylation site and the nt 2157 3′ splice site allows a balance to be maintained between mRNAs *a* and *f* (10, 11).

In the present communication, we have analyzed new E3 virus mutants in attempts to identify additional *cis*-acting sequences that determine E3 pre-mRNA processing. One set of mutants has deletions in the vicinity of the nt 372 5′ splice site that is used for the nt 372 to 768 splice in E3. Deletion of intron sequences at nt 391 to 437 (*dl*731) and nt 477 to 666 (*dl*733) (10) did not affect the nt 372 to 768 splice. We conclude that these intron sequences, and presumably the majority of intron sequences, can be deleted without affecting the efficiency of the nt 372 to 768 splice.

The deletions in *dl*726 and *dl*727, which remove intron sequences to within 1 or 2 nt, respectively, of the nt 372 5′ splice site, both abolish splicing at this site. Thus, the intron sequences required for splicing at the nt 372 site are located between 2 bp (deleted in *dl*727) and 18 bp (deleted in *dl*731, which uses this site) downstream from the nt 372 site. A consensus sequence for 5′ splice sites (see Fig. 2) is essential for splicing (30); no doubt *dl*726 and *dl*727 are defective in splicing because portions of the consensus sequence are deleted.

The deletions in *dl*722 and *dl*728 remove the nt 372 site and activate cryptic 5′ splice sites at nt 428 and nt 446, respectively. Activation of cryptic 5′ splice sites by mutations in the 5′ splice consensus sequence is quite common (30). Domenjoud et al. (21), while studying the E3 nt 372 to 768 splice in vitro, found that mutation of the nt 372 site activated the cryptic 5′ splice site at nt 446 but not the one at nt 428. In our in vivo experiments, the nt 428 site is preferred over the nt 446 site because the former was activated in *dl*722, which retains both these cryptic 5′ splice sites. Many studies have indicated that when there is a choice between alternative potential 5′ splice sites, the site with the greatest similarity to the consensus sequence is usually preferred (30). The E3 nt 372 site matches the consensus sequence most closely, followed by the nt 446 site and then the nt 428 site (Fig. 2); thus, the preferred use of the nt 428 site over the nt 446 site in *dl*722 cannot be explained on the basis of match to the consensus sequence. *dl*726 and *dl*727, which are almost completely defective in splicing, have the cryptic sites at nt 428 and nt 446 deleted. These mutants retain nearby GT's (Fig. 2), but they are not used as cryptic sites.

Although similarity to the 5′ splice consensus sequence and complementarity to U1 small nuclear RNA are important in 5′ splice site selection, a variety of studies have indicated that the sequence context of the 5′ splice is important in selection (30). For the E3 nt 372 5′ splice site, Domenjoud et al. (21) reported that, in an in vitro splicing reaction, deletion of sequences near the 3′ end of the nt 1 to 372 exon reduces use of the nt 372 5′ splice site and increases use of the nt 446 cryptic site. They presented evidence that these sequences may form a hairpin, and they proposed that these sequences function to enhance use of the nt 372 5′ splice site and to prevent use of the nt 446 cryptic 5′ splice site. It remains to be determined whether this putative splice selector element functions in vivo in virus-infected cells, but it could explain why the nt 428 cryptic 5′ splice site is preferred over the nt 446 site in *dl*722.

With regard to whether the nt 372 to 768 splice affects downstream alternative pre-mRNA processing events, *dl*726 and *dl*727, which do not excise the first intron, still accumulate the same E3 mRNAs as the wild type. This indicates that excision of the first intron is not obligatory for cleavage and/or polyadenylation at the E3A and E3B sites, for use of the alternative nt 951 and nt 1017 5′ splice sites, or for use of the alternative nt 2157 and nt 2880 3′ splice sites. However, the frequency of use of the E3A polyadenylation site and of the alternative 5′ and 3′ splice sites is reduced, so that unspliced mRNA *c* plus *i* is increased about threefold in abundance, and mRNA *c* becomes the most abundant mRNA. This suggests that the nt 372 to 768 splice, although not obligatory, does increase the efficiency of the downstream processing sites. However, the reciprocal does not appear to be true, i.e., the downstream processing events are not required for the nt 372 to 768 splice. This conclusion is based upon our observation that mutations which prevent use of the E3A polyadenylation site and/or the nt 2157 3′ splice site do not affect the nt 372 to 768 splice (4, 5, 7–11).

The role that splicing plays in transport of mRNA to the cytoplasm is unclear; some mRNAs need not be spliced, whereas with some other mRNAs, defects in splicing prevent transport (43, 46). With *dl*726 and *dl*727, mRNAs *a* and *c* are unspliced, yet they are transported to the cytoplasm with reasonable efficiency (although *dl*727 mRNA levels are reduced compared with wild-type levels). Thus, for these E3 mRNAs, splicing is not obligatory for transport.

Region E3 is embedded within the major late transcription unit. The L4 poly(A) addition site has its AAUAAA component at nt 595 within the E3 nt 372 to 768 intron (8, 15, 22, 41). E3 mRNAs that use this L4 site are not usually detected early after infection (1, 8), although a scarce nuclear RNA designated E3-CP1, which initiates at nt +1 in E3 and uses the L4 site, has been described (24). This L4 site is, of course, used efficiently late after infection by the major late pre-mRNA. Adami and Nevins (1) have used transfected plasmids and shown that the L4 site is used efficiently whenever a splice can occur upstream in the pre-mRNA, as is the situation during late stages of infection. They also presented evidence that deletion of either the nt 372 or nt 768 splice site, which prevented the nt 372 to 768 splice, led to increased use of the L4 poly(A) addition site, resulting in abundant synthesis of the equivalent of the E3-CP1 RNA. In our experiments, mutants *dl*726 and *dl*728, which do not carry out the nt 372 to 768 splice, did not make a detectable mRNA (or nuclear pre-mRNA) terminating at the L4 site during early stages of infection. The discrepancy between our results and those of Adami and Nevins (1) is probably due to the fact that we used virus mutants and they used transfected plasmids or possibly due to differences in the deletions.

The poly(A) addition sites at the E3A poly(A) addition site are located 17 to 29 nt downstream of an AUUAAA se-

quence (6, 10, 11). Only about 40% of E3 mRNAs terminate at the E3A site. If the E3A site were more efficient, then mRNAs *c*, *e*, *f*, and *h* (all of which use the E3B site) would not be synthesized in proper quantity, nor would the 10.4-, 14.5-, and 14.7-kDa proteins which are encoded by mRNAs *f* and *h*. Therefore, inefficient use of the E3A poly(A) addition site is beneficial to the virus. We have shown here that mutation of the AUUAAA sequence to AAUAAA (*pm*761), which is the usual poly(A) addition signal, increased mRNA *a* levels about twofold and decreased mRNA *c* levels, lending support to the idea that the E3A poly(A) addition signal may have evolved to be intrinsically inefficient. In vitro studies on cleavage and polyadenylation have indicated that AUUAAA is about 20% (58) to 70% (49) as efficient as AAUAAA.

Our region III (nt 2488 to 3002) mutations (*dl*759 and *dl*763) decrease mRNA *a* levels about two- to threefold and specifically increase mRNA *f* levels. Deletion of nt 2229 to 2436 has no effect (data not shown). mRNA *c* was not affected significantly. It is possible that region III includes a pause or partial termination signal for RNA polymerase, which would facilitate better use of the E3A poly(A) addition site used by mRNA *a*. mRNA *f* levels may be increased for the same reason that they are increased by deletions in region II, i.e., deletions in region III, by decreasing the ability of cleavage and/or polyadenylation factors to bind to the E3A site, result in increased binding of splicing factors to the nt 2157 3′ splice site for mRNA *f*. An RNA polymerase pause/termination signal has been described recently for simian virus 40 (16) and adenovirus (17). It is also possible that region III contains splice-suppressing sequences analogous to those in region I.

Mutants *dl*758 and *dl*762 have a region IV (nt 2904 to 3251) phenotype, i.e., the mRNA *a* level is increased about two- to threefold, those of mRNAs *d* and *e* are increased, and that of mRNA *f* is decreased. This phenotype is somewhat in accord with the idea that region IV contains a sequence that facilitates use of the E3B poly(A) addition site; deletion of this sequence provides increased opportunity for the E3A site to be used to form mRNA *a*. Possibly analogous upstream elements that are required for efficient use of the downstream poly(A) addition site have been identified in the adenovirus L1 site (19), the simian virus 40 late site (13), and the human immunodeficiency virus type 1 site (20, 57). Also, Russnak and Ganem (48) have identified multiple sequences 5′ to the polyadenylation signal that mediate differential poly(A) addition sites used in hepatitis B virus.

We now have a map of the *cis*-acting sequences involved in alternative pre-mRNA processing in the E3 region. This map will enable us to pursue two different approaches to studying adenovirus gene expression and function. First, the mechanism by which those *cis*-acting sequences function can be more easily investigated. Second, it is now possible to design virus mutants to overproduce specific E3 mRNAs and proteins coded by them to study the properties and functions of these proteins.

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