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Adenovirus Mutants with Splice-Enhancing Mutations in the E3 Complex Transcription Unit Are Also Defective in E3A RNA 3′-End Formation

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Region E3 of adenovirus encodes about 10 overlapping mRNAs with different spliced structures. The mRNAs are 5′ coterminal and form two major 3′-coterminal families termed E3A and E3B. As a group, the mRNAs have two 5′ splice sites and four or five 3′ splice sites. We previously described a novel class of virus mutants with deletions that enhance distant upstream and downstream 5′ and 3′ splice sites in region E3 (S. L. Deutscher, B. M. Bhat, M. H. Pursley, C. Cladaras, and W. S. M. Wold, Nucleic Acids Res. 13:5771–5788, 1985). We now report that two of these mutants, *dl*710 and *dl*712, are defective in RNA 3′-end formation at the E3A site. This result was surprising because the deletions in *dl*710 and *dl*712 are upstream of the putative signal for E3A RNA 3′-end formation. The explanation that we favor for this result is that the enhanced splicing activity in these mutants results in the splicing out of the E3A 3′-end site from the RNA precursor before the E3A 3′ ends have a chance to form.

Most tumor virus genes and many cellular genes are organized into "complex" transcription units (10, 20). In a complex transcription unit, RNA precursors form alternate mRNAs and usually alternate proteins, depending on the selection of the RNA 3′-end site or the splice sites or both. This differential RNA processing is often tissue specific or developmentally regulated. Little is known about this important aspect of posttranscriptional gene regulation.

We have analyzed early region E3 of adenovirus (Fig. 1) as a model to understand complex transcription units (3, 4, 6, 7, 11, 24). Using viable virus mutants, we have mapped sequences required for RNA 3′-end formation at the E3A site (4) and for splicing at the nucleotide (nt) 2157 3′ splice site (3). The E3A 3′-end site has an ATTAAA sequence at nt 2161 to nt 2166 and poly(A) addition sites clustered near nt 2183. In our previous study (4), E3A RNA 3′-end formation was abolished in virus mutants with the following deletions: (i) Δ(1654-2207), which removes ATTAAA and the poly(A) addition sites, (ii) Δ(2044-2167), which removes ATTAAA but leaves the poly(A) addition sites, and (iii) Δ(2173-2237), which leaves ATTAAA but removes the poly(A) addition sites. Thus, ATTAAA as well as downstream sequences, including the poly(A) addition sites, are required for E3A RNA 3′-end formation. No effect on E3A RNA 3′-end formation was observed in mutants with Δ(2092-2138) or Δ(2070-2141) deletions, i.e., deletions 22 nts and 19 nts, respectively, upstream of ATTAAA (4). We have also discovered a novel class of mutants that enhances the 951→2157 splice or the 951→2830 splice or both (11). We now report that two of these mutants, *dl*710 and *dl*712 (Fig. 1), are defective in RNA 3′-end formation at the E3A site. This finding was unexpected because the deletions in *dl*710 [Δ(1679-2123)] and *dl*712 [Δ(1691-2122)] are 38 nts and 39 nts, respectively, upstream of ATTAAA at the E3A site. These results provide insights into RNA processing in E3.

The E3A 3′ ends synthesized by *rec*700 (i.e., wild-type virus), *dl*710, and *dl*712 were analyzed by the nuclease gel procedure (2) with ^{32}P-labeled RNA probes (19). Two probes, a *Hin*dIII probe and an *Hpa*II probe, were used for the *rec*700 analysis. These probes and the fragments expected from the analysis are shown in Fig. 2. Bands of ~825 (medium arrow), ~1,085 (thin arrow), and 287 (thin arrow) nts were observed when *rec*700 RNA was analyzed with the *Hin*dIII probe (Fig. 2, lane e). The ~825-nt band corresponds to mRNA *a*, with 3′ ends at the E3A site, the ~1,085-nt band corresponds to mRNAs *b* and *c*, which are colinear with the probe, and the 287-nt band corresponds to mRNA *f*, with a 3′ splice at nt 2157. With the *Hpa*II probe (Fig. 2, lane g), bands of ~269 (medium arrow), 529 (thin arrow), and 287 (thin arrow) nts were observed. The ~269-nt band corresponds to the E3A 3′ ends for mRNAs *a* and *d*, the 529-nt band corresponds to mRNAs *b* and c, and the 287-nt band corresponds to mRNA *f*.

*dl*710 RNA was analyzed with *Sst*I and *Hin*dIII probes, and *dl*712 RNA was analyzed with *Hin*dIII, *Bam*HI, and *Hae*II probes (see the legend to Fig. 2 for descriptions of the probes). The positions expected for bands corresponding to the E3A 3′ ends for mRNA *a* are indicated by the thick arrows in Fig. 2. Specifically, *dl*710 should have yielded bands of 794 nts for the *Sst* I probe (lane j) and 390 nts for the *Hin*dIII probe (lane 1), and *dl*712 should have yielded bands of 463 nts for the *Hin*dIII probe (lane o) and 219 nts for the *Hae*II probe (lane s). Bands of these sizes, if present at all, were very much reduced as compared with the bands for the E3A 3′ ends in *rec*700.

The other expected E3 mRNAs were detected in these analyses. Bands corresponding to mRNAs *b* and *c*, which are colinear with the probes, and a prominent heterogeneous band of 287 nts, corresponding to mRNA *f*, were observed in the RNAs from both mutants and with all probes. These bands are indicated by the thin arrows in lanes j, l, o, q, and s of Fig. 2. With all probes, the 287-nt band was much more abundant than in *rec*700 RNA because the 951→2157 splice is enhanced in *dl*710 and *dl*712 (11).

There are at least three interesting explanations for the virtual absence of E3A 3′ ends in *dl*710 and *dl*712 mRNAs. First, the sequences deleted could contain an RNA 3′-end signal. This seems unlikely because the Δ(2092-2138) and

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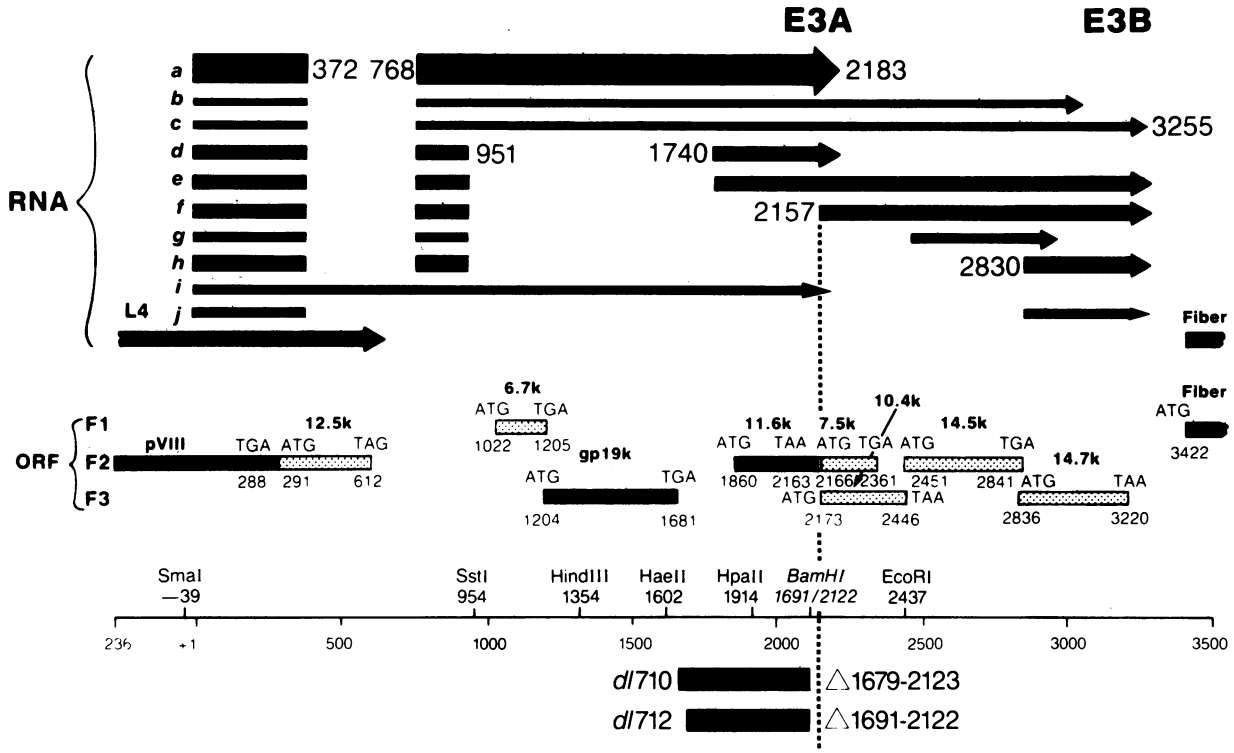


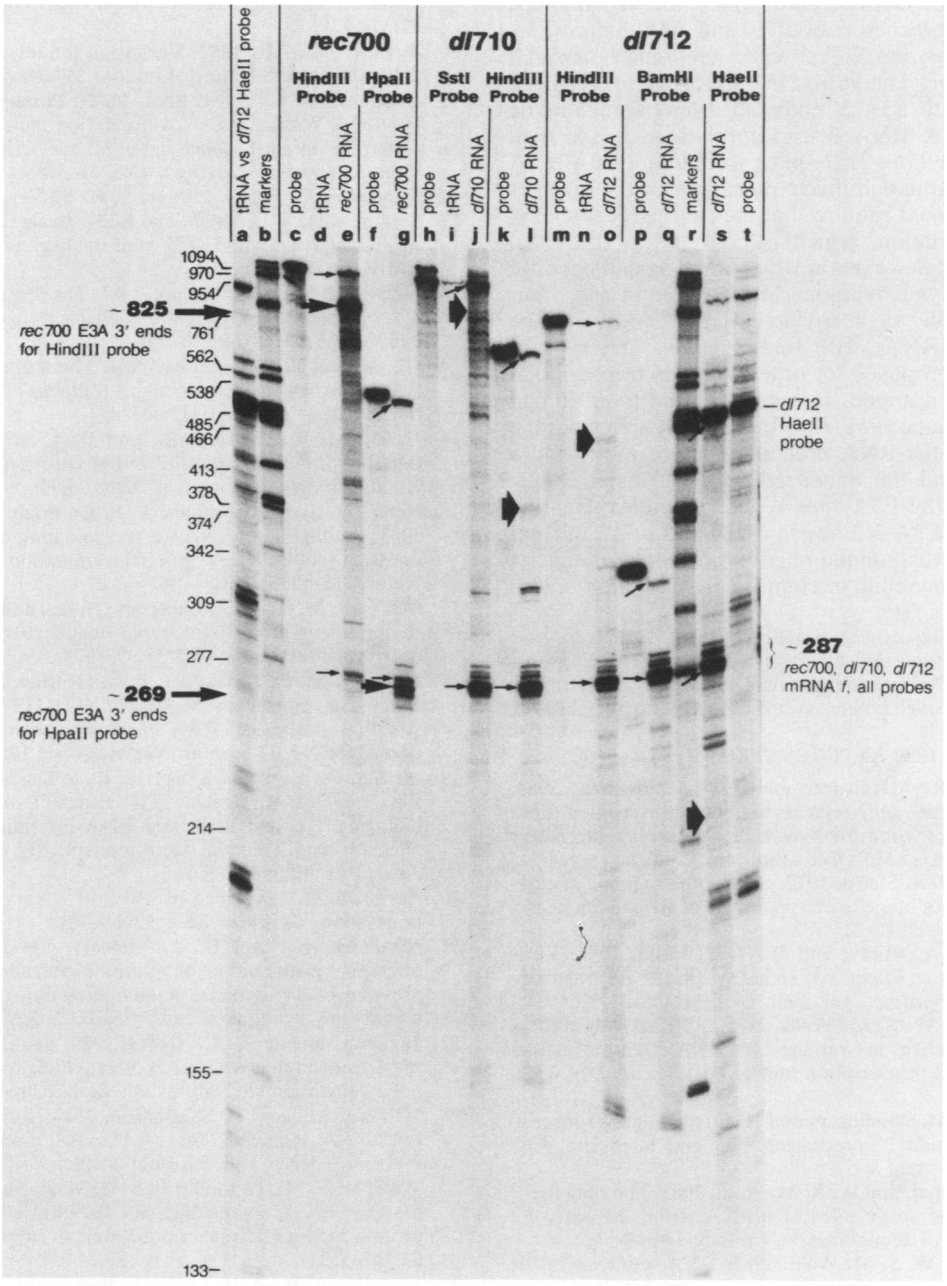
FIG. 1. Schematic representation of the E3 transcription unit of adenovirus 2 and of the sequences deleted in virus mutants *dl*710 and *dl*712. Nucleotide +1 is the first major transcription initiation site. Open reading frames (ORFs) F1, F2, and F3 are shown. Black bars indicate proven proteins, and stippled bars indicate proteins that we propose exist (7). Arrows *a* through *j* represent the spliced structures of the E3A and E3B 3′-coterminal families of mRNAs. Original references and evidence in support of this schematic diagram are given in references 6 and 7. The isolation of *dl*710 and *dl*712 is described in reference 11. *dl*710 has one and *dl*712 has six *Bam*HI linkers (CGGGATCCCG) in their deletions.

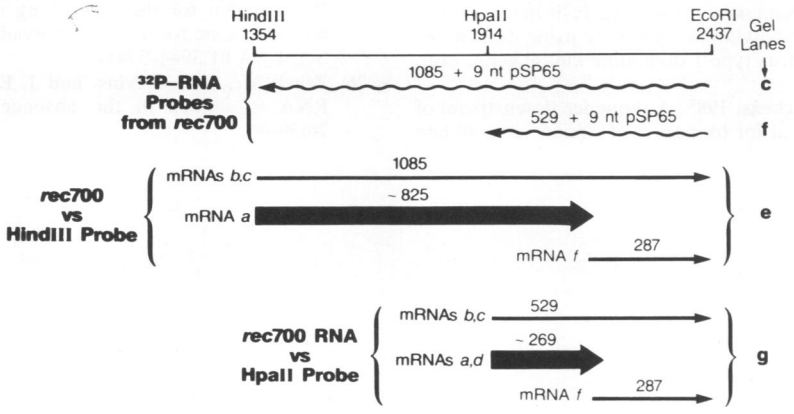
Δ(2070-2141) deletions did not affect E3A RNA 3′-end formation (4). Furthermore, it has been shown in simian virus 40 (13, 22) and in certain expression vectors (8, 9, 14, 17, 18, 21, 23, 25) that a relatively small region that includes AATAAA and downstream sequences is necessary and sufficient for RNA 3′-end formation.

The second explanation relates to the general mechanism of RNA 3′-end formation in eucaryotes (reviewed in reference 5). There is much evidence that transcription continues for ~700 to 4,000 nts past the poly(A) addition site and that the 3′ ends are formed by endonucleolytic cleavage and polyadenylation. The deletions in *dl*710 and *dl*712 could affect the overall tertiary structure of the RNA precursor such that cleavage does not occur at the E3A site.

The third explanation, and the one that we favor, is that the enhanced splicing at the nt-951 5′ splice site precludes E3A RNA 3′-end formation. This could occur in two different ways, depending on how the E3A 3′ ends are actually formed in wild-type virus. In the one case, E3A 3′ ends could be formed by cleavage of the RNA precursor before

FIG. 2. Defective RNA 3′-end formation at the E3A site in mutants *dl*710 and *dl*712. Shown is an RNase-gel analysis of *rec*700 (i.e., wild-type virus), *dl*710, and *dl*712 RNAs. The schematic diagram at the bottom depicts the *rec*700 ^{32}P-labeled RNA probes used as well as the RNase-resistant bands obtained from the analysis of *rec*700 RNA. In the gel, boldface numbers indicate RNase-resistant bands, and lightface numbers indicate ^{32}P-labeled RNA size markers. Thin arrows in the gel indicate RNase-resistant bands corresponding to mRNAs *b* and *c*, which are colinear with the probes, or to mRNA *f*, with a 3′ splice at nt 2157. Medium arrows indicate bands corresponding to *rec*700 E3A 3′ ends for mRNAs *a* and *d*. Thick arrows indicate the positions expected for *dl*710 and *dl*712 E3A 3′ ends. Probes were prepared by in vitro transcription with SP6 polymerase (19). With each virus, the probe substrate was the *Eco*RI-*Sma*I fragment (nts 2437 to −39) (Fig. 1) cloned in pSP65 (19). For *rec*700, the *Hin*dIII and *Hpa*II probes were prepared by cleaving at the *Hin*dIII (nt 1354) and *Hpa*II (nt 1914) sites, respectively, before transcription. Similarly, the *dl*710 *Sst*I and *Hin*dIII probes were prepared by cleaving at the *Sst*I (nt 954) and *Hin*dIII (nt 1354) sites, and the *dl*712 *Hin*dIII, *Bam*HI, and *Hae*II probes were prepared by cleaving at the *Hin*dIII (nt 1354), *Bam*HI (nt 1691/2122), and *Hae*II (nt 1602) sites. The *Bam*HI (1691/2122) site shown in italics in Fig. 1 resulted from the ligation of *Bam*HI linkers into the deletion in *dl*712. All probes contained 9 nts of pSP65 sequences at their 5′ termini. Each virus RNA was analyzed with probes prepared from the corresponding mutant plasmid DNA. After the probes were prepared, the RNase-gel analyses with 30 μg of cytoplasmic RNA were done as described previously (3, 4, 11). Although most of the probes consisted of one band and gave no background when analyzed against tRNA, this reproducibly was not the case for the *dl*712 *Hae*II probe (lanes a and t). Thus, most of the bands present in lane s (*Hae*II probe versus *dl*712 RNA) are background bands, because they were also observed with tRNA (lane a). Nevertheless, the *Hae*II data in lane s clearly show the 479-nt band (thin arrow), which represents mRNAs *b* and *c*, which are colinear with the probe, the 287-nt band (thin arrow), which represents mRNA *f*, with a 3′ splice at nt 2157, and the absence of a 219-nt band (thick arrow), which would represent the E3A 3′ ends of mRNA *a*.


rec700
dl710
dl712
HindIII Probe
HpaII Probe
SstI Probe
HindIII Probe
HindIII Probe
BamHI Probe
HaeII Probe
tRNA vs dl712 HaeII probe
markers
probe
tRNA
rec700 RNA
dl710 RNA
dl712 RNA
a b c d e f g h i j k l m n o p q r s t
1094
970
954
~825
rec700 E3A 3' ends for HindIII probe
761
562
538
485
466
413
378
374
342
309
277
~269
rec700 E3A 3' ends for HpaII probe
214
155
133
dl712 HaeII probe
~287
rec700, dl710, dl712 mRNA f, all probes


HindIII 1354
HpaII 1914
EcoRI 2437
Gel Lanes
32P-RNA Probes from rec700
1085 + 9 nt pSP65
c
529 + 9 nt pSP65
f
rec700 vs HindIII Probe
mRNAs b,c
1085
mRNA a
~825
mRNA f
287
e
rec700 RNA vs HpaII Probe
mRNAs b,c
529
mRNAs a,d
~269
mRNA f
287
g

transcription reaches the E3B site. If the 951→2830 splice occurs in the RNA precursors of *dl*710 and *dl*712 before E3A 3′-end cleavage, then the E3A 3′-end site would be spliced out of the precursor. The 951→2157 splice has never been seen in mRNAs with E3A 3′ ends, so it may somehow be antagonistic to E3A RNA 3′-end formation. If so, then enhancement of the 951→2157 splice would limit E3A RNA 3′-end formation. Although this explanation for the *dl*710 or *dl*712 phenotype would require that splicing precede cleavage and polyadenylation, which is contrary to the usual situation (20), examples exist in which splicing can occur in the absence of polyadenylation (15, 20, 26). In the other case, mRNA *c*, with polyadenylated E3B 3′ ends, may be the precursor of mRNAs with E3A 3′ ends. This type of scenario has been proposed for other complex transcription units (1, 12, 16). If it applies to region E3 and to *dl*710 and *dl*712, then the enhanced 951→2830 splice may splice out the E3A 3′ ends from the RNA precursor before they have a chance to form, and the enhanced 951→2157 splice may inhibit cleavage at the E3A site. Whatever the explanation for the absence of E3A 3′ ends in *dl*710 and *dl*712, further characterization of this unique phenotype should illuminate aspects of RNA processing in complex transcription units.

The mutant virus plaques were isolated by N. Takemori.

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