A Small Deletion Distant from a Splice or Polyadenylation Site Dramatically Alters Pre-mRNA Processing in Region E3 of Adenovirus

BHEEM M. BHAT AND WILLIAM S. M. WOLD*

Institute for Molecular Virology, Saint Louis University School of Medicine, St. Louis, Missouri 63100

The E3 complex transcription unit of adenovirus encodes overlapping mRNAs (*a* to *i*) with different exon structures. The major mRNAs are *a* (~40% of the total) and *c* (~15%), which are spliced once, and *f* (~15%) and *h* (~25%), which are spliced twice. mRNA *a* uses the upstream E3A polyadenylation site, and the other mRNAs use the downstream E3B polyadenylation site. We analyzed virus deletion mutants to identify sequences important in alternative pre-mRNA processing in region E3. Our main finding is that a 64-base-pair deletion in *dl*742 causes mainly mRNAs *f* and *h* to be formed. mRNAs *a* and *c* are barely made. *dl*742 does not delete either a splice site or a polyadenylation site. Thus, the sequences deleted must function in alternative pre-mRNA processing independently of the signals at the actual splice and polyadenylation sites. The lack of synthesis of mRNA *a* by *dl*742 does not appear to result from a defect in the E3A polyadenylation signal but rather from an increase in splicing activity which results in the synthesis of doubly spliced mRNAs *f* and *h* at the expense of singly spliced mRNAs *a* and *c*. This suggests, in the wild-type situation, that the frequency of use of the E3A versus the E3B polyadenylation site may be determined by the rate of splicing, as well as, presumably, the rate of cleavage-polyadenylation at the E3A site.

Most tumor virus genes and many cellular genes are organized into complex transcription units in which different mRNAs are formed by alternative pre-mRNA processing (reviewed in references 12, 15, and 20). Very little is known about how alternative pre-mRNA processing pathways are selected. Complex and simple transcription units are similar in that short consensus sequences at the 5' and 3' splice sites and at the lariat branchpoint site provide *cis*-acting splicing signals (reviewed in reference 15). Also, signals for pre-mRNA cleavage and polyadenylation are located near the polyadenylation site and include an AATAAA (or related sequence) and sequences immediately downstream (reviewed in reference 7). However, it seems likely that complex transcription units contain additional information which determines the pre-mRNA processing pathways.

A few genetic studies have identified sequences that might be specifically involved in alternative splicing. Deletions near the 3' end of the first exon for simian virus 40 small t antigen mRNA reduced splicing of that mRNA (18). Deletions in exon 1 for simian virus 40 late mRNAs affected the use of alternative downstream 5' and 3' splice sites, and it was suggested that sequences at the 5' end of the pre-mRNA might influence alternative splicing (24). Deletions in the intron for murine leukemia virus *env* mRNA inhibited splicing (17). Deletions in the intron of adenovirus E1A 13S mRNA inhibited splicing of that mRNA, and it was suggested that a signal may exist in this intron which affects the efficiency of splicing of the 13S mRNA versus that of the overlapping 12S and 9S mRNAs (25). The simian virus 40 early and late, murine leukemia virus, and E1A transcription units have a single polyadenylation site. Alternative pre-mRNA processing has also been studied in the immunoglobulin μ-chain complex transcription unit, which has two polyadenylation sites. The efficiency of use of the upstream

(secreted) versus the downstream (membrane-bound) polyadenylation site is determined by the cell type. Some studies have indicated that the efficiency of cleavage-polyadenylation at the upstream site determines whether μ_s or μ_m mRNA will be made (11, 19). Another study suggested that regulation of μ_s or μ_m synthesis is achieved by competition between cleavage-polyadenylation of the μ_s site and splicing of the $C_\mu 4$ and μ_m exons (23). Still another study concluded that selection of the polyadenylation site is determined primarily by its proximity to the promoter (22).

We used virus deletion mutants to analyze pre-mRNA processing in the E3 complex transcription unit of adenovirus. E3 encodes overlapping mRNAs that use one of two polyadenylation sites termed E3A and E3B (Fig. 1). Our objective was to identify sequences that determine the frequency with which these mRNAs are formed. *dl*702, *dl*713, *dl*719, and *dl*712 (Fig. 1) have been partially characterized (2–6, 13). These deletions have the following effects on the accumulation of the major mRNAs *a*, *c*, *f*, and *h*. *dl*702 has no effect. *dl*713 deletes the ATTAAA element of the E3A polyadenylation signal and the 3' splice site at nucleotide (nt) 2157; mRNAs *a* and *f* are not made, and *c* and *h* are made in abundance. This is the phenotype to be expected a priori from a mutant with this deletion. *dl*719 deletes the essential sequences downstream of the ATTAAA element of the E3A polyadenylation signal. mRNA *a* is not made. Surprisingly, mRNAs *c* and *h* are not made in abundance as in *dl*713; rather, virtually only mRNA *f* is produced. Somehow, the deletion greatly increases use of the 2157 3' splice site. The *dl*712 deletion causes essentially only mRNAs *f* and *h* to be formed. mRNA *a* is barely made, even though the deletion is 44 base pairs (bp) upstream of the ATTAAA element of the E3A polyadenylation signal. Thus, we identified two phenotypic groups of mutants that have novel effects on alternative pre-mRNA processing, one group exemplified by *dl*719, in which mRNA *f* is made
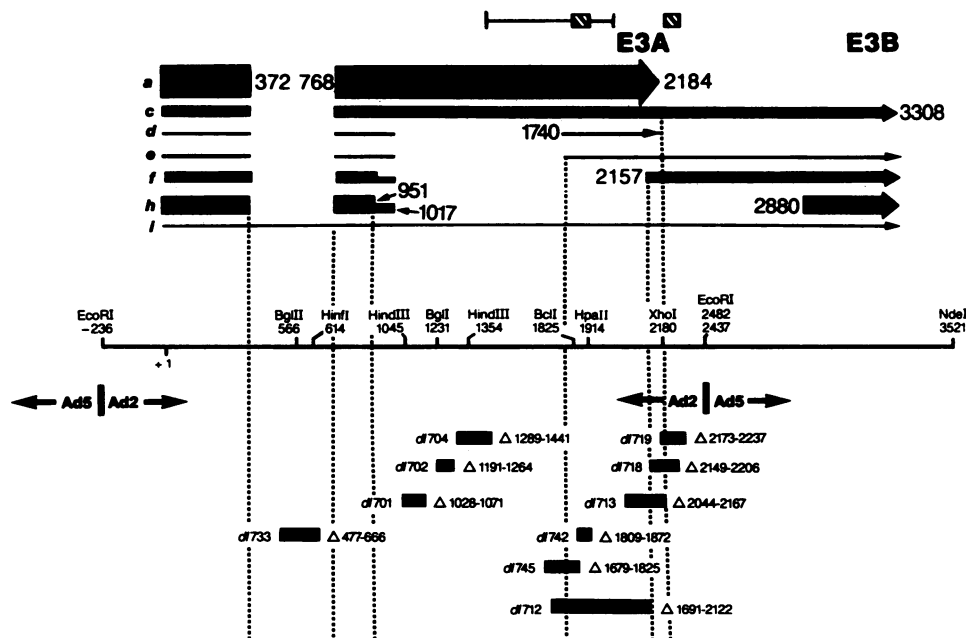
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* Corresponding author.

FIG. 1. Schematic of the E3 transcription unit in rec700 and of the deletion mutants. rec700 is the wild-type version of the mutants. E3 in rec700 has Ad2 sequences between the EcoRI sites at nt −236 and 2437 and Ad5 sequences elsewhere. Ad2 numbers are used from nt −236 to 2437, and Ad5 numbers are used downstream from nt 2437 (see reference 10). Nt +1 is the transcription initiation site. Restriction sites are numbered at the 5' base pair in the recognition sequence. Arrows a to i indicate the spliced structures of the mRNAs (8). mRNA a is ~40% of the total, c is ~15%, f is ~15%, h is ~25%, and d and e are scarce (8, 10). The hatched bars near the top indicate sequences that affect alternative pre-mRNA processing and are deleted in the dl742 and dl719 groups of mutants.

almost exclusively, and a second group exemplified by dl712, in which almost all pre-mRNA is processed into mRNAs f and h. These two phenotypic groups are unique to region E3 and are very different from any mutants or constructs mentioned in the previous paragraph. In this paper we describe mutants dl733, dl701, dl704, dl745, dl742, and dl718. The most interesting new mutants are dl742 and dl745, which are in the same phenotypic group as dl712 in the sense that the spliced mRNAs are made at the expense of mRNAs a and c. We show with dl745 that the E3A polyadenylation signal is fully functional, which suggests that the phenotype is due to splice enhancement. Importantly, the 64-bp deletion in dl742 does not remove a classic pre-mRNA processing signal such as a splice site or a polyadenylation site. Thus, the region deleted must be important in alternative pre-mRNA processing.

MATERIALS AND METHODS

Isolation of plasmid and virus mutants. Methods for the isolation of virus mutants have been previously described (26). The mutants have adenovirus type 5 (Ad5) sequences from genomic map position 0 to 76, Ad2 sequences from 76 to 86, and Ad5 sequences from 86 to 100. Ad2 and Ad5 are closely related in region E3 (10). rec700 (Fig. 1) is the wild-type version of the mutants.

Nuclease gel analysis of mRNA. Infection of KB cells and extraction of cytoplasmic mRNA have been previously described (9). mRNA extracted at 6 h postinfection (no DNA or protein inhibitors) was used in all experiments except for that described in Fig. 4A, lane c, for which RNA extracted at 21 h was used. Nuclease gel analysis (1) was done with RNases T_1 and A, with ^{32}P-labeled RNA probes prepared with SP6 polymerase (21) and with 6% sequencing gels,

exactly as previously described (3). Thirty micrograms of cytoplasmic RNA was used. In all of the gels, the probes were in excess, so the results are quantitative. The plasmids used to prepare the probes have been previously described (2).

RESULTS

Use of the 951 5' splice site is specifically enhanced in the dl742 and dl719 phenotypic groups of mutants. The effects of the deletions on the accumulation of E3 mRNAs were characterized by the nuclease gel procedure with a variety of ^{32}P-labeled RNA probes. We first assayed the 1-to-372 exon which is present in most E3 mRNAs. This exon was found in equal abundance in all of the mutants (data not shown). This indicates that none of the deletions detectably affect either E3 transcription or the stability or transport to the cytoplasm of any of the abundant mRNAs. This is an important point to appreciate. For example, the deletions in dl712 and dl742 greatly reduce mRNAs a and c and correspondingly increase mRNAs f and h. The reduction in mRNAs a and c cannot be attributed to their decreased stability or transport, because this would be reflected in a marked decrease in abundance of the 1-to-372 exon in cytoplasmic mRNA. Since the dl712 and dl742 deletions (or any of the other deletions) do not affect the abundance of the 1-to-372 exon, the decrease in mRNAs a and c results from preferential synthesis of mRNAs f and h.

We next examined use of the 768 3' splice site and the 951 and 1017 5' splice sites. Use of these splice sites yields three overlapping exons. mRNAs a and c have the 768-to-E3A/B exon, which extends from the 3' splice at nt 768 to the 3' end at either the E3A or the E3B polyadenylation site. mRNAs d to h have the 768-to-951 and 768-to-1017 exons, which extend from the 3' splice site at nt 768 to the 5' splice site at

either nt 951 or 1017. One or another of these latter two exons is spliced to one of the 3′ splice sites at nt 1740 (mRNAs *d* and *e*), nt 2157 (mRNA *f*), and nt 2880 (mRNA *h*).

These three exons were assayed with a *Hin*dIII-*Hin*fI probe, nt 1045 to 614 (Fig. 2). Since almost all E3 mRNAs use the 768 3′ splice site, this assay measures the frequency with which the 951 and 1017 5′ splice sites are used. Four nuclease-protected fragments, of 436, 283, 250, and 184 nt, were obtained from *rec*700 mRNA (lane b) (*rec*700 is an Ad5-Ad2-Ad5 recombinant that is the parent of the mutants [26]). The 436-nt band corresponds to mRNA *i*, which is colinear with the probe. The 283-nt band represents the abundant mRNAs *a* and *c*. The 250- and 184-nt bands are derived from the 768-to-1017 and 768-to-951 exons, respectively, in mRNAs *d* to *h*. Similar results were obtained with Ad2 (lane a). Similar results were also obtained with *dl*733, *dl*701, *dl*702, *dl*704 (lanes c to f), *dl*713 (lane j), and *dl*718 (lane k), indicating that these deletions do not affect use of the 5′ splice sites at nt 951 and 1017. The 383-nt band from *dl*733 (lane c) and the 260-nt band from *dl*701 (lane d) arise from the discontinuity between the wild-type probe and the deletion mutant mRNAs.

The deletions in *dl*712, *dl*745, and *dl*742 have a marked effect on the relative use of the 951 and 1017 5′ splice sites. The 184-nt band representing the 768-to-951 exon is very abundant (lane g, h, and i), indicating that the 951 5′ splice

site is used very efficiently. As is necessary with increased use of the 951 site, the 250- and 283-nt bands derived from the 768-to-1017 and 768-to-E3A/B exons are correspondingly reduced. Thus, use of the 951 5′ splice site is specifically enhanced to the exclusion of the 1017 5′ splice site. Also, small deletions, such as the 64-bp deletion in *dl*742, can exert a profound effect on pre-mRNA processing.

The pattern with *dl*719 was similar to that obtained with *dl*712, *dl*745, and *dl*742, i.e., the 768-to-951 exon was very elevated and the other exons were reduced (Fig. 2, lane l). Thus, even though *dl*719 is in a different phenotypic group from the *dl*742 class, its deletion also specifically enhances the 951 5′ splice site to the exclusion of the 1017 site.

Use of the 2157 3′ splice site is enhanced and use of the E3A polyadenylation site is virtually eliminated in *dl*712 and *dl*742. Use of the 2157 3′ splice site and the E3A polyadenylation site is shown in Fig. 3. The probe extends from the *Eco*RI site to the *Hpa*II site, nt 2437 to 1914. *rec*700 yielded three sets of nuclease-protected fragments, 529 nt, 287 nt, and a doublet of 270 and 277 nt (lane a). The 529-nt band corresponds to mRNAs *c* and *i*, which are colinear with the probe. The 287-nt band represents the 2157-to-E3B exon in mRNA *f*. The 270- and 277-nt bands are derived from the 768-to-E3A exon in mRNA *a*.

*dl*733, *dl*701, and *dl*704 (lanes b to d) gave the same pattern as *rec*700, consistent with the conclusions from Fig. 2 that
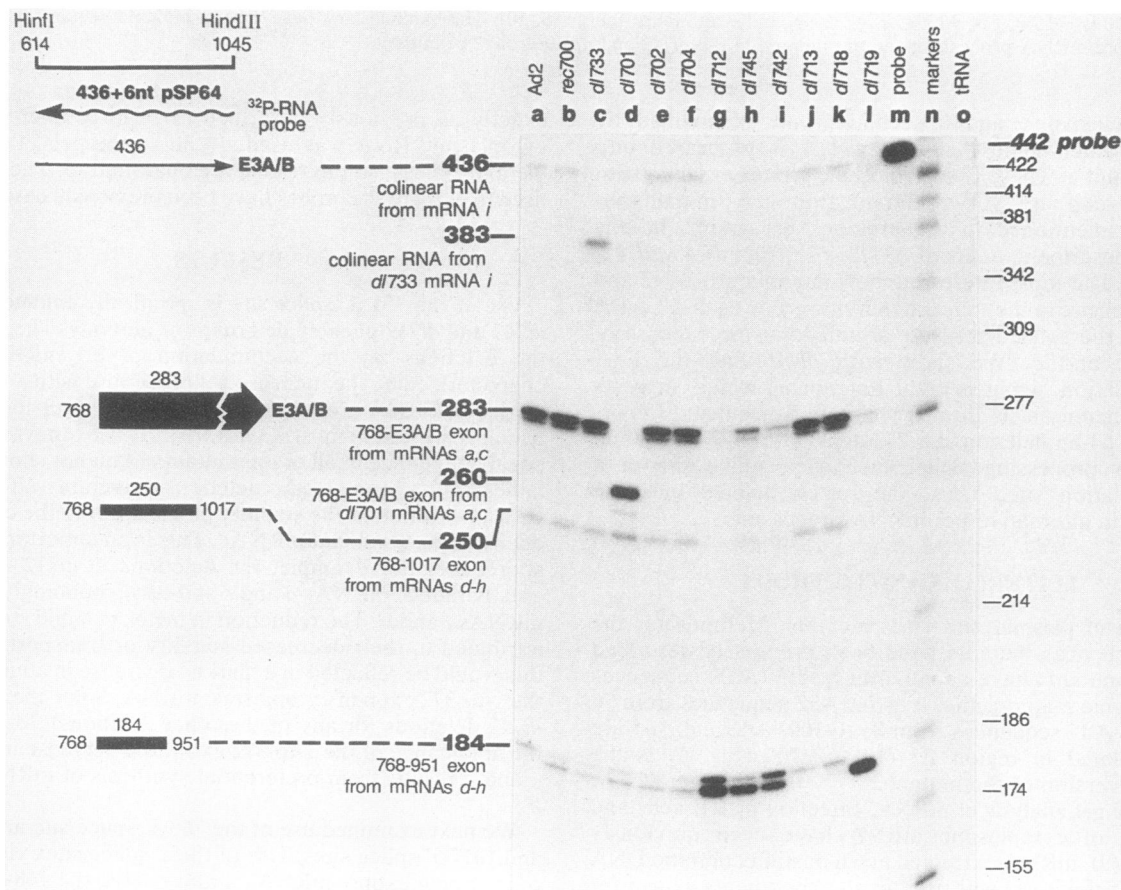

FIG. 2. Nuclease gel analysis for use of the 3′ splice site at nt 768 and the 5′ splice sites at nt 951 and 1017. The schematic indicates the probes used, as well as the RNase-resistant fragments observed in the analysis. In the gel, heavy roman type indicates RNase-resistant fragments, medium italic type indicates probes, and light roman type indicates ^{32}P-labeled RNA size markers. The probe was prepared by cleavage at the *Hin*fI (614) site before transcription.
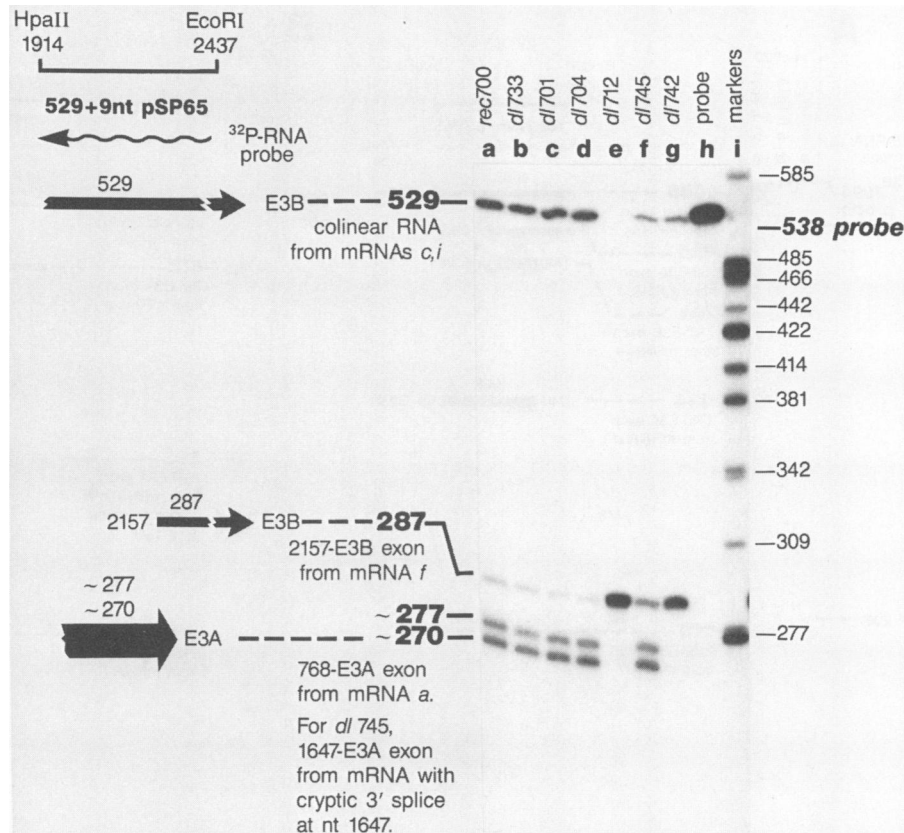
FIG. 3. Nuclease gel analysis for use of the 2157 3' splice site and the E3A polyadenylation site. The probe was prepared by cleavage at the *Hpa*II (1914) site before transcription. See the legend to Fig. 2 for details.

the deletions do not affect pre-mRNA processing. *dl*712 and *dl*742 produced large quantities of the 287-nt band (lanes e and g), reflecting increased use of the 2157 3' splice site for mRNA *f*. *dl*742 produced only a trace of the 270- and 277-nt bands, establishing that mRNA *a* with 3' ends at the E3A site is barely made. The synthesis of mRNA *a* by *dl*712 cannot be addressed by this gel because of the discontinuity between the deletion and the wild-type probe. However, we have shown that, as is the case with *dl*742, *dl*712 makes very little mRNA *a* (2, 5). The important conclusion from Fig. 3 is that the deletion in *dl*742 enhances use of the 2157 3' splice site and virtually eliminates use of the E3A polyadenylation site, even though the deletion is ~300 bp upstream of these sites.

The E3A polyadenylation signal is functional in *dl*745. One explanation for the *dl*742 and *dl*712 results is that the deletions remove an element of the E3A polyadenylation signal. A second explanation is that the deletions enhance splicing such that the E3A site is spliced out of the pre-mRNA precursor. We show below that the E3A polyadenylation signal is as functional in *dl*745 as it is in *rec*700; since *dl*745 is in the same phenotypic group as *dl*742 and *dl*712, this suggests that the latter explanation is probably correct. As shown in Fig. 3, lane f, *dl*745 produced the 270- and 277-nt bands indicative of mRNAs with 3' ends derived from the E3A site. These mRNAs are very abundant, comparable to mRNA *a* (compare the 270- and 277-nt bands in lane f with those in lanes a to d). However, these mRNAs in *dl*745 cannot be mRNA *a* alone because, as shown in Fig. 2, the 768-to-E3A/B exon is much reduced and the 768-to-951 exon is elevated. *dl*745 formed the 287-nt band from the 2157-to-E3B exon in mRNA *f* at levels intermediate

between those of *rec*700 and *dl*742. Thus, use of the 2157 3' splice is increased, but not to the extent of *dl*742 and *dl*712.

To determine the structures of the E3A-terminated mRNAs formed by *dl*745, the region between the E3A site and the 951 and 1017 5' splice sites was analyzed. One such analysis, with *rec*700, *dl*745, and *dl*742, is shown in Fig. 4. The probes extend from the *Eco*RI site to the *Hin*dIII site, nt 2437 to 1354. The probe for each mutant was prepared from the corresponding plasmid, so the probes (and some protected fragments) varied in size according to the deletion. *rec*700 yielded three bands of 1085, 825, and 287 nt (Fig. 4A, lane b). The 1,085-nt band corresponds to the colinear mRNAs *c* and *i*, the 825-nt band corresponds to the 768-to-E3A exon of mRNA *a*, and the 287-nt band corresponds to the 2157-to-E3B exon of mRNA *f*. No bands of 444 and 704 nt, reflecting use of the 1740 3' splice site, are visible in this gel (such bands appear faintly in some gels). We have reported that this splice is scarce during early stages of infection but is amplified late after infection (6). The results with late RNA (lane c) provide a comparison with the early RNA (lane b). The 704-nt band corresponds to the 1740-to-E3B exon of mRNA *e*, and the 444-nt band corresponds to the 1740-to-E3A exon of mRNA *d*.

*dl*745 early RNA yielded four bands: 941, 681, 393, and 287 nt (Fig. 4B, lane b). The 941-nt band corresponds to the colinear mRNAs *c* and *i*. The 681-nt band is derived from the 768-to-E3A exon of mRNA *a*. This band is reduced compared with the equivalent 825-nt 768-to-E3A band in *rec*700 (Fig. 4A, lane b), consistent with the data in Fig. 2 showing that mRNA *a* is reduced. The 287-nt band corresponds to the 2157-to-E3B exon of mRNA *f*. This band is about two- to
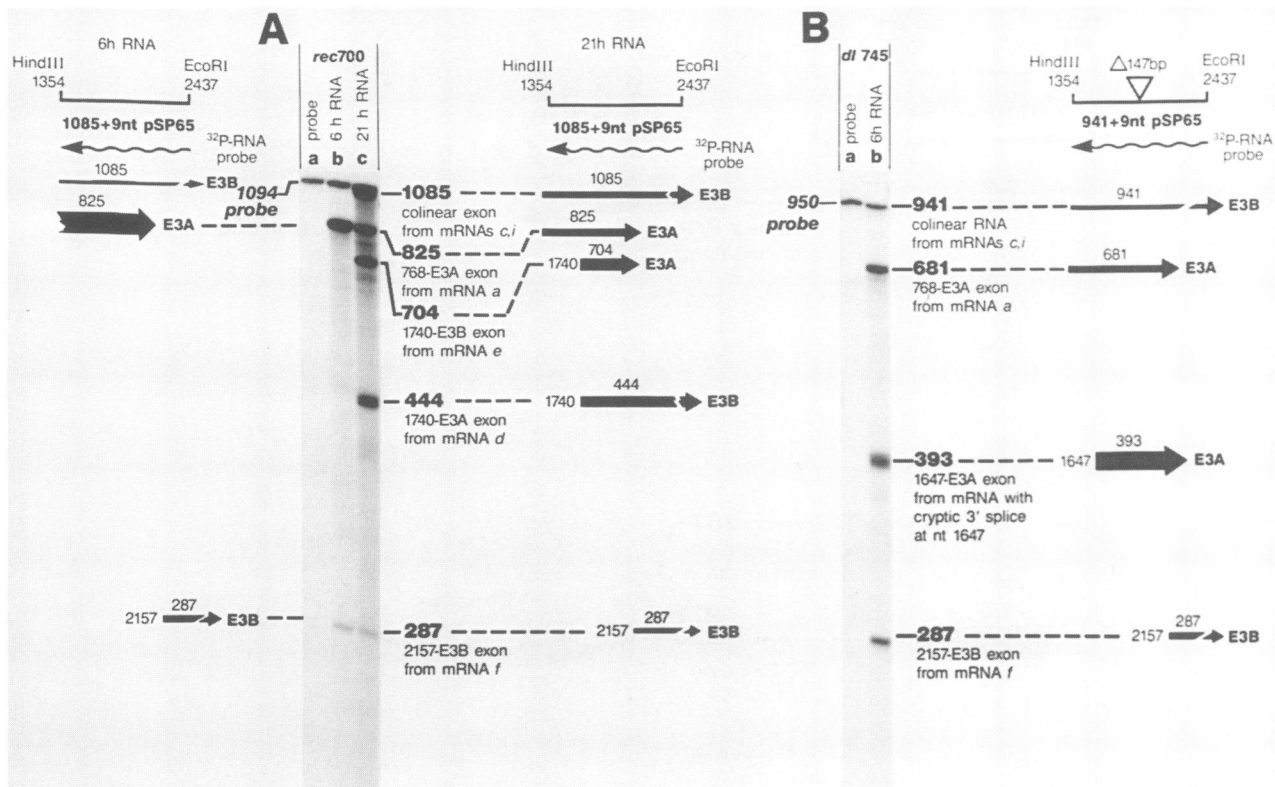
FIG. 4. Nuclease gel assay showing that *dl*745 makes an mRNA with a cryptic 3' splice site at nt 1647 and that the E3A polyadenylation signal is barely used in *dl*742. *rec*700 RNA was extracted at 6 or 21 h postinfection. *dl*745 and *dl*742 RNAs were extracted at 6 h postinfection. The probe used for each mutant RNA was prepared from the corresponding mutant plasmid cleaved at the *Hin*dIII (1354) site. The probes and some of the RNase-resistant fragments vary in size because of the deletions. Panels A, B, and C are from the same gel and gel exposure.
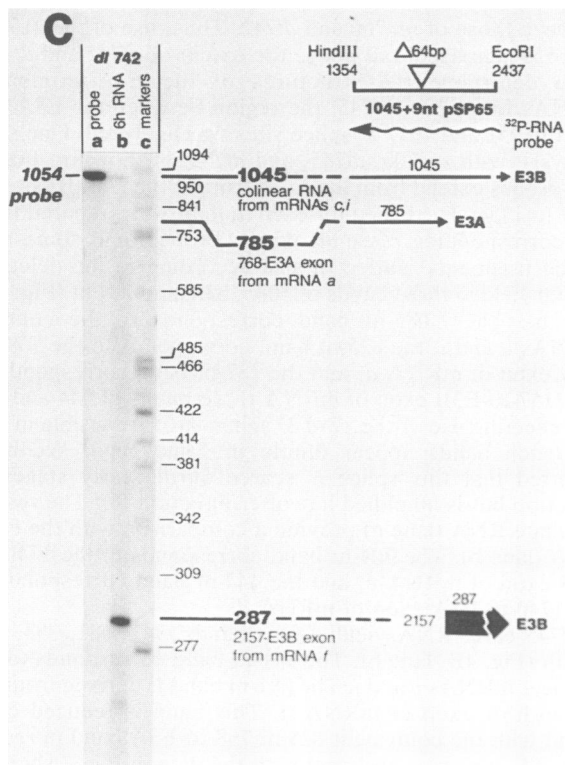
threefold more abundant than in *rec*700 (also shown in Fig. 3), indicating increased use of the 2157 3' splice site. The abundant 393 band does not derive from use of the 1740 3' splice site, because this site is deleted. Accordingly, this band must result from use of a cryptic 3' splice site. There is an AG at nt 1647 (10, 16); use of this AG as a 3' splice site would yield a 1647-to-E3A exon of 393 nt. We conclude that the E3A-terminated mRNAs made by *dl*745 (Fig. 3, lane f) consist of both this cryptic mRNA and mRNA *a*. The abundant cryptic mRNA reflects the splice-enhancing phenotype in *dl*745 (Fig. 2, lane h).

*dl*742 early RNA produced only one major band, of 287 nt, corresponding to the 2157-to-E3B exon of mRNA *f* (Fig. 4C, lane b). This band was more intense than in *rec*700 and *dl*745 (also shown in Fig. 3). The 785-nt band from the 768-to-E3A exon of mRNA *a* and the 1,045-nt band from the colinear mRNAs *c* and *i* were both very faint. There was no indication that *dl*742 uses the 1740 3' splice site or the cryptic 3' splice site at nt 1647 that is used by *dl*745. These results confirm the conclusions from Fig. 2 and 3 that *dl*742 makes large amounts of mRNA *f* and trace amounts of mRNAs *a* and *c*.

Use of the 2880 3' splice site is enhanced in *dl*712, *dl*742, and *dl*745. Use of the 2880 3' splice site and the E3B poly-
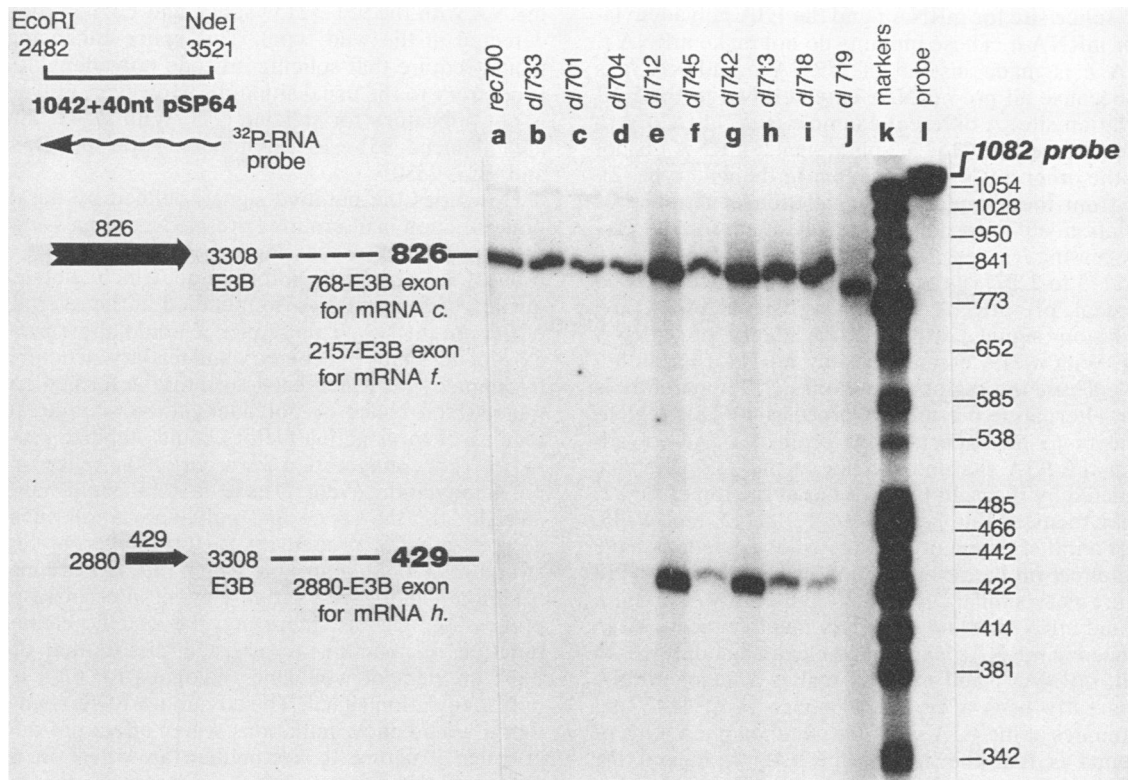
FIG. 5. Nuclease gel assay for use of the 2880 3' splice site and the E3B polyadenylation site. The probe plasmid was cleaved at the EcoRI site before transcription.

adenylation site is shown in Fig. 5. The probe extends from the NdeI site to the EcoRI site, nt 3521 to 2482. A strong band of 826 nt and a weak band of 429 nt were obtained from rec700 (lane a). The 826-nt band is derived from the 768-to-E3B exon in mRNA c, the 2157-to-E3B exon in mRNA f, and the unspliced mRNA i. The 429-nt band is from the 2880-to-E3B exon in mRNA h. dl733 (lane b), dl701 (lane c), and dl704 (lane d) were similar to rec700, again showing that these deletions do not affect pre-mRNA processing.

dl712 and dl742 (Fig. 5, lanes e and g) formed large amounts of both the 826- and 429-nt bands; this is indicative of the greatly increased frequency of the 951→2157 and 951→2880 splices for mRNAs f and h. Use of the 2880 3' splice is increased in dl745 (429-nt band in lane f), although not nearly to the extent of dl712 and dl742. Most of the 826-nt band in dl745 is derived from mRNA f, which is increased about two- to threefold (Fig. 3). The main reason that use of the 2880 and 2157 3' splice sites in dl745 is not as frequent as in dl712 and dl742 is that many of the dl745 pre-mRNAs are processed into the cryptic mRNA.

dl713 and dl718 (Fig. 5, lanes h and i) also formed elevated amounts of the 826- and 429-nt bands as compared with rec700. This reflects the abundant synthesis of E3B-terminated mRNAs which results from loss of the E3A polyadenylation signal. The 826-nt band is derived from mRNAs c and i, as indicated by the data in Fig. 2. mRNA f is not made, because the 2157 3' splice site is deleted. Importantly, although the 2880-to-E3B exon for mRNA h is increased relative to rec700, it is much less abundant than in dl712 and dl742. As discussed previously (2), mRNA h is increased in this class of mutants because of the increased

abundance of E3B-terminated pre-mRNAs rather than because of a specific increase in splicing activity.

Only the 826-nt band was detected in dl719 (Fig. 5, lane j). Again, this is the result of almost exclusive synthesis of mRNA f.

DISCUSSION

The sequences deleted in the dl742 and dl719 groups are important in alternative pre-mRNA processing. We constructed virus mutants to identify signals important in alternative pre-mRNA processing. We operationally defined such signals as sequences whose deletion dramatically affects the differential accumulation of E3 mRNAs in a manner that is not obvious. These signals need not necessarily involve splice or polyadenylation sites, where deletion of the site obviously eliminates the synthesis of the mRNA that uses the site and results in a corresponding nonspecific increase in the other mRNAs.

Our current collection of mutants, altogether, spans about two-thirds of E3. Most of the sequences deleted do not fit the definition of a signal for alternative pre-mRNA processing. Some deletions do not include a splice or polyadenylation site. Mutants in this category are dl733, dl701, dl702, and dl704 (Fig. 1). Other such mutants are dl703 (Δ1211 to 1249), dl716 (Δ2093 to 2139) (2), and dl753 (Δ2229 to 2436) (H. Brady and W. S. M. Wold, unpublished data). With all of these mutants, there is no detectable effect on the accumulation of E3 mRNAs. Other deletions remove a splice or polyadenylation site, but the effect of the deletion is what should be anticipated. For example, dl713 and dl718 delete

the 2157 3' splice site for mRNA f and the E3A polyadenylation site for mRNA a. These mutants do not make mRNA f, and mRNA c is made instead of mRNA a. mRNA h is increased because all pre-mRNAs extend down to the E3B polyadenylation site. A different example is dl714 (Δ2070 to 2141), which deletes the 2157 3' splice signal; mRNA f is not made, but the other mRNAs are similar to the wild type (2). It is clear from these mutants that deletion of a splice or polyadenylation site does not necessarily affect overall pre-mRNA processing.

dl719 (Δ2173 to 2237) is a type of mutant in which deletion of a classical pre-mRNA processing signal, the E3A polyadenylation signal, dramatically affects pre-mRNA processing. With dl719, essentially only mRNA f is synthesized (2, 3). Thus, the region deleted in dl719 apparently is involved in alternative pre-mRNA processing. This region, which appears to be important for both cleavage-polyadenylation at the E3A site and splicing at the 2157 3' splice site, is depicted by the right hatched bar at the top of Fig. 1.

The major focus of this paper is dl712, dl742, and dl745, members of another group of mutants whose deletions have a profound effect on the accumulation of E3 mRNAs. dl712 and dl742 are very similar; essentially only mRNAs f and h are made, and mRNAs a and c are very much reduced. dl745 is also reduced in mRNAs a and c and elevated about two- to threefold in mRNAs f and h. dl745 makes a major mRNA which apparently uses a cryptic 3' splice at nt 1647 and which terminates at the E3A site. The common phenotype of these mutants is that the spliced mRNAs (f, h, and the cryptic mRNA) are made at the expense of mRNAs a and c.

It is important to note that dl742 does not delete a splice or polyadenylation site. dl712 and dl745 delete the 1740 3' splice site, but this is a minor splice site early after infection. Clearly, the sequences deleted in these mutants fit the definition of a signal for alternative pre-mRNA processing. Most of the crucial sequences for this putative signal appear to be within the 64-bp deletion in dl742 (Δ1809 to 1872), because the dl742 phenotype is nearly as strong as that of dl712. The dl745 (Δ1679 to 1825) phenotype is weaker than dl742, which suggests that the dl745 deletion is near the 5' boundary of the signal. The extreme 5' and 3' boundaries are nt 1441 and 2044, i.e., deletions in dl704 and dl713, respectively, which lack this phenotype. The location of this signal is depicted by the left hatched bar at the top of Fig. 1.

How do the deletions in the dl742 group exert their effect? One possibility is that the deletions remove an essential component of the E3A polyadenylation signal. This would eliminate mRNA a and should increase mRNAs f and h. However, mRNA c should also increase, as in dl713 and dl718, and this was not observed. Also, there is no precedent to suggest that a component of the E3A polyadenylation signal would be located 299 bp upstream of the ATTAAA element, as would have to be the case with dl742. But the most convincing argument against a defect in E3A 3'-end formation is that dl745 forms E3A 3' ends normally (Fig. 3). dl745 is in the same group as dl742 and dl712, i.e., its deletion is in the same region and it has a splice-enhancing phenotype. By analogy, therefore, the E3A polyadenylation signal is also probably functional in dl742 and dl712, even though it is not used. If so, then the most obvious explanation for the phenotype is that the deletions enhance splicing activity. With dl742 and dl712, the 951→2157 and 951→2880 splices are enhanced such that use of the E3A site is prevented (5). For the 951→2880 splice, the E3A site would be spliced out of the precursor. For the 951→2157 splice, the splice would somehow prevent E3A 3'-end formation (no

mRNA with the 951→2157 splice and E3A 3' ends has been detected in the wild type). This splice-enhancing scenario would require that splicing precede polyadenylation, which is contrary to the usual situation. However, polyadenylation is not obligatory for splicing (27). With dl745, three splices are enhanced: 951→1647 (a major cryptic splice), 951→2157, and 951→2880.

How does the putative signal, defined by the dl742 deletion, function in alternative pre-mRNA processing? Alternative pre-mRNA processing can be viewed as a stochastic process in which the information which dictates the frequency of each mRNA is embodied in the overall sequence of the pre-mRNA. This sequence could allow pre-mRNA to exist in alternative secondary and tertiary structures, and the frequency with which each structure is formed could determine which splice or polyadenylation sites are used. The binding of trans-acting factors could stabilize pre-mRNA in the correct configuration for a particular splice or cleavage-polyadenylation event. These factors could have different affinities for the splice and polyadenylation sites; this, together with the propensity to form different higher-order structures, could determine which mRNAs are made. There is another, more speculative view of alternative pre-mRNA processing. That is, there may be specific elements which function to maintain a particular distribution of mRNAs. Such an element would be analogous to, e.g., a cleavage-polyadenylation signal. The advantage of such an element is that it would allow mutations which affect pre-mRNA higher-order structure to accumulate anywhere in pre-mRNA (except in the element), but these mutations would not affect alternative pre-mRNA processing. This element could function by controlling pre-mRNA higher-order structure or perhaps the assembly of pre-mRNA into processing complexes. The element could also act in concert with trans-acting factors.

Our results seem more consistent with the element view than with the stochastic view. According to the element view, most deletions would have little effect on the distribution of mRNAs. Deletions of major splice or polyadenylation sites would have predictable effects. Only deletions in the control elements, e.g., the 64 bp deleted in dl742 (and the 65 bp deleted in dl719), would have dramatic and a priori unpredictable effects. This is what we observed. To accommodate our results to the stochastic view, we must assume that deletions in much of E3 do not affect pre-mRNA higher-order structure, factor binding, or complex formation (14) in a manner which influences the balance between the different processing pathways. At this stage, we cannot distinguish between these two views, but whichever view is correct, we presume that the selective pressure on the region deleted in dl742 (i.e., the biological function of this region) is to suppress splicing so that mRNAs a and c would predominate. Both of these mRNAs encode gp19K, the major E3 protein. It should also be emphasized that, whatever the explanation for the dl742 phenotype, the dl742 deletion is novel in the sense that it identifies a small region, distant from a splice or polyadenylation site, which clearly is important in alternative pre-mRNA processing. Since the region is small, we should be able to design experiments to decipher how the sequences function.

ACKNOWLEDGMENTS

LITERATURE CITED

1. **Berk, A. J., and P. A. Sharp.** 1978. Structure of the adenovirus 2 early mRNAs. Cell **14:**695–711.
2. **Bhat, B. M., H. A. Brady, M. H. Pursley, and W. S. M. Wold.** 1986. Deletion mutants that alter differential RNA processing in the E3 complex transcription unit of adenovirus. J. Mol. Biol. **190:**543–557.
3. **Bhat, B. M., H. A. Brady, and W. S. M. Wold.** 1985. Virus deletion mutants that affect a 3' splice site in the E3 transcription unit of adenovirus 2. Mol. Cell. Biol. **5:**2405–2413.
4. **Bhat, B. M., and W. S. M. Wold.** 1985. ATTAAA as well as downstream sequences are required for RNA 3'-end formation in the E3 complex transcription unit of adenovirus. Mol. Cell. Biol. **5:**3183–3193.
5. **Bhat, B. M., and W. S. M. Wold.** 1986. Adenovirus mutants with splice-enhancing mutations in the E3 complex transcription unit are also defective in E3A RNA 3'-end formation. J. Virol. **57:**1155–1158.
6. **Bhat, B. M., and W. S. M. Wold.** 1986. Genetic analysis of mRNA synthesis in adenovirus region E3 at different stages of productive infection by RNA-processing mutants. J. Virol. **60:**54–63.
7. **Birnstiel, M. L., M. Busslinger, and K. Strub.** 1985. Transcription and 3' processing: the end is in site! Cell **41:**349–359.
8. **Chow, L. T., T. R., Broker, and J. B. Lewis.** 1979. Complex splicing patterns of RNAs from the early regions of adenovirus 2. J. Mol. Biol. **134:**265–303.
9. **Cladaras, C., B. Bhat, and W. S. M. Wold.** 1985. Mapping the 5' ends, 3' ends, and splice sites of mRNAs from the early E3 transcription unit of adenovirus 5. Virology **140:**44–54.
10. **Cladaras, C., and W. S. M. Wold.** 1985. DNA sequence of the E3 transcription unit of adenovirus 5. Virology **140:**28–43.
11. **Danner, D., and P. Leder.** 1985. Role of an RNA cleavage/poly(A) addition site in the production of membrane-bound and secreted IgM mRNA. Proc. Natl. Acad. Sci. USA **82:**8658–8662.
12. **Darnell, J. D., Jr.** 1982. Variety in the level of gene control in eukaryotic cells. Nature (London) **297:**365–371.
13. **Deutscher, S. L., B. M. Bhat, M. H. Pursley, C. Cladaras, and W. S. M. Wold.** 1985. Novel deletion mutants that enhance a distant upstream 5' splice in the E3 transcription unit of adenovirus 2. Nucleic Acids Res. **13:**5771–5788.
14. **Gallinaro, H., A. Sittler, and M. Jacob.** 1986. *In vivo* splicing of the premRNAs from early region 3 of adenovirus-2: association of precursors, intermediates and products with hnRNP. Nucleic Acids Res. **14:**4171–4185.
15. **Green, M. R.** 1986. Pre-mRNA splicing. Annu. Rev. Genet. **20:**671–708.
16. **Hérissé, J., G. Courtois, and F. Galibert.** 1980. Nucleotide sequence of the *Eco*RI D fragment of adenovirus 2 genome. Nucleic Acids Res. **8:**2173–2192.
17. **Hwang, L.-H. S., J. Park, and E. Gilboa.** 1984. Role of intron-contained sequences in formation of Moloney murine leukemia virus *env* mRNA. Mol. Cell. Biol. **4:**2289–2297.
18. **Khoury, G., P. Gruss, R. Dhar, and C.-J. Lai.** 1979. Processing and expression of early SV40 mRNA: a role for RNA conformation in splicing. Cell **18:**85–92.
19. **Kobrin, B. J., C. Milcarek, and S. L. Morrison.** 1986. Sequences near the 3' secretion-specific polyadenylation site influence levels of secretion-specific and membrane-specific IgG2b mRNA in myeloma cells. Mol. Cell. Biol. **6:**1687–1697.
20. **Leff, S. E., M. G. Rosenfeld, and R. M. Evans.** 1986. Complex transcriptional units: diversity in gene expression by alternative RNA processing. Annu. Rev. Biochem. **55:**1091–1117.
21. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12:**7035–7056.
22. **Nishikura, K., and G. A. Vuocolo.** 1984. Synthesis of two mRNAs by utilization of alternative polyadenylation sites: expression of SV40-mouse immunoglobulin μ chain gene recombinants in Cos monkey cells. EMBO J. **3:**689–699.
23. **Peterson, M. L., and R. P. Perry.** 1986. Regulated production of μm and μs mRNA requires linkage of the poly(A) addition sites and is dependent on the length of the μ5 μm intron. Proc. Natl. Acad. Sci. USA **83:**8883–8887.
24. **Somasekhar, M. B., and J. E. Mertz.** 1985. Exon mutations that affect the choice of splice sites used in processing the SV40 late transcripts. Nucleic Acids Res. **13:**5591–5609.
25. **Ulfendahl, P. J., U. Pettersson, and G. Akusjärvi.** 1985. Splicing of the adenovirus-2 E1A 13S mRNA requires a minimal intron length and specific intron signals. Nucleic Acids Res. **13:**6299–6315.
26. **Wold, W. S. M., S. L. Deutscher, N. Takemori, B. M. Bhat, and S. C. Magie.** 1986. Evidence that AGUAUAUGA and CCAAGAUGA initiate translation in the same mRNA in region E3 of adenovirus. Virology **148:**168–180.
27. **Zeevi, M., J. R. Nevins, and J. E. Darnell, Jr.** 1981. Nuclear RNA is spliced in the absence of poly(A) addition. Cell **26:**39–46.