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ATTAAA As Well As Downstream Sequences Are Required for RNA 3′-End Formation in the E3 Complex Transcription Unit of Adenovirus

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We mapped the location of the E3A RNA 3′ end site in the E3 transcription unit of adenovirus 2. The procedure used was nuclease-gel analysis with ^{32}P-labeled RNA probes. The poly(A) addition sites were microheterogeneous and were located ~17 to 29 nucleotides downstream from an ATTAAA sequence. To identify the sequences that make up the E3A RNA 3′ end signal, we constructed five viable virus mutants with deletions in or near the E3A RNA 3′ end site. The mutants were analyzed for E3A RNA 3′ end formation in vivo. No effect was observed from a 47-base-pair (bp) deletion (*dl*716) or a 72-bp deletion (*dl*714) located 22 and 19 nucleotides, respectively, upstream of the ATTAAA. In contrast, E3A RNA 3′ end formation was abolished by (i) a 554-bp deletion (*dl*708) that removes both the ATTAAA and the poly(A) addition sites, (ii) a 124-bp deletion (*dl*713) that removes the ATTAAA but leaves the poly(A) addition sites, and (iii) a 65-bp deletion (*dl*719) that leaves the ATTAAA but removes the poly(A) addition sites. These results indicate that the ATTAAA, as well as downstream sequences, including the poly(A) addition sites, are required for E3A RNA 3′ end formation.

The mechanism by which polyadenylated RNA 3′ ends are formed is not known. Transcription continues past the poly(A) addition site in most transcription units that have been analyzed, which suggests that the RNA 3′ end is formed by endonucleolytic cleavage and polyadenylation of the RNA precursor rather than by transcription termination (reviewed in reference 48). Transcription may terminate in a general area ~700 to 4,000 nucleotides (nt) past the poly(A) addition site, depending on the transcription unit (12, 19–21, 28, 31, 35, 40, 54). Cleavage and polyadenylation could be a concerted reaction, or polyadenylation could rapidly follow cleavage (37, 46). Nothing is known about the factors that carry out the cleavage reaction except that small nuclear RNAs may be involved (6, 46).

Some of the *cis*-acting signals for RNA 3′ end formation are beginning to be defined. Most polyadenylated RNAs have AATAAA or a similar sequence (see reference 62) located 11 to 30 nt upstream from the 3′ end (52) (for simplicity, we will use T in place of U when discussing sequence). Deletion of the AATAAA prevents 3′ end formation (15, 17). A variety of base substitutions in the AATAAA greatly reduces the efficiency of 3′ end formation (30, 39, 45, 49, 61, 62). These studies suggested that AATAAA is required for RNA cleavage rather than polyadenylation, because the small fraction of molecules that were cleaved were also polyadenylated (see reference 45). However, in an in vitro study in which cleavage did not occur and the free 3′ ends of runoff transcripts were polyadenylated, AATAAA was required for polyadenylation (38).

AATAAA alone cannot be the only 3′ end signal because this sequence is found in regions where RNA 3′ ends are not normally formed (3, 13, 35, 50, 53, 64). Recent genetic studies indicate that regions downstream from AATAAA (or its functional analog) and the poly(A) addition site are required for efficient 3′ end formation (25, 42, 43, 55, 56, 65), perhaps through the formation of a specific RNA secondary structure (43, 55, 65).

Histone mRNA 3′ end formation shares many features with that of poly(A)$^+$ mRNAs, even though most histone mRNAs are not polyadenylated. The 3′ ends of histone mRNAs are generated by processing rather than by transcription termination (9, 33, 51). Histone mRNAs terminate at a conserved stem and loop structure, which seems to be required for 3′ end formation (8). A conserved CAAGAAAGA spacer sequence is also essential (24), and ~50 to 80 nt of spacer sequences (i.e., sequences downstream from the 3′ end site) are required for maximum processing efficiency (8). For 3′ end processing, a small nuclear RNA, termed U7 RNA, is required, which has complementarity to the histone terminal stem and loop structure (23, 58).

We are studying the E3 complex transcription unit of adenovirus (Ad) (see references 13 and 14) by using a genetic approach with viable virus mutants to define *cis*-acting signals that control splicing and 3′ end selection of the E3 mRNAs. E3 encodes about 10 5′ coterminal mRNAs with different spliced structures (see Fig. 1). These mRNAs form two major families with coterminal 3′ ends termed E3A and E3B. In this paper, we describe the deletion mapping of sequences that are required to form RNA 3′ ends at the E3A site. We report that E3A RNA 3′ ends were not formed by (i) mutants with deletion of an ATTAAA sequence located 23 to 18 nt upstream of the major E3A 3′ end and (ii) a mutant with a deletion of sequences 7 to 71 nt downstream of the ATTAAA and including the poly(A) addition sites.

MATERIALS AND METHODS

Cells, viruses, and RNA extraction. Procedures for the maintenance of KB cells, Ad preparation, and plaque assays have been previously described (27). For RNA preparation, suspension cultures of KB cells were infected with mutants and maintained in cycloheximide (25 μg/ml) from 3 to 9 h

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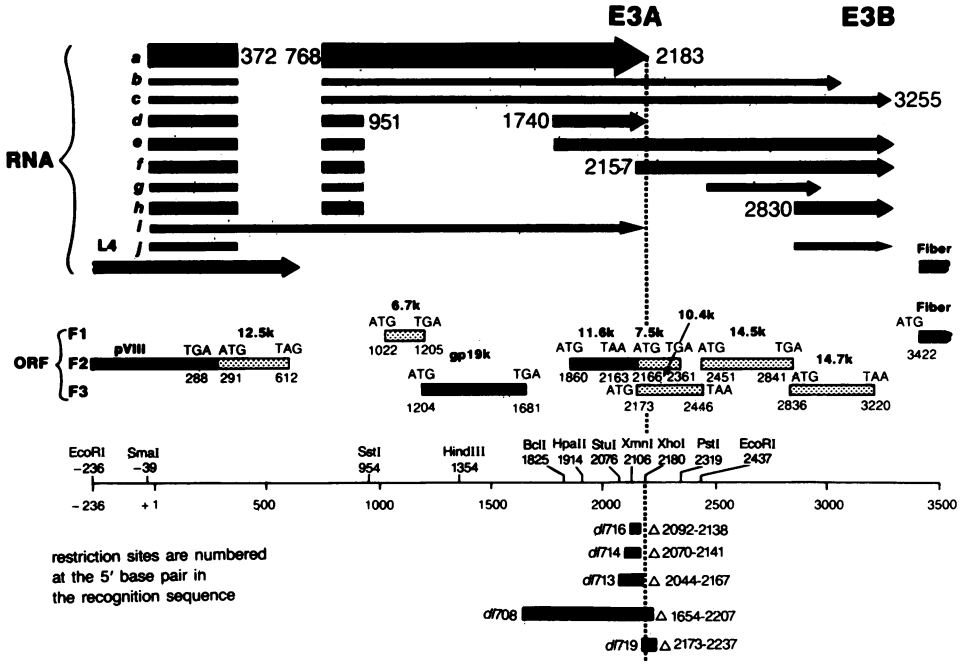

FIG. 1. Schematic representation of the E3 transcription unit of Ad2 and of deletion mutants *dl*716, *dl*714, *dl*713, *dl*708, and *dl*719. The numbers represent nucleotide numbers in the transcription unit (14, 29). Nucleotide +1 is the first major transcription initiation site (4, 13). Open reading frames (ORFs) 1 (F1), 2 (F2), and 3 (F3) are shown. Black bars indicate proven proteins, and stippled bars indicate proteins that we propose to exist (14). Proteins pVIII and fiber are late structural proteins coded by the L4 and fiber mRNAs, respectively. Arrows a through j represent the spliced structures of the E3A and E3B 3′ coterminal families of mRNAs as determined by electron microscopy (11, 32). The RNA 3′ end and splice sites have been determined by sequence analysis of cDNA clones of E3 mRNAs (1, 2, 36, 57) and by fingerprinting (18). Evidence in support of this schematic is discussed in references 13 and 14.

postinfection (63). Cells were lysed (63), and cytoplasmic RNA was extracted (16).

Isolation of plasmid and virus mutants. Deletions were made in the Ad2 *Eco*RI D fragment cloned in pBR322. For *dl*708, *dl*714, and *dl*716, we used a mutant of pBR322 which has the sequences between the *Cla*I and *Pvu*II sites (59) deleted. For *dl*713 and *dl*719, we used wild-type pBR322. Deletions were made by cleaving at the *Bcl*I (nt 1825), *Stu*I (nt 2076), *Xmn*I (nt 2106), or *Xho*I (nt 2180) site, deleting with BAL 31 exonuclease, and recircularizing (34). With *dl*708, *Bam*HI linkers (CGGGATCCCG, New England BioLabs, Inc.) were ligated to the plasmid before recircularizing. The deletions were sequenced by the procedure of Maxam and Gilbert (41).

To transfer plasmid mutations to the viral genome, plasmids were cleaved with *Eco*RI, digested with bacterial alkaline phosphatase, and ligated to *Eco*RI-cleaved Ad5 terminal protein-DNA complex. The terminal protein-DNA complex was prepared as previously described (10). The ligation mixture was transfected into KB cells (26) with a glycerol shock at 5 h posttransfection (22). Well-separated plaques were expanded into virus stocks. Virus stocks were screened for the expected genomic composition and for the expected deletion by isolating virus ^{32}P-labeled DNA from the Hirt supernatant (10), cleaving with *Hin*dIII, electrophoresing on 0.8% agarose gels, and autoradiographing the dried gel. The mutants are Ad5-Ad2-Ad5 recombinants that contain a deletion. Accordingly, they are named with a *rec dl* prefix, e.g., *rec dl*716. For brevity, we use only the *dl* notation.

Nuclease-gel analysis of cytoplasmic RNA. Cytoplasmic RNA samples were analyzed by the nuclease-gel procedure (7), with ^{32}P-labeled RNA probes prepared by in vitro transcription with SP6 RNA polymerase (44). The plasmid substrates for *rec*700, *dl*708, *dl*713, *dl*714, and *dl*719 were prepared by excising the *Sma*I-*Eco*RI fragment (nt −39 to 2437; see Fig. 1) from the *Eco*RI-D–pBR322 clone that was originally used to construct these virus mutants and cloning between the *Sma*I and *Eco*RI sites in the polylinker of pSP65 (44). The plasmid substrate for *dl*716 was prepared by cloning the *Bcl*I-*Pst*I fragment (nt 1825 to 2319) between the *Bam*HI and *Pst*I sites of pSP64 (44). Each plasmid clone was used to prepare probe for the analysis of RNA from cells infected by the corresponding virus mutant.

Synthesis of ^{32}P-labeled RNA probes was done as described before (44) with 80 μM [α-^{32}P]UTP (New England Nuclear Corp.). RNasin, SP6 polymerase, pSP64, and pSP65 were obtained from Promega Biotech, and RNase-free DNase was obtained from Worthington Diagnostics.

Hybridizations and RNase digestions were essentially as described before (44). Cytoplasmic RNA (30 μg) was mixed with 10^6 Cerenkov cpm of probe and hybridized at 40°C for 12 to 16 h. Samples were digested with 40 μg of RNase A plus 10 U of RNase T1 (both from Sigma) per ml at 30°C for 1 h in a buffer containing 0.3 M NaCl (44). Samples were electrophoresed on 6% sequencing gels (14).

RESULTS

Construction of Ad deletion mutants. Deletions were made in the cloned Ad2 *Eco*RI D fragment. *Eco*RI-D is located at position 76 to 83 in the Ad2 genomic map, and it represents nt −236 to 2437 in the Ad2 E3 transcription unit (14, 29). The sequences deleted in the five mutants analyzed in this paper are indicated in Fig. 1 (see also Fig. 5).

The plasmid deletions were transferred to the Ad genome as outlined in Fig. 2. The Ad2 *Eco*RI D fragment containing the deletion was ligated between the Ad5 *Eco*RI A and B fragments to generate a viable virus genome. The virus designated *rec*700 was also constructed by using unmutated Ad2 *Eco*RI-D to serve as a wild-type control for the mutants. *rec*700 and the mutants were analyzed by *Hin*dIII digestion of viral ^{32}P-labeled DNA. As expected, *rec*700 DNA had all Ad5 *Hin*dIII fragments except *Hin*dIII-B (map position 72.8 to 89.1, Fig. 3). In place of Ad5 *Hin*dIII-B, *rec*700 DNA had two fragments, one that comigrates with Ad2 *Hin*dIII-*H* and another designated *Hin*dIII-*E'* (indicated by the dot in Fig. 3). The mutant DNAs had the same fragments as *rec*700 DNA, except for *Hin*dIII-*E'* (indicated by dots), which was variable in size with each mutant because it contained the deletion. We concluded that DNAs from *rec*700, as well as all deletion mutants, had the expected genomic composition.

The E3A RNA 3' end site is located approximately at nucleotide 2183 in the E3 transcription unit of Ad2. In a previous study, we used the nuclease-gel procedure with a 3' end-labeled probe to map the major E3A RNA 3' end site to nt 2227 in the E3 transcription unit of Ad5 (13). We now report that the majority of E3A 3' ends form at or near the equivalent position in Ad2, i.e., nt 2183. Two ^{32}P-labeled RNA probes were used (see the schematic in Fig. 4). The *Hin*dIII probe contains 1,085 nt of Ad2 sequences, from the *Eco*RI (nt 2437) site to the *Hin*dIII (nt 1354) site (Fig. 1; Fig. 4, lane c). The *Hpa*II probe contains 529 nt of Ad2 sequences, from the *Eco*RI (nt 2437) site to the *Hpa*II (nt 1914) site (Fig. 1; Fig. 4, lane c). Both probes have 9 nt of pSP65 sequences at their 5' termini.

The *Hin*dIII probe was hybridized to *rec*700 (i.e., wild-type) cytoplasmic mRNA and digested with RNase, and the products were resolved on a sequencing gel. A major heterogeneous band of ~825 nt, as well as bands of 1,085 and 287 nt, were observed (Fig. 4, lane a and schematic). The 1,085-nt band corresponds to mRNAs b and c (Fig. 1), which are colinear with the probe. The 287-nt band corresponds to mRNA f, which has a 3' splice site at nt 2157 (B. Bhat, H. Brady, and W. Wold, unpublished data). The ~825-nt band corresponds to mRNA a, with 3' ends at or near the E3A site at nt 2183. With the *Hpa*II probe, a major heterogeneous band of ~269 nt, as well as bands of 529 and 287 nt, were seen (Fig. 4, lane d and schematic). The 529-nt band corresponds to mRNAs b and c, which are colinear with the probe, the 287-nt band corresponds to mRNA f with the 2157-nt 3' splice, and the ~269-nt band corresponds to mRNA a with 3' ends at the E3A site. The heterogeneity in the 3' end band probably reflects both RNase nibbling into the probe-mRNA hybrids as well as genuine heterogenity in the mRNA 3' ends. The DNA sequence of the E3A 3' end site is given at the top of Fig. 5. As indicated by the medium-sized arrows, three different 3' ends have been identified in cDNA clones (1, 36), and these all differ from the major 3' end site suggested by our nuclease-gel data for Ad2 (Fig. 4) and Ad5 (13). The important point for this paper is that the majority of E3A 3' ends form in the vicinity of nt 2183. The various 3' end sites are located ~17 to 29 nt downstream from an ATTAAA sequence (Fig. 5).

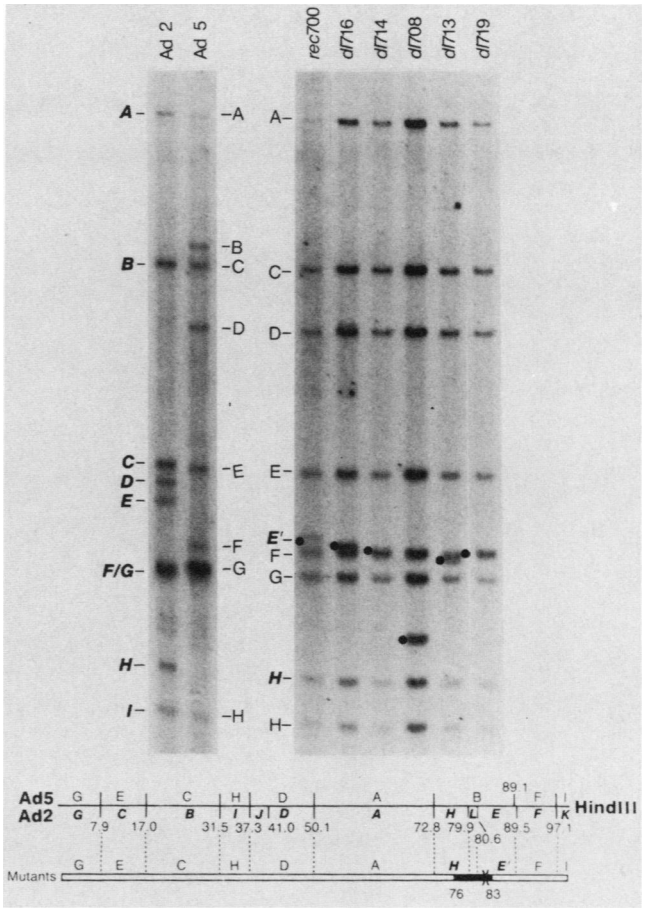

FIG. 3. *Hin*dIII digest of ^{32}P-labeled viral DNA. All mutants have the expected Ad5-Ad2-Ad5 recombinant genomic structure. ^{32}P-labeled viral DNA was digested with *Hin*dIII, electrophoresed on an agarose gel, and autoradiographed. Ad5 fragments are in roman type, and Ad2 fragments are in bold italics. Restriction maps are given at the bottom. The *Hin*dIII *E'* fragment (indicated by dots in the gel) contains the deletion (indicated by X in the schematic) in the mutants.

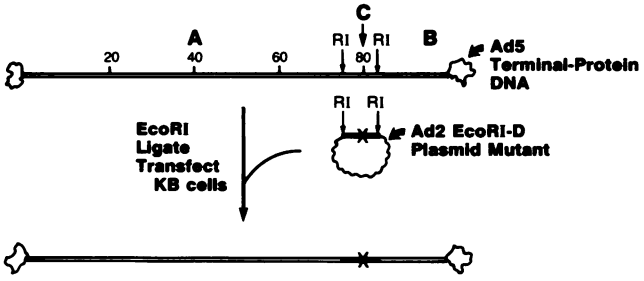

FIG. 2. Method used to construct virus mutants. Mutations were made in a plasmid consisting of the Ad2 *Eco*RI D fragment (map positions 76 to 83) cloned in pBR322. After DNA sequencing of the mutation, the mutated *Eco*RI D fragment was ligated between the Ad5 *Eco*RI A and B fragments and then transfected into KB cells. Plaques were picked, expanded into stocks, and screened for the expected mutation (Fig. 3).

Virus mutants had the expected deletions. Figure 5 presents the deletions in the plasmids used to construct the virus mutants. The nuclease-gel data in Fig. 6 confirm that the virus mutants have these same deletions. In these experiments, the *Hin*dIII and *Hpa*II probes prepared from *rec*700 DNA (Fig. 4, schematic) were hybridized to mRNA from

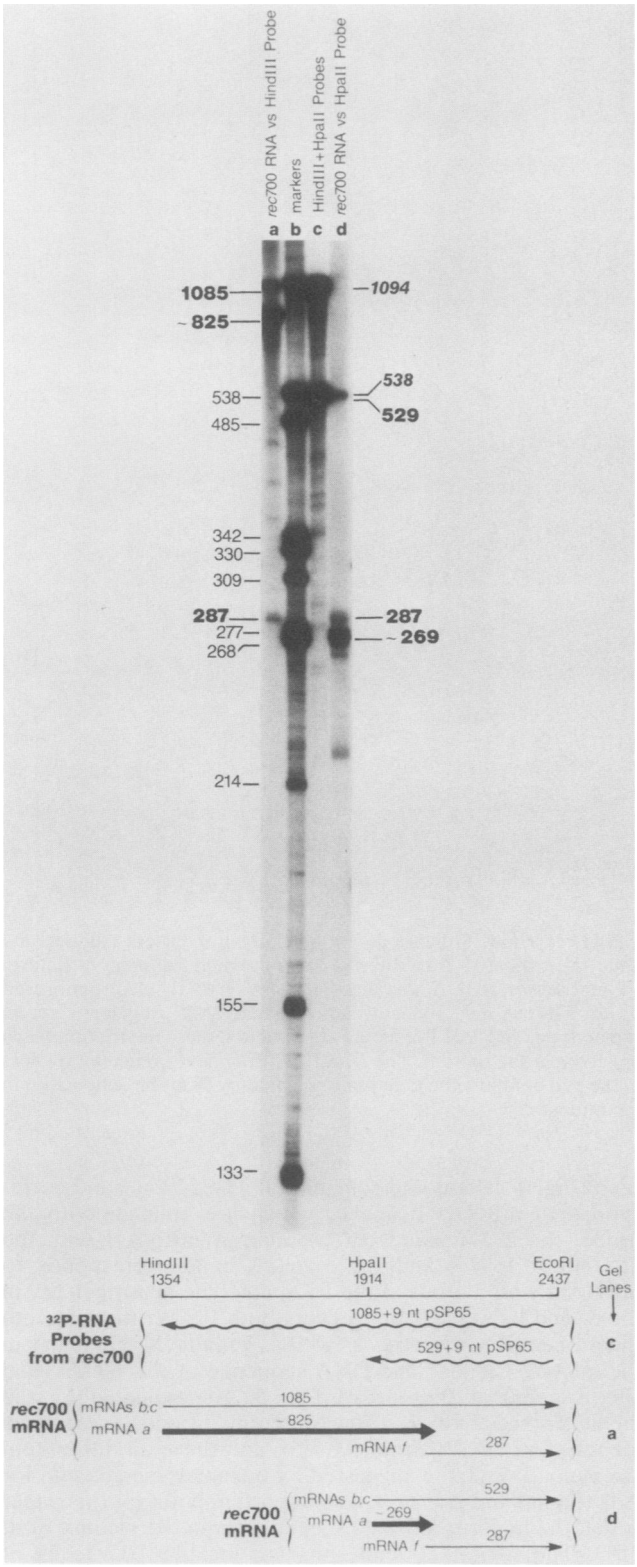

FIG. 4. Nuclease-gel analysis of mRNA from cells infected by *rec*700 wild-type virus. The schematic at the bottom presents the probes used as well as the RNase-resistant fragments observed in the analysis. In the gel, heavy roman type indicates RNase-resistant fragments, italics indicate probes, and light roman type indicates ^{32}P-labeled RNA size markers. Probes were prepared by in vitro transcription with SP6 polymerase. The probe substrate was the *rec*700 *Eco*RI-*Sma*I fragment (nt 2437 to −39) (Fig. 1) cloned in pSP65. The *Hin*dIII and *Hpa*II probes were made by cleaving at those sites before transcription. The probes contain 9 nt of pSP65 sequences at their 5′ ends. Lanes: a, *Hin*dIII probe versus *rec*700 RNA; b, Markers; c, *Hin*dIII and *Hpa*II probes; d, *Hpa*II probe versus *rec*700 RNA.

cells infected by the mutants. RNase should digest the probe in the single-stranded loop in the hybrid between the probe and mRNAs b and c. Two probe fragments should survive RNase digestion: one from the 3′ end of the probe to the 5′ boundary of the deletion, and the other from the 3′ boundary of the deletion to the 5′ end of the probe. This is what was observed. The fragments detected are described in the legend to Fig. 6. We concluded that the virus mutants had the expected deletions.

Nuclease-gel analysis of virus mutants for E3A RNA 3′ end formation. Cytoplasmic mRNA from cells infected by the deletion mutants was analyzed for E3A 3′ ends. For *dl*714, *dl*713, *dl*708, and *dl*719, the *Hin*dIII and *Hpa*II probes were used as in the *rec*700 analysis (Fig. 4), except that they were cognate probes prepared from mutant plasmid DNA. For *dl*716, *Bcl*I and *Hpa*II probes were used. These probes are illustrated at the bottom of Fig. 7 and are explained in the legend.

Figure 7 presents data for *dl*716 and *dl*714, which have deletions on the 5′ side of the E3A 3′ end sites as well as an ATTAAA sequence (Fig. 5). With *dl*716, major heterogeneous bands of ~311 and ~222 nt were observed with *Bcl*I (Fig. 7A, lane c) and *Hpa*II (lane f) probes, respectively. These bands represent the E3A 3′ ends for mRNA a. Bands of 452 (lane c) and 363 (lane f) nt were also observed; these correspond to mRNAs b and c, which are colinear with the probes. The 168-nt band obtained with both probes corresponds to mRNA f, which has the 3′ splice at nt 2157. With *dl*714, prominent heterogeneous bands of ~753 and ~197 nt were produced from *Hin*dIII (Fig. 7B, lane b) and *Hpa*II (lane e) probes, respectively. Again, these bands represent the 3′ ends for mRNA a. The 1,013- (Fig. 7B, lane b) and 457-nt (lane e) bands represent mRNAs b and c. A band of 287 nt for mRNA f was not found in *dl*714 RNA with either probe, because the deletion abolishes splicing at the nt 2157 3′ splice site (B. Bhat, H. Brady, and W. Wold, unpublished data). We concluded that the deletions in *dl*716 and *dl*714 have no discernable effect on the formation of E3A 3′ ends.

Figure 8 shows the results with *dl*713 and *dl*708; *rec*700 was included in this experiment to serve as a positive control. *rec*700 and *dl*713 were analyzed with the same cognate *Hin*dIII and *Hpa*II probes as described previously. *dl*708 was analyzed with the cognate *Hin*dIII probe, as well as a cognate *Sst*I probe, which extends from the *Eco*RI (nt 2437) site to the *Sst*I (nt 954) site (Fig. 1). *rec*700 RNA gave the same prominent heterogeneous bands of ~825 (Fig. 8, arrow in lane d) and ~269 (arrow in lane f) nt that were identified in Fig. 4 as corresponding to the E3A 3′ ends for the *Hin*dIII and *Hpa*II probes. The 1,085-, 529-, and 287-nt *rec*700 bands (indicated by arrows in Fig. 8) were described previously for Fig. 4. *dl*708 has both the E3A poly(A) addition sites as well as an ATTAAA sequence deleted (Fig. 5). No bands corresponding to E3A 3′ ends were detected in *dl*708 RNA with either the *Sst*I (Fig. 8, lane n) or the *Hin*dIII (lane p) probe. The bands of 936 (arrow in lane n) and 541 (arrow in lane p) nt represent mRNAs that are colinear with the probes. We concluded that the deletion in *dl*708 abolished E3A RNA 3′ end formation. *dl*713 has the ATTAAA sequence deleted but retains almost all of the downstream

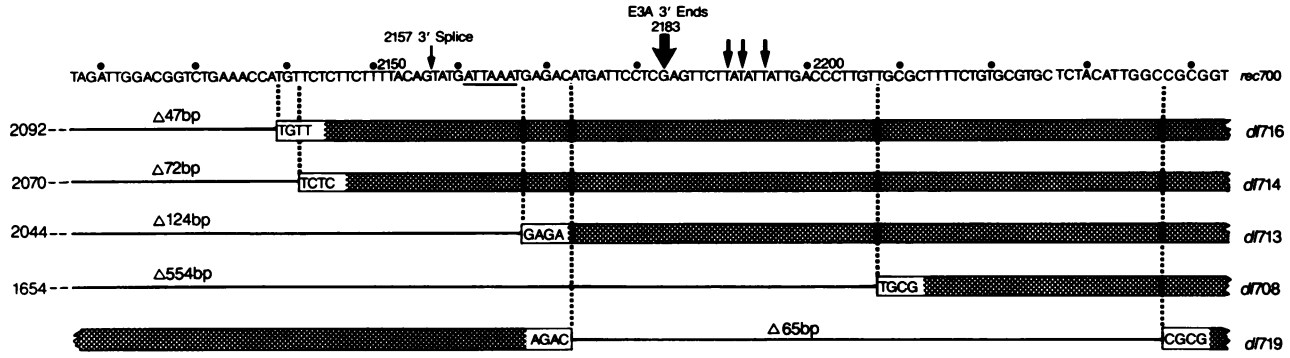

FIG. 5. DNA sequence of the region near the E3A RNA 3' end site and of the sequences deleted in the virus mutants.

sequences, including the poly(A) addition sites (Fig. 5). As with *dl*708, no bands corresponding to E3A 3' ends were obtained with either the *Hin*dIII (Fig. 8, lane i) or *Hpa*II (lane h) probes. The positions where the E3A 3' ends should have appeared in the gel are indicated by the thick arrows near the top of lane i and the bottom of lane k. The 961- (Fig. 8, arrow in lane i) and 405-nt (arrow in lane k) bands are RNAs that are colinear with the probes. We concluded that the deletion in *dl*713 abolished E3A RNA 3' end formation, probably by destroying an E3A 3' end signal. Most likely ATTAAA (Fig. 5) is part of the signal.

*dl*719 has a 65-base-pair deletion that removes sequences from 7 to 71 nt downstream of the ATTAAA and including the poly(A) addition sites (Fig. 5). No bands corresponding to E3A 3' ends were obtained from *dl*719 mRNA with either the *Hin*dIII (Fig. 9, lane b) or *Hpa*II (lane c) probes. The strong 222-nt band represents mRNA f with the 3' splice at nt 2157; this splice is enhanced in *dl*719 mRNA, apparently because the nt 951 5' splice site is enhanced (B. Bhat, H. Brady, and W. Wold, unpublished data). The 222-nt band represents the same 3' splice as the 287-nt band observed with *rec*700 (Fig. 4, lanes a and d; Fig. 8, lanes d and f), but it is smaller because of the deletion in *dl*719. The strong bands of ~130 nt (Fig. 9, lanes b and c) were reproducibly obtained from *dl*719 mRNA (Fig. 6, lane f). These bands cannot represent mRNA 3' ends, because the bands are the same size with both the *Hin*dIII and *Hpa*II probes. The bands could represent a cyptic 3' splice or perhaps a cryptic miniexon. However, there are no AGs or GT-AG combinations (47) in the DNA sequence between the deletion and the *Eco*RI (nt 2437) site that would yield a ~133-nt fragment if they were used for cryptic splicing. Whatever the explanation for the ~130-nt bands, it is clear from Fig. 9 that *dl*719 is defective in 3' end formation at the E3A site.

DISCUSSION

We mapped the E3A RNA 3' end site to nt ~2183 in the E3 transcription unit of Ad2. To identify the sequences that make up the E3A RNA 3' end signal, we constructed viable virus mutants with deletions in the proximity of the E3A 3' end site, and we analyzed the effects of these deletions on E3A 3' end formation in vivo. E3A 3' ends were not formed by *dl*708, which has the poly(A) addition sites as well as an upstream ATTAAA sequence deleted. We conclude that information for E3A 3' end formation resides within the deletion (Δ1654 to 2207) in *dl*708. Results obtained with the other mutants allow for finer mapping of this information. It is almost certain that the deletions in *dl*708, *dl*713, and *dl*719 that prevent E3A RNA 3' end formation exert their effects in *cis*, not in *trans*, e.g., by affecting the functions of the 11,600-molecular-weight (11.6K) protein or the putative 10.4K or 7.5K protein or both (Fig. 1). This is because E3A 3' ends were formed normally by *dl*714, despite its having a large deletion in the 11.6K gene and despite its inability to synthesize the 10.4K-7.5K proteins because it does not make the nt 2157 3' splice to form mRNA f, which encodes these proteins. The sequences that make up the putative E3A RNA 3' end signal are discussed below.

ATTAAA probably is essential for E3A RNA 3' end formation. The formation of 3' ends at the E3A site was abolished by the deletion in *dl*713 (Δ2044 to 2167) but was unaffected by the deletions in *dl*716 (Δ2092 to 2138) and *dl*714 (Δ2070 to 2141). These results indicate that the sequences within nt 2142 to 2167, which are present in *dl*714 but not in *dl*713, contain a signal for 3' end formation at the E3A site. Most likely, the ATTAAA at nt 2161 to 2166 (Fig. 5) is this signal. The closely related sequence AATAAA is a 3' end signal (see Introduction), which apparently directs endonucleolytic cleavage of the RNA precursor (45). ATTAAA is found in place of AATAAA near the poly(A) addition site of about 12% of mRNAs (62), which suggests that it is a functional analog of AATAAA. Our results provide direct evidence that this is probably the case. ATTAAA is found at the equivalent position in Ad5 (13), which suggests that there is a biological basis for the use of this sequence rather than AATAAA.

Sequences downstream from ATTAAA are essential for E3A RNA 3' end formation. The formation of 3' ends at the E3A site was abolished by the deletion in *dl*719 (Δ2173 to 2237). This 65-base-pair deletion in the region 7 to 71 nt downstream from ATTAAA removes all known E3A poly(A) addition sites. We propose that some of the sequences deleted in this mutant form part of the E3A 3' end signal. These sequences could be part of the same signal as that represented by ATTAAA, or they could represent a distinct signal which functions, e.g., to form an appropriate secondary or tertiary structure for cleavage or to bind *trans*-acting factors that catalyze cleavage.

In addition to the analysis of the E3A site described in this paper, one other 3' end site has been studied by deletion mutagenesis in viable virus, that for the simian virus 40 late transcription unit. Fitzgerald and Shenk (17) originally reported that deletion of the AATAAA sequence prevented 3' end formation at the normal site but that most deletions downstream from AATAAA, including deletion of the natural poly(A) addition site, simply altered the poly(A) addition site to a distance ~11 to 19 nt downstream from AATAAA. They concluded that AATAAA fixes the position of the

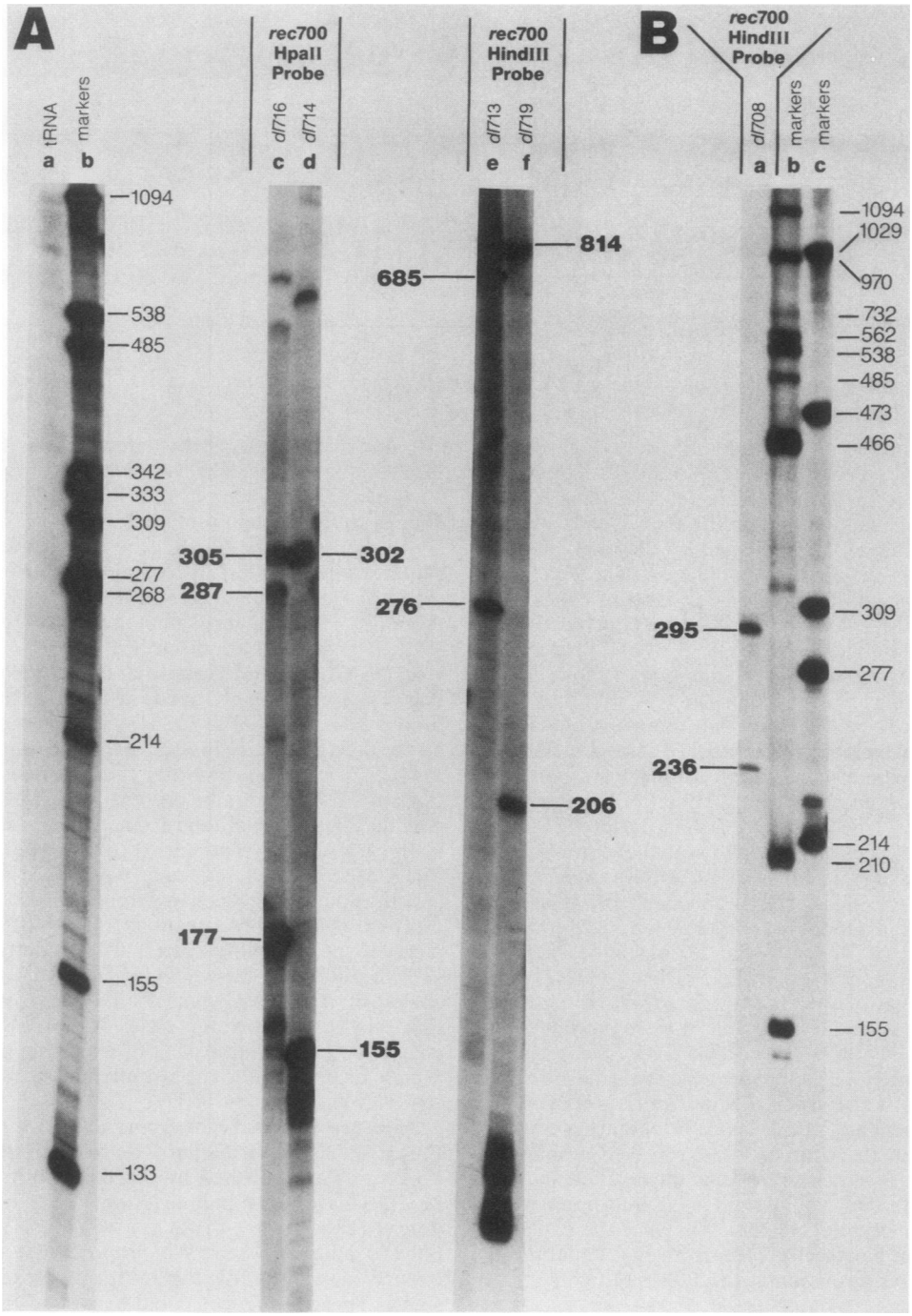

FIG. 6. Evidence that the virus mutants have the expected deletions. RNA from mutant virus-infected cells was analyzed by the nuclease-gel procedure with the *rec*700 *Hin*dIII or *Hpa*II probe. The *rec*700 probes are described in the legend to Fig. 4. (A) All lanes were from the same gel: a, *Hin*dIII probe versus tRNA; b, markers; c, *Hpa*II probe versus *dl*716 RNA (The 177-nt band is a probe fragment from the *Hpa*II site [nt 1914] to the deletion at nt 2092, and the 305-nt band is a probe fragment from the deletion at nt 2138 to the *Eco*RI site [nt 2437]. The 287-nt fragment represents mRNA f with the 3′ splice at nt 2157 [see also Fig. 4].); d, *Hpa*II probe versus *dl*714 RNA (The 155- and 302-nt bands are probe fragments from the *Hpa*II site [nt 1914] to the deletion [nt 2070] and from the deletion [nt 2141] to the *Eco*RI site [nt 2437], respectively); e, *Hin*dIII probe versus *dl*713 RNA (The 685- and 276-nt bands are probe fragments from the *Hin*dIII site [nt 1354] to the deletion [nt 2044] and from the deletion [nt 2167] to the *Eco*RI site [nt 2437], respectively); f, *Hin*dIII probe versus *dl*719 RNA (The 814- and 206-nt bands are probe fragments from the *Hin*dIII site [nt 1354] to the deletion [nt 2173] and from the deletion [nt 2237] to the *Eco*RI site [nt 2437], respectively. The two strong bands of ~130 nt were also observed with the *dl*719 cognate probe and are discussed together with Fig. 9.). (B) Lanes: a, *Hin*dIII probe versus *dl*708 RNA (The 295- and 236-nt bands are probe fragments from the *Hin*dIII site [nt 1354] to the deletion [nt 1654] and from the deletion [nt 2207] to the *Eco*RI site [nt 2437], respectively); b and c, markers.

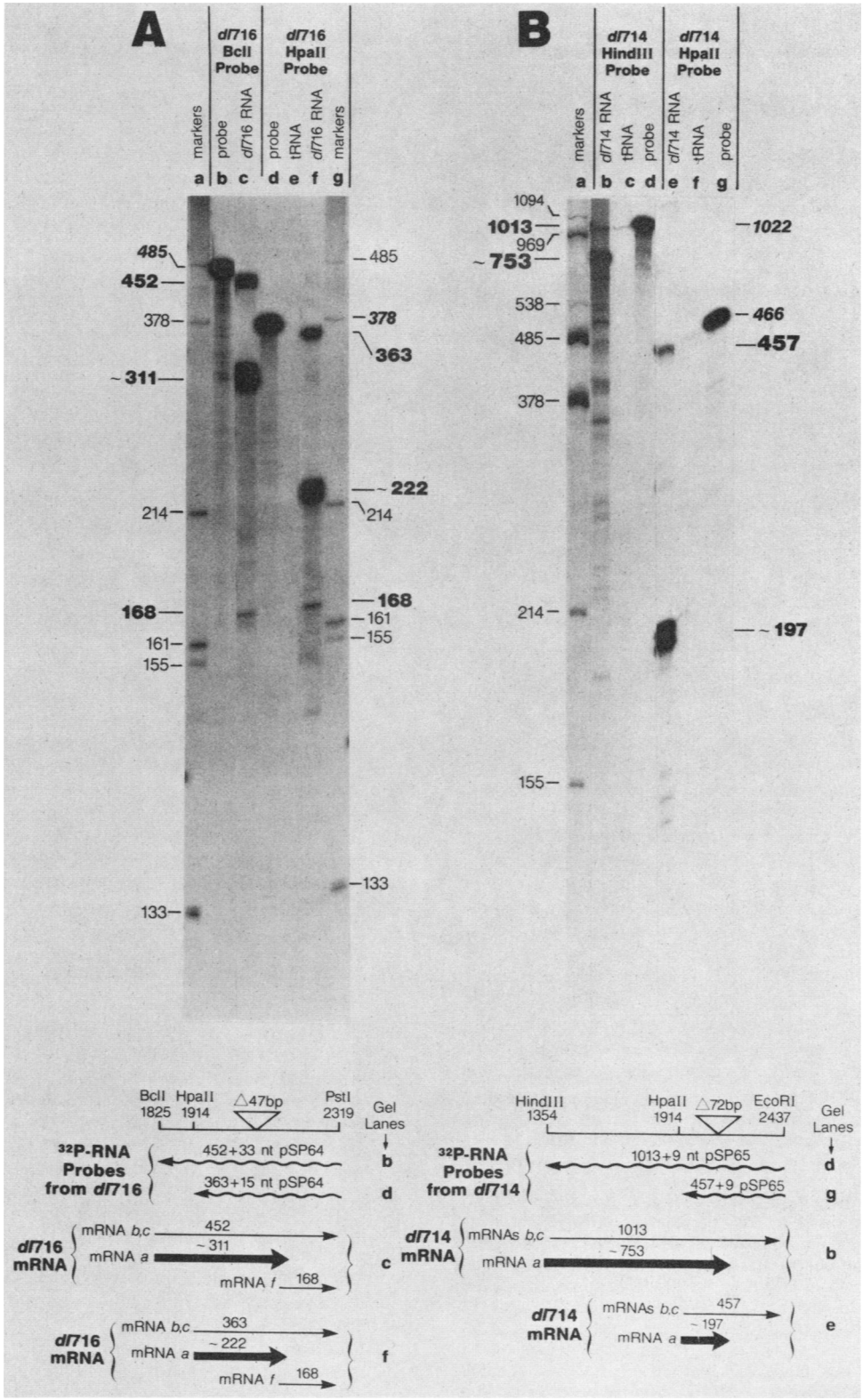

FIG. 7. Nuclease-gel analysis of mRNA from cells infected by *dl*716 (A) and *dl*714 (B). RNase-resistant fragments are indicated by heavy roman type, probes by italics, and markers by light roman type. Each RNA was analyzed with probe prepared from its own DNA. The substrate for the *dl*716 probes was the *dl*716 *Pst*I-*Bcl*I fragment (nt 2319 to 1825) cloned between the *Pst*I and *Bam*HI sites of pSP64. The *Bcl*I probe was prepared by cleaving at the *Eco*RI site in the pSP64 polylinker before transcription. This probe contains 33 nt of pSP64 sequences, 15 nt at its 5′ terminus, and 18 nt at its 3′ terminus. The *Hpa*II probe was prepared by cleaving at the *Hpa*II site before transcription; it contains 15 nt of pSP64 sequences at its 5′ terminus. Lanes: a, markers; b, *Bcl*I probe; c, *Bcl*I probe versus *dl*716 RNA; d, *Hpa*II probe; e, *Hpa*II probe versus tRNA; f, *Hpa*II probe versus *dl*716 RNA; g, markers. For *dl*714, the same probes were used as for *rec*700 (Fig. 4), except that they were prepared from *dl*714 DNA. Lanes: a, markers; b, *Hin*dIII probe versus *dl*714 RNA; c, *Hin*dIII probe versus tRNA; d, *Hin*dIII probe; e, *Hpa*II probe versus *dl*714 RNA; f, *Hpa*II probe versus tRNA; g, *Hpa*II probe.

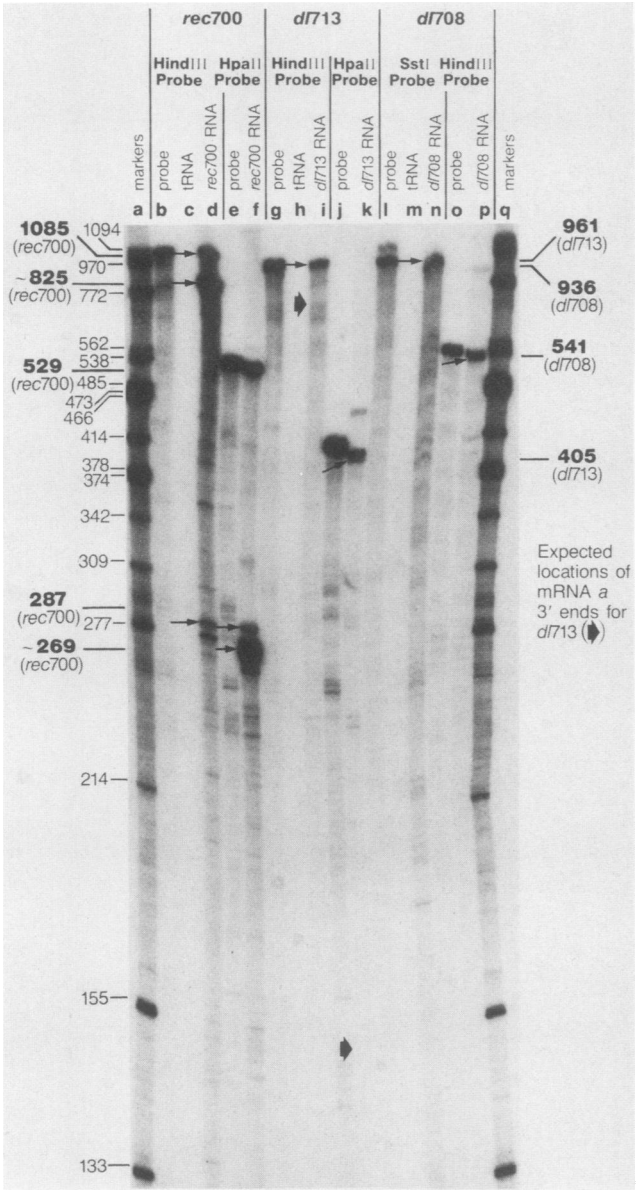

FIG. 8. Nuclease-gel analysis of mRNA from cells infected by *rec*700, *dl*713, or *dl*708. RNase-resistant fragments are indicated by heavy roman type and by medium arrows in the gel. Markers are indicated by light roman type. The same *Hin*dIII and *Hpa*II probes were used as described for *rec*700 in the legend to Fig. 4, except that they were prepared from mutant DNA as indicated. The *Sst*I probe was prepared by cleaving the plasmid substrate at the *Sst*I (nt 954) site before transcription with SP6 polymerase. Each RNA was analyzed with its own cognate probe. Lanes: a, markers; b, *rec*700 *Hin*dIII probe; c, *rec*700 *Hin*dIII probe versus tRNA; d, *rec*700 *Hin*dIII probe versus *rec*700 RNA; e, *rec*700 *Hpa*II probe; f, *rec*700 *Hpa*II probe versus *rec*700 RNA; g, *dl*713 *Hin*dIII probe; h, *dl*713 *Hin*dIII probe versus tRNA; i, *dl*713 *Hin*dIII probe versus *dl*713 RNA; j, *dl*713 *Hpa*II probe; k, *dl*713 *Hpa*II probe versus *dl*713 RNA; l, *dl*708 *Sst*I probe; m, *dl*708 *Sst*I probe versus tRNA; n, *dl*708 *Sst*I probe versus *dl*708 RNA; o, *dl*708 *Hin*dIII probe; p, *dl*708 *Hin*dIII probe versus *dl*708 RNA; g, markers.

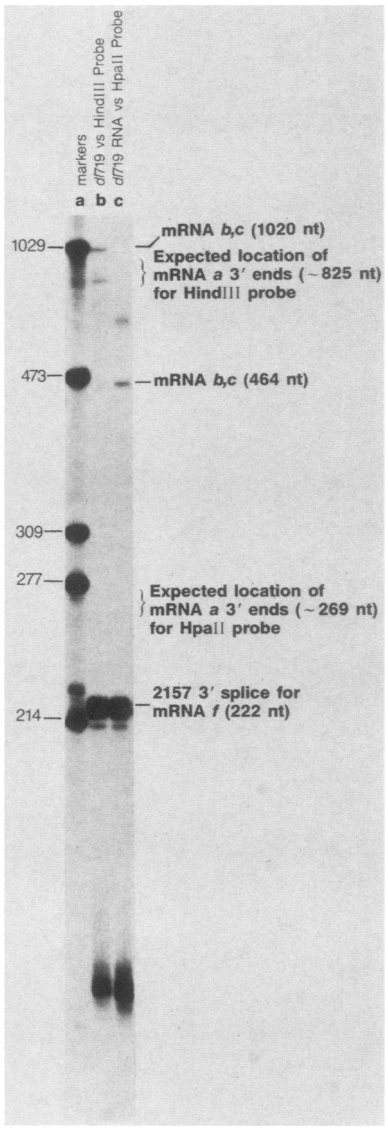

FIG. 9. Nuclease-gel analysis of mRNA from cells infected by *dl*719. The *Hin*dIII and *Hpa*II probes (see the legend to Fig. 4) were prepared from *dl*719 DNA. These probes are the 1,029- and 473-nt fragments, respectively, shown in lane a. Lanes: a, markers; b, *dl*719 *Hin*dIII probe versus *dl*719 RNA; c, *dl*719 *Hpa*II probe versus *dl*719 RNA.

poly(A) addition site and that the actual sequence at the site may not be crucial. However, they also suggested that elements other than AATAAA might be involved in the 3′ end signal, because AATAAA is not unique to 3′ end sites. Sadofsky and Alwine (55) studied these same viable simian virus 40 mutants and concluded that deletion of sequences between 3 and 60 nt downstream from AATAAA decreased the efficiency at which 3′ ends were formed near the normal site. Instead, extended transcripts occurred with 3′ ends at a number of distinct sites.

Several transcription units expressed in vivo in expression vectors have also been shown to require sequences downstream from AATAAA or its analog for efficient or accurate RNA 3′ end formation or both. Hepatitis surface antigen RNA required sequences between 30 and 45 nt downstream from a TATAAA sequence (56). Ad E2A RNA required AATAAA plus between 34 and 49 nt downstream sequences (43). Bovine growth hormone RNA 3′ ends formed normally with AATAAA plus 103 nt of downstream sequences; how-

ever, mutant genes with only 20, 29, or 32 base pairs of sequences downstream from AATAAA, but retaining the natural poly(A) addition site, formed 3' ends efficiently at discrete sites either 5' or 3' to the natural site (65). Thus, deletion of sequences immediately downstream from the natural poly(A) addition site affected the site of 3' end formation but not its efficiency. With rabbit β-globin RNA, AATAAA plus 51 nt downstream were sufficient for 3' end formation, but a deletion that leaves 15 nt downstream from AATAAA and removes the natural poly(A) addition site abolished 3' end formation near this site (25). With a vector containing a herpes simplex virus type 1 immediate early RNA 3' end site, RNA 3' end formation occurred efficiently with AATAAA plus 64 nt downstream; however, RNA 3' end formation was reduced in efficiency by a deletion that leaves 34 nt downstream, and it was abolished by a deletion that removes the natural poly(A) addition site and leaves 20 nt downstream (42).

In our analyses with *dl*708, *dl*713, and *dl*719, we did not observe cryptic poly(A) addition sites, either nearby the AATAAA or at new sites downstream. Indeed, our preliminary experiments indicate that the vast majority of transcripts use the E3B 3' end site.

The studies discussed above, as well as our results reported here, indicate that sequences downstream from AATAAA (or its analog) are required for efficient 3' end formation. However, the effects that have been observed from the deletions varied somewhat. Some deletions reduced 3' end formation at the appropriate distance downstream from AATAAA (or its analog) to undetectable levels (this paper; 25, 42, 43, 56). Other deletions in this region appear to have had a less pronounced effect on the efficiency of 3' end formation, although many of them altered the poly(A) addition site (17, 55, 65). These differences in results suggest that the sequences downstream from AATAAA and the poly(A) addition site may vary in the way that they participate in RNA 3' end formation.

Sequences near poly(A) addition sites do not have profoundly conserved sequence homologies or potentials for RNA secondary structure. Nevertheless, similarities exist. CAYTG is located immediately upstream from the poly(A) addition site in some transcription units or immediately downstream from the site in other transcription units (5, 6). Berget (6) has presented a model in which both CAYTG and AATAAA might form base pairs with U4 small nuclear RNA to form a 3' end processing substrate. McLaughlan et al. (42) reported that YGTGTTYY is located ~24 to 30 nt downstream from AATAAA in 67% of transcription units. This sequence is similar to that noted by Taya et al. (60). Le Moullec et al. (36) pointed out that sequences of the form $(T)_n\ (A)_p\ (T)_q$ (n, p, q >1) are found 4 to 24 nt beyond many poly(A) addition sites. It is interesting that the sequences deleted in *dl*719 (Fig. 5) contain an example of each of these different consensus sequences. Regarding the CAYTG consensus, TTCCTCG is found at nt 2177 to 2183 on the 5' side of the major poly(A) addition site. This would be a class I site as defined by Berget (6). Also, TATTG (nt 2195 to 2199) and CCTTG (nt 2202 to 2206) are found in tandem immediately 3' to the three E3A poly(A) addition sites that have been identified from cDNA clones (1, 36). Regarding the YGTGTTYY consensus, CGAGTTCT is found at nt 2182 to 2189, which includes the major E3A poly(A) addition site. Regarding the $(T)_n\ (A)_p\ (T)_q$ sequence, TTATATTATT is found at nt 2189 to 2198, downstream from the major E3A poly(A) addition site and including the sites found in cDNA clones. Whether any of these consensus-related sequences are indeed RNA 3' end signals remains to be established by small deletion and point mutagenesis.

Several authors have suggested that secondary structure could be important in RNA 3' end formation in eucaryotes. There is strong evidence that this is true for histone 3' ends (8). In those examples in which this question has been examined for poly(A)+ RNAs, deletions that destroy the potential for the postulated RNA secondary structures affected the efficiency or specificity of 3' end formation (43, 55, 65). Although we are able to postulate certain imperfect hairpin loop structures that could form in RNA coded from the E3A site, it seems unlikely that they would be formed in vivo unless stabilized by unknown RNA or protein factors or both.

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