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Duplication of Functional Polyadenylation Signals in Polyomavirus DNA Does Not Alter Efficiency of Polyadenylation or Transcription Termination

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We constructed viable insertion mutants of polyomavirus that contain duplications of the nucleotide sequences surrounding the polyadenylation sites for both E- and L-strand RNAs. Our results showed that formation of poly(A)$^+$ 3′ termini of L-strand mRNAs requires sequence elements located between 12 and 87 nucleotides downstream of AAUAAA. No more than 19 nucleotides upstream and 44 nucleotides downstream of AAUAAA are required for polyadenylation of E-strand mRNAs. Our results and those of others suggest that there are three distinct sequence elements required for mRNA 3′ end formation: AAUAAA and two downstream elements. An insertion mutant containing two adjacent functional polyadenylation signals produced E-strand and L-strand mRNAs with 3′ ends at both sites. However, the overall level of polyadenylation of L-strand RNAs was not increased over the low (10 to 25%) levels seen with wild-type virus. Neither was the efficiency of termination of L-strand transcription increased in mutant virus-infected cells. We conclude that factors required for both polyadenylation and transcription termination are limiting in polyomavirus-infected mouse cells.

The formation of polyadenylated 3′ ends of mRNAs in higher eucaryotes involves cleavage of a precursor RNA shortly downstream of the hexanucleotide AAUAAA or a closely related sequence (28, 34, 38, 44). During transcription of mRNA-coding genes, RNA polymerase II progresses at least 1 kilobase (kb) beyond the polyadenylation site before terminating and releasing the RNA chain (18, 20, 24). Cleavage and polyadenylation of this precursor RNA takes place within 1 to 2 min of its synthesis (3, 35). Cleavage takes place on the growing RNA chain before termination has occurred during transcription of adenovirus late genes (35). In other genes it is not known whether cleavage and polyadenylation precede or follow termination of transcription.

The hexanucleotide AAUAAA is essential for efficient formation of polyadenylated 3′ termini of mRNAs (16, 23, 33, 48). It has recently been shown that additional nucleotide sequences downstream of AAUAAA are required (9, 30, 31, 40, 49); however, the number of these additional elements and their exact composition is not known. The nature of the nucleotide sequences that signal termination of transcription by RNA polymerase II remains obscure.

There is circumstantial evidence suggesting that polyadenylation and termination of transcription are linked. During the late phase of infection of mouse cells by polyomavirus, RNA polymerase II does not efficiently terminate transcription of the L DNA strand (1). We have recently determined that about 50% of RNA polymerases terminate during each traverse of the 5.3-kb circular viral DNA (46). Polyadenylation of viral RNA is also inefficient; only 10 to 25% of L-strand transcripts become polyadenylated during the late phase (3). In contrast, during the early phase of infection, transcription of the E DNA strand is efficiently terminated, and E-strand transcripts are efficiently polyadenylated (2, 4). Transcription of simian virus 40 DNA in productively infected monkey cells is efficiently terminated (18); these transcripts are also efficiently polyadenylated and exported to the cytoplasm as mRNAs (12). However, when simian virus 40 DNA is injected into *Xenopus* oocytes, RNA molecules larger than genome length are produced, and polyadenylation of these transcripts is inefficient (32).

The apparent linkage between efficiency of polyadenylation and transcription termination suggests that a common signal, or a common cellular factor, is involved in both processes. If a common signal is involved, it may be that this signal is weak and therefore poorly recognized by the cellular machinery when both polyadenylation and termination are inefficient. Duplication of such a signal should simultaneously increase the efficiency of both processes. If, however, polyadenylation and termination are inefficient because a cellular factor required for both processes is present in limiting amounts, then duplication of the signal should have little or no effect on either process. We constructed viable insertion mutants of polyomavirus that contain a second functional polyadenylation signal. In the course of these experiments, we obtained information on the nucleotide sequence requirements for polyadenylation of both E- and L-strand RNAs. Although mRNA 3′-end formation is correctly signalled by both polyadenylation sites, neither the overall efficiency of polyadenylation nor that of termination is increased compared to that of wild-type virus. These results suggest that inefficient polyadenylation and termination of late polyomavirus transcripts is a result of limiting amounts of a cellular factor(s) required for these processes.

MATERIALS AND METHODS

Virus, cells, and materials. Polyomavirus strain AT3 (W. C. Skarnes, D. C. Tessier, and N. H. Acheson, manuscript in preparation), closely related to the A3 strain, was used in all experiments. Ten-day-old baby mice were purchased from Canadian Breeding Farms. Primary baby mouse kidney (BMK) cells and Swiss mouse 3T6 fibroblasts were grown in

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Dulbecco modified Eagle medium (Flow Laboratories, Inc., McLean, Va.). S1 nuclease was obtained from Boehringer Mannheim Biochemicals (Dorval, Quebec, Canada); T4 DNA ligase and Klenow fragment of DNA polymerase were from New England Nuclear Corp. (Boston, Mass.) and Pharmacia, Inc. (Piscataway, N.J.), respectively. Oligo(dT)-cellulose was purchased from Collaborative Research, Inc. (Waltham, Mass.). ^{32}P-labeled deoxynucleoside triphosphates and [5-^{3}H]uridine were purchased from ICN Pharmaceuticals Inc. (Irvine, Calif.) and Amersham Corp. (Arlington Heights, Ill.), respectively. Restriction nucleases were obtained from Amersham, Boehringer Mannheim, New England BioLabs, Inc. (Beverly, Mass.), and New England Nuclear Corp.

RNA preparation. Cell fractionation and RNA extraction were as previously described (1). Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography as described by Aviv and Leder (6; see also reference 3).

Transfection and plaque assays. For transfection experiments, cesium chloride-purified plasmid DNA was cleaved with *Eco*RI to release linear polyomavirus DNA. The digested DNA was diluted in a sterile solution of DEAE-dextran (200 μg/ml) in Dulbecco modified Eagle medium containing 50 mM Tris hydrochloride, pH 7.4. Confluent BMK cells in 60-mm petri dishes were covered with 2 ml of this solution containing 400 ng of DNA and incubated at 37°C and 5% CO_2 for 6 h. The DNA solution was removed by suction, and the cells were washed with Dulbecco modified Eagle medium and covered with 5 ml of fresh Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. Plates were incubated at 37°C and 5% CO_2, and the extent of cytopathic effect was monitored over a period of about 9 days.

For plaque assays, serial dilutions of *Eco*RI-digested DNA were made in the same DEAE-containing medium, and 50, 10, or 1 ng of DNA per ml was used to transfect confluent BMK cells (three plates per dilution). The procedure was the same as above, except that cells were overlaid with 8 ml of Eagle minimum essential medium (Flow Laboratories) containing 0.9% Bacto-Agar (Difco Laboratories, Detroit, Mich.) and 10% fetal bovine serum. The plates were supplemented with 3 to 4 ml of the same mixture after 4 days of incubation at 37°C and 5% CO_2. After 8 days of incubation, cells were stained overnight with 4 to 5 ml of a 0.02% solution of neutral red. Individual plaques were isolated for production of virus stocks.

Construction of ins 1 and ins 2. Polyomavirus strain AT3 DNA was digested with *Hpa*II to generate eight fragments, one of which (fragment 6; 376 nucleotides) contains the AATAAA signals on both the E and L strands (Fig. 1). Fragment 6 was blunt ended with the Klenow fragment of DNA polymerase and cleaved with *Alu*I to generate 279- and 97-nucleotide fragments to which *Sal*I linkers (New England BioLabs) were ligated. Small linker oligomers were removed by passage through a NACS ion exchange column (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), and the remaining DNA fragments were treated with *Sal*I to remove multiple linkers and to create *Sal*I sticky ends. The resulting DNA was cloned into the unique *Sal*I site of pBR322. Ampicillin-resistant, tetracycline-sensitive recombinants were screened for the presence of the 97-nucleotide *Alu*I-*Hpa*II fragment which contains the AATAAA sequences. A somewhat shorter fragment was recovered from a recombinant plasmid (clone 24) by cleavage with *Sal*I. DNA sequencing (29) confirmed that the inserted fragment had lost 16 nucleotides at one end and extended from nucleotide 2896 to 2976.

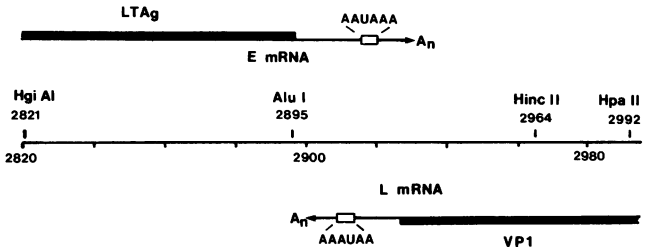

FIG. 1. Representation of region of polyomavirus DNA in which the 3′ ends of E- and L-strand mRNAs map. Nucleotide numbers are from Soeda et al. (41). The sites of cleavage of selected restriction endonucleases are shown. Lines above and below the DNA segment represent E and L mRNAs, whose polyadenylated 3′ ends are denoted by arrowheads (E mRNA, nucleotide 2930; L mRNA, nucleotide 2900 [21, 26]). The thickened portions represent open reading frames: LTAg, large T antigen; VP1, major viral capsid protein. AAUAAA sequences at nucleotides 2915 to 2920 on E mRNA and at nucleotides 2913 to 2908 on L mRNA are shown.

Polyomavirus DNA was cleaved with *Eco*RI and cloned into the unique *Eco*RI site of the plasmid pMK16.1 (a derivative of pMK16 [25] in which the *Sal*I site has been deleted). The recombinant plasmid, pMK-A-12, was cleaved at its unique *Bgl*I site at nucleotide 92 in polyomavirus DNA. The linearized DNA was blunt ended with DNA polymerase I (Klenow fragment) and ligated to *Sal*I linkers. The ligated DNA was subsequently transfected into *Escherichia coli* DH-1 cells, and colonies on kanamycin plates were screened for the presence of a *Sal*I site in place of the *Bgl*I site. One such isolate, pMK-S-20, gave rise to viable virus when linear polyomavirus DNA was released by *Eco*RI digestion and transfected onto baby mouse kidney cells. This virus contained a *Sal*I site at the position previously occupied by the *Bgl*I site. These experiments were carried out by N. Acheson and H. Abdelwahed.

pMK-S-20 DNA was cleaved with *Sal*I and ligated to the 81-nucleotide *Sal*I-linkered fragment 2895 to 2976. The recombinant plasmids were used to transform competent DH-1 cells, and colonies on kanamycin plates were screened for the presence of the insert by restriction endonuclease digestion. The orientation of the inserted fragment was also determined by restriction analysis, and the structures of ins 1 and ins 2 were confirmed by DNA sequencing (Fig. 2). Both mutants were viable in transfection and plaque assays (see above).

Construction of ins 3. The pBR322 recombinant (clone 24) containing the previously inserted 81-nucleotide fragment was digested with *Hinc*II to release a slightly shorter (69-nucleotide) fragment extending from nucleotides 2896 to 2964. This fragment was ligated to pMK-A-12 DNA linearized by partial digestion with *Hinc*II. Transfected DH-1 cells were screened for the presence of recombinant plasmids containing the 69-nucleotide fragment inserted between nucleotides 2964 and 2965 in polyomavirus DNA. Only recombinants in which the L strand of the insert was joined to the L strand of the genome (ins 3; Fig. 2) were viable in transfection and plaque assays.

Construction of ins 4. Polyomavirus DNA was digested with *Hgi*AI, and the 470-nucleotide fragment 5 (nucleotides 2821 to 3286) was eluted from a 7% polyacrylamide gel. After generating blunt ends with the Klenow fragment of DNA polymerase I, we ligated the fragment to *Sal*I linkers as above. The fragment was subsequently digested with *Hinc*II, and the resulting 144-nucleotide fragment (nucleotides 2821 to 2964) was eluted from a 7% polyacrylamide gel and ligated

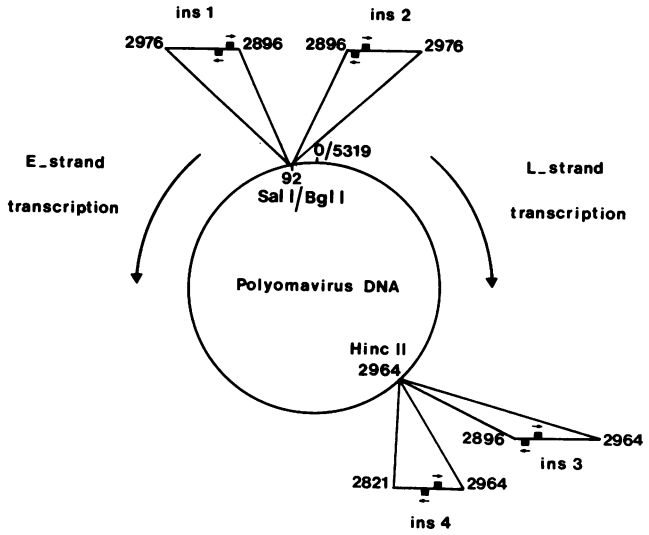

FIG. 2. Structure of polyomavirus viable insertion mutants. All mutants contain a duplication of sequences surrounding the two adjacent AATAAAs, one on each DNA strand, indicated by black rectangles. The small arrows denote the orientation (5′ to 3′) of each AAUAAA in RNA transcripts. In ins 1, the L strand of the insert is joined to the L strand of the viral genome. The insert contains 12 nucleotides downstream of the AATAAA in the direction of L-strand transcription. ins 2 carries the same fragment in the opposite orientation, such that the E strand of the insert is joined to the L strand of the viral genome. The insert in this orientation contains 56 nucleotides downstream of AATAAA in the direction of L-strand transcription. In both ins 3 and ins 4, the L strand of the insert is joined to the L strand of viral genome. This insertion is upstream of the normal L-strand polyadenylation site. The inserts contain 12 (ins 3) or 87 (ins 4) nucleotides downstream of AATAAA in the direction of L-strand transcription.

to linearized pMK-A-12 as above. After transformation of DH-1 cells, colonies on kanamycin plates were screened by restriction analysis, and those containing an insert at the *Hin*cII site at nucleotide 2964 were further analyzed to determine the orientation of the inserted fragment. As described above, only mutants in which the L strand of the insert is joined to the L strand of the genome were viable in transfection and plaque assays. The structure of ins 4 (Fig. 2) was confirmed by DNA sequencing, which revealed that the *Hgi*AI end of the fragment had no *Sal*I linker attached and therefore that the *Hin*cII site at this junction was not reconstituted.

RNA blotting. RNA blotting was carried out essentially as described by Thomas (43). Cytoplasmic poly(A)$^+$ RNA (5 μg) was glyoxal denatured at 50°C for 1 h (11) and loaded onto a 1.1% agarose slab gel (195 by 160 by 3 mm) containing 10 mM sterile phosphate buffer, pH 7.0. Electrophoresis was carried out at 90 V and 34 mA for 3 h, with recirculation of buffer. Capillary transfer to nitrocellulose (BA-85; Schleicher & Schuell, Inc., Keene, N.H.) was for 38 h at room temperature in 20× SSC (3 M NaCl plus 0.3 M trisodium citrate). The nitrocellulose was baked in a vacuum oven at 80°C for 2 h, after which the glyoxal adduct was removed by treating the blot with 50 mM Tris hydrochloride (pH 8.0) at 100°C. The blot was prehybridized at 42°C for 16 h in the buffer specified by Thomas (43) and hybridized at the same temperature for 48 h to a labeled single-stranded DNA probe prepared as described below. After washing, blots were exposed to Kodak XAR-5 film at −70°C.

The single-stranded DNA probe hybridized to RNA blots was ^{32}P-labeled fd103 bacteriophage DNA (22) containing the L strand of polyomavirus *Pst*I fragment 3 (M. McNally, M.Sc. thesis, McGill University, Montreal, Quebec, 1984). In vivo labeling was carried out as described by Ray and Schekman (39). M2167 bacteria were grown to about 2×10^8 cells per ml in dephosphorylated L broth and infected with phage particles at a multiplicity of 10 PFU per cell. At the same time, 0.1 mCi of inorganic $^{32}PO_4^{3-}$ per ml was added. After 5 h of growth with agitation at 37°C, phage particles were recovered by precipitation with 2% (wt/vol) polyethylene glycol. Phage particles were subsequently purified on a discontinuous CsCl step gradient (50), and phage DNA was extracted with hot phenol followed by ethanol precipitation. A sample of purified labeled DNA was counted in Atomlight (New England Nuclear Corp.); specific activity was 88×10^3 cpm/μg.

S1 nuclease mapping. DNA of plasmids containing ins 3 or ins 4 was digested with *Hpa*II, and the fragments containing the duplicated polyadenylation signals were eluted from a 7% polyacrylamide gel. The recessed 3′ ends of each fragment were labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP. The labeled DNA was digested with restriction nucleases which cut very close to one of the labeled ends (see figures), resulting in probes labeled on only one strand. E-strand probes were further strand separated.

Cytoplasmic poly(A)$^+$ RNA extracted from infected cells was hybridized with the appropriate labeled DNA probe in 0.4 M NaCl–1 mM EDTA–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)–80% formamide (see figure legends for time and temperature). Hybridization mixtures were then diluted 10-fold with ice-cold S1 buffer (300 mM sodium acetate [pH 4.6], 4.5 mM $ZnSO_4$) containing 300 U of S1 nuclease per ml (1 U digests 1 μg of DNA in 30 min at 37°C) and incubated for 30 min at 30°C. After ethanol precipitation, pellets were suspended in 15 μl of 100% formamide, denatured at 85°C for 5 min, and quickly chilled. Bromophenol blue loading buffer (5 μl) was added, and samples were immediately loaded on a 7% acrylamide–8 M urea gel and electrophoresed for the times indicated in each experiment.

Measurement of proportion of viral RNA that is polyadenylated. Virus-infected cells were labeled for 10 min with 500 μCi of [5-^{3}H]uridine per ml per 100-mm petri dish at 28 h after infection. Poly(A)$^+$ or poly(A)$^-$ nuclear RNA was denatured together with an excess of single-stranded M13 DNA containing the L strand of *Hpa*II fragment 1 (nucleotides 2991 to 4409) in 50% formamide–10 mM PIPES (pH 6.8) at 85°C for 10 min. Samples were adjusted to 25% formamide–0.4 NaCl–10 mM PIPES (pH 6.8) and hybridized at 37°C for 5 h. Samples were then adjusted to 4% formamide–1 M NaCl–2 mM EDTA–2 mM PIPES–50 mM Tris hydrochloride (pH 7.5), and DNA-RNA hybrids were collected by loading onto 13-mm-diameter nitrocellulose filters (maximum, 10 μg of DNA per filter). Filters were washed with 1 M NaCl, treated with 20 μg of RNase A per ml in 0.4 M NaCl–50 mM Tris hydrochloride (pH 2.5) for 30 min at 37°C, further washed in 1 M NaCl, dried, and counted in a toluene-based scintillation fluid.

RESULTS

Polyadenylation of L-strand RNA does not occur within fragment 2896 to 2976 inserted near the origin of DNA replication. The overlapping 3′ ends of polyomavirus E- and L-strand RNAs are polyadenylated at nucleotides 2930 and

2900, respectively (21, 26) (Fig. 1). We isolated a DNA fragment (nucleotides 2896 to 2976) that contained both these polyadenylation sites as well as their associated AATAAA sequences. We inserted this 81-nucleotide fragment into a unique *Sal*I site that we had previously created within the polyomavirus genome by addition of *Sal*I linkers to the *Bgl*I site, at nucleotide 92 (see Materials and Methods). This site lies between the origin of DNA replication and the initiation site for E-strand transcription, within a region known to be nonessential for virus growth (8). Two selected mutants, ins 1 and ins 2 (Fig. 2), carry the insert in each of the two orientations, approximately 2.8 kb downstream of the normally used L-strand polyadenylation site. Both mutants are viable, as expected.

NIH mouse 3T6 cells were infected with wild-type or mutant virus, and RNA was extracted 28 h postinfection. Cytoplasmic poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography, glyoxalated, and analyzed by agarose gel electrophoresis followed by blotting onto nitrocellulose (see Materials and Methods). Both mutants were fully functional in producing L-strand mRNAs for the three capsid proteins (Fig. 3, lanes 1 and 3) when compared with wild-type virus (lane 2). The 1.6-kb RNA codes for VP1, the most abundant capsid protein (most intense band on autoradiogram); the 2.2- and 2.5-kb bands represent mRNA species for capsid proteins VP3 and VP2, respectively (26). All these RNA species are heterogeneous in length, owing to different numbers of A residues at their 3′ ends and to different numbers of spliced leader sequences at their 5′ ends (26, 27).

Unspliced L-strand mRNA polyadenylated at the inserted site was expected to be about 5.3 kb long, taking into account the distance from the major transcription initiation site at nucleotide 5128 (21, 26, 46) to the polyadenylation site in the insert, as well as the length of the poly(A) tail. Spliced mRNAs lacking the introns removed in mVP2 or mVP3 were expected to be 5.0 or 4.4 kb long. No viral mRNA species longer than 2.5 kb could be detected (Fig. 3), even when the blots were exposed for much longer periods (not shown). Lanes 4 and 5 show that RNA molecules up to 13 kb in length were efficiently transferred to nitrocellulose in this experiment. We conclude that the inserted DNA fragment did not function in either orientation to generate 3′ ends of stable cytoplasmic poly(A)$^+$ RNA.

Polyadenylation of L-strand RNA does not occur within fragment 2896 to 2964 inserted adjacent to the normal polyadenylation site. We wanted to test whether the fragment we inserted at the *Sal*I site contained insufficient sequences to function as a polyadenylation signal or did not function because of its altered position in the viral genome. Therefore, we inserted fragment 2896 to 2964 immediately adjacent to its normal position, at the *Hin*cII cleavage site located between nucleotides 2965 and 2964 (see Materials and Methods and Fig. 2). In this mutant, designated ins 3, the insert lies upstream of the normally used L-strand polyadenylation site and exactly reproduces the sequences between nucleotides 2964 and 2896. Thus, coding sequences for VP1 are not interrupted, and the mutant is viable. The insert contains 12 nucleotides downstream of AATAAA in the direction of L-strand transcription, as does ins 1.

3T6 cells were infected with wild-type or ins 3 virus, and RNA was extracted 28 h postinfection. Cytoplasmic poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography and analyzed, in this case, by S1 nuclease mapping (10, 47). A DNA fragment spanning the entire duplicated region was prepared from ins 3 DNA. RNAs were hybridized to this DNA fragment, and S1 nuclease-resistant DNA

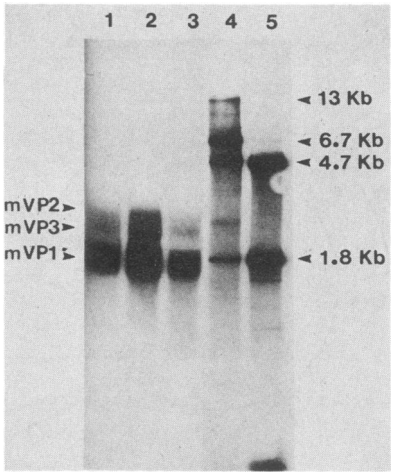

FIG. 3. Analysis of cytoplasmic poly(A)$^+$ RNA from mouse 3T6 cells infected with wild-type or mutant viruses. Cytoplasmic poly(A)$^+$ RNA (5 μg) from cells infected with ins 2 (lane 1), wild type (lane 2), or ins 1 (lane 3) was glyoxal denatured and subjected to electrophoresis. RNAs were transferred to nitrocellulose, which was hybridized with ^{32}P-labeled single-stranded bacteriophage fd103 DNA containing an insert of the L strand of polyomavirus DNA fragment *Pst*I-3 (nucleotides 4225 to 3313). This DNA is therefore complementary to L-strand mRNAs. Nuclear (lane 4) or cytoplasmic (lane 5) RNA (0.5 μg) from uninfected 3T6 cells, labeled in vivo for 7 h with $^{32}PO_4^{3-}$, was subjected to electrophoresis and transferred as a test of the efficiency of transfer of high-molecular-weight RNA to nitrocellulose.

was analyzed by electrophoresis on a denaturing urea-polyacrylamide gel.

RNA from wild-type-infected cells gave rise to an S1-resistant DNA fragment 92 nucleotides long, as expected (Fig. 4A, lane 3). This fragment corresponds to the distance from the labeled 3′ end to the normally used L-strand polyadenylation site at nucleotide 2900 (21, 26). RNA from ins 3-infected cells gave rise to an S1-resistant DNA fragment 166 nucleotides long (Fig. 4A, lane 4), which corresponds to the distance from the labeled 3′ end of the probe to the normally used polyadenylation site (Fig. 4B). RNA polyadenylated at the inserted site was expected to protect a DNA fragment 92 nucleotides long. This species was not observed in mutant-infected cells. A mixture of mutant and wild-type RNAs (lane 5) gave rise to both S1-resistant fragments, showing that the shorter RNA is not displaced in DNA-RNA hybrids by the longer RNA under our conditions.

These results allow us to conclude that the inserted 69-nucleotide fragment in ins 3 does not contain sufficient sequences to function as a polyadenylation signal, in the direction of L-strand transcription. Since the insert exactly reproduces all nucleotide sequences normally present upstream of the AATAAA, this fragment must be lacking essential sequences downstream of the AATAAA. Therefore, nucleotide sequences further than 12 nucleotides downstream of this AATAAA are required for formation of an mRNA polyadenylated 3′ end.

Polyadenylation of L-strand RNA occurs within fragment 2821 to 2964 inserted adjacent to the normal polyadenylation site. It remained for us to demonstrate that an analogous insert containing more of the sequences downstream of AATAAA would function as a signal in 3′-end formation. We therefore inserted an *Hgi*AI-*Hin*cII fragment, extending from nucleotides 2821 to 2964, into the same *Hin*cII site

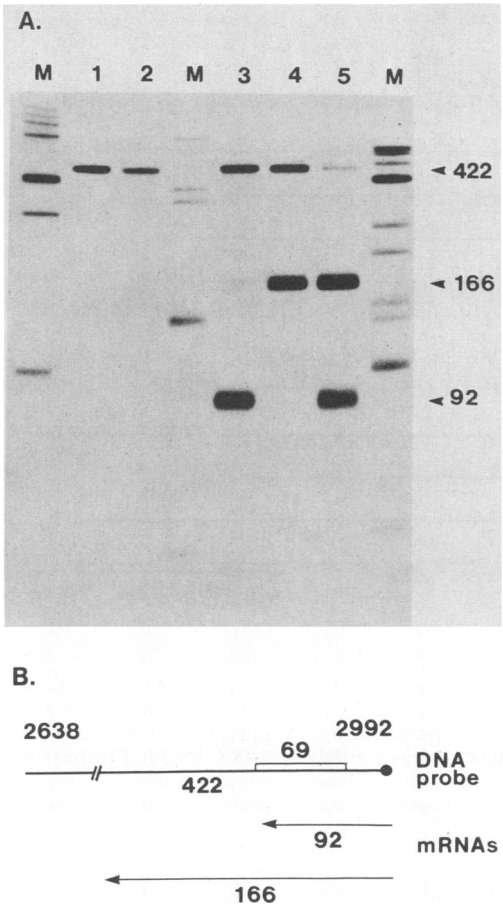

FIG. 4. S1 nuclease mapping of L-strand mRNAs from 3T6 cells infected with wild-type and ins 3 virus. (A) Autoradiogram of S1 mapping gel. Lane 1 contained 4 ng of double-stranded ^{32}P[DNA] probe alone. Each hybridization reaction received 80 ng of [^{32}P]DNA probe and 2 μg of the appropriate cytoplasmic polyadenylated RNA (except lane 2 which received no RNA): wild-type RNA (lane 3), ins 3 RNA (lane 4), and a mixture of 2 μg of wild type plus 2 μg of ins 3 RNA (lane 5). Hybridization was carried out at 48°C for 2 h. Hybrids were treated with S1 nuclease as described in Materials and Methods, and S1-resistant DNA fragments were analyzed on a 7% polyacrylamide–8 M urea gel. Electrophoresis was for 18 h at 45 V and 11 mA. The 422-nucleotide band in all lanes represents renatured probe. The molecular weight markers (lanes M) are, from left to right: ^{32}P-labeled polyomavirus DNA fragments from *Hpa*II, *Hin*fI, or *Hpa*II plus *Hin*fI digests. (B) Structure of the 3′-end-labeled DNA fragment used as a probe in this experiment. Nucleotide numbers above the line identify the positions of the ends of the DNA probe; the insert is shown as an elongated rectangle. The labeled end of the probe is marked by a filled circle. Lengths of the probe and the expected S1-resistant fragments are shown below the lines. Arrowheads designate expected positions of polyadenylated 3′ ends of mRNAs.

between nucleotides 2965 and 2964 (Fig. 2). In this mutant, called ins 4, the insert (144 nucleotides) contains 87 nucleotides downstream of AATAAA in the direction of L-strand transcription, instead of 12 nucleotides as in ins 3.

Late cytoplasmic poly(A)$^+$ RNAs from cells infected with wild-type or ins 4 virus were analyzed by S1 nuclease mapping (Fig. 5). In this case, the 3′-end-labeled probe was prepared from ins 4 DNA and was 497 nucleotides long. RNA from wild-type-infected cells protected a DNA fragment 92 nucleotides long, as expected (Fig. 5A, lane 2). RNA from ins 4-infected cells (Fig. 5A, lane 3) protected two DNA fragments: a 235-nucleotide fragment protected by RNA polyadenylated at the normally used site, shifted 144 nucleotides downstream from its usual position; and a 92-nucleotide fragment protected by RNA polyadenylated at the inserted site. We did not attempt to quantitate the proportion of RNA molecules polyadenylated at each of the two sites; however, it appears that the inserted site functions somewhat less efficiently than the normally used site. It should be noted that ins 4 mRNAs are less abundant than wild-type or ins 3 mRNAs; this is a reproducible finding. These experiments demonstrate that nucleotide sequences between 12 and 87 nucleotides downstream of the L-strand AATAAA are required to signal correct formation of a polyadenylated 3′ end on polyomavirus mRNA.

Polyadenylation of E-strand RNA occurs within the insert in both ins 3 and ins 4. We also analyzed E-strand mRNAs transcribed in ins 3- and ins 4-infected 3T6 cells during the late phase of the lytic cycle. DNA fragments spanning the entire duplicated region were prepared from ins 3 and ins 4 DNAs (Fig. 6B). The 3′ ends of the E strands of these fragments were labeled, and the DNA strands were separated. This was made necessary because of the low concentrations of E-strand mRNAs present in infected cells; even small amounts of renaturation of the DNA probe rendered detection of DNA protected by E-strand mRNAs difficult.

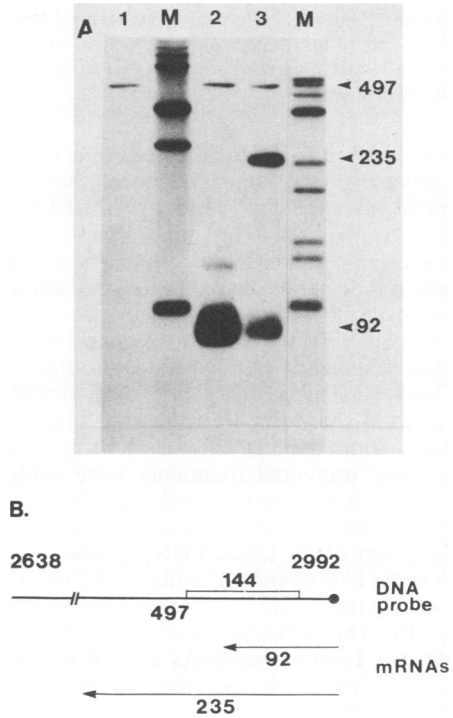

FIG. 5. S1 nuclease mapping of L-strand mRNAs from 3T6 cells infected with wild-type or ins 4 virus. (A) Autoradiogram of S1 mapping gel. Each hybridization reaction received 150 ng of ^{32}P-labeled probe and 6.5 μg of the appropriate cytoplasmic polyadenylated RNA (except lane 1 which received no RNA): wild-type RNA (lane 2); ins 4 RNA (lane 3). Hybridization was at 48°C overnight. Electrophoresis was for 18 h at 45 V and 12 mA. The 497-nucleotide band in all lanes represents renatured probe. The molecular weight markers (lanes m) are, from left to right: ^{32}P-labeled polyomavirus DNA fragments from *Hpa*II or *Hpa*II plus *Hin*fI digests. (B) Structure of the 3′-end-labeled DNA fragment used as a probe in this experiment. See the legend to Fig. 4B for details.

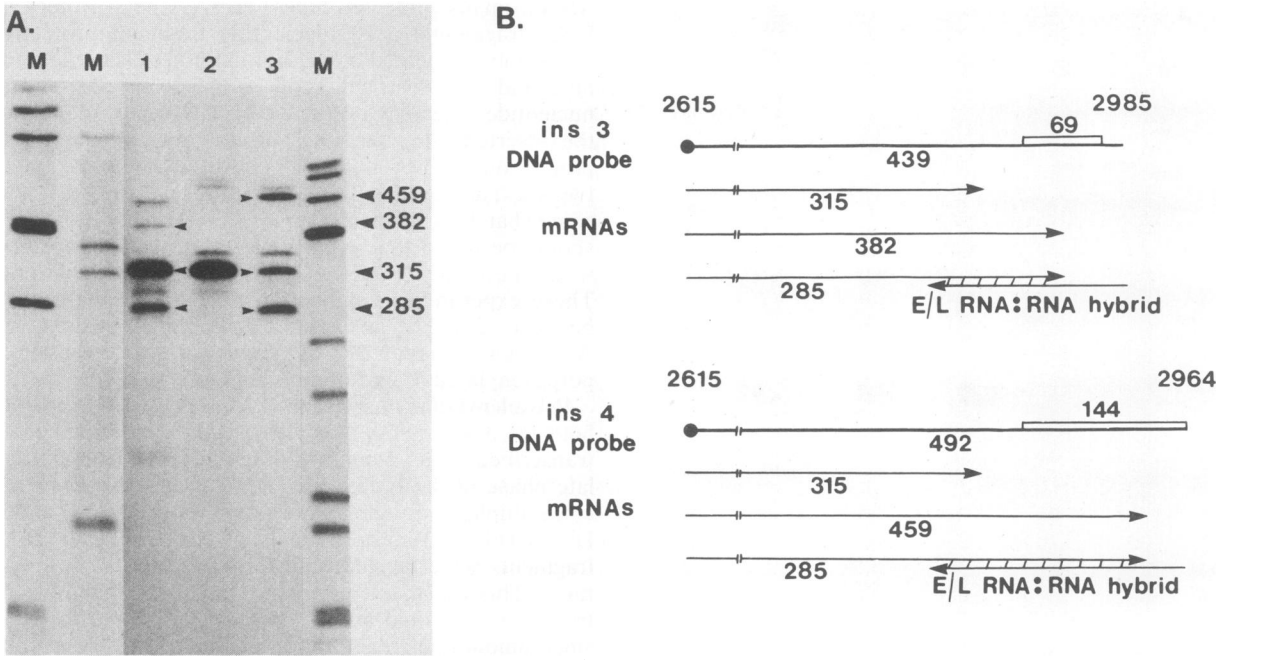

FIG. 6. S1 nuclease mapping of E-strand mRNAs from 3T6 cells infected with wild-type, ins 3, or ins 4 virus. (A) Autoradiogram of S1 mapping gel. Each hybridization reaction received approximately 30 ng of strand-separated ^{32}P-labeled DNA probe and 5 μg of the appropriate cytoplasmic polyadenylated RNA: ins 3 RNA hybridized with ins 3 DNA probe (lane 1); wild-type RNA hybridized with ins 4 DNA probe (lane 2); ins 4 RNA hybridized with ins 4 DNA probe (lane 3). Hybridization was carried out at 48°C for 3 h. Electrophoresis was for 22 h at 45 V and 10 mA. The molecular weight markers (lanes m) are, from left to right: ^{32}P-labeled polyomavirus DNA fragments from *Hpa*II, *Hin*fI, or *Hpa*II plus *Hin*fI digests. (B) Structure of the 3′-end-labeled DNA fragments used as probes in this experiment. See the legend to Fig. 4B for details.

Cytoplasmic poly(A)$^+$ RNA from cells infected with wild-type virus gave rise to a major 315-nucleotide S1-resistant DNA fragment, as expected, when hybridized to the ins 4 probe (Fig. 6A, lane 2). The minor bands in this lane probably represent residual full-length and S1-nicked probe; each of these minor bands accounts for only about 0.1% of the input radioactivity (data not shown).

RNA from cells infected with ins 3 gave rise to a major 315-nucleotide fragment when hybridized to the ins 3 probe (Fig. 6A, lane 1). This corresponds to E-strand mRNAs polyadenylated at the normally used site, upstream of the insert. Besides some residual full-length probe (439 nucleotides), two other protected fragments were detected. The 382-nucleotide fragment corresponds to RNA polyadenylated within the insert. The 285-nucleotide fragment arises from displacement of the labeled DNA probe by hybridization between the overlapping 3′ ends of the most extended E-strand and L-strand RNAs produced by ins 3 (see diagram, Fig. 6B). This RNA-RNA hybridization occurs frequently because L-strand mRNAs are 10 to 20 times as abundant as E-strand mRNAs during the late phase (7, 17). The overlap between the 3′ ends of these mRNAs is 99 nucleotides for ins 3, 174 nucleotides for ins 4, and only 30 nucleotides for wild-type virus. Little or no such "shadow" fragment was formed in hybridizations with wild-type RNA, presumably because the 30-nucleotide overlap did not form a stable RNA-RNA hybrid (24 of these 30 base pairs are A · U pairs). This suggests that all of the 285-nucleotide protected DNA fragment arises from hybrids containing E-strand mRNAs polyadenylated within the insert. Therefore, the sum of the 285- and 382-nucleotide bands should be proportional to the level of polyadenylation within the insert. It is clear that RNAs polyadenylated at the normally used site are more abundant that those polyadenylated within the insert of ins 3.

RNA from cells infected with ins 4 gave rise to approximately equal amounts of 459-, 315-, and 285-nucleotide protected DNA fragments when hybridized with the ins 4 probe (Fig. 6A, lane 3). Three additional minor bands in this lane are also present in lane 2 and are probably residual full-length and S1-nicked probe (see above). In analogy to the results with ins 3, the 315-nucleotide fragment corresponds to mRNA polyadenylated at the normally used site, and the sum of the 459- and 285-nucleotide fragments corresponds to mRNA polyadenylated within the insert. It is notable that RNAs polyadenylated within the insert are more abundant than those polyadenylated at the normally used site, in contrast to results with ins 3. As with L-strand mRNAs, ins 4 E-strand mRNAs are less abundant than wild-type or ins 3 E-strand mRNAs.

The nucleotide sequences of both ins 3 and ins 4 are identical to those of wild-type virus up to nucleotide 2964, in the direction of E-strand transcription (Fig. 2). We conclude that this region, which includes 44 nucleotides downstream of AATAAA, contains sufficient information to signal correct 3′-end formation of E-strand mRNA. The insert of ins 3 begins at nucleotide 2896; therefore no more than 19 nucleotides upstream of AATAAA are required for 3′-end formation. However, ins 4 appears to polyadenylate a larger proportion of its mRNA within the insert than does ins 3. This may mean that the presence of more upstream sequences within the ins 4 insert increases the efficiency of its use. Alternatively, the polyadenylation signals within both inserts may function with the same efficiency, but mRNAs polyadenylated within the ins 4 insert may be more stable than those polyadenylated within the ins 3 insert. This would

TABLE 1. Proportion of viral L-strand nuclear RNA that is polyadenylated

Virus	RNA fraction	Virus-specific RNA (10^3 cpm/petri dish)[a]		% of RNA polyadenylated[b]
		2 μg of DNA	5 μg of DNA	
Wild type	A^-	88.3	88.2	12.8
	A^+	13.2	12.6	
ins 4	A^-	20.9	23.1	13.1
	A^+	3.2	3.5	

[a] Samples of ^{3}H-labeled RNAs were hybridized to either 2 or 5 μg of single-stranded cloned DNA as described in the text. Counts per minute hybridized are expressed as petri dish equivalent to allow comparison among samples.
[b] Calculated by using average value from both hybridizations.

lead to accumulation of higher relative steady-state levels of the ins 4 mRNA.

Effect of duplicated polyadenylation signal on efficiency of polyadenylation of L-strand RNAs. Having identified a functional polyadenylation signal, we proceeded to analyze the effect of a duplicated signal on the overall efficiency of polyadenylation of L-strand RNA. We knew that only a small proportion of L-strand RNA is polyadenylated in the late phase of productive infection by wild-type virus (3). We reasoned that if polyadenylation was inefficient because the signal is inherently weak, addition of a second copy of the signal should increase the overall level of polyadenylation.

3T6 cells were infected at the same time with either wild-type or ins 4 virus, and RNA was labeled with [^{3}H]uridine for 10 min at 28 h after infection. We chose a 10-min labeling period for the following reasons. Polyadenylation takes place within 1 to 2 min of the synthesis of an RNA precursor (3); the rate of incorporation of [^{3}H]uridine into viral and cellular RNA becomes constant by 5 min after the beginning of labeling (2); some labeled viral mRNAs begin to be exported to the cytoplasm as early as 15 min after the beginning of labeling (2). Therefore, to measure relative rates of polyadenylation in the nucleus, labeling should be done for more than 5 but less than 15 min. Poly(A)$^+$ and poly(A)$^-$ nuclear RNAs were hybridized in solution with an excess of single-stranded M13 DNA containing the L strand of polyomavirus *Hpa*II fragment 1. This fragment covers the distal two-thirds of the region which codes for L-strand mRNAs.

The results (Table 1) indicate that the same proportion of viral nuclear RNA (about 13% in this experiment) is polyadenylated in wild-type- and ins 4-infected cells. Therefore, the presence of a second functional polyadenylation signal on the L DNA strand did not increase the overall efficiency of polyadenylation of L-strand RNA. Instead, the same total number of polyadenylation events is now distributed among two separate sites. This finding suggests that the formation of 3′ ends of polyomavirus mRNAs is limited not by the strength of the polyadenylation signals but rather by the capacity of the cellular machinery to recognize the appropriate signals.

Effect of duplicated polyadenylation signal on efficiency of termination of L-strand transcription. We describe in detail elsewhere (46) a method for measuring the efficiency of transcription termination in polyomavirus. This method involves quantitation of transcripts made by RNA polymerases passing through the promoter region on the L strand of viral DNA. Hybridization of in vivo pulse-labeled RNA to DNA fragments encompassing the L-strand promoter allows us to distinguish RNAs made by recently initiated polymerases from RNAs made by polymerases passing through the promoter region after having already completed at least one traverse of the circular viral DNA molecule. The former give rise to RNA-DNA hybrids shorter than the DNA fragment used, and the latter give rise to full-length RNA-DNA hybrids, after RNase and S1 nuclease treatment to remove single-stranded RNA and DNA. The frequency of termination per traverse of the viral genome can be calculated from the relative amounts of these two classes of hybrids, since only RNA polymerases which have not terminated during a genome traverse will give rise to the full-length DNA-RNA hybrids.

Twenty-eight hours after infection with ins 4 virus, 3T6 cells were labeled with [^{3}H]uridine for 2 min. Nuclear RNA was hybridized with an excess of single-stranded DNA from an M13 bacteriophage clone of polyomavirus *Hpa*II fragment 3L, which extends from 191 nucleotides upstream to 718 nucleotides downstream of the major L-strand transcription start site at nucleotide 5128 (45, 46). Hybrids were treated with RNase T_1 and S1 nuclease and analyzed by gel electrophoresis followed by scintillation counting of gel slices. The ratio of radioactivity in less-than-full-length hybrids (newly initiated transcripts) to that in full-length hybrids was approximately 1 in several independent experiments with ins 4 virus (Table 2). Results described elsewhere (46) establish that this ratio is identical at the same time after infection with wild-type virus. In both cases, we calculate that approximately 50% of the RNA polymerases which initiate transcription at the L-strand promoter terminate during each traverse of the polyomavirus genome. Thus, the presence of a second functional polyadenylation signal has no measurable effect on the efficiency of termination of L-strand transcription by RNA polymerase II.

DISCUSSION

Nucleotide sequences required for mRNA 3′-end formation. By analyzing viable insertion mutants of polyomavirus, we showed that formation of polyadenylated 3′ termini of L-strand mRNAs requires a sequence element located somewhere between 12 and 87 nucleotides downstream of AAUAAA. Our results also showed that no more than 19 nucleotides upstream and 44 nucleotides downstream of AAUAAA are required for correct polyadenylation of E-strand mRNAs.

Similar findings have recently been reported for several other mammalian genes and viruses (19, 30, 31, 40, 49). However, the nature of the sequence element(s) downstream of AAUAAA required for 3′-end formation remains obscure. Berget's analysis (9) of some 60 mammalian genes revealed

TABLE 2. Efficiency of transcription termination on L strand of ins 4 DNA

Expt	Full-length hybrids		Less-than-full-length hybrids		% Termination[b]
	cpm	cpm/100 U residues[a]	cpm	cpm/100 U residues	
1	581	245	368	201	45
2	988	417	615	339	44
3	1,486	627	1,368	755	54

[a] Counts per minute were divided by the number of U residues present in each RNA class, determined from the sequence of polyomavirus DNA and the known 5′ and 3′ ends of each RNA, and then multiplied by 100.
[b] Percent termination per genome traverse was calculated as 100 × (counts per minute per 100 U residues in less-than-full-length hybrids) ÷ (counts per minute per 100 U residues in full-length + less-than-full-length hybrids) (46).

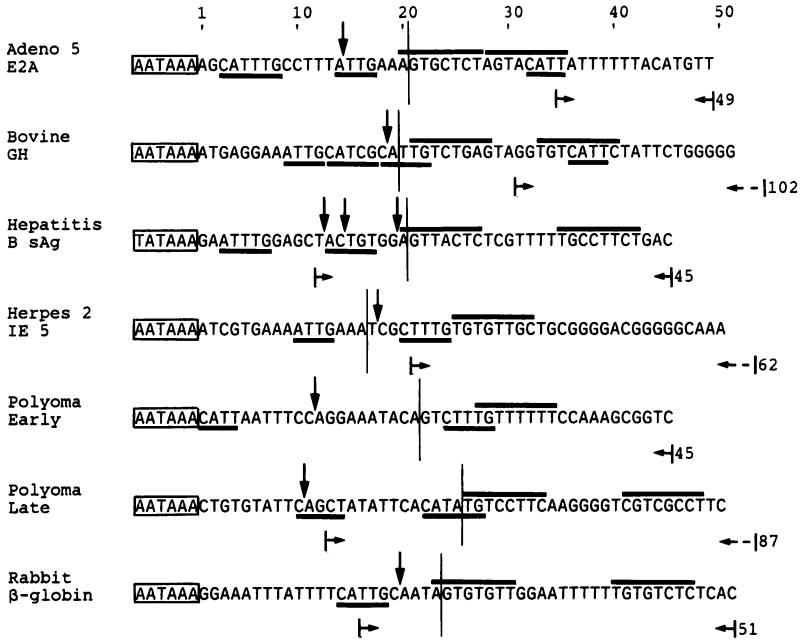

FIG. 7. Comparison of nucleotide sequences downstream of AAUAAA. Shown are polyadenylation signal regions from genes in which sequence requirements for in vivo 3′-end formation have been established by deletion analysis. Nucleotide sequences are shown as the DNA strand of the same polarity as mRNA, in 5′ to 3′ orientation. Boxes denote AATAAA or related sequences; vertical arrows denote sites of mRNA 3′-end formation; underlining denotes CAPyUG homologies; overlining denotes PyGTGTTPyPy homologies; rightward arrows denote the downstream site most distant from AATAAA at which 3′-extending deletions were found to partly or completely inhibit mRNA 3′ end formation; leftward arrows denote the site closest to AATAAA at which 3′-extending deletions were found not to affect 3′ end formation; numbers next to leftward arrows indicate the last nucleotide before the deletion. Sequences are divided into an A+T-rich half and a C+T-rich half by thin vertical lines. References for data are: adenovirus type 5 E2A, 30; bovine growth hormone, 49; hepatitis B s-antigen, 40; herpesvirus type 2 IE5, 31; rabbit β-globin, 19; polyomavirus E and L, this paper.

that sequences related to the pentanucleotide CAPyUG are always present within the first 25 nucleotides downstream of the hexanucleotide AAUAAA in functional polyadenylation sites. She suggested that these two conserved sequence blocks might hybridize to complementary sequences within the small nuclear RNA U4, leading to recognition of the correct cleavage site by an endonuclease. Evidence that a small nuclear ribonucleoprotein is involved in this process has been obtained by inhibiting in vitro polyadenylation with anti-small nuclear ribonucleoprotein antibodies (34). On the other hand, McLauchlan et al. (31; see also reference 42) detected a different consensus sequence, PyGTGTTPyPy, located from 20 to 40 nucleotides downstream of AAUAAA. Related sequences are present in 67% of some 100 polyadenylation sites analyzed; many of the genes which lack homologous sequences contain T-rich regions in the same area.

Figure 7 shows nucleotide sequences downstream of AAUAAA for those genes in which deletion analysis has established sequence requirements for polyadenylation in vivo. We would like to point out several common features of these sequences. (i) Correct mRNA 3′-end formation requires, in addition to AAUAAA, sequence elements within the first 40 to 50 nucleotides downstream of this hexanucleotide. This is shown by the loss of function when deletions are made within this distance downstream of AAUAAA (Fig. 7, rightward arrows), and the retention of function when deletions are made beyond 40 to 50 nucleotides downstream of AAUAAA (leftward arrows). (ii) Within the proximal half of this region, up to about 25 nucleotides downstream of AAUAAA, each of the genes shown contains at least one four- or five-nucleotide homology (underlined) to the CAPyUG consensus sequence of Berget (9). The site of cleavage and polyadenylation is also in the proximal half. (iii) Within the distal half of this region, from about 25 to 50 nucleotides downstream of AAUAAA, each of the genes shown contains at least one homology (overlined) to the PyGTGTTPyPy consensus sequence of McLauchlan et al. (31). Two homologies are present in five of the seven genes analyzed. (iv) Nucleotide sequences in the proximal half of this region tend to be rich in A+T (average, 68% A+T) and poor in C (average, 14% C). In contrast, sequences in the distal half tend to be rich in C+T (average, 62% C+T) and poor in A (average, 11% A). These figures were calculated by considering the A residue closest to nucleotide 25 to be the end of the proximal half and nucleotide 45 to be the end of the distal half.

Taken together, these observations suggest that there may be three distinct sequence elements in polyadenylation signals: (i) AAUAAA or closely related sequences; (ii) a feature within the next 25 nucleotides downstream of AAUAAA, which could be either homology to CAPyUG or simply an A+T-rich, C-poor region; and (iii) a feature between 25 and 50 nucleotides downstream, which could be either homology to PyGTGTTPyPy, or simply a pyrimidine-rich, A-poor region. Strong homologies to CAPyUG and PyGTGTTPyPy are not universally found in all functional polyadenylation signals (9, 31). It may be that recognition of these signals involves a complex interaction of factors such as U4 RNA which recognize specific sequences (AAUAAA) and other

factors which recognize base composition or resulting RNA structure rather than sequence per se. An example of such a factor is the termination factor Rho of *E. coli*, which interacts with pyrimidine-rich regions of RNA that contain relatively little secondary structure (37).

3′-end formation at multiple polyadenylation sites. We have previously shown that RNA polymerase can traverse the entire L strand of the circular, 5.3-kb polyomavirus genome up to four times before terminating transcription (3). A fraction (10 to 25%) of these RNAs are polyadenylated, at a site near nucleotide 2900. Some RNAs are polyadenylated at the first site encountered, 2.2 kb from the 5′ end; others are polyadenylated at the second site, 5.3 kb beyond; others at the third site, etc. The result is a family of polyadenylated RNAs of length 2.2 + n(5.3) kb, where n can be an integer from 0 to at least 3 (3). In such a system it is necessary that upstream polyadenylation signals not be recognized with 100% efficiency so that downstream sites are used. This could be accomplished either by means of a polyadenylation signal which is inherently weak (see, e.g., reference 14) or by modulation of the polyadenylation machinery so that it does not efficiently recognize signals.

We reasoned that if the polyadenylation machinery is fully functional but the L-strand polyadenylation signal is weak, insertion of a second copy of the signal should double the overall level of polyadenylation. Our results show that, although both signals function in mRNA 3′-end formation, the duplication has no measurable effect on the proportion of L-strand transcripts which are polyadenylated during the late phase of virus infection. We are led to conclude that the polyadenylation machinery is unable to function efficiently in this system. Perhaps one of the factors involved in 3′-end formation is present in limiting amounts. A consequence of this would be that polyadenylation signals known to be efficiently recognized in other systems should work less well in polyomavirus-infected mouse cells.

Fitzgerald and Shenk (16) also found that insertion of an L-strand polyadenylation signal upstream of its normal site in simian virus 40 DNA led to approximately equal use of both sites in virus-infected cells. The inserted fragment contained approximately 120 nucleotides upstream and 110 nucleotides downstream of the AAUAAA and presumably included a complete polyadenylation signal. These authors did not compare overall levels of polyadenylation of RNA during infections with mutant or wild-type viruses. Therefore, it is not clear whether the simian virus 40 polyadenylation signal is inherently weak or whether the machinery does not efficiently recognize the first signal encountered.

In contrast, Gil and Proudfoot (19) have shown that insertion of an intact polyadenylation signal upstream of the normal signal in the rabbit β-globin gene led to exclusive use of the upstream signal during transient expression assays in HeLa cells. Partial inactivation of the upstream signal by deletion led to the use of both sites, and further deletions in the upstream signal allowed the normal signal to be used exclusively. In this system it appears that the polyadenylation signal is inherently strong and that the machinery efficiently recognizes the first signal exposed during the process of transcription.

Relationship between polyadenylation and termination of transcription. One of the goals of the experiments presented here was to test the hypothesis that polyadenylation and termination of transcription are specified by a common signal. It is clear that RNA polymerase II can continue transcription for 1 to 2 kb beyond polyadenylation sites (18, 24); thus, termination and polyadenylation do not take place at the same site. However, it may be that recognition of a polyadenylation signal leads to subsequent termination of transcription. This could occur if one of the factors involved in recognition of polyadenylation signals was associated with RNA polymerase II during elongation and dissociated upon binding to its recognition site. RNA polymerase could then change from an elongation mode to a termination mode and subsequently terminate at multiple sites 1 to 2 kb downstream (13, 20).

We found that the presence of a duplicated polyadenylation signal in polyomavirus DNA has no measurable effect on either polyadenylation or termination efficiency. This neither proves nor disproves our hypothesis, but rather suggests that some factor required for both processes is limiting during the late phase of polyomavirus infection. As this paper was being prepared, Falck-Pedersen et al. (15) reported that insertion of a polyadenylation signal plus some 1.5 kb of downstream sequences from the mouse β-globin gene into the adenovirus E1A gene led to termination of transcription within that gene during the early phase of adenovirus infection. The precise boundaries of the nucleotide sequences for transcription termination in the mouse β-globin gene insert remain to be defined. Interestingly, termination within this insert was much less efficient during the late phase than during the early phase of adenovirus infection (15). Previous results suggested that termination of transcription of adenovirus late genes is efficient in the early phase but not in the late phase of infection (5, 35, 36). It is possible that a viral gene product is responsible for the reduced level of transcription termination during the late phase, in both polyomavirus- and adenovirus-infected cells.

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LITERATURE CITED

1. **Acheson, N. H.** 1978. Polyomavirus giant RNAs contain tandem repeats of the nucleotide sequence of the entire viral genome. Proc. Natl. Acad. Sci. USA **75**:4754–4758.
2. **Acheson, N. H.** 1981. Efficiency of processing of viral RNA during the early and late phases of productive infection by polyoma virus. J. Virol. **37**:628–635.
3. **Acheson, N. H.** 1984. Kinetics and efficiency of polyadenylation of late polyomavirus nuclear RNA: generation of oligomeric polyadenylated RNAs and their processing into mRNA. Mol. Cell. Biol. **4**:722–729.
4. **Acheson, N. H., and F. Mieville.** 1978. Extent of transcription of the E strand of polyomavirus DNA during the early phase of productive infection. J. Virol. **28**:885–894.
5. **Akusjarvi, G., and H. Persson.** 1981. Controls of RNA splicing and termination in the major late adenovirus transcription unit. Nature (London) **292**:420–426.
6. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA **69**:1408–1412.
7. **Bar-Shavit, R., O. Laub, and Y. Aloni.** 1978. The frequencies of transcription from the E- and L-strands of polyoma DNA. J. Gen. Virol. **39**:357–360.
8. **Bendig, M. M., and W. R. Folk.** 1979. Deletion mutants of polyoma virus defining a nonessential region between the origin of replication and the initiation codon for early proteins. J.

Virol. **32**:530–535.
9. **Berget, S. M.** 1984. Are U4 small nuclear ribonucleoproteins involved in polyadenylation? Nature (London) **309**:179–182.
10. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
11. **Carmichael, G. G., and G. K. McMaster.** 1980. The analysis of nucleic acids in gels using glyoxal and acridine orange. Methods Enzymol. **65**:380–391.
12. **Chiu, N. H., M. F. Radonovich, M. M. Thoren, and N. P. Salzman.** 1978. Selective degradation of newly-synthesized nonmessenger simian virus 40 transcripts. J. Virol. **28**:590–599.
13. **Citron, B., E. Falck-Pedersen, M. Salditt-Georgieff, and J. E. Darnell.** 1984. Transcription termination occurs within a 1000 base pair region downstream from the poly(A) site of the mouse β-globin (major) gene. Nucleic Acids Res. **12**:8723–8731.
14. **Cole, C. N., and G. M. Santangelo.** 1983. Analysis in Cos-1 cells of processing and polyadenylation signals by using derivatives of the herpes simplex virus type 1 thymidine kinase gene. Mol. Cell. Biol. **3**:267–279.
15. **Falck-Pedersen, E., J. Logan, T. Shenk, and J. E. Darnell.** 1985. Transcription termination within the E1A gene of adenovirus induced by insertion of the mouse β-major globin terminator element. Cell **40**:897–905.
16. **Fitzgerald, M., and T. Shenk.** 1981. The sequence of 5′-AAUAAA-3′ forms part of the recognition site for polyadenylation of late SV40 mRNAs. Cell **24**:251–260.
17. **Flavell, A. J., and R. Kamen.** 1977. Strand-specific transcription of polyoma virus DNA late during productive infection. J. Mol. Biol. **115**:237–242.
18. **Ford, J. P., and M.-T. Hsu.** 1978. Transcription pattern of in vivo-labeled late simian virus 40 RNA: equimolar transcription beyond the mRNA 3′ terminus. J. Virol. **28**:795–801.
19. **Gil, A., and N. J. Proudfoot.** 1984. A sequence downstream of AAUAAA is required for rabbit β-globin mRNA 3′-end formation. Nature (London) **312**:473–474.
20. **Hagenbüchle, O., P. K. Wellauer, D. L. Cribbs, and U. Schibler.** 1984. Termination of transcription in the mouse α-amylase gene Amy-2^a occurs at multiple sites downstream of the polyadenylation site. Cell **38**:737–744.
21. **Heiser, W. C., and W. Eckhart.** 1982. Polyoma virus early and late mRNAs in productively infected mouse 3T6 cells. J. Virol. **44**:175–188.
22. **Herrmann, R., K. Neugebauer, E. Pirkl, H. Zentgraf, and H. Schaller.** 1980. Conversion of bacteriophage fd into an efficient single-stranded DNA vector system. Mol. Gen. Genet. **177**:231–242.
23. **Higgs, D. R., S. E. Y. Goodbourn, J. Lamb, J. B. Clegg, D. J. Weatherall, and N. J. Proudfoot.** 1983. α-Thalassaemia caused by a polyadenylation signal mutation. Nature (London) **306**: 398–400.
24. **Hofer, E., and J. E. Darnell.** 1981. The primary transcription unit of the mouse βglobin gene. Cell **23**:585–593.
25. **Kahn, M., R. Kolter, C. Thomas, D. Figurski, R. Meyer, E. Remault, and D. R. Helinski.** 1979. Plasmid cloning vehicles derived from plasmids ColE1, F, R6K, and RK2. Methods Enzymol. **68**:268–280.
26. **Kamen, R., J. Favoloro, and J. Parker.** 1980. Topography of the three late mRNAs of polyomavirus which encode the virion proteins. J. Virol. **33**:637–651.
27. **Legon, S., A. J. Flavell, A. Cowie, and R. Kamen.** 1979. Amplification in the leader sequence of "late" polyomavirus mRNAs. Cell **16**:373–388.
28. **Manley, J. L., P. A. Sharp, and M. L. Gefter.** 1982. RNA synthesis in isolated nuclei: processing of adenovirus serotype 2 late messenger RNA precursors. J. Mol. Biol. **159**:581–599.
29. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
30. **McDevitt, M. A., M. J. Imperiale, H. Ali, and J. R. Nevins.** 1984. Requirement of a downstream sequence for generation of a poly(A) addition site. Cell **37**:993–999.
31. **McLauchlan, J., D. Gaffney, J. L. Whitton, and J. B. Clements.** 1985. The consensus sequence YGTGTTYY located downstream from the AATAAA signal is required for efficient formation of mRNA 3′ termini. Nucleic Acids Res. **13**:1347–1368.
32. **Miller, T. J., D. L. Stephens, and J. E. Mertz.** 1982. Kinetics of accumulation and processing of simian virus 40 RNA in *Xenopus laevis* oocytes injected with simian virus 40 DNA. Mol. Cell. Biol. **2**:1581–1594.
33. **Montell, C., E. F. Fisher, M. H. Caruthers, and A. J. Berk.** 1983. Inhibition of RNA cleavage but not polyadenylation by a point mutation in mRNA 3′ consensus sequence AAUAAA. Nature (London) **305**:600–605.
34. **Moore, C. L., and P. A. Sharp.** 1984. Site-specific polyadenylation in a cell-free reaction. Cell **36**:581–591.
35. **Nevins, J. R., and J. E. Darnell, Jr.** 1978. Steps in the processing of Ad2 mRNA: poly(A)$^+$ nuclear sequences are conserved and poly(A) addition precedes splicing. Cell **15**:1477–1493.
36. **Nevins, J. R., and M. C. Wilson.** 1981. Regulation of adenovirus-2 gene expression at the level of transcriptional termination and RNA processing. Nature (London) **290**:113–118.
37. **Platt, T., and D. G. Bear.** 1983. Role of RNA polymerase, rho factor, and ribosomes in transcription termination, p. 123–161. *In* J. Beckwith, J. Davies, and J. Gallant (eds.), Gene function in prokaryotes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Proudfoot, N. J., and G. G. Brownlee.** 1976. 3′ non-coding region sequences in eukaryotic messenger RNA. Nature (London) **263**:211–214.
39. **Ray, D. S., and R. W. Schekman.** 1969. Replication of bacteriophage M13. I. Sedimentation analysis of crude lysates of M13-infected bacteria. Biochim. Biophys. Acta **179**:398–407.
40. **Simonsen, C. C., and A. D. Levinson.** 1983. Analysis of processing and polyadenylation signals of the hepatitis B virus surface antigen gene by using simian virus 40-hepatitis B virus chimeric plasmids. Mol. Cell. Biol. **3**:2250–2258.
41. **Soeda, E., J. R. Arrand, N. Smolar, J. E. Walsh, and B. E. Griffin.** 1979. Coding potential and regulatory signals of the polyoma virus genome. Nature (London) **283**:445–453.
42. **Taya, Y., R. Devos, J. Tavernier, H. Cheroutre, G. Engler, and W. Fiers.** 1982. Cloning and structure of the human immune interferon-γ chromosomal gene. EMBO J. **1**:953–958.
43. **Thomas, P. S.** 1980. Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. Methods Enzymol. **100**:255–266.
44. **Tosi, M., R. A. Young, O. Hagenbüchle, and U. Schibler.** 1981. Multiple polyadenylation sites in a mouse α-amylase gene. Nucleic Acids Res. **9**:2313–2323.
45. **Treisman, R., and R. Kamen.** 1981. Structure of polyoma virus late nuclear RNA. J. Mol. Biol. **148**:273–301.
46. **Tseng, R. W., and N. H. Acheson.** 1986. Use of a novel S1 nuclease RNA-mapping technique to measure efficiency of transcription termination on polyomavirus DNA. Mol. Cell. Biol. **6**:1624–1632.
47. **Weaver, R. F., and C. Weissmann.** 1979. Mapping of RNA by a modification of the Berk-Sharp procedure: the 5′ termini of 15S β-globin mRNA precursor and mature 10S β-globin mRNA have identical map coordinates. Nucleic Acids Res **7**:1175–1193.
48. **Wickens, M., and P. Stephenson.** 1984. Role of the conserved AAUAAA sequence: four AAUAAA point mutants prevent messenger RNA 3′ end formation. Science **226**:1045–1051.
49. **Woychik, R. P., R. H. Lyons, L. Post, and F. M. Rottman.** 1984. Requirement for the 3′ flanking region of the bovine growth hormone gene for accurate polyadenylation. Proc. Natl. Acad. Sci. USA **81**:3944–3948.
50. **Yamamoto, K. R., B. Alberts, R. Benzinger, L. Lawhorne, and G. Treiber.** 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. Virology **40**:734–744.