JOURNAL OF VIROLOGY, Dec. 1987, p. 3754–3758
0022-538X/87/123754-05$02.00/0

Vol. 61, No. 12

Polyomavirus Early Region Alternative Poly(A) Site: 3′-End Heterogeneity and Altered Splicing Pattern

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Received 14 May 1987/Accepted 14 August 1987

The position of an alternative polyadenylation [poly(A)] site at the 3′ end of the polyomavirus middle T antigen (T-Ag) coding sequences suggests the possibility of a functional role for this site in early gene regulation. The fine structure of this alternative poly(A) site was determined by cDNA sequence and 3′ S1 analyses. Cleavage-poly(A) was found to be heterogeneous, occurring at multiple CA dinucleotides downstream from the AATAAA signal sequence. About 50% of the alternative poly(A) takes place upstream from the middle T-Ag stop codon. In addition, the pattern of splicing of transcripts with the alternative poly(A) site differed from that with the major poly(A) site at the end of the early region. The ratio of the small and middle T-Ag splices to the large T-Ag splice for the alternative poly(A)$^+$ mRNAs is about 2.5 times that found for mRNAs with the major poly(A) site. The altered splicing pattern and 3′-end heterogeneity of the alternative poly(A)$^+$ mRNAs would result in preferential translation of small T-Ag (to a greater degree) and middle T-Ag over large T-Ag at later times in the polyomavirus lytic cycle.

The polyomavirus (Py) early region is a complex transcription unit whose primary nuclear transcripts are differentially spliced to produce at least three mRNAs encoding the large, middle, and small T antigens (T-Ags) (24). Large T-Ag, which is the best studied of the three T-Ags, is a nuclear phosphoprotein with DNA-binding activity. Large T-Ag is required for the initiation of viral DNA synthesis (9, 11), although in mouse 3T6 cells either small T-Ag or a truncated form of middle T-Ag is required in addition to large T-Ag to elicit DNA synthesis (2, 27). Large T-Ag can negatively regulate transcription of the Py early region by binding to DNA sequences in the transcriptional control region (4). An active large T-Ag is required for efficient transformation of primary cells in vitro and the formation of tumors in vivo (10, 22) and is required for the initiation but not the maintenance of transformation in established cells (11, 17). The membrane-associated middle T-Ag is the Py oncogene (28) and has an associated protein kinase activity which may be due to the c-*src* protein with which it can complex (5). Little is known about the activity of the small T-Ag, which appears to reside in the cytoplasm, although the small or middle T-Ag or both are thought to have a role in VP1 phosphorylation before virus assembly (12).

Py early region transcription is complicated not only by differential splicing pathways, but also by the existence of two polyadenylation [poly(A)] sites. The major site, situated just downstream of the large T-Ag translational stop codon, is used by the majority of all three predominant early region mRNAs (16). An alternative site is signalled by an AATAAA sequence close to the end of the middle T-Ag coding sequences. The hamster papovavirus nucleotide sequence, which also appears to contain overlapping reading frames for its three early region proteins, also contains an alternative poly(A) site at the end of its middle T-Ag coding region (7), indicating the apparent conservation of this signal. In contrast, simian virus 40, which has no middle T-Ag analog, lacks an alternative poly(A) processing site, despite the presence of an AATAAA consensus sequence within the large T-Ag reading frame (3).

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In most Py-transformed rodent cell lines the alternative poly(A) site is used only at a relatively low level (16). Even in a majority of those transformed cells in which the major poly(A) site is lost as a result of recombination with cellular DNA, the Py early region transcripts utilize a cellular poly(A) signal in preference to the alternative poly(A) signal (16, 23). Conversely, in exceptional Py-transformed cell lines such as 53-Rat, viral early region transcripts are cleaved and polyadenylated almost exclusively at the alternative poly(A) site (16), showing that under certain circumstances this site is able to function efficiently. Early region transcription utilizing the alternative poly(A) site has not been studied in detail, although when measured it appeared to be at a relatively low level (about 10% early Py mRNA) (16). In this report we present a more detailed analysis of the early region transcripts utilizing the alternative poly(A) site during the lytic cycle. We found differences in the splicing pattern and a 3′-end heterogeneity at the site of poly(A) of such transcripts which would result in preferential translation of small T-Ag (to a greater degree) and middle T-Ag over large T-Ag at later times in the lytic cycle.

MATERIALS AND METHODS

Viral infection of 3T6 cells. Mouse 3T6 fibroblasts (5×10^7 per bioassay tray [24.5 by 24.5 cm]) were infected with A2 strain Py at a multiplicity of 50 PFU per cell in 10 ml of serum-free Dulbecco modified Eagle medium with frequent rocking in a 37°C incubator. The incubator was flushed periodically with CO_2 to maintain a relatively acid pH. After 90 min, the cells were refed with 100 ml of Dulbecco modified Eagle medium plus 5% horse serum and incubation was continued at 37°C.

Isolation of 53-Rat total cellular RNA and poly(A)$^+$ species selection. Total cellular RNA was prepared by guanidinium isothiocyanate lysis and was fractionated into poly(A)$^+$ and poly(A)$^-$ components by chromatography on oligo(dT)-cellulose as described by Maniatis et al. (18). The yield of poly(A)$^+$ RNA varied from 2 to 5% of the total RNA.

Formaldehyde-agarose gel electrophoresis of RNA and Northern (RNA) blotting. The procedures used for electrophoresis and blotting were modified from those presented by

Maniatis et al. (18). A 200-ml gel was cast which contained 1% (wt/vol) agarose in 1× MOPS-E (20 mM 3-[*N*-morpholinepropanesulfonic acid], 1 mM EDTA, 5 mM sodium acetate [pH 7.0])–6.8% (wt/vol) formaldehyde. A 2-μg sample of poly(A)$^+$ RNA was prepared in 1× MOPS-E–6.8% (wt/vol) formaldehyde–50% (vol/vol) formamide and heated at 55°C for 15 min, after which 0.1 volume of RNA loading buffer (50% [vol/vol] glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol) was added per sample before loading on the gel.

The gel was electrophoresed in 1× MOPS-E–6.8% (wt/vol) formaldehyde at 30 mA overnight with the buffer circulating by means of a peristaltic pump. The gel was run until the bromophenol blue had travelled 75% of its length and was then soaked in water for 5 min, followed by 50 mM NaOH for 20 to 30 min to partially hydrolyze the RNA for ease of transfer during blotting. The gel was then neutralized by soaking in 0.1 M Tris chloride (pH 7.4) for 20 to 30 min and finally soaked in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 60 min. The gel was laid on a glass plate, and the RNA was transferred to nitrocellulose without the need for a reservoir. The dried, baked filter was prehybridized and subsequently hybridized to a random-primed DNA probe in 50% formamide at 45°C. The filter was washed sequentially in 2× SSC–0.5% sodium dodecyl sulfate at 68°C (twice) and then in 0.2× SSC–0.5% sodium dodecyl sulfate at 68°C before air drying and exposure to Kodak XAR-5 film with an intensifying screen (Cronex Lightning-Plus) at −70°C. Relative proportions of each mRNA species were determined by densitometric scanning of the film with a Joyce-Loebl Chromoscan 3.

An RNA size marker ladder (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was run in an isolated slot on the gel. The marker track was then cut off, blotted, and stained by soaking in 5% (vol/vol) acetic acid for 15 min and then in 0.5 M sodium acetate (pH 5.4)–0.04% (wt/vol) methylene blue for 5 min. The filter was then rinsed with water to reveal the RNA.

Random oligonucleotide priming. For random priming (8), the gel-purified Py *Bgl*I-*Eco*RI restriction fragment (50 ng, 17 μl) was denatured by boiling for 5 min, and after briefly chilling on ice, 5 μl of OLB (250 mM Tris hydrochloride [pH 8.0], 25 mM $MgCl_2$, 100 μM each dTTP and dGTP, 1 M HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 6.6], 1 mg of pd(N)$_6$ [sodium salt] per ml), 1 μl of bovine serum albumin (10 mg/ml), 2.5 μl each of [α-^{32}P]dC and dATP (300 Ci/mmol), and 2 U of large-fragment DNA polymerase I (Klenow polymerase) were added. Incubation was at room temperature for 3 h. Unincorporated nucleotides were removed by column chromatography over Sephadex G-75 with 0.1 M NaCl–10 mM Tris–1 mM EDTA (pH 7.5).

S1 nuclease mapping. Single-stranded, 3'-end-labeled DNA probes were prepared as described previously (18). Total poly(A)$^+$ RNA (2 μg) with 40 μg of yeast RNA carrier was annealed to 20,000 cpm of each of the probes, and the resulting hybrids were treated with S1 nuclease as described previously (29). The S1-resistant products were fractionated on a 50% (wt/vol) urea–4% polyacrylamide gel.

cDNA synthesis. Oligo(dT)-primed first-strand synthesis was initiated by adding 10 μl of oligo(dT) (1 mg/ml), 5 μl of Tris hydrochloride (1 M, pH 8.3 at 42°C), 5 μl of $MgCl_2$ (0.2 M), and 14 μl of KCl (1 M) to 10 μg of 53-Rat poly(A)$^+$ RNA in 32 μl. After the mixture was heated to 65°C for 10 min and briefly chilled on ice, the following were added: 1 μl of dithiothreitol (1 M), 2.5 μl of RNasin, 5 μl each of [α-^{32}P]dC and dATP (3,000 Ci/mmol), 2.5 μl each of dC and dATP (25 mM), 5 μl each of dG and dTTP (25 mM), and 5 μl of super avian myeloblastosis virus reverse transcriptase. Reverse transcription was performed for 2 h at 42°C, after which time unincorporated nucleotides were removed by Sephadex G-75 chromatography with 100 mM NaCl–0.1% sodium dodecyl sulfate in 10 mM Tris–1 mM EDTA (pH 7.5). Labeling the cDNA to a relatively low specific activity in this way provides a useful means to detect small quantities of product at later stages. A small sample of the reverse-transcribed product was run out on a sequencing-type gel to assess its average size. Dextran (1 μg) was added as carrier, and the nucleic acid was precipitated with ethanol after the addition of ammonium acetate to 2 M. The samples were resuspended in 2 M ammonium acetate, reprecipitated, and washed with 70% (vol/vol) ethanol before further use.

Second-strand synthesis was performed by the method of Gubler and Hoffman (14) in SSS buffer (20 mM Tris chloride [pH 7.4], 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 100 mM KCl, 0.15 mM β-NAD, 40 μM each deoxynucleoside triphosphate, 50 μg of bovine serum albumin), using 8.5 U of *Escherichia coli* RNase H per ml, 230 U of DNA polymerase I per ml, and 10 U of *E. coli* ligase per ml. Reaction mixtures were incubated at 12°C for 60 min, followed by 22°C for 60 min. Reactions were stopped by adding EDTA to 20 mM and sodium dodecyl sulfate to 0.1% (wt/vol) final concentrations. Samples were extracted twice with phenol and once with chloroform and ethanol precipitated twice in the presence of 2 M ammonium acetate.

The double-stranded cDNA was suspended in T4 DP buffer (33 mM Tris-acetate [pH 7.9], 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, 0.1 mg of bovine serum albumin per ml) and treated with T4 DNA polymerase for 5 min at 37°C. Subsequently, deoxynucleoside triphosphates were added to a final concentration of 200 μM, and the incubation was continued at 37°C for a further 30 min. Samples were extracted with phenol and then chloroform and ethanol precipitated in the presence of 2 M ammonium acetate.

*Eco*RI linkers were then ligated to the repaired cDNA, and excess linkers were removed with *Eco*RI. cDNA was purified away from linkers by agarose gel electrophoresis and size selected to be greater than 70 base pairs in length. The cDNA was then cloned into the *Eco*RI site of λgt10 (15) and screened by hybridization to a random-primed Py probe. cDNA inserts of interest were all subsequently subcloned into M13mp18 or -19 and sequenced.

DNA sequence analysis. DNA sequence analysis was performed by the dideoxy chain termination method with recombinant M13 templates grown in *E. coli* JM103 as described previously (1), with the modification that templates were protein extracted twice with 10 mM Tris–1 mM EDTA (pH 7.5)-saturated phenol and once with chloroform before use.

RESULTS

Northern analysis of Py early region mRNAs utilizing the major or alternative poly(A) site. The sizes and amounts of Py early region transcripts utilizing either the alternative or the major poly(A) site (Fig. 1) were assessed at different times during the lytic cycle after infection of mouse 3T6 cells with the A2 strain of Py. The relative abundance and overall splicing pattern of early region mRNAs were initially determined by Northern blotting (Fig. 2). Early region mRNA from the Py-transformed cell line 53-Rat, which exclusively

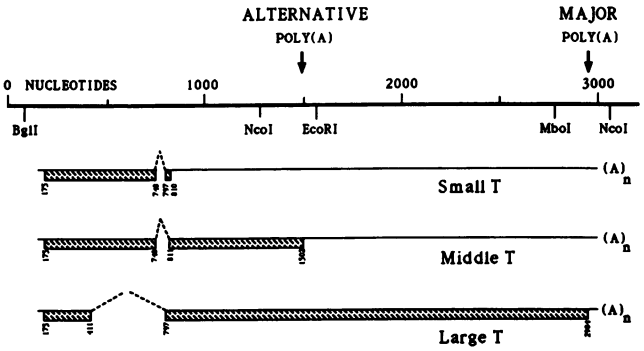

FIG. 1. A linearized representation of the Py early region showing the positions of the known differential RNA-processing sites. Nucleotide numbering is adapted from Griffin et al. (13), taking into account the differences in sequence described by Deininger et al. (6). The three differentially spliced predominant early mRNAs encoding small, middle, and large T-Ags (coding regions boxed) are aligned with the map. The positions of the major and alternative poly(A) sites are indicated, along with the restriction sites used in this study.

utilizes the alternative poly(A) site, was analyzed in parallel for comparison (Fig. 2). This Northern analysis gave rise to two main points of interest. One is the time course of appearance of the Py RNA utilizing the alternative poly(A) site; RNA utilizing the alternative site was only detected at relatively late times after infection, accounting for about 15% of the early mRNAs at 22 and 26 h. The failure to detect alternatively poly(A)$^+$ species at early times may result, in part at least, from the relatively low total signal.

A second point of interest concerns the splicing pattern of the early region mRNAs polyadenylated at the two different sites. At 22 h the ratios of small-plus-middle T-Ag spliced mRNA to large T-Ag spliced mRNA were 0.5 for the major poly(A) site and 1.3 for the alternative poly(A) site (Fig. 2). Thus, the splicing pattern associated with the alternative site would, in comparison with that of the major site, favor the production of small or middle T-Ag or both, with relatively less of the large T-Ag splice, which would not give rise to a known protein product, being produced. As the alternative poly(A) site occurs in the middle of the large T-Ag coding region (Fig. 1), no preexisting translational termination codon exists in the large T-Ag reading frame before the end of the large T-Ag spliced mRNA. This is consistent with the fact that no large T-Ag-related proteins have been reported from the translation of the alternatively spliced large T-Ag mRNAs, either in vivo or in vitro, from the lytic cycle or from 53-Rat cells (16, 17).

3′-End heterogeneity of early region mRNAs polyadenylated at the alternative poly(A) site. The positions of cleavage-poly(A) at the major and alternative poly(A) sites were initially investigated by 3′ S1 mapping (Fig. 3). The 289-nucleotide (nt) *Mbo*I-*Nco*I fragment (Py nts 2771 to 3060) was protected to give a single band of 171 ± 1 nt, showing poly(A) of mRNAs at the major site to be at a single position at Py nt 2942 ± 1. This is 17 ± 1 nts downstream from the AATAAA consensus sequence (Fig. 4). In contrast to the homogeneity at the major site, heterogeneous 3′ ends were detected in mRNAs polyadenylated at the alternative

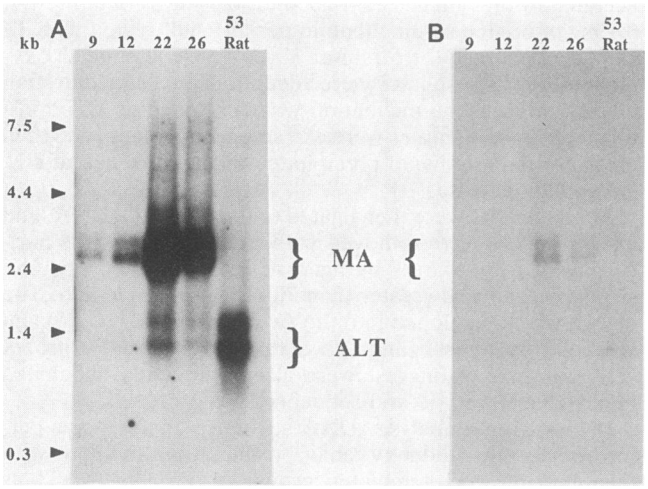

FIG. 2. Py early region mRNAs expressed during lytic infection of mouse 3T6 cells and in the Py-transformed cell line 53-Rat (17). (A) Total poly(A)$^+$ RNA (2 μg) from cells harvested at 9, 12, 22, and 26 h after infection of 3T6 cells with Py A2 or from 53-Rat cells was subjected to 1% agarose–formaldehyde gel electrophoresis. After blotting onto nitrocellulose, the RNAs were probed with a *Bgl*I-*Eco*RI fragment of the Py early region corresponding to nts 87 to 1562, which was labeled to high specific activity by random oligonucleotide priming. Early region mRNAs cleaved at the major poly(A) site (MA) and the alternative site (ALT) each appear as a doublet. Within each doublet, the upper band corresponds to the sum of species spliced in the small and middle T-Ag reading frames, whereas the lower band reflects splicing in the large T-Ag reading frame. Py mRNAs from 53-Rat cells are almost exclusively polyadenylated at the alternative site (16). Size markers were provided by an RNA ladder (Bethesda Research Laboratories). kb, Kilobase. (B) A shorter exposure of panel A showing the splicing pattern of the major species present at 22 and 26 h postinfection.

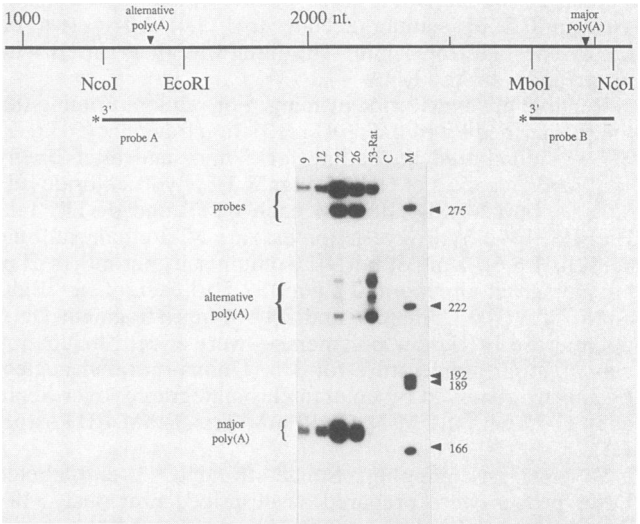

FIG. 3. High-resolution 3′ S1 mapping of Py early mRNAs. End-labeled probes A and B span the alternative and major poly(A) sites, respectively. RNAs [2 μg of poly(A)$^+$] from Py A2-infected mouse 3T6 cells harvested at 9, 12, 22, and 26 h postinfection and from 53-Rat cells both give a triplet of bands corresponding to alternatively poly(A)$^+$ mRNA 3′ ends at Py nts 1495 ± 1, 1506 ± 1, and 1514 ± 1. Transcripts utilizing the major early poly(A) site give a single band which corresponds to mRNA 3′ ends at Py nt 2942 ± 1 (see text). Note that the major site is absent from the actively transcribed early region in 53-Rat. A negative control (C) was provided by annealing to 40 μg of yeast RNA alone. Size markers in nucleotides (M) were end-labeled *Dde*I fragments of a *Bam*HI linear clone of Py in the plasmid vector pAT153.

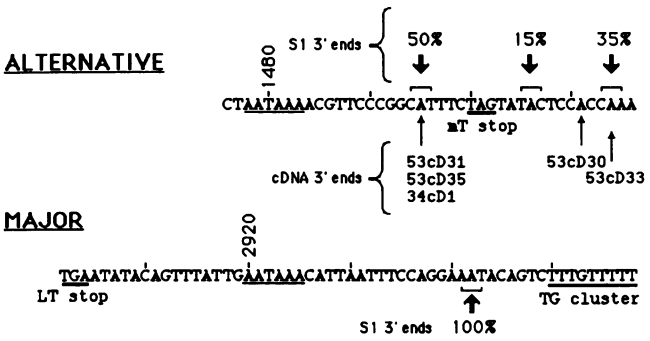

FIG. 4. Positions of cleavage-poly(A) sites in 53-Rat cDNAs. Oligo(dT)-primed cDNAs were synthesized from 53-Rat total poly(A)$^+$ RNA and were cloned into λgt10. In the upper half of the figure the positions of cleavage-poly(A) for the five clones sequenced are shown in relation to the AATAAA consensus sequence and the TAG middle T-Ag translational termination codon. The positions and relative abundances of 3′ ends determined by densitometric scanning of 3′ S1 mapping data are indicated above the sequence. The lower half of the figure shows the deduced position of cleavage-poly(A) at the major site, in relation to the AATAAA consensus and TGA stop codon in the large T-Ag reading frame.

poly(A) site (Fig. 3). Identical heterogeneous 3′ ends were also found for mRNAs from 53-Rat in which the early region transcripts almost exclusively utilize the alternative poly(A) site (Fig. 3). Protection of the 287-nt *Nco*I-*Eco*RI probe (Py nts 1279 to 1566) gave three predominant fragments of 216 ± 1, 227 ± 1, and 235 ± 1 nts, mapping the 3′ ends of RNAs utilizing the alternative poly(A) signal to three predominant positions (Fig. 3). About 50% is polyadenylated 12 ± 1 bases downstream from the AATAAA at Py nucleotide 1495 ± 1, 15% is polyadenylated at Py nucleotide 1506 ± 1, and 35% is polyadenylated at Py nucleotide 1514 ± 1 (Fig. 4). These proportions did not vary between mRNAs harvested at 22 or 26 h and mRNA from 53-Rat cells.

This method for mapping sites of poly(A) may be slightly imprecise since it is known that S1 can digest away (nibble) a few nucleotides past the true end of an RNA-DNA heteroduplex. Consequently, sequence analysis of the site of poly(A) of cDNAs derived from early region mRNAs utilizing the alternative poly(A) site was undertaken to determine the exact locations of the poly(A) tracts. Since the pattern of alternatively poly(A)$^+$ mRNAs in the Py lytic cycle was identical to that observed with 53-Rat, as judged by S1 mapping (Fig. 3), this transformed cell line was used as a convenient source of mRNAs for the cDNA cloning. The results of sequence analysis of the points of poly(A) of five independent cDNA clones are shown in Fig. 4 and confirm that the S1 result is not artifactual. Three cDNA ends were located at Py nt 1495, and one was located at Py nt 1514; these are the two major sites identified by S1 mapping. The fifth cDNA end, at Py nt 1511, corresponds to one of the minor heterogeneous ends detected by S1 mapping (Fig. 3). Thus, both S1 mapping and cDNA analyses show that about 50% of the mRNAs utilizing the alternative poly(A) site are polyadenylated upstream from the middle T-Ag stop codon (Fig. 4) and presumably are not capable of directing the translation of a functional middle T-Ag protein.

DISCUSSION

The Py early region contains two poly(A) signals, the major and alternative signals. The use of these signals by different Py early region mRNAs at different times during the lytic cycle in mouse 3T6 cells has been measured. In addition, the location of the site(s) of poly(A) directed by each signal has been determined.

The major poly(A) site is used by at least 85% of the Py early region mRNAs. About twice as many major site poly(A)$^+$ mRNA molecules contain the large T-Ag splice as contain the small and middle T-Ag splices (Fig. 2). The environment of the major poly(A) site is similar in its layout to a wide variety of viral and cellular poly(A) sites, with cleavage-poly(A) occurring at a single site (Fig. 3) 17 ± 1 nts (Py nt 2942 ± 1) downstream from the AATAAA consensus sequence (24) and just upstream from a T+G-rich element (20) (Fig. 4).

The alternative poly(A) site is used by up to 15% of the Py early mRNAs. This maximal level of utilization was observed at later times (22 to 26 h postinfection) in the cycle; at earlier times (9 to 12 h postinfection) alternative poly(A) was not detected. The ratio of small and middle T-Ag splices to large T-Ag splices for the alternatively poly(A)$^+$ mRNAs is about 2.5 times that observed for mRNAs utilizing the major site (Fig. 2). The sites of poly(A) governed by the alternative signal are heterogeneous (Fig. 3), occurring at at least three distinct sites between Py nts 1495 and 1514 (Fig. 4). It is not known if this heterogeneity reflects the absence of a T+G-rich element downstream from the signal. Heterogeneity with respect to the site of poly(A) governed by a single poly(A) signal is unusual but has been described for a few other transcription units (25, 26, 30).

Whereas the major poly(A) signal is located downstream from the large T-Ag reading frame stop codon at the end of the Py early region, the alternative poly(A) signal is located in the middle of the early region upstream from the stop codon of the middle T-Ag reading frame (Fig. 4). Thus, none of the alternatively poly(A)$^+$ mRNAs could produce a functional large T-Ag; it would not be expected that a truncated form of large T-Ag would be efficiently translated as no preexisting stop codon exists in the large T-Ag reading frame before the ends of the alternatively poly(A)$^+$ RNAs. Interestingly, poly(A) at Py nt 1506 would result in the creation of a TAA stop codon in the large T-Ag reading frame, and such an mRNA could code for a truncated large T-Ag of 35 kilodaltons. It would be expected that such a protein might be present only in small amounts, as S1 mapping suggests that only 15% of the alternatively poly(A)$^+$ mRNAs are polyadenylated at Py nt 1506 and this site was not detected by the cDNA analysis (Fig. 4). In addition the alternatively poly(A)$^+$ mRNAs contain less of the large T-Ag splice in comparison with RNAs cleaved at the major site (Fig. 2).

The biological consequences of these results are that while all alternatively poly(A)$^+$ species could, if appropriately spliced, direct the synthesis of small T-Ag, only the 50% that are poly(A)$^+$ downstream from the middle T-Ag stop codon could encode middle T-Ag. Transcripts cleaved at the alternative poly(A) site and spliced in the large T-Ag reading frame do not appear to give rise to a truncated large T-Ag-related protein; by analogy, transcripts spliced in the middle T-Ag frame and polyadenylated at nt 1495 would not be expected to direct the synthesis of a truncated middle T-Ag. It will be interesting to discover whether or not cleavage at one or the other of the alternative positions is associated with a particular combination of splice sites.

Although no function has yet been ascribed to middle T-Ag through studies of the lytic cycle in in vitro tissue culture systems, this does not appear to be the case for viral replication in vivo in the animal. Sequences spliced out of

the large T-Ag mRNA but retained in the mRNAs for small and middle T-Ags have been shown to be essential for viral growth in the mouse (19). Indeed, it is difficult to imagine why the virus should conserve the middle T-Ag reading frame if the protein has no function in its life cycle in the mouse. A possible explanation for the high degree of DNA sequence homology between the alternative poly(A) site of Py and the corresponding region of the hamster papovavirus (7) is that the AATAAA sequence encodes important amino acids of both the middle and large T-Ag proteins, in overlapping reading frames. This situation may constitute an evolutionary trap, in that the possibilities for divergence at the level of DNA sequence may be very limited by a requirement to conserve the two protein sequences. A much more attractive hypothesis would be that small and middle T-Ag synthesis might be regulated in vivo by control of poly(A) at the alternative site. Further studies of the use of the alternative poly(A) site during virus growth in the mouse are required to support this hypothesis.

ACKNOWLEDGMENTS

We thank Clive Dickson and Nic Jones for helpful discussions during the preparation of this manuscript.

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