Requirement of A-A-U-A-A and adjacent downstream sequences for SV40 early polyadenylation

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ABSTRACT

RNA mapping experiments and chloramphenicol acetyltransferase assays were used to analyze polyadenylation in COS cells of transcripts from derivatives of pSV2-neo and pSV2-cat, in which the SV40 early poly(A) signal has been modified. Neither the sequence A-A-U-A-A nor the sequences located immediately downstream from it in the SV40 early gene appear to function by themselves as a poly(A) signal. When combined, however, these two elements form a poly(A) signal whose efficiency and specificity closely resemble those of the wild type signal. The addition of six nucleotides between the A-A-U-A-A sequence and the poly(A) site has no detectable effect on the efficiency or site of polyadenylation. Deletion of the 60 nucleotides immediately upstream from the hexanucleotide also has no detectable effect on polyadenylation. Therefore, A-A-U-A-A and sequences downstream from it appear to be sufficient for SV40 early polyadenylation.

INTRODUCTION

The biosynthesis of functional mRNA in eukaryotic cells involves complex post-transcriptional modifications which include 5' capping, internal methylation, removal of intervening sequences, and polyadenylation at the 3' end. The formation of the mRNA 3' end involves endonucleolytic cleavage of a larger precursor followed by the addition of 50 to 200 adenylate residues (1,2). An important question regarding the process of polyadenylation is the elucidation of sequences required for proper cleavage of the primary transcript.

The highly conserved sequence A-A-U-A-A, which usually appears 10-30 nucleotides upstream from the cleavage site (3), has been shown to be necessary for 3' end formation (4-8). Since this sequence can be found at sites other than mRNA 3'ends, additional sequences must be required for polyadenylation. Lai et. al. (9) identified conserved sequences downstream from the poly(A) site, which McLauchlan et. al. (16) fitted to the consensus sequence Y-G-T-G-T-T-Y-Y (Y = C or T). Numerous recent studies have shown that se-

quences located downstream from the hexanucleotide A-A-U-A-A-A are necessary for cleavage and/or polyadenylation, since deletion of these sequences markedly reduces the efficiency or shifts the site of poly(A) addition (10-21). In the case of the SV40 early polyadenylation signal, at least 18 nucleotides downstream from the cleavage site are required for efficient and accurate polyadenylation (18,20).

In this report, transient expression analyses in transfected COS cells were used to test whether the sequences located downstream of the A-A-T-A-A-A of SV40 early can restore polyadenylation activity to a clone that contains a synthetic hexanucleotide but lacks 60 nucleotides of upstream sequences. No polyadenylation was observed when the SV40(E) hexanucleotide and sequences upstream from it were deleted, or when the entire wild type poly(A) signal was replaced by a sequence containing a synthetic A-A-T-A-A-A. However, polyadenylation is restored when the synthetic A-A-T-A-A-A and the sequences that follow the hexanucleotide in SV40 early are combined. The data also show that deletion of sequences upstream from the A-A-T-A-A-A has no detectable effect on polyadenylation. Finally, an insertion of 6 bases between the poly(A) addition site and the A-A-T-A-A-A does not alter the fidelity of the cleavage/polyadenylation reaction.

MATERIALS AND METHODS

Cells

COS cells (22) were maintained in Dulbecco's modified Eagle's medium (GIBCO), 10% fetal calf serum (KC Biological, Lenexa, KS), and 5% CO₂ in air at 37°C. Recombinant plasmids were grown in *Escherichia coli* RR1, except for pUC8 (23) which was grown in TB1, an *r_k*⁻ strain of JM83 (24).

Enzymes and Oligonucleotides

Restriction endonucleases and modifying enzymes were obtained from New England Biolabs or Bethesda Research Laboratories. S1 nuclease was purchased from Bethesda Research Laboratories. Oligonucleotides were synthesized by OCS Labs, Denton, TX.

Construction of Plasmids

pSVN-2, pSV2-neo Δ 109, and pSVN-3 are derivatives of pSV2-neo (25) (Fig. 1A). First, XhoI linkers were added at the HpaI site of pSV2-neo (position 4848), 82 bp upstream from the SV40 early poly(A) site, and at the HindIII site of pUC8 (23). To generate pSVN-1, the 882 bp XhoI-EcoRI fragment from pSV2-neo, which contains the SV40 early poly(A) site, was replaced with a 36 bp XhoI-EcoRI segment containing the polycloning sites of pUC8.

Second, pSVN-2 was constructed by insertion of the 12 bp oligonucleotide

between the XhoI and Sall sites in the polycloning region of pSVN-1.

pSV2-neo Δ 109 was constructed from pSV2-neo by Bal 31 digestion from the HpaI site at 4848 (Fig.1A) and insertion of a XhoI linker at the deletion endpoint. These endpoints are 42 bp upstream of the HpaI site (-124) and 67 bp downstream of the HpaI site (-15). The hybrid clone pSVN-3 was constructed by insertion of the 820 bp XhoI-EcoRI fragment from pSV2-neo Δ 109 between the Sall and EcoRI sites of pSVN-2.

In order to utilize chloramphenicol acetyltransferase activity as an alternative assay of polyadenylation, the neo region of pSVN-2, pSV2-neo Δ 109, and pSVN-3 was replaced by the cat region of pSV2-cat (26). This was done by inserting a XhoI linker at the HpaI site of pSV2-cat, forming pSV2-cat-1, followed by exchange of the HindIII-XhoI fragment of this plasmid with the corresponding fragment of pSVN-2, pSV2-neo Δ 109, and pSVN-3. The resultant clones are pSVC-2, pSV2-cat Δ 109 and pSVC-3, respectively. Transfection of COS cells with pSV2-cat-1 yielded the same CAT activity as transfection with pSV2-cat (data not shown).

Transfections and CAT Assays

Transfections were performed according to the DEAE-dextran procedure of Luthman and Magnusson (27) except the chloroquine treatment was omitted. For each transfection, 2.8 μ g of DNA were added per 150 mm plate and 1.0 μ g per 90 mm plate. CAT activity was measured in COS cell extracts as described by Gorman et al. (26). Protein concentration in the extract was determined by the method of Lowry et al. (28).

RNA Preparation

Total cell RNA was prepared 48 hr after transfection by the guanidinium isothiocyanate procedure of Chirgwin et al. (29) and the centrifugation method of Glisin et al. (30). Total cell RNA was separated into poly(A)⁺ and poly(A)⁻ fractions by oligo(dT)-cellulose chromatography (31).

S1 Nuclease Mapping

Restricted DNA fragments were labeled at their 3' or 5' ends according to Maniatis et al. (32). To obtain probes labeled at only one strand, labeled DNAs were digested with a second restriction endonuclease, followed by purification from 1% agarose gels (33). Conditions for S1 nuclease mapping were as described by Favaloro et al. (34). The probe utilized in the S1 nuclease experiment depicted in Fig. 3 was purified from a 12% acrylamide-urea sequencing gel and eluted as described by Maxam and Gilbert

C.

Figure 1A. Map of pSV2-neo (5729 bp). Sequences are derived from SV40 (open boxes), the neo gene of Tn5 (closed box), and pBR322 (solid line). Numbering begins at the EcoRI site. The 5' end, poly(A) site, and intervening sequence of the early orientation transcript are shown.

Figure 1B. Schematic Diagram of pSV2-neo, pSVN-2, pSV2-neoΔ109, and pSVN-3. The plasmid pSVN-2 contains a 30 bp oligonucleotide containing the sequence A-A-T-A-A-A at position -75 in place of the 882 bp HpaI-EcoRI fragment of pSV2-neo. pSV2-neoΔ109 is a bidirectional deletion mutant which lacks 109 bp (42 bp upstream and 67 downstream from the HpaI site of pSV2-neo) and contains a XhoI linker at the deletion end point. pSVN-3 contains the XhoI-EcoRI fragment of pSV2-neoΔ109 cloned immediately 3' to the A-A-T-A-A-A sequence in pSVN-2 at position -15. The junction between the SalI site of pSVN-2 and the XhoI site of pSV2-neoΔ109 at position -15 is depicted as S/X.

Figure 1C. Nucleotide Sequences Surrounding the Poly(A) Sites of pSV2-neo, pSVN-2, pSV2-neoΔ109 and pSVN-3. Synthetic oligonucleotides are underlined. A-A-T-A-A-A's are indicated by the filled boxes above thesequence, and the SV40 early downstream element as defined by Hart et al. (18,20) is indicated by the stippled boxes above the sequence. The A residue of the SV40 early poly(A) addition site defines position +1 and is indicated by the arrow.

(35). Conditions for S1 nuclease mapping of short fragments were according to McDevitt et al. (12). The DNA sequencing ladder in Fig. 3 was obtained by nucleotide sequencing of the 3' end labeled probe according to Maxam and Gilbert (35).

RESULTS

Previous studies have established that SV40 early polyadenylation requires sequences that reside immediately downstream of the poly(A) site (15,18,20). Recent studies by Hart et al. (18,20) have defined a critical element located within the first 18 nucleotides downstream from the poly(A)

site. Deletion of these sequences reduced the frequency of cleavage at the wild type site to about 5%.

To test whether the A-A-U-A-A and sequences downstream from it are sufficient to form a functional poly(A) addition signal, the clones pSVN-2, pSV2-neoΔ109, and pSVN-3 were constructed from pSV2-neo (Fig.1A). Early orientation transcripts of pSV2neo initiate at nucleotide 2580, proceed through the neo gene and are polyadenylated at the SV40 early poly(A) site at 4929 (25). In this paper, the A residue at 4930, immediately after the cleavage site, is defined as +1 (Fig. 1C). The -82 to +799 fragment from pSV2-neo, which contains the SV40 early poly(A) site and neighboring regions, was replaced by a synthetic oligonucleotide containing an A-A-T-A-A-A to form pSVN-2 (Figs. 1B, C). This plasmid also lacks 60 bp of sequences which normally reside upstream of the distal A-A-T-A-A-A of the SV40 early signal (Fig. 1C). The plasmid pSV2-neoΔ109 was generated from

Figure 2. S1 Nuclease Mapping of 3' Ends. RNA was prepared from COS cells 48 hr after transfection with pSVN-2 and pSVN-3. 20 μ g of yeast tRNA (Y), 20 μ g of total RNA (T), 2 μ g of poly A⁺ RNA (A⁺) or 20 μ g of poly A⁻ RNA (A⁻) were hybridized to the indicated probes at 42°C. S1 nuclease digestions were at 25°C and protected fragments were analyzed in 5% polyacrylamide-urea sequencing gels. The probe itself (P) was electrophoresed and size markers (M) were ϕ X174 DNA digested with HaeIII and 3' end labeled. **PANEL A:** Probe was the 601 bp RsaI-XmnI fragment of pSVN-2 that was 3' labeled at the RsaI site. The bracket next to the gel indicates the region where a band would be expected if the A-A-U-A-A-A of pSVN-2 was functional as a poly(A) signal. **PANEL B:** Probe was the 256 bp RsaI-BamHI fragment from pSVN-3 that was 3' labeled at RsaI. In lane 14, total RNA prepared from COS cells after co-transfection with equimolar amounts of pSV2-neo and pSVN-3 was analyzed. The band at 205 nucleotides maps the poly(A) site of pSVN-3 RNA and the band at 174 nucleotides maps the discontinuity between pSV2-neo RNA and the probe.

pSV2-neo by bidirectional deletion with nuclease Bal 31 from the HpaI site. This plasmid lacks 42 bp upstream and 67 bp downstream from the HpaI site (-124 to -15 in pSV2-neo, Figs. 1B, C). Both A-A-T-A-A's of the SV40 early sequence (located at -51 to -46 and -22 to -17) were deleted, but all sequences following the distal A-A-T-A-A were retained. To construct the hybrid plasmid pSVN-3, the entire region downstream from the distal A-A-T-A-A was removed from pSV2-neo Δ 109 as a XhoI-EcoRI fragment, and placed adjacent to the synthetic A-A-T-A-A of pSVN-2 (between the SalI and EcoRI sites). The spacing between the A-A-T-A-A and SV40 downstream sequences is increased by 6 bp as a result of the SalI/XhoI junction at position -15 (Fig. 1B, C). This plasmid, as does pSVN-2, lacks 60 bp of SV40 wild type sequences located upstream of the A-A-T-A-A. The nucleotide sequence in the region surrounding the poly(A) signal of each of the constructions is shown in Fig. 1C. RNA isolated from cells transfected with these plasmids show correct 5' ends at the SV40 early initiation site as determined by S1 nuclease mapping (data not shown).

S1 Nuclease Analysis of 3' Ends

To map the 3' ends of transcripts generated from pSVN-2 and pSVN-3, COS cells were transfected with each of the plasmids and RNA prepared 48 hours later. DNA probes for S1 nuclease mapping were prepared from each plasmid as described in materials and methods. No defined 3' ends were observed in total, poly(A)⁺, or poly(A)⁻ pSVN-2 RNAs (Fig. 2A, lanes 3-5). This indicates, as expected, that the sequence A-A-U-A-A by itself does not function as a poly(A) signal. The same result is observed with pSV2-neo Δ 109 (data not shown), indicating that sequences located downstream from the distal A-A-U-A-A are not sufficient to serve as a poly(A) signal. In the case of pSVN-3 RNA, a band mapping at 205 ± 5 nucleotides is observed in both total and poly(A)⁺ RNAs (Fig. 2B, lanes 9 and 10). This indicates that pSVN-3 transcripts are cleaved and the poly(A) tract is added 205 ± 5 nucleotides from the position of the label at the Rsa I site. In the construct pSVN-3, the wild type site of poly(A) addition is located 207 nucleotides from the Rsa I site. Thus, RNA derived from cells transfected with pSVN-3 contain a polyadenylated 3' end that maps to within a few nucleotides of the wild type site.

Comparison of Steady-State mRNA Levels

Analysis of RNA from cells transfected with equimolar amounts of pSV2-neo and pSVN-3 revealed two bands of equal intensity (Fig. 2B, lane 14). The band at 174 nucleotides maps the discontinuity between pSV2-neo RNA and the probe, which was generated from pSVN-3 DNA. The band at 205

Table I
Chloramphenicol Acetyltransferase Activity of Transfected COS Cells

<u>Plasmid</u>	<u>Specific Activity</u>	<u>Relative Activity</u>
pSV2-cat	0.93	1.00
pSV2-cat Δ 109	0.21	0.23
pSVC-2	0.23	0.25
pSVC-3	0.87	0.94

COS cells were transfected for 48h with the plasmids indicated and extracts were prepared in triplicate. Assays contained 50 μ g of protein and a time course (0-40 min) was followed for each extract. Specific activity is expressed as nmoles chloramphenicol acetylated per min per mg protein. The standard for the relative activity calculation is the pSV2-cat value.

nucleotides maps the poly(A) site of pSVN-3 RNA. The probe for this experiment was end-labeled, and the observed bands were similar in size and base composition. Therefore, no difference in the steady-state level of pSV2-neo and pSVN-3 RNA is apparent.

To confirm this observation by an alternative assay, the poly(A) signal of pSV2-cat(26) was replaced by the poly(A) signals of pSV2-neo Δ 109, pSVN-2 and pSVN-3, generating pSV2-cat Δ 109, pSVC-2 and pSVC-3, respectively. Cells transfected with pSV2-cat Δ 109 or pSVC-2, which contain defective poly(A) signals, exhibited chloramphenicol acetyltransferase (CAT) activities that were 23-25% of that observed in cells transfected with pSV2-cat (Table 1). The reduced, but significant level of CAT activity reflects the formation of polyadenylated RNA at cryptic sites in flanking pBR322 sequences (11, 42) and may also reflect the translation of non-polyadenylated transcripts. In contrast, cells transfected with pSVC-3, which contains the reconstructed poly(A) signal, exhibited the same level of CAT activity as cells transfected with pSV2-cat (Table 1). Therefore, transfection of COS cells with pSVC-3 results in the accumulation of as much translatable mRNA as transfection with pSV2-cat.

Assuming that transcripts polyadenylated at the intact SV40 early poly(A) site or the reconstructed poly(A) site do not differ significantly in stability, the co-transfection and CAT assay data strongly suggest that the wild-type and reconstructed poly(A) signals function with equal efficiency.

Precise Mapping of the pSVN-3 Poly(A) Site

To determine if the poly(A) site of pSVN-3 RNA indeed maps to the wild type site, a more precise mapping experiment was done with a shorter DNA

Figure 3. Precise Mapping of the pSVN-3 Poly(A) Site. 30 μ g of yeast tRNA (lanes 1-4), and 30 μ g of total pSVN-3 RNA (lanes 5-8) were hybridized to a 86 nucleotide fragment of pSVN-3 DNA that was 3' end labeled at the XhoI site, digested with BamHI, and purified as a single-stranded probe. Hybridizations were performed at 25°C (a), 37°C (b), 42°C (c) and 52°C (d). Samples were treated with S1 nuclease according to McDevitt et al. (12) and analyzed in a 12% polyacrylamide-urea sequencing gel. The 86 bp XhoI-BamHI fragment was sequenced according to Maxam and Gilbert (35) to serve as size markers (lanes 9-12). The upper strand of the sequence represents the coding strand and has the same sequence as the mRNA. The lower strand, whose sequence appears on the gel, is the template strand. The A-A-T-A-A-A sequence, the wild type SV40 early poly(A) site (36), and the downstream element as defined by Hart et al. (18, 20) are underlined.

probe and using its nucleotide sequence as size markers. Reddy et al. (36) demonstrated that cleavage of SV40 early RNA occurs after the C residue located 16 nucleotides downstream from the A-A-U-A-A-A (Fig. 1C). However, the primer extension assay that was used could not differentiate whether the A residue that follows the C comes from the poly(A) tract or the actual

transcript. Therefore, the SV40 early poly(A) site is located either 16 or 17 nucleotides from the A-A-U-A-A-A.

When the hybridization temperature was 37°C, the poly(A) site of pSVN-3 RNA maps to a C residue located 26 nucleotides from the A-A-U-A-A-A (37 nucleotides from the XhoI site) (Fig. 3, lane 7). A doublet of bands, which maps to the A and U residues located 23 and 24 nucleotides from the A-A-U-A-A-A (34 and 35 nucleotides from the XhoI site), is observed when the hybridization temperature was 42°C or 52°C (Fig. 3, lanes 5 and 6). These results show that the poly(A) site in pSVN-3 RNA maps to or within 3 nucleotides downstream of the wild type site (36). If the hybridization temperature was 25°C, a ladder of bands occurs below the C residue that is located 28 nucleotides from the XhoI site (Fig. 3, lane 8). This ladder also appears when yeast RNA was analyzed as a control (lane 4) and is an S1 mapping artifact. Hence, cleavage and polyadenylation of pSVN-3 RNA occurs at, or immediately downstream from, the SV40 poly(A) site, even though the spacing between the A-A-U-A-A-A and the wild type site was increased by six nucleotides.

DISCUSSION

The data suggest that neither the sequence A-A-U-A-A-A nor the sequences located downstream from it can by themselves function as a poly(A) signal. However, a functional poly(A) signal is reconstituted when the A-A-U-A-A-A and the downstream sequences are combined. Previous studies have demonstrated that the conserved hexanucleotide A-A-U-A-A-A is required for polyadenylation. If this sequence is altered by deletion or point mutations, polyadenylation is virtually abolished (4,7). However, this sequence cannot be the only determinant for polyadenylation because many A-A-U-A-A-A sequences located within transcripts molecules are not utilized. For example, the chicken ovalbumin gene has eight hexanucleotides, but only the most 3' one is utilized as part of a poly(A) signal (37). In addition, mRNAs with alternative 3' ends, such as those that encode membrane-bound or secreted immunoglobulin μ chains (38), result from differential cleavage of primary transcripts at either of two poly(A) sites (41). Each poly(A) site is located 10-20 nucleotides downstream from an A-A-U-A-A-A. Therefore, the A-A-U-A-A-A sequence by itself cannot specify the cleavage site because there would be no capability for differential poly(A) selection.

The lack of discrete 3' ends in pSVN-2 RNA (Fig. 2A) and pSV2-neo Δ 109 RNA (data not shown), as well as the measurement of baseline CAT activity in

cells transfected with pSVC-2 and pSV2-cat Δ 109 (Table I), indicate that little or no polyadenylation occurs in transcripts from these DNAs. Although the possibility of negative effects from neighboring sequences in these constructs cannot be eliminated, the data suggest that neither the hexanucleotide, nor the sequences downstream from it in SV40 early, can by themselves specify a poly(A) signal. However, in the constructs pSVN-3 and pSVC-3, these two elements combine to form a functional poly(A) signal whose efficiency and specificity resemble those of the wild type signal. This is demonstrated by the equivalent CAT activity of cells transfected with pSVC-3 and pSV2-cat (Table I) and by the equal intensities of the pSV2-neo and pSVN-3 bands in the co-transfection experiment (Fig. 2B, lane 14). Moreover, the wild type site of polyadenylation is preserved, within experimental error, in pSVN-3 (Fig. 3). The regeneration of a functional poly(A) signal in pSVN-3 and pSVC-3 shows that both the A-A-U-A-A-A and the sequences downstream from it in SV40 early are needed in conjunction for polyadenylation. These results are in agreement with those of Conway *et al.* on the SV40 late poly(A) signal (17) and Hart *et al.* on the adenovirus E2A and SV40 early poly(A) signals (18,20). In all of these genes, deletion of downstream sequences inhibits polyadenylation.

The data also indicate that deletion of sequences upstream from the A-A-U-A-A-A has no detectable effect on polyadenylation, since pSVN-3 lacks 60 base pairs of SV40 sequences that are located upstream of the distal hexanucleotide. This result is consistent with the data of others. With an *in vitro* system that was deficient in cleavage activity, Manley *et al.* showed that removal of sequences upstream of the SV40 early distal A-A-U-A-A-A had no effect on addition of poly(A) to the 3' ends of RNA substrates (15). In the adenovirus E3A transcription unit, removal of portions of the region upstream of the variant hexanucleotide A-U-U-A-A-A had no effect on polyadenylation (21).

Earlier experiments, in which shifts in the SV40 late cleavage site were observed if nucleotides between the A-A-U-A-A-A and the wild type cleavage site were deleted, suggested that cleavage/polyadenylation preferentially occurred at a set distance from the hexanucleotide (4). However, we show that insertion of 6 nucleotides between the A-A-U-A-A-A and the SV40 early poly(A) site has no detectable effect on either polyadenylation efficiency or site selection. If selection of the cleavage/polyadenylation site was dependent mainly on the number of nucleotides from the A-A-U-A-A-A sequence, the poly(A) site of pSVN-3 RNA should have occurred 6 nucleotides

upstream of that observed. Base specificity therefore appears to be an important determinant in selection of the site of cleavage/polyadenylation. A similar result was reported by Wickens et al. (7). They showed that insertion of an 8 nucleotide linker midway between the A-A-U-A-A-A and the wild type cleavage site of the SV40 late signal also had no detectable effect on polyadenylation efficiency or site selection. The specificity of the cleavage site might involve sequences having homology to C-A-(C/U)-U-G, which frequently occur in regions adjacent to poly(A) sites (39,40). In SV40 early, the sequence C-A-C-U-G is located 6 nucleotides upstream from the poly(A) site (Fig. 1C), and in SV40 late, the sequence C-A-A-U-U-G is located 2 nucleotides downstream from the poly(A) site (40). For both cases in which nucleotides were inserted between the A-A-U-A-A-A and the poly(A) site, the relationship between the C-A-(C/U)-U-G sequence and the C-A at the poly(A) site was undisturbed.

The present results show that the sequences downstream from the SV40 early hexanucleotide can function with a synthetic A-A-U-A-A-A. Hart et al. (18) demonstrated that sequences located downstream of the poly(A) site in adenovirus E2A transcripts can function in place of those of SV40 early. These results, together with the observation that sequences upstream from the A-A-U-A-A-A appear not to be required, suggest that A-A-U-A-A-A and downstream sequence elements are sufficient for polyadenylation.

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