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A sequence downstream of A-A-U-A-A-A is required for formation of simian virus 40 late mRNA 3′ termini in frog oocytes

(mRNA processing/polyadenylylation)

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ABSTRACT **The 3′ terminus of simian virus 40 late mRNA is formed by nucleolytic cleavage of an mRNA precursor. In this report we show that efficient cleavage requires a sequence in the 3′ flanking region, "downstream" of the highly conserved A-A-U-A-A-A sequence. This sequence is not required for polyadenylylation itself or for accurately positioning the 3′ terminus that is formed.**

Formation of the 3′ terminus of many eukaryotic mRNAs involves post-transcriptional processing not transcription termination (1, 2). mRNA precursors are processed nucleolytically to generate a 3′ terminus to which polyadenylic acid is then added. The formation of the 3′ terminus (the polyadenylylation site) involves an endonucleolytic step (3) and so is referred to as cleavage. The enzymes responsible for cleavage have not been characterized and may include both endo- and exoribonucleases. Similarly, the primary cleavage site in the pre-mRNA is not known.

The highly conserved sequence, A-A-U-A-A-A, found near the polyadenylylation site of nearly all eukaryotic mRNAs (4, 5), is necessary for cleavage (5–9). A-A-U-A-A-A point mutations (5–7) or deletions (8) prevent the formation of mRNA 3′ termini. Although the A-A-U-A-A-A sequence is necessary for cleavage, it is not sufficient; the sequence is found in the middle of several genes, where no 3′ terminus is formed.

A small [220 base pair (bp)] region of simian virus 40 (SV40), spanning the polyadenylylation site of late mRNAs, is necessary and sufficient for cleavage and polyadenylylation in frog oocytes (5). All signals other than A-A-U-A-A-A required for cleavage must reside within those 220 bp (5). In this report, we describe an effort to identify those additional signals in which we tested defined portions of the 220-bp region for their ability to support cleavage in frog oocytes.

METHODS

Oocyte injection, RNA preparation and oligo(dT)-cellulose chromatography, and nuclease S1 mapping were carried out as described (5).

Plasmid Constructions. Deletion derivatives of pSVL−141/+79 (ref. 5; see map in Fig. 1) were constructed by treating that plasmid with either *Hin*dIII (3′ deletion; Fig. 3) or with *Hpa* I (internal deletion; Fig. 4), digesting with BAL-31 exonuclease, and resealing the plasmid. pSVL−141/−7 was constructed by cloning the small *Bam*HI/*Hin*dIII (position −141 to position −7) fragment of pSVL1 (5), which corresponds to the *Bam*HI/*Hpa* I fragment of pSVL−141/−7 with a *Hin*dIII linker at the *Hpa* I site, into *Bam*HI/*Hin*dIII-cleaved pBR322. [pSVL1, and therefore pSVL−141/−7, contains an *Eco*RI linker at the *Alu* I site of SV40 (position 2652; ref. 10). It has been shown that this linker does not effect cleavage (5)].

Preparation of Hybridization Probes. The M13 templates used for probe synthesis (see below) were prepared by cloning either the *Bam*HI/*Hin*dIII fragments of the internal deletion plasmids or the *Bam*HI/*Fnu*DII fragments of 3′ deletion plasmids into an appropriate M13 replicative form DNA. The *Fnu*DII site used for the 3′ deletions is position 4258 in pBR322 (11).

Single-stranded uniformly labeled hybridization probes were prepared by the "prime cut" probe method (5, 12). In brief, a "sequencing primer" was hybridized to an M13 clone carrying the region of interest (see above) and then extended by using DNA polymerase in the presence of [^{32}P]dATP. Products were cleaved with *Bam*HI to generate uniform 3′ ends of the probe and denatured. The probe was then purified by electrophoresis on a sequencing gel (13). The 5′ end of each probe (i.e., the primer) is not homologous to the plasmid injected into oocytes; this "tag" distinguishes undigested probe from probe hybridized to unprocessed RNA in nuclease S1 mapping experiments.

Positioning 3′ Termini. The location of RNA 3′ termini (see Figs. 3*A* and 4*A*) was determined by nuclease S1 mapping, using, as molecular weight markers, either an appropriate sequencing ladder or end-labeled *Msp* I-cleaved pBR322. The resolution of this method is plus or minus two bases.

RESULTS

Structure of the Parental Template. The structure of the parental plasmid used in this study is diagrammed in Fig. 1. It contains a 220-bp fragment of SV40 DNA that spans the polyadenylylation site of late mRNAs, cloned into pBR322 (5). The SV40 fragment includes 141 bases before the polyadenylylation site and 79 bases beyond, and so the plasmid is designated pSVL−141/+79. Upon injection into oocytes, pSVL−141/+79 is transcribed by RNA polymerase II from "promoters" in pBR322. It has previously been shown that the "fused" pBR322/SV40 transcripts that result are cleaved and polyadenylylated as efficiently as transcripts of wild-type SV40 (5).

The A-A-U-A-A-A sequence and the polyadenylylation site are indicated in the sequence shown in Fig. 1. The cytidine preceding the poly(A) stretch is designated −1; the following adenosine is +1.

3′ Flanking Sequence Required for Efficient Cleavage. To determine whether sequences downstream of the A-A-U-A-A-A sequence are essential for cleavage, a derivative of pSVL−141/+79 was constructed that lacks all 79 bp of the 3′ flanking region and 6 bp upstream of the polyadenylylation site but retains the A-A-U-A-A-A (diagrammed in Fig. 2*A*). The same pBR322 sequence is joined to SV40 in both plasmids. Oocytes were injected with this derivative

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Abbreviations: bp, base pair(s); SV40, simian virus 40.

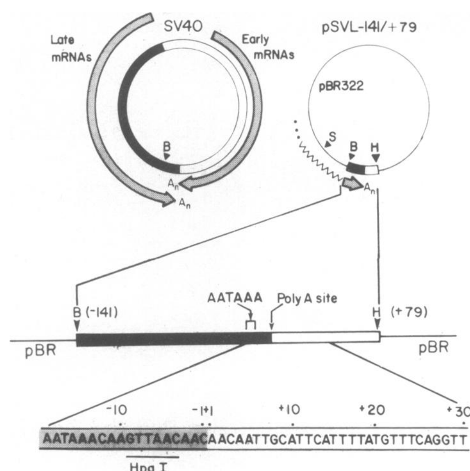


FIG. 1. Structure of the parental plasmid, pSVL−141/+79. (*Upper Left*) Diagram of SV40, indicating early and late mRNAs. (*Upper Right*) pSVL−141/+79: the region of SV40 contained in late mRNA is shown in black; the 3′ flanking region is white. This convention is also used in Figs. 2–4. The sequence of SV40 surrounding the polyadenylylation site is shown, with the numbering system used in this paper indicated. B, H, and S, *Bam*HI, *Hin*dIII, and *Sal*I sites.

(pSVL−141/−7) or with unmodified pSVL−141/+79 and, 6 hr later, RNA was prepared. Cleavage efficiency (defined here as the ratio of cleaved to total RNA) was determined by nuclease S1 mapping. Cleaved RNA can be distinguished from RNA that has not been cleaved by the length of a labeled DNA probe that it protects from digestion by S1 nuclease (Fig. 2*B*).

pSVL−141/+79 transcripts protect two fragments (Fig. 2*B*). One corresponds to RNA having a 3′ terminus at the normal position and the other, to RNA that has not been cleaved. Ninety-five per cent of the RNA has been cleaved, as judged by the relative intensity of the fragments (determined by microdensitometry and corrected for differences in the length of the fragments; see Fig. 3 for details). In contrast, with pSVL−141/−7, the relative intensity of the two protected fragments is reversed: only 20% of the RNA is cleaved. The 3′ terminus of cleaved pSVL−141/−7 RNAs is shifted 22 bases downstream of the normal 3′ terminus, into pBR322. We infer that a region downstream of A-A-U-A-A-A is required for efficient cleavage.

Deletions Downstream of the Polyadenylylation Site. To define the essential region of the 3′ flanking sequence more precisely, a family of plasmids was constructed that retained various lengths of SV40 sequence beyond A-A-U-A-A-A (Fig. 3*A*). To analyze cleavage from these templates, two sets of nuclease S1 mapping experiments were performed. In the first (Fig. 3*B*), the hybridization probe contained SV40 sequence from nucleotide −141 to nucleotide +79. As a result, the length of probe protected by noncleaved RNA (NC, dark arrowheads) varies with the extent of the deletion, while RNA cleaved at the normal position should yield a 143-base fragment (C, open arrowheads). Templates that retain more than 26 bp beyond the polyadenylylation site direct efficient cleavage: pSVL−141/+26, +33, and +62 are at least 90% as efficient as pSVL−141/+79 (Figs. 3*A* and *B*). In contrast, pSVL−141/+9 transcripts are ≈40% as efficient (Figs. 3*A* and *B*).

In the second group of experiments (Fig. 3*C*), we analyzed plasmids retaining <9 bp of 3′ flanking sequence, using a different hybridization probe for each template. Each probe contains the entire SV40 sequence plus adjacent pBR322 sequences from the relevant plasmid. Since different pBR322 sequences are joined to the SV40 sequence in each template, the lengths of the probes vary, as do the lengths of each probe protected by unprocessed RNA. RNA cleaved at the normal position should always protect a 143-base fragment.

We analyzed eight deletion templates using these probes (Fig. 3*C*). Whereas transcripts of pSVL−141/+9, +4, −4a, −4b, and −7 are processed inefficiently, transcripts of pSVL−141/+26 are processed as efficiently as those of pSVL−141/+79. Cleavage efficiencies are given in Fig. 3*A*, normalized to a pSVL−141/+79 efficiency of 1.0.

The assignment of particular bands to cleaved RNA is confirmed by the observation that these bands are generated by polyadenylylated, but not by nonpolyadenylylated, RNA (data not shown). [With pSVL−141/−4a, polyadenylylated RNA generates a 135-base band (open arrow in Fig. 3*C*), while nonpolyadenylylated RNA protects three bands: a prominent band of 166 bases (dark arrow) and two less intense bands of 161 and 156 bases. The minor bands could result either from artifactual S1 cleavage or from nonpolyadenylylated RNA with a 3′ end at an abnormal position.]

Three lines of evidence suggest that the processing inefficiency of plasmids having <9 bases of 3′ flanking sequence is due to their lack of a SV40 sequence rather than to the presence of an inhibitory sequence in pBR322. First, the plasmids join different pBR322 sequences to SV40 sequences, yet all yield a single, consistent result: plasmids retaining the SV40 sequence from +9 to +26 process efficiently while those that lack that sequence do not. Second, in pSVL−141/+79 and pSVL−141/−7, the same pBR322 sequences are joined to different SV40 endpoints yet only pSVL−141/+79 cleaves efficiently. Third, in another pair of plasmids—pSVL−141/−4a and pSVL−141/−4b—the same SV40 deletion endpoint is joined to different pBR322

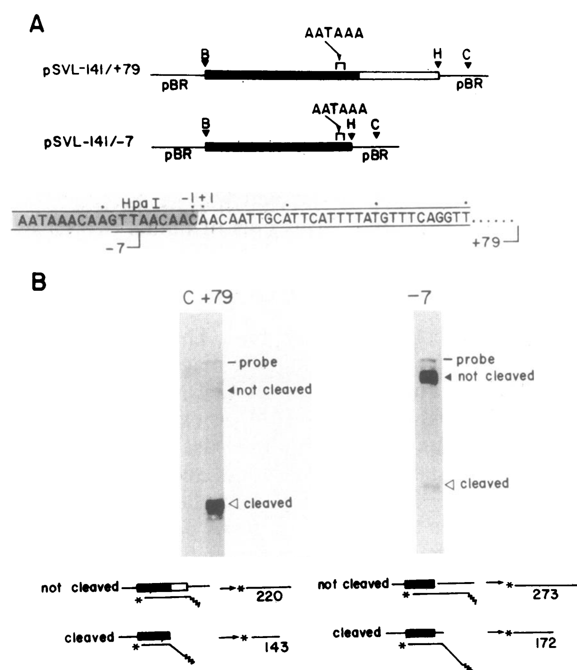


FIG. 2. Removal of sequences from −7 to +79 reduces cleavage efficiency. The structure of pSVL−141/−7 is shown, relative to that of pSVL−141/+79, using the same conventions as in Fig. 1. R indicates the *Eco*RI site of pBR322. Nuclease S1 mapping was carried out using two oocytes' worth of RNA from oocytes that had been injected with 5 ng of pSVL−141/+79, pSVL−141/−7, or with buffer only (C). "Cleaved" and "not cleaved" indicate bands of probe protected from S1 digestion by cleaved or not cleaved RNA. Two different probes were used, as diagrammed at the bottom of the figure. The pSVL−141/+79 probe contains the −141 to +79 region; the pSVL−141/−7 probe contains the −141 to −7 region of SV40 plus 105 adjacent nucleotides of pBR322. The probes are uniformly labeled and single-stranded.

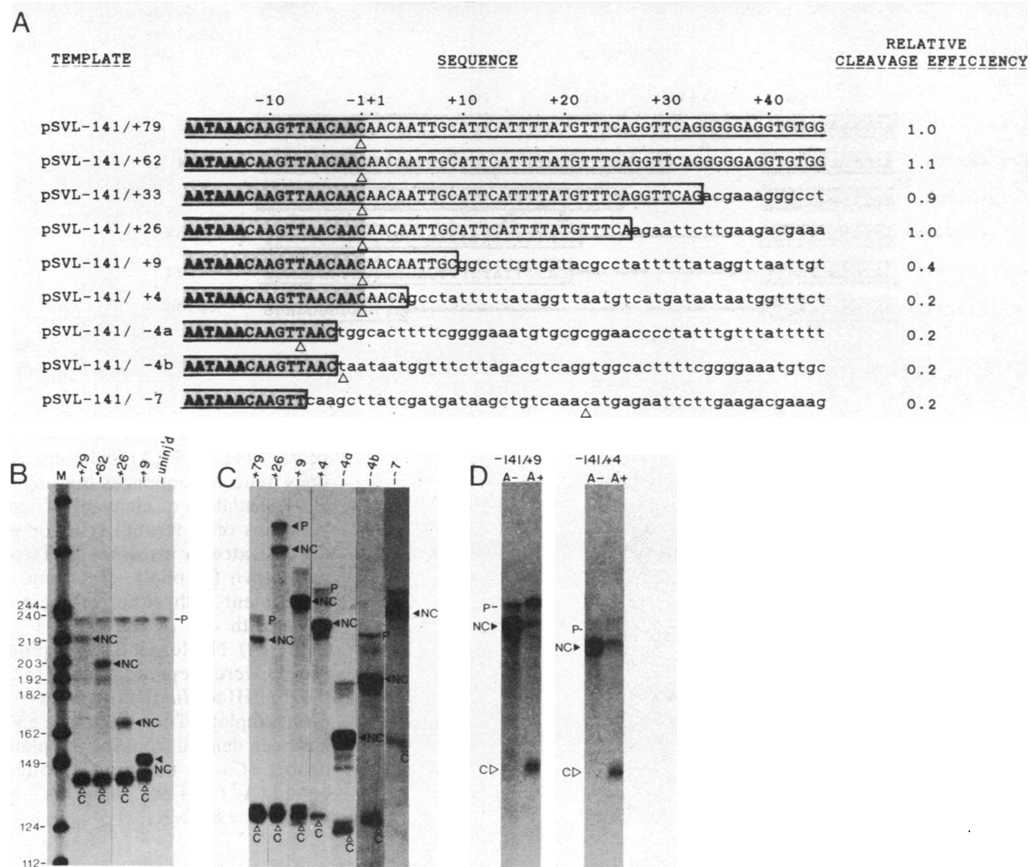


FIG. 3. A region 9–26 bases beyond the polyadenylylation site is required for efficient cleavage. (*A*) Sequence of templates. Capital letters indicate SV40 sequence, small letters indicate pBR322 sequence. Positions of the 3′ termini (plus or minus two bases) are indicated by arrowheads under the sequences. To measure cleavage efficiency, autoradiograms were scanned using a laser scanning microdensitometer (Zeineh model S1-504-XL). Two or more exposures were scanned for each template. Relative cleavage efficiency was then calculated as follows: optical density (OD) of cleaved band ÷ [(OD of cleaved band + OD of uncleaved band) × (*C*)], where *C* is a factor that corrects for the difference in the number of labeled adenosine residues protected by cleaved and uncleaved RNAs. (The probes are uniformly labeled with [^{32}P]dATP.) *C* varies from 0.5 to 0.95. Efficiencies shown are an average of at least two independent injection experiments. Independent determinations differed by <5% for all templates. pSVL−141/+79 was used as a standard in each experiment; its cleavage efficiency varied from 85 to 98%. (*B*) Nuclease S1 mapping using a probe spanning position −141 to position +79. Open arrowheads, cleaved RNA (C); dark arrowheads, noncleaved RNA (NC); P, undigested probe; M, markers prepared by "filling-in" the 3′ termini of *Msp* I-digested pBR322 with [^{32}P]dCTP. (*C*) Nuclease S1 mapping using a different, homologous probe from each template. Each probe was prepared from an M13 clone containing a fragment of the relevant plasmid from the *Bam*HI site, through all of the SV40 sequence, through the flanking pBR322 sequence, to the *Fnu*DII site in pBR322 (position 4258). (*D*) pSVL−141/+9 and pSVL−141/+4 transcripts were fractionated by oligo(dT)-cellulose chromatography. Polyadenylylated (A^+) and nonpolyadenylylated (A^-) fractions were assayed by S1 mapping, using the same probes as in *C*.

sequences. Both of these plasmids direct cleavage with comparable, low efficiency.

Although the region between +4 and +26 is required for efficient cleavage, it is not required for polyadenylylation of the small amount of RNA that has been cleaved (Fig. 3*D*). Thus, RNA prepared from oocytes injected with pSVL−141/+9 or +4 was chromatographed over oligo(dT)-cellulose, and polyadenylylated (A^+) and nonpolyadenylylated (A^-) fractions were assayed by nuclease S1 mapping. With each template, >80% of the cleaved RNA, and little of the uncleaved precursor, is retained by oligo(dT)-cellulose.

The +4 to +26 region also is not required for correctly positioning the 3′ terminus. pSVL−141/+9 and +4 form 3′ termini—albeit inefficiently—at the same site as +79, +62, +33, +26, and wild-type SV40 (5). In contrast, deletion templates that lack the normal polyadenylylation site—pSVL−141/−4a, −4b, and −7—form 3′ termini that are shifted relative to wild-type SV40. The shifts are clear despite the imprecision of S1 mapping (roughly four bases). The magnitude and direction of the shift vary with the template examined.

Internal Deletions. To define the 5′ "boundary" of the functional downstream region, a group of deletion plasmids of pSVL−141/+79 was constructed, in which each contains a gap in the 3′ flanking sequence. All of the gaps have the same 5′ boundary (−7) but they have different 3′ boundaries (Fig. 4*A*). Each plasmid lacks the normal polyadenylylation site but contains the A-A-U-A-A-A sequence. We designate these plasmids by the regions of SV40 they contain; for example, pSVL−141/−7:+4/+79 lacks the 9 bp from −6 to +3 inclusive. For simplicity, in the text such a plasmid is referred to simply as pSVL−7:+4. The efficiency with which each plasmid directs cleavage was determined by injection into oocytes and S1 mapping, using a different homologous hybridization probe for each template (see Fig. 4 for details). The probes contain the entire SV40 region of each plasmid. As a result, their length and the length of fragments protected by unprocessed RNA vary with the template analyzed.

Deletion of the polyadenylylation site itself results in a decrease in cleavage efficiency; transcripts of pSVL−7:+4 and pSVL−7:+8 are cleaved 40% as efficiently as those of pSVL−141/+79 (Fig. 4*A*). The resulting 3′ termini, formed at "cryptic" sites not normally used in SV40, are efficiently

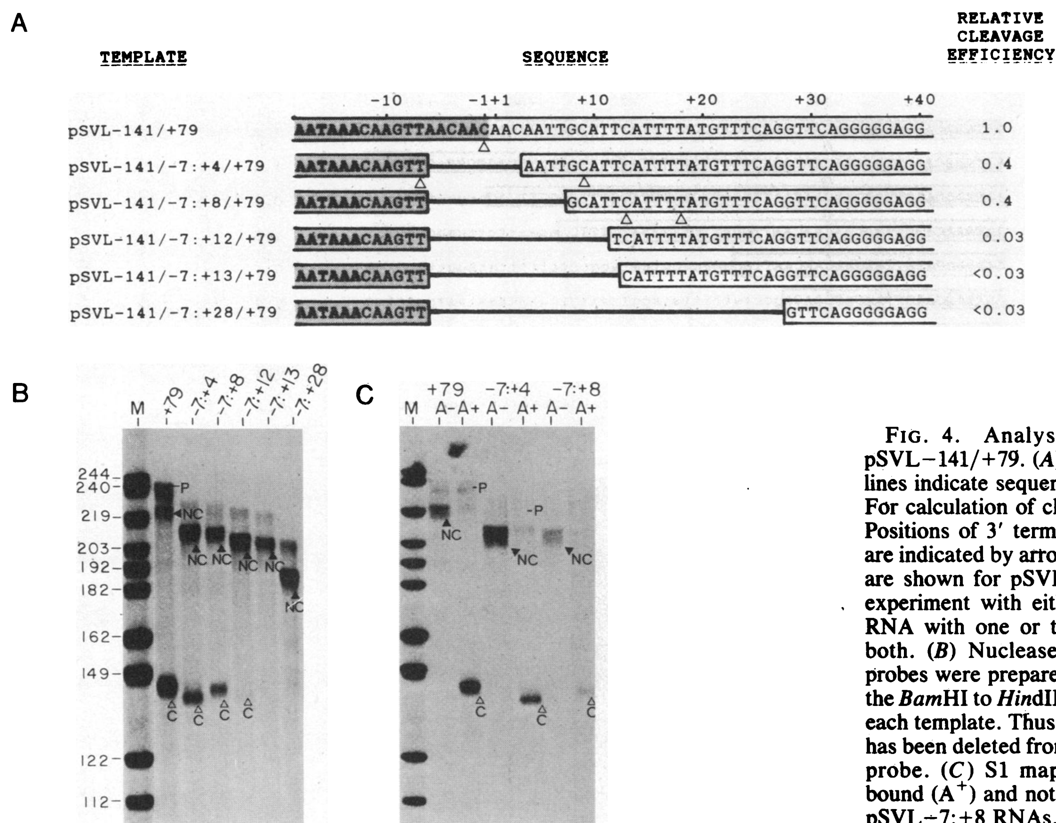


FIG. 4. Analysis of internal deletions of pSVL−141/+79. (A) Structure of templates. Dark lines indicate sequences missing in each template. For calculation of cleavage efficiency, see Fig. 3. Positions of 3′ termini (plus or minus two bases) are indicated by arrowheads. Two different 3′ ends are shown for pSVL−7:+4 and −7:+8; any one experiment with either of these templates yields RNA with one or the other 3′ terminus but not both. (B) Nuclease S1 mapping. Hybridization probes were prepared from M13 clones containing the *Bam*HI to *Hin*dIII fragment (−141 to +79) from each template. Thus, the more SV40 sequence that has been deleted from the template, the shorter the probe. (C) S1 mapping of oligo(dT)-cellulose-bound (A^{+}) and not bound (A^{-}) pSVL−7:+4 and pSVL−7:+8 RNAs.

polyadenylylated (Fig. 4C). Removal of an additional 4 bp of 3′ flanking sequence from pSVL−7:+8 results in a dramatic decrease in cleavage. Cleaved RNA is barely detectable from pSVL−7:+12, indicating that cleavage is no more than 10% as efficient as pSVL−7:+8 (Fig. 4 A and B). Similarly, pSVL−7:+13 and pSVL−7:+28 produce no detectable cleaved RNA. These data corroborate the existence of critical sequences downstream of A-A-U-A-A-A. Furthermore, they indicate that at least one important element lies in the immediate vicinity of the polyadenylylation site itself (−6 to +3).

DISCUSSION

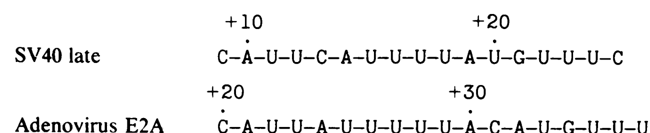
The experiments reported here identify a region, in addition to the A-A-U-A-A-A sequence, that is necessary for efficient cleavage. The small amount of cleaved RNA produced from templates lacking this region is polyadenylylated, indicating that the region is not required for polyadenylylation itself (Fig. 3D). Similarly, this region is not required for accurately positioning the 3′ terminus (Fig. 3C).

Our data do not reveal the biochemical function of this region. It could be recognized directly by the cleavage enzyme, or it could signal a pause in transcription that is obligatory for 3′ end formation *in vivo*. Endonucleolytic scission could occur within this region or elsewhere. Our kinetic data (5) indicate that this region is not a transcription terminator in oocytes.

Our results define the approximate location of the functional element(s) downstream of the A-A-U-A-A-A sequence. Deletions of the 3′ flanking region (Fig. 3) indicate that part of at least one element lies between 9 and 26 bases beyond the polyadenylylation site. Interpretation of the internal deletions (Fig. 4) is more complex, since removal of the polyadenylylation site (pSVL−7:+4 and +8) reduces cleavage efficiency to 40% of control, while removal of an additional four bases of the 3′ flanking region reduces cleavage to no more than 3%. The data suggest the existence of two "elements" affecting cleavage efficiency—one in the immediate vicinity of the polyadenylylation site (−6 to +3) and another further downstream (with a boundary between +9 and +12). Point mutations will be required to position the element(s) precisely. Nevertheless, both sets of data show that the region downstream of A-A-U-A-A-A is required for efficient cleavage. For convenience, we will refer to the region between +4 and +26 as a downstream element, but we recognize that more than one functional element may lie in this region.

The first report of the necessity for 3′ flanking sequences in the formation of a eukaryotic mRNA 3′ terminus involved histone H2A mRNA (14). The generality of this observation was unclear, since, unlike most mRNAs, histone mRNA lacks the A-A-U-A-A-A sequence and is not polyadenylylated. However, McDevitt *et al.* (15) recently identified a downstream element in adenovirus E2A pre-mRNA. This mRNA, like SV40 late mRNA, contains the A-A-U-A-A-A sequence and is polyadenylylated. The sequence of the critical regions of these two "typical" pre-mRNAs, as defined by progressive removal of the 3′ flanking sequence (Fig. 3 and ref. 15) are similar, as shown below.

Two sequence similarities merit comment. First, both elements contain a C-A-U-U tetranucleotide. Berget (16) has proposed that 3′ end formation requires that U4 small nuclear RNA base pair with SV40 late pre-mRNAs. A C-A-U-U tetranucleotide (or C-A-A-U-U), and the A-A-U-A-A-A sequence, may be involved in that hypothetical U4–pre-mRNA interaction (16). Since the region +3 to +16 contains C-A-A-U-U and two copies of C-A-U-U, it might be difficult to reveal the function of C-A-U-U by point mutations in any one such sequence.



Second, both elements contain a five-base sequence complementary to A-A-U-A-A-A. Since not all genes contain such a sequence near the polyadenylylation site, the sequence *per se* probably is not required. Nevertheless, a stem structure involving the A-A-U-A-A-A sequence still might be necessary; base pairing could occur either between the pre-mRNA and a small nuclear RNA, as Berget (16) has suggested, or between two regions of the pre-mRNA. Intramolecular base pairing is required for the formation of histone mRNA 3′ termini (14, 17). The role, if any, of a stem formed between A-A-U-A-A-A and the downstream element in SV40 can be tested by using previously described A-A-U-A-A-A point mutants that prevent cleavage (5).

We have noted that of five genes tested for cleavage in oocytes—SV40 late (18), bovine growth hormone (unpublished work), thymidine kinase (*TK*; R. Harland, personal communication; unpublished work), and human α- and β-globin (D. Melton, personal communication)—the two that direct efficient cleavage (SV40 late and β-globin) contain a five-base complement of the A-A-U-A-A-A sequence near the polyadenylylation site, and the three that do not direct efficient cleavage (α-globin, *TK*, and growth hormone) do not contain such a sequence. Although five genes is too small a sample to draw a general conclusion, perhaps oocytes can cleave only one class of pre-mRNAs—those that form an intramolecular stem. Processing the other class of pre-mRNAs may require an additional component that oocytes lack.

Studies of SV40 mutants in monkey cells are consistent with the existence of a downstream element. Sadofsky and Alwine (19) have proposed that a sequence 3–60 bases beyond the polyadenylylation site is crucial. Furthermore, Fitzgerald and Shenk (1981) were unable to isolate viable deletion mutants that lack sequences from +9 to +26. The *dl*1265 deletion of Cole *et al.* (20) is viable yet lacks sequences from +7 to +60. However, Sadofsky and Alwine (19) have shown that *dl*1265 probably does reduce cleavage efficiency since cells infected with *dl*1265 accumulate transcripts that extend beyond the polyadenylylation site. In both frog oocytes and monkey cells, deletion of the polyadenylylation site (e.g., pSVL−7/+4) decreases cleavage efficiency (19) and shifts the 3′ terminus that is formed (8). The magnitude of the apparent decrease in cleavage efficiency is greater in monkey cells (19) than in oocytes.

3′ Termini formed from different deletion templates do not lie a precisely fixed distance from either the A-A-U-A-A-A sequence or the downstream element. When the pre-mRNA contains a normal polyadenylylation site, 3′ termini are formed at that site; when the pre-mRNA lacks a normal polyadenylylation site, 3′ termini are shifted. Depending on the template, 3′ termini may lie either in pBR322 sequences (pSVL−141/−4b and −7) or at "cryptic" SV40 sites (pSVL−7:+4, +8, and +13). The magnitude of the shift varies; it is greatest (22 bases downstream of wild type) in pSVL−141/−7.

The downstream region affects cleavage efficiency, not accuracy, since pSVL−141/+9 and pSVL−141/+4 form 3′ termini at the normal site. In contrast, with the bovine growth hormone gene, deletion of sequences downstream of the polyadenylylation site shifts the 3′ terminus that is formed but does not reduce cleavage efficiency (21).

Although it is clear that the region between −6 and +26 contains one or more elements required for efficient cleavage, deletion analysis of the sort we have carried out cannot distinguish whether the processing activity recognizes primary sequence or higher order structure nor can it precisely locate the downstream element(s). These two goals may be achieved through the analysis of a large collection of point mutants or through a direct analysis of higher order structure in the −141/+26 domain of the pre-mRNA.

Note: Gil and Proudfoot (22) have reported that cleavage of β-globin pre-mRNA requires sequences between −5 and +31.

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