**A Functionally Redundant Downstream Sequence in SV40 Late Pre-mRNA Is Required for mRNA 3'-End Formation and for Assembly of a Precleavage Complex in Vitro**

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David Zarkower* and Marvin Wickens

From the Department of Biochemistry, College of Agriculture and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706

In eukaryotes, mRNA 3' termini are formed by endonucleolytic cleavage of a long primary transcript and polyadenylation of the new end. Here we show that sequences downstream of the poly(A) site are required for cleavage of simian virus 40 (SV40) late pre-mRNAs in vitro in a crude nuclear extract from HeLa cells. The critical sequences are functionally redundant: extensive deletions or substitutions of downstream sequences prevent cleavage, but small substitutions do not. This functional redundancy is not due to a repetition of the same sequence. Either two or more different sequences can promote cleavage, or a single element exists which is long and diffuse.

Although pre-mRNAs transcribed from certain genes require a U- or UG-rich sequence downstream of the poly(A) site for efficient cleavage, SV40 does not. Removal of these sequences from SV40 late pre-mRNAs does not significantly reduce cleavage efficiency.

Downstream sequences also are required for formation of a specific precleavage complex between SV40 pre-mRNA and components present in the extract. Mutant RNAs that are cleaved efficiently form such complexes, while those that are cleaved inefficiently do not. Based on these and previous results (Zarkower, D., and Wickens, M. (1987b) EMBO J. 6, 4185-4192), we propose that a critical role of the region downstream of the poly(A) site is to facilitate formation of a specific precleavage complex in which cleavage subsequently occurs.

Formation of the 3' terminus of most eukaryotic mRNAs involves endonucleolytic cleavage of a long precursor RNA and the addition of approximately 250 adenylate residues to the newly generated 3'-end (polyadenylation). The cleavage reaction requires at least two sequences in the pre-mRNA: the sequence AAUAAA, which lies upstream of the polyadenylation site (poly(A) site), and the "downstream element(s)," which lies beyond the poly(A) site. The sequence AAUAAA is located 6-30 bases from the poly(A) site of virtually all mRNAs. Mutations in this sequence greatly reduce cleavage efficiency in vivo (Fitzgerald and Shenk, 1981; Gil and Proudfoot, 1983; Montell et al., 1983; Higgins et al., 1984; Wickens and Stephenson, 1984) and in vitro (Hart et al., 1985; Zarkower et al., 1986) and prevent polyadenylation of RNAs which end at the poly(A) site (Manley et al., 1985; Zarkower et al., 1986).

The region downstream of the poly(A) site is essential for cleavage of several different cellular and viral mRNAs in vitro (reviewed in Birnstiel et al., 1985). In some pre-mRNAs, a modestly conserved U-rich or UG-rich element appears to be critical (Hart et al., 1985; McLauchlan et al., 1986). Because the exact sequence can vary, it has been proposed that differential poly(A) site selection may depend on selective recognition of specific downstream sequences by cellular factors (McDevitt et al., 1984).

Histone mRNAs differ from most other mRNAs in that they lack AAUAAA and usually are not polyadenylated. However, cleavage of histone mRNAs, like that of other mRNAs, requires elements both upstream and downstream of the cleavage site (Birchmeier et al., 1982; Georgiev and Birnstiel, 1985).

We have shown previously that the region downstream of the poly(A) site is required for efficient cleavage of SV40 late pre-mRNA in frog oocytes (Conway and Wickens, 1985). In this report we demonstrate that the same region is required for cleavage in vitro in an extract of HeLa cell nuclei. We examine which sequences within this region are essential by analyzing a collection of deletion and substitution ("linker scanning") mutants. Finally, we demonstrate that downstream sequences are required for formation of a specific precleavage complex between the pre-mRNA and processing factors in the extract.

**MATERIALS AND METHODS**

**Plasmids and RNA Substrates**

3'-Deletions—pSVL-141/+70 (Wickens and Stephenson, 1983) was cleaved with HindIII at +70, treated with Bal-31 exonuclease, and resealed with 8-base pair XbaI linkers (CTCTAGAG). Deletion end points were determined by dideoxynucleotide sequencing (Sanger and Coulson, 1978). The small BamHI/XbaI (−141 to deletion end point) fragment from each mutant was cloned into BamHI/XbaI pBR322 in which an XbaI linker had been inserted into the HindIII site. The resulting plasmids (designated pSV−141/−1 etc.; Fig. 2) contain SV40 sequences from position −141 (nucleotide 2533, numbering as in Tooze, 1981) to various end points. For SP6 RNA polymerase transcription, the small BamHI/EcoRI fragment was inserted into BamHI/EcoRI pSP64 (Melton et al., 1984). The resulting clones, designated pSP−141/−1, etc., when linearized with EcoRI and transcribed with SP6 RNA polymerase yield RNAs containing SV40 sequences from −141 to the various end points followed by a 3'-terminal 32 bases of vector-derived sequence.

**Linker Scanning Mutants—pSV−141/−70** was cleaved at −7 with
HpaI, treated with Bal-31 exonuclease, and resealed with XbaI link-ers. The resulting 5’-deletion end points were determined by dideoxy-nucleotide sequencing. The small XbaI/HindIII fragments of 5’-deletion mutants were cloned into XbaI/HindIII 3’-deletion plasmids (see above), generating a family of plasmids containing SV40 se-quences from −141 to +70 with an XbaI linker sequence replacing a small region. These plasmids are designated pLS+X/+Y, where X is the base of SV40 adjacent to the 5’-side of the linker sequence and Y is the base adjacent to the 3’-side.

**Long Substitution Mutants**—pLS−1/+27A contains a 23-base polylinker sequence inserted between positions −1 and +27 of SV40. pLS−1/+27B contains a 32-base pair synthetic oligonucleotide (gift of C. Donnelly, University of Wisconsin-Madison) inserted at the same position. Both plasmids are otherwise identical to pSV−141/+70. The sequence of the relevant region of each is shown in Fig. 5.

Long and short substitution mutants were cloned into BamHI/HindIII ISPS6 as BamHI/HindIII fragments to generate templates for SP6 transcription.

**Preparation of DNA Substrates**

DNA was incubated with SP6 RNA polymerase as described (Melton et al., 1984; Zarkower and Wickens, 1987a). Deletion mutants were transcribed from EcoR1-cleaved templates (see above). Substitution mutants were transcribed from templates cleaved at +55 with Drai.

Full-length transcripts were eluted from a polyacrylamide gel slice by soaking overnight in 0.5 M ammonium acetate, 0.1% sodium dodecyl sulfate, and 0.5 mM EDTA at 25 °C, and precipitated with ethanol.

**Preparation of Nuclear Extract and Processing in Vitro**

Nuclear extract was prepared from HeLa cells by the method of Dignam et al. (1983) except that MgCl2 was omitted from buffer A. 0.125 mM EDTA was included in buffer D, extract was dialyzed for 3.5 h, and phenylmethylsulfonyl fluoride was omitted from all buffers. Standard reactions contained 5.5 μl of nuclear extract, 40 mM KCl, 20 mM phosphocreatine (Sigma), 0.1 mM ATP, 2.8% polyvinyl alco-hol, 1 mM 3'-dATP (Sigma), and 1–5 fmol of labeled pre-mRNA in a total volume of 12.5 μl.

RNA was isolated and analyzed as described (Zarkower and Wick-ens, 1987a). Cleavage was quantitated by laser microdensitometry of autoradiograms.

**Oocyte Injection**

Oocyte injection, RNA preparation, and nucleic S1 mapping were performed as described (Wickens and Stephenson, 1983). The hybridization probe used to map deletion mutants was a uniformly labeled single-stranded DNA containing sequences complementary to SV40 from −141 to +70 and a noncomplementary 5’-portion that distin-guishes undigested probe from probe protected by uncleaved RNA (Conway and Wickens, 1985). This probe was used to analyze all deletion mutant RNAs except −141/+7 RNA. To map that RNA, the hybridization probe used to map deletion mutants is a uniformly labeled probe protected by uncleaved RNA from −141 to +70 and a noncomplementary 5’-portion that distin-guishes undigested probe from probe protected by uncleaved RNA.

**Gel Retardation Assay**

Complexes between labeled RNA and protein components were identified by a “gel retardation” assay (Dahlberg et al., 1983; Konarska and Sharp, 1986; Pikielny and Rosbash, 1986; Zhang and Cole, 1987). This assay is described in detail in Zarkower and Wickens (1987a).

Briefly, nuclear extract reactions are stopped by treatment with 5 mg/ml heparin at 0 °C for 10 min and then analyzed by electropho-resis through a 4% acrylamide nonnondenaturing gel (acrylamide/N,Nmethylen bisacrylamide ratio of 80:1). Electrophoresis was performed at 4 °C and at 15 V/cm. Both the gel and the buffer contain 45 mM Tris, 45 mM boric acid, and 0.6 mM EDTA.

**RESULTS**

**Downstream Sequences Are Required for Cleavage in Vitro**—In DNA-injected oocytes, the sequence between +5 and +26 is required for efficient cleavage (Conway and Wickens, 1985).

To determine whether a similar sequence is required in vitro, we compared cleavage of labeled −141/+7 and −141/+23 RNAs in a crude extract of HeLa cell nuclei (Dignam et al., 1983; Moore and Sharp, 1985). The two RNAs contain SV40 sequences from 141 nucleotides upstream of the poly(A) site to either 7 or 23 nucleotides downstream (Fig. 1A). Both include, at their 3’ termini, 32 nucleotides of vector-derived sequence; this segment was included to minimize end effects which might interfere with processing nonspecifically (by nonspecific protein binding to RNA 3’ termini, for example (Conway and Wickens, 1987a)). Each RNA was incubated in nuclear extract containing 3'-dATP, which prevents polyad-enylation by acting as a chain terminator (Moore and Sharp, 1985). Cleavage efficiency was determined by gel electrophoresis of deproteinized RNA. To ensure that the assay was quantitative, we determined cleavage efficiency both as a function of time (Fig. 1B) and of RNA concentration (Fig. 1C).

The kinetic analysis demonstrates that RNA containing 23 nucleotides of SV40 downstream is cleaved more rapidly than RNA with only seven nucleotides (Fig. 1B). During the first 10 min of incubation, when the rate of cleavage is constant, −141/+23 RNA is cleaved 24 times more rapidly than −141/+7 RNA. After 10 min, 80% of the −141/+23 RNA has been cleaved; in contrast, even after 40 min, less than 10% of the −141/+7 RNA has been cleaved.

Since cleavage is saturable by substrate (Zarkower and Wickens, 1987a), accurate comparison of two RNAs requires that they both be assayed at subsaturating concentrations. We, therefore, assayed cleavage of labeled −141/+7 and −141/+23 RNAs using a 60-fold range of RNA concentration, at a fixed time, 20 min (Fig. 1C). To aid in quantitation, equal amounts of radioactivity were loaded onto each gel lane. At the lowest amount of RNA tested (2.5 fmol), 75% of −141/+23 RNA (lane 1) but only 5% of −141/+7 RNA (lane 4) is cleaved.

The experiments presented in Fig. 1 demonstrate that, in vitro, an RNA containing the SV40 sequence from +8 to +23 is cleaved more rapidly than one which lacks this sequence. Based on the kinetic and substrate concentration data, all subsequent determinations of cleavage efficiency were performed at short times (10 min or less) using subsaturating amounts of RNA (1–10 fmol).

**Deletions Leaving Less Than 23 Bases of Downstream Sequence Reduce Cleavage in Vitro**—To define more precisely which downstream sequences are required in vitro, we ana-lyzed a family of mutant RNAs (Fig. 2A). These “deletion RNAs” contain different lengths of downstream sequences, ranging from 0 to 70 nucleotides. RNAs with less than 55 bases of SV40 downstream sequence contain, at their 3’ termini, 32 nucleotides of vector-derived sequence to minimize end effects (see above). Cleavage of each RNA was analyzed after 5 and 10 min of incubation (Fig. 2B); from these data, rates of cleavage were determined. In Fig. 2C, these rates are normalized to −141/+70 RNA.

RNAs which retain 23 or more bases of SV40 sequence beyond the poly(A) site are cleaved efficiently (Fig. 2B, lanes 6–8 and 14–16). As additional SV40 sequences are deleted, cleavage efficiency drops; an RNA with no downstream sequences (−141−1 RNA, Fig. 2B, lanes 1 and 9) is cleaved 30-fold less efficiently than −141/+70 RNA (Fig. 2B, lanes 8 and 16).

**Comparison of Cleavage in Vitro and in DNA-injected Oocytes**—To determine whether the same sequences are required in vivo, plasmid DNAs containing 7, 13, 15, 23, or 70 bases downstream of the poly(A) site were injected into oocytes
Redundant Downstream Element and Precleavage Complex

A

5' AAUAACAAGUUAACAAACUACAUUGCAUUUAUGUUCucuagagaucauguaagcugcaaaacaggaauuc 3'

-141/+23 RNA:

5' AAUAACAAGUUAACAAACUACAUUGCAUUUAUGUUCucuagagaucauguaagcugcaaaacaggaauuc 3'

-141/+7 RNA:

B

-141/+23 RNA -141/+7 RNA

C

-141/+23 RNA -141/+7 RNA

FIG. 1. Cleavage of -141/+23 and -141/+7 RNAs. A, structure of substrate RNAs. The sequences of both RNAs, from AAUAAA to their 3' termini, are shown. SV40 sequences are capitalized; vector-derived sequences are lower case. The poly(A) site is indicated by arrowheads. B, kinetic analysis. 25 fmol of each RNA was incubated in nuclear extract containing 3'-dATP, in a total volume of 62.5 μl. At the times indicated above each lane, 12.5 μl of each reaction was removed, deproteinized, and analyzed by gel electrophoresis. C, RNA titration. 2.5 fmol (lanes 1 and 4), 25 fmol (lanes 2 and 5), or 150 fmol (lanes 3 and 6) of each RNA was incubated in nuclear extract containing 3'-dATP in a total volume of 12.5 μl. After 20 min of incubation, each reaction was analyzed. An equal amount of radioactivity was loaded onto each gel lane to simplify quantitation.

(Fig. 3). Each DNA contains 141 nucleotides of SV40 sequence upstream of the poly(A) site, as did the RNAs analyzed in Fig. 2. The injected templates are transcribed by RNA polymerase II from promoter-like sequences in the vector (Mertz and Gurdon, 1977; Wickens and Stephenson, 1984). Cleavage of the fused pBR322/SV40 mRNA precursors was assayed by nuclease S1 mapping, as diagrammed in Fig. 3A. Cleaved RNA protects a probe fragment of 143 nucleotides, whereas RNA that has not been cleaved protects a larger probe fragment, the length of which depends on the length of SV40 sequence it contains.

As in vitro, RNAs retaining at least 23 bases of downstream SV40 sequence are cleaved efficiently: 90% of the RNA transcribed from -141/+70 and -141/+23 templates is cleaved (lanes 1 and 2). In contrast, only 60% of -141/+15 RNA (lane 3), 30% of -141/+13 RNA (lane 4), and a trace amount of -141/+7 RNA (lane 5) is cleaved.

The effect of each deletion in oocytes and in vitro is com-
Redundant Downstream Element and Precleavage Complex

A

-141/-70 RNA

-141/-55 RNA

-141/-23 RNA

-141/-15 RNA

-141/-13 RNA

-141/-9 RNA

-141/-7 RNA

-141/-1 RNA


B

5 minutes

10 minutes

C

Cleavage Rate (relative to -141/+70)

-141/-1 RNA

-141/+7 RNA

-141/+9 RNA

-141/+13 RNA

-141/+15 RNA

-141/+23 RNA

-141/+55 RNA

-141/+70 RNA

FIG. 2. Downstream sequences are required for efficient cleavage in vitro. A, structure of mutant RNAs. The sequence of each RNA from AAUAAA to its 3' terminus is shown. SV40 sequences are capitalized; vector-derived sequences are lower case. The natural poly(A) site, where present, is indicated by an arrowhead. B, cleavage of 3' deletion RNAs. 10 fmol of each RNA was incubated in nuclear extract containing 3'-dATP in a total volume of 25 µl. After 5 min (lanes 1-8) or 10 min (lanes 9-12), 12.5 µl of each reaction was removed and RNA prepared and analyzed. RNAs are in the same order as in A: lanes 1 and 9, -141/-1 RNA; lanes 2 and 10, -141/+7 RNA; lanes 3 and 11, -141/+9 RNA; lanes 4 and 12, -141/+13 RNA; lanes 5 and 13, -141/+15 RNA; lanes 6 and 14, -141/+23 RNA; lanes 7 and 15, -141/+55 RNA; lanes 8 and 16, -141/+70 RNA. C, cleavage rate of each RNA relative to -141/+70 RNA. The fraction of recovered RNA that was cleaved was determined from the autoradiograms in B, and the rate of cleavage was calculated and normalized to that of -141/+70 RNA.

pared in Fig. 3B. The oocyte data of Conway and Wickens (1985) are included. Qualitatively, each deletion behaves similarly in vitro (triangles) and in oocytes (circles). We conclude that the dependence of cleavage on sequences between the poly(A) site and 23 bases beyond, observed in the injection assay in vivo, is reproduced faithfully in the in vitro assay.

Short Substitutions of Downstream Sequences Do Not Reduce Cleavage in Vitro—To identify critical nucleotides between +1 and +23, we analyzed a family of mutant RNAs in which short regions of SV40 sequence have been replaced by an 8-nucleotide XbaI linker sequence (CUCUAGAG). The relevant sequence of each RNA is shown in Fig. 4A. These “linker scanning” mutants (McKnight, 1980) are named by the positions of SV40 on either side of the linker insertion: for example, in LS +7/+14 RNA, an XbaI linker sequence replaces positions +8 through +13 of SV40. Any one mutation replaces only a short region of SV40 sequence; as a group, however, the mutations replace the entire region from -6 to +26.

Each RNA was incubated in extract containing 3'-dATP. After 5, 10, 15, and 30 min, aliquots were removed to determine the fraction of RNA which had been cleaved (Fig. 4B). RNAs were analyzed in two groups; -141/+55 RNA was included in both groups to provide a standard. All of the mutant RNAs, with the exception of LS -7/+4 and LS -1/+9, are cleaved as rapidly as wild type -141/+55 RNA (Fig. 4B). LS -7/+4 RNA is cleaved 40% as rapidly and LS -1/+9 70% as rapidly. These reductions probably occur because the cleavage site has been changed, not because the downstream element is defective.1 Regardless, neither substitution reduces cleavage 20-fold, as occurs with deletion mutants which lack the downstream region entirely (Figs. 1 and 2). We conclude that no single short sequence lying between +1 and +23 is responsible for downstream element function.

Long Substitutions Reduce Cleavage Efficiency—In the following experiments we test the hypothesis that two or more short sequences between +1 and +23 are sufficient to direct efficient cleavage. In this view, deletion of all downstream sequences prevents cleavage because it removes all of the elements; conversely, short substitution mutants are cleaved because they still contain at least one sufficient sequence.

We analyzed two mutant RNAs in which the entire region from the poly(A) site, +1, to +26 has been replaced with prokaryotic sequences. These mutants, designated LS -1/+27A and LS -1/+27B, differ only in the sequence which has been inserted; both lack SV40 sequence between +1 and +26. Cleavage of the two LS -1/+27 RNAs was analyzed after 5, 10, 15, and 20 min of incubation in the nuclear extract (Fig. 5B). For comparison, -141/+55 RNA, LS -1/+14 RNA,

1 M. Sheets, V. Bardwell, and M. Wickens, unpublished data.
Prepared 5 h after injection of each template. Hybridization probes were uniformly labeled and single-stranded and are complementary large substitution mutants, though clearly defective, are efficiently; in contrast, both LS -1/+27 RNAs are cleaved inefficiently (lane 2).

We conclude that the region between +1 and +27 is redundant; either half is sufficient to direct cleavage. Furthermore, since even the LS -1/+27 linker-scanning mutants are more efficient than a deletion which lacks all downstream sequences (e.g. -141/+7), we infer that sequences between +28 and +55 are able to enhance cleavage.

Downstream Sequences Facilitate Formation of a Precleavage Complex—Prior to cleavage, a complex forms between the pre-mRNA and processing factors (Zhang and Cole, 1987; Skolnik-David et al., 1987; Zarkower and Wickens, 1987b). We have shown previously that this precleavage complex is involved in the cleavage reaction by four criteria: 1) pre-mRNAs containing AAUAAA mutations do not form the complex (Conway and Wickens, 1987; Zarkower and Wickens, 1987b); 2) formation of the complex is prevented by an anti-AAUAAA oligonucleotide annealed to the substrate, but not by control anti-sense oligonucleotides; 3) the extract components which form the complex are saturable by functional substrate; and 4) the formation of this complex, which occurs in the absence of ATP, precedes cleavage (Zarkower and Wickens, 1987b). In the following experiments, we analyze whether, like AAUAAA, downstream sequences are required for the formation of this specific precleavage complex.

Formation of the precleavage complex was assayed by "gel retardation" (Dahlberg et al., 1969; Konarska and Sharp, 1986; Pikielny and Rosbash, 1986). Reactions containing labeled RNA were loaded onto a nondenaturing acrylamide gel without prior deproteinization. The specific precleavage complex migrates slower than both naked RNA and nonspecific RNA-protein complexes.

As soon as any RNA, regardless of sequence, is added to extract, it enters a nonspecific complex which migrates slower than naked RNA. As expected, -141/+55 RNA forms this nonspecific complex at 0 min (Fig. 7). By 10 min, the specific precleavage complex is detected as well. RNA which extends to +23 forms as much specific complex as -141/+55 RNA. In contrast, RNAs which contain SV40 sequence from -141 to either -1, +7, +9, +13, or +15 form only low levels of the specific complex. (The low levels of complex are detectable in longer exposures of the autoradiogram in Fig. 7 (net shown).)

Thus, the region downstream of the poly(A) site is required for formation of the precleavage complex.

Fig. 8 presents an identical analysis of substitution mutants. LS +1/+14, LS +9/+20, and LS +15/+27 RNAs, each of which is cleaved as well as wild type RNA, form the precleavage complex efficiently (Fig. 8). LS -7/+4 is cleaved 3-fold less well than wild type (Fig. 4) and forms slightly less complex (Fig. 8). The long substitution mutations, LS -1/+27A and LS +1/+27B, which are cleaved poorly (Figs. 5 and 6) form little complex (Fig. 8).

Qualitatively the ability of each RNA to form the precleavage complex parallels the efficiency with which it is cleaved. This strongly suggests that the role of sequences downstream of the poly(A) site is, at least in part, to facilitate formation of an essential precleavage complex.

**DISCUSSION**

Deletion of sequences downstream of the poly(A) site prevents cleavage of SV40 late pre-mRNA in vitro. However,
Redundant Downstream Element and Pre-cleavage Complex

A

-141/+55 RNA
LS -7/+4 RNA
LS -1/+9 RNA
LS +7/+14 RNA
LS +9/+20 RNA
LS +11/+20 RNA
LS +13/+20 RNA
LS +15/+27 RNA

B

not cleaved

cleaved

-141/+55 RNA
-7/+4 RNA
-1/+9 RNA
+7/+14 RNA
+9/+20 RNA
+11/+20 RNA
+13/+20 RNA
+15/+27 RNA

FIG. 4. Short downstream substitutions do not prevent cleavage in vitro. A, structure of RNA substrates. A partial sequence of each RNA is shown. Each RNA extends from -141 to +55. Black bars represent XbaI linker sequence. B, cleavage of mutant RNAs. 20 fmol of each RNA was incubated in nuclear extract containing 3'-dATP in a total volume of 50 μl. After 5, 10, 15, and 30 min of incubation, 12.5 μl of each reaction was removed and the RNA prepared and analyzed. RNAs were analyzed in two groups: -141/+55, LS-7/+4, LS-1/+9, and LS+7/+14; and -141/+55, LS+9/+20, LS+11/+20, LS+13/+20, and LS+15/+27. Below each group is plotted the fraction of recovered RNA that is cleaved versus time of incubation.

Cleavage is relatively unaffected by replacement of any short downstream sequence (this paper) or by the chemical modification of any single downstream nucleotide (Conway and Wickens, 1987). We infer that the functional element is not a single short sequence. At least two mechanistic interpretations of this conclusion can be considered. The downstream region may consist of multiple distinct elements, each of which is sufficient to direct cleavage. Although the sequences from +1 to +14 and from +15 to +27 are quite different (see Fig. 1), either one can function in the absence of the other (compare LS -1/+14 and LS +15/+27 to the LS -1/+27 mutants). Thus, in this hypothesis, very divergent sequences must be able to support cleavage. Alternatively, the downstream region could contain a single large element which none of our short substitution mutations disrupts sufficiently to prevent cleavage.

SV40 late pre-mRNAs differ significantly from other pre-mRNAs with respect to which downstream sequences are required. First, unlike other pre-mRNAs (McDevitt et al., 1984; Zhang et al., 1986; Gil and Proudfoot, 1987), SV40 late pre-mRNA does not require a UG-rich downstream sequence for cleavage. This difference is most dramatically illustrated by a comparison of our data with the recently published results of Zhang and Cole (1987) on herpes simplex virus thymidine kinase pre-mRNA. Mutations in thymidine kinase pre-mRNA, analogous to those analyzed here, exhibit very different properties in the in vitro assay; replacement of a short UG-rich sequence of thymidine kinase significantly reduces cleavage and complex formation (Zhang and Cole, 1987). A consensus sequence for the UG-rich element has been proposed (McLauchlan et al., 1988). SV40 late pre-mRNAs contain only one such sequence downstream of the poly(A) site,
Redundant Downstream Element and Pre-cleavage Complex

Fig. 5. Long substitutions reduce cleavage efficiency in vitro. A, structure of RNA substrates. RNAs contain SV40 sequence from -141 to +55. Black bars indicate substituted bases. Lower case white letters indicate the foreign "linker" sequence. B, cleavage of mutant RNAs. Reactions were performed as in Fig. 4. After 5, 10, 15, and 20 min of incubation, 12.5 μl of each reaction was removed and the RNA analyzed. The fraction of recovered RNA that is cleaved versus time of incubation is plotted. Relative rates of cleavage were calculated based on the 5- and 10-min incubations.

Fig. 6. Downstream region is redundant in oocytes. Nuclease S1 mapping of RNA from oocytes injected with DNA encoding either short linker scanning mutants (LS-1/+9, LS-1/+14, LS+15/+27), a long substitution mutant (LS-1/+27A), or wild type RNA (-141/+70). RNA was prepared and analyzed by nuclease S1 mapping as in Fig. 3. Each RNA was mapped with a different homologous probe; thus, the sizes of probe fragments protected by uncleaved RNA vary. The uppermost band in each lane is undigested full-length probe. The fraction of RNA that is cleaved, relative to -141/+70 RNA, is tabulated to the right.

positioned between nucleotides +18 and +25 (see below).

Consensus YGUGUYY
SV40 late UAGGUUUC

Since LS +15/+27 RNA, which lacks this sequence, is cleaved efficiently, this element cannot be the sole sequence directing cleavage of SV40 pre-mRNAs. Through similar reasoning, we conclude that SV40 late pre-mRNA also differs from β-globin pre-mRNA, in which two sequence elements, one UG-rich and the other U-rich, both are required for cleavage (Gil and Proudfoot, 1987).

These differences between pre-mRNAs suggest that downstream sequences might be exploited to regulate cleavage, such that one site is used in preference to another, as has been proposed previously (McDevitt et al., 1984). This possibility is also suggested by the observation that the specific downstream sequences required for cleavage of SV40 late pre-mRNAs vary with the cells used in the assay. The region between +1 and +27, which is critical in both oocytes and HeLa cell nuclear extracts, is relatively unimportant for cleavage in monkey cells infected with SV40 (Fitzgerald and Shenk, 1981; Sadofsky et al., 1985). Rather, in infected monkey cells, cleavage is reduced 3-fold by deletion of a sequence further downstream between +47 and +55 (Sadofsky et al., 1985). In oocytes and in HeLa cell extracts, removal of these sequences has no effect on cleavage of an otherwise wild type RNA (Fig. 3). Only when sequences from +1 to +26 are missing can any activity of this region, albeit slight, be detected; LS -1/+27 RNAs are cleaved more efficiently than an RNA lacking all downstream sequences. The variability in which downstream sequence is critical in different cells may reflect a difference in the factors and signals used to process the pre-mRNA. We speculate that the redundancy observed in SV40 late pre-mRNA could have evolved to expand the host range of the
for downstream sequences is artifactual; they conclude that deletions downstream of the B-globin poly(A) site prevent cleavage because an inhibitory prokaryotic sequence is brought near AAUAAA, not because a crucial eukaryotic sequence is missing. In this view, most random sequences must be inhibitory, since a wide variety of genes and constructs, each juxtaposing a different foreign sequence to the poly(A) site, demonstrates the requirement for the authentic downstream region (reviewed in Birnstiel et al., 1985). In particular, in SV40 late pre-mRNA, six different foreign sequences have been used to replace the authentic downstream region, and in each case cleavage is prevented (Fig. 2; Conway and Wickens, 1985). Thus, the downstream sequence must possess special features, missing in most sequences, which enable cleavage to proceed. Therefore, although our data are consistent with those of Mason et al. (1986), we strongly favor the alternative interpretation that the downstream region is essential but is functionally redundant.

Mutations which replace sequences at or within 10 nucleotides of the polyadenylation site, for example, LS−1/+14 and LS−1/+10, modestly reduce cleavage efficiency. This reduction is probably a consequence of altering the sequence which must be cleaved rather than of removing the downstream element. Whereas removal of the downstream element reduces cleavage 20-fold (Figs. 1 and 2), these mutations reduce cleavage less than 2-fold. Furthermore, these mutations all replace the nucleotides within which cleavage normally occurs (Sheets et al., 1987); as a result, cleavage occurs at new positions within the foreign sequence, shifted by one to three nucleotides relative to wild type (not shown).

Only RNAs that are efficiently cleaved promote formation of a precleavage complex; whereas mutant RNAs carrying only short substitutions permit cleavage and complex formation, RNAs carrying either deletions or long substitutions are not cleaved and do not form complexes. Although the isolated downstream region does not detectably form specific complexes in the gel retardation assay nor compete for cleavage (not shown), downstream sequences are required for complex formation and are protected from RNase T1 digestion in the precleavage complex (Humphrey et al., 1987). Together, these results strongly suggest that one essential function of downstream sequences is to promote formation of a precleavage complex.

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DELETIONS

SUBSTITUTIONS

Fig. 7. Formation of a specific precleavage complex requires downstream sequences. 2 fmol of each uniformly labeled RNA was incubated in 12.5 µl of nuclear extract for 10 min under conditions which permit complex formation but reduce the rate of cleavage dramatically (see "Materials and Methods" and accompanying paper). Reactions were stopped by addition of heparin and analyzed by electrophoresis through a nondenaturing gel. "Nonspecific" indicates the position of the nonspecific complex, and "specific" indicates the position of the specific precleavage complex. Length of incubation before heparin treatment is indicated beneath each lane. "+55" denotes −141/+55 RNA. The end points of the deletion mutants are indicated above each lane. "Origin" indicates the position of the gel slots.

Fig. 8. Long, but not short, substitutions prevent complex formation. The short and long substitution mutants from Figs. 4 and 5 were analyzed as in Fig. 7. "Nonspecific" indicates the position of the nonspecific complex, and "specific" indicates the position of the specific precleavage complex. Length of incubation before heparin treatment is indicated beneath each lane.
Redundant Downstream Element and Precleavage Complex

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