Point mutations in AAUAAA and the poly (A) addition site: effects on the accuracy and efficiency of cleavage and polyadenylation *in vitro*

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ABSTRACT

Three sequences in the vicinity of poly (A) addition sites are conserved among vertebrate mRNAs. We analyze the effects of single base changes in each position of AAUAAA and in the nucleotide to which poly (A) is added on 3′ end formation *in vitro*. All 18 possible single base changes of the AAUAAA sequence greatly reduce addition of poly (A) to RNAs that end at the poly (A) addition site, and prevent cleavage of RNAs that extend beyond. The magnitude of reduction varies greatly with the position changed and the base introduced. For any given mutation, cleavage and polyadenylation are reduced to similar extents, strongly suggesting that the same factor interacts with AAUAAA in both reactions. Mutations at and near the conserved adenosine to which poly (A) is added disturb the accuracy, but not the efficiency, of 3′ end formation. For example, point mutations at the conserved adenosine shift the 3′ end of the most abundant 5′ half-molecule downstream by a single nucleotide. The mechanism by which these mutations might exert their effects on the precision of 3′ end formation are discussed.

INTRODUCTION

Most eukaryotic mRNAs possess a poly (A) tract at their 3′ termini. Addition of the poly (A) occurs in the nucleus and involves two sequential steps: cleavage of the primary transcript and the subsequent addition of poly (A) to the newly formed 3′ end (reviewed in 1–4). Both reactions can be faithfully reconstituted *in vitro* by incubating synthetic mRNA precursors in a crude extract of HeLa cell nuclei (5–7).

Three conserved sequences are located in the vicinity of the cleavage site of vertebrate mRNAs: (1) the AAUAAA sequence, (2) the nucleotide to which poly (A) is added (which generally is an adenosine), and (3) the region downstream of this nucleotide. Here we focus on AAUAAA and the nucleotide to which poly (A) is added (i.e., the poly (A) addition site).

The sequence AAUAAA, typically located 15 to 25 nucleotides upstream of the poly (A) addition site, is very highly conserved (8–10). Single base substitutions in AAUAAA reduce the efficiency of cleavage (11–15). They also interfere with the addition of poly (A) to RNAs the end at or near the natural poly (A) addition site (15–18). Here, we generate all 18 possible single base changes in AAUAAA. We quantitate and compare their effects on cleavage and polyadenylation. The results demonstrate that every position is required, and that the extent of reduction varies with the base substituted.

Addition of the poly (A) tail normally is coupled to cleavage of an RNA precursor. The cleavage reaction leaves a 3′ hydroxyl group on the nucleotide to which poly (A) is then added, one base at a time (1–4). In the two mRNAs that have been analyzed in detail, the nucleotide to which poly (A) is added is an adenosine (20,21). This feature appears to be moderately conserved: 70% of natural vertebrate mRNAs possess an A at this position (1; see Methods). Thus, in most mRNAs, the first nucleotide of the poly A tail apparently is derived from the mRNA precursor, not from post-transcriptional processing.

In vitro, cleavage of a synthetic mRNA precursor generates two 'half-molecules' (6,15,19–21). The 5′ half-molecule ends at the poly (A) addition site. The 3′ half molecule comprises a family of RNAs that differ only at their 5′ termini. Each carries a 5′ terminal phosphate. The 5′ end of the longest 3′ half-molecule lies at the poly (A) addition site (20,21). One simple interpretation of these data is that cleavage occurs at the polyadenylation site and is followed by 5′ to 3′ exonucleolytic digestion of the 3′ half molecule. Alternatively, cleavage could occur at each of the sites downstream of the poly (A) site and be followed by 3′ to 5′ trimming to generate the unique 3′ end of the 5′ half-molecule.

In this report we analyze the effect, on cleavage, of single base substitutions at and near the natural poly (A) addition site. We assess their effects both on cleavage efficiency and on the precise location of the 3′ end of the 5′ half-molecules that result. Previous studies strongly suggest that the identity of the terminal nucleotide has little effect on poly (A) addition *per se* (32). Here we

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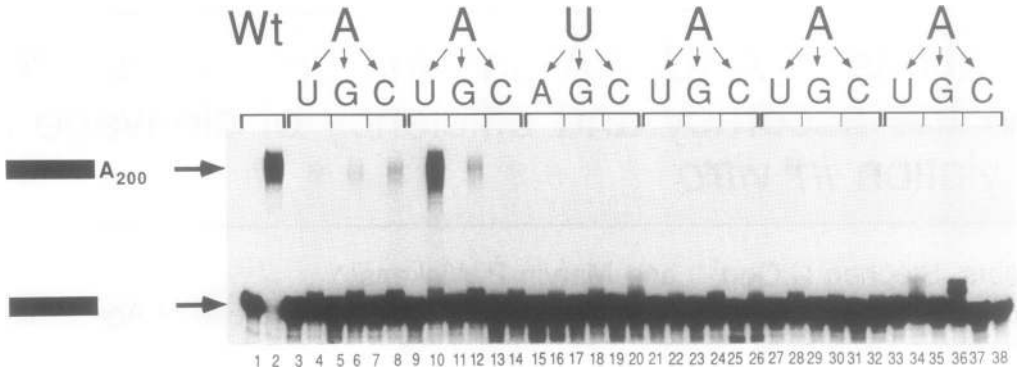

FIGURE 1. Each base in AAUAAA is required for POLY (A) addition. Labeled −58/+7 RNA was incubated in nuclear extract for 30 minutes. The products were analyzed by electrophoresis through a 15% polyacrylamide gel. Each pair of lanes represents RNA before (odd numbered lanes) and after (even numbered lanes) incubation in extract. The position in AAUAAA that is changed is indicated above each pair of lanes. For example, the RNA analyzed in lanes 3 and 4 contains UAUAAA rather than AAUAAA. A black box to the left of the figure indicates the substrate.

demonstrate that its identity is important in determining precisely where the 3′ end of the 5′ half molecule will lie. This result is discussed in light of the two possible mechanisms by which the 3′ end may be formed.

MATERIALS AND METHODS

Plasmids and RNA substrates

A 128 nucleotide fragment of SV40 spanning the poly (A) addition site of virion protein (late) mRNAs (nucleotides 2533 to 2720 of the SV40 genome [numbering as in ref 23]), was derived from pSPSV−141/+70 (12) and cloned into the *Hin*dIII site of pGEM3Z(f+) generating pSPSV−58/+70. This template was cleaved with *Bsm*I 9 nucleotides past the poly (A) site and the two nucleotide 3′ overhang removed with T4 DNA polymerase treatment. This DNA was transcribed to produce −58/+7 RNA. This 77 nucleotide RNA contains 12 nucleotides of vector sequence followed by 65 nucleotides of SV40 sequence. −58/+55 RNA was generated from pSPSV−58/+70 template cleaved with *Dra*I. This 125 nucleotide RNA contains the same 12 nucleotides of vector sequence followed be 113 nucleotides of SV40 sequence. (For sequences, see Fig. 6.)

Mutations in the AATAAA sequence or the cleavage site of pSPSV−58/+70 were generated by oligonucleotide-directed mutagenesis (24). RNAs generated from these templates are identical to −58/+7 except for the changes in AAUAAA and a G to C change at the −9 position. This latter change does not affect processing, and facilitates our assay to locate the 3′ terminus of the 5′ half-molecule (i.e., Fig. 6.).

RNA synthesis *in vitro*

Truncated DNA templates were transcribed *in vitro* as described (25) except the reactions contained 0.1 mM GTP, 1.2 mM GpppG and 50 to 500 μCi of [^{32}P] nucleoside triphosphate. Full length RNAs were purified by elution from urea-containing acrylamide gels (26).

Preparation of nuclear extract and processing *in vitro*

Nuclear extract was prepared by the method of Dignam et al (27), except that $MgCl_2$ was omitted from Buffers A and D. RNA (10 fmol or less) was incubated at 30°C in a 12.5 μl reaction containing 5.5 μl of extract (equivalent to approximately 10^6 cells) and additional components. The final concentrations of the various components (including the contributions of the extract) were as follows: 44 mM KCl, 8.8 mM Hepes (pH 7.6), 0.44 mM EDTA, 2.2 mM DTT, 8.8% glycerol, 20 mM

Sequence	Polyadenylation (% of AAUAAA)		Cleavage (% of AAUAAA)	
	Av.	S.D.	Av.	S.D.
AAUAAA	100		100	
UAUAAA	17	± 3.0	-	
GAUAAA	11	± 1.0	30	± 2.8
CAUAAA	18	± 6.4	28	± 1.4
AUUAAA	77	± 4.7	66	± 6.4
AGUAAA	29	± 8.1	30	± 7.2
ACUAAA	11	± 6.0	11	± 1.8
AAAAAA	4.6	± 3.7	-	
AAGAAA	6.0	± 1.0	-	
AACAAA	4.0	± 2.0	-	
AAUUAA	2.3	± 0.6	-	
AAUGAA	4.3	± 0.6	-	
AAUCAA	4.0	± 1.7	N.D.	
AAUAUA	10	± 2.3	-	
AAUAGA	3.3	± 1.5	2.0	± 2.8
AAUACA	11	± 2.3	-	
AAUAAU	4.7	± 2.3	N.D.	
AAUAAG	1.7	± 0.6	-	
AAUAAC	3.7	± 1.5	-	

FIGURE 2. Polyadenylation and cleavage efficiencies point mutations in AAUAAA. The fraction of each substrate that was polyadenylated or cleaved was quantitated by scanning densitometry using data from at least three experiments (see Materials and Methods). In each case the percentage of the precursor converted to product was calculated and normalized to the value obtained with AAUAAA-containing RNA. For the AAUAAA-containing RNA, the proportion of the RNA that received poly A varied from 76 to 91% in different experiments. The column S.D. lists standard deviations. The symbol (−) indicates that this mutation was not tested for cleavage efficiency. N.D. indicates that cleavage was not detectable with this mutation.

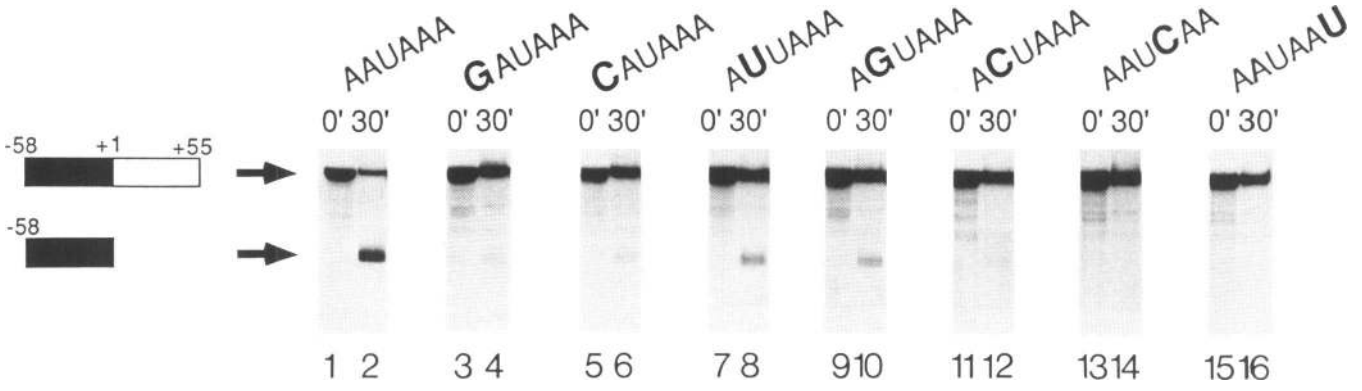

FIGURE 3. AAUAAA is required for cleavage. Labeled −58/+55 RNA was incubated in extract containing 1mM 3'dATP for the times indicated above each lane. The products were analyzed by electrophoresis through a 15% polyacrylamide gel. Lanes 1 and 2, AAUAAA-containing RNA; lanes 3 and 4, GAUAAA-containing RNA; lanes 5 and 6, CAUAAA-containing RNA; lanes 7 and 8, AUUAAA-containing RNA; lanes 9 and 10, AGUAAA-containing RNA; lanes 11 and 12, ACUAAA-containing RNA, lanes 13 and 14, AAUCAA-containing RNA; lanes 15 and 16, AAUAAU-containing RNA.

phosphocreatine, 100 μM ATP, and 2.8% polyvinyl alcohol. In some experiments polyadenylation was inhibited by including 1mM 3' dATP. After incubation, the RNA was purified as described (15).

Polyacrylamide gel electrophoresis

Electrophoresis through 6%, 10% or 15% polyacrylamide gels containing 7 M urea was performed as described (28).

Quantitation and scanning densitometry

For quantitation, films were scanned with a laser microdensitometer. Several exposures of the same gel were used. Films were flashed to ensure linearity of response. This was confirmed by comparing the optical densities of the images to optical densities obtained with known amounts of radioactivity, and by using a Betascope Blot Analyzer (Betagen, Waltham, MA).

RNAse T1 treatment

RNAs were mixed with 20 μg tRNA, and incubated with 10 units RNAse T1 for 30 mins at 37°C, in 10 mM Tris-HCl, pH 8.0, in a 3 μl reaction.

Computer analysis of vertebrate polyadenylation sites

To assess the conservation of AAUAAA among vertebrate mRNAs, 269 vertebrate cDNA sequences from the EMBL data base were analyzed. Only a single member of a closely related gene family (e.g., globins) was included. The criterion used to consider the sequence a genuine 3' end was solely that the cDNA clone terminate in oligo (A). Although this criterion is imperfect, in every case AAUAAA (or a close analog) was found between 4 and 50 nucleotides from the oligo (A) tail. In 75% of the cases, the inferred poly (A) addition site lies between 15 and 25 nucleotides from the U in AAUAAA; in 88% of the cases, AAUAAA and the poly (A) addition site are 10 to 35 nucleotides apart.

The consensus sequence for AAUAAA derived by this analysis is given in the Discussion. Variant sequences that were observed are indicated below, together with their frequency of occurrence (given as a percentage of the 269 sequences examined).

	A	A	U	A	A	U
A	**98**	**86**	0.8	**98**	**95**	**96**
U	0.8	12	**98**	0.5	3.4	1.5
G	1.1	1.9	0.4	1.5	0	0.7
C	0.4	0.7	0.8	0.4	1.5	2.3

Certain mRNAs possess multiple AAUAAA sequences. (The most dramatic case is that of rat cytochrome P-450 mRNA (29), with 13 consecutive and uninterrupted repeats of AAUAAA.) In such mRNAs, only the AAUAAA closest to the poly (A) addition site was considered.

To assess the conservation of the nucleotide to which poly (A) is added, we examined genomic sequences of all 63 of the 269 mRNAs for which genomic sequences were available. By comparing genomic and cDNA sequences, we inferred the identity of the nucleotide to which poly (A) apparently had been added. In those cases in which the start of the poly (A) coincided with an A in the genomic sequence, that A was considered to be the nucleotide to which poly (A) is added post-transcriptionally. (This assumption was based on detailed anaylsis of adenovirus L3 (20) and SV40 late mRNA (21) mRNAs, which demonstrated that poly (A) post-transcriptionally to an A present in the precursors.) The 'consensus sequence' derived by this analysis is given below, together with the frequency of occurrence of all nucleotides near the poly (A) addition site (designated +1).

					−1	+1				
	X	X	X	X	c	A	X	X	X	X
A	46	30	33	17	13	**71**	32	17	22	19
U	16	17	40	43	17	13	24	41	24	33
G	19	17	11	25	11	5	22	19	22	19
C	19	35	16	14	**59**	11	22	22	32	24

Thus, CA is the most common dinucleotide to which poly (A) is added.

RESULTS

Every base in AAUAAA is required for polyadenylation

−58/+7 RNA containing AAUAAA receives poly (A) with high efficiency in the nuclear extract (30). After 30 minutes incubation in extract, more than 85% of the substrate is converted into higher molecular weight products (Fig. 1, lanes 1 and 2). These products result from the addition of poly (A), as shown by their retention on oligo (dT) cellulose (data not shown). Polyadenylation of this RNA proceeds without cleavage (30).

To examine the requirement for AAUAAA in polyadenylation

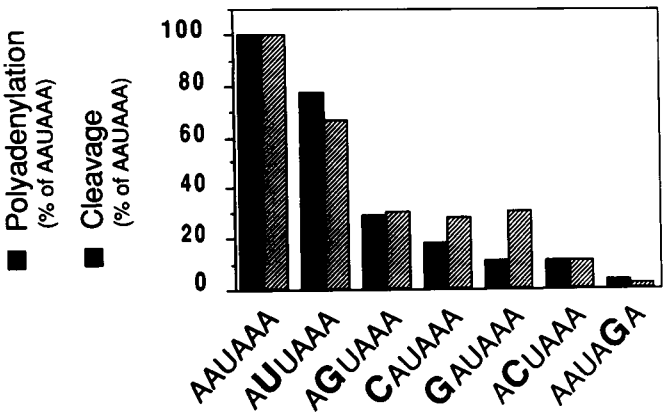

FIGURE 4. Comparison of the effects of AAUAAA mutations on cleavage and on poly (A) addition. Values are taken from Fig. 2.

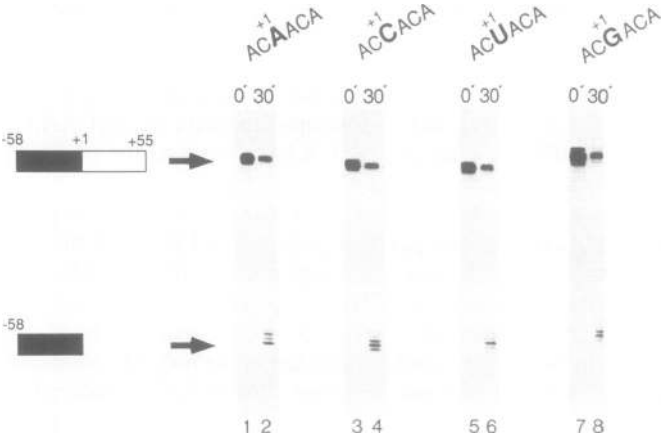

FIGURE 5. Point mutations at the poly (A) addition site do not prevent cleavage. Labeled −58/+55 RNA was incubated in extract containing 1mM 3′dATP for the times indicated above each lane. The products were analyzed by electrophoresis through a 6% polyacrylamide gel. Lanes 1 and 2, +1A-containing RNA (i.e. the natural sequence); lanes 3 and 4, +1C-containing RNA; lanes 5 and 6, +1U-containing RNA; lanes 7 and 8, +1G-containing RNA. For reference the six nucleotides from −2 to +4 in each RNA are indicated above each pair of lanes. The termini of the 5′ half-molecules in this assay and in the assays shown in Figures 6, 7, 8 and 10 differ slightly, because poly (A) addition was blocked with 3′dATP in this experiment, but with 1 mM EDTA in those experiments. 1 mM 3′dATP permits a small amount of A-addition, but 1 mM EDTA prevents it nearly entirely.

we generated all 18 single nucleotide variations in this sequence by oligonucleotide-directed mutagenesis. Each of the 18 point mutations reduce polyadenylation efficiency (Fig. 1, lanes 3−38). The extent of reduction varies (Figs. 1 and 2). For example, AUUAAA-containing RNA is polyadenylated 80% as efficiently as is AAUAAA-containing RNA, while AAUAAG depressed polyadenylation more than 50-fold. We conclude that each base in AAUAAA is required for poly (A) addition, but that quantitatively, the magnitude of the requirement varies with the position in the sequence and the base that is introduced.

Similar effects on polyadenylation and on cleavage

To determine, quantitatively, the extent to which different mutations in AAUAAA impair cleavage, we prepared seven

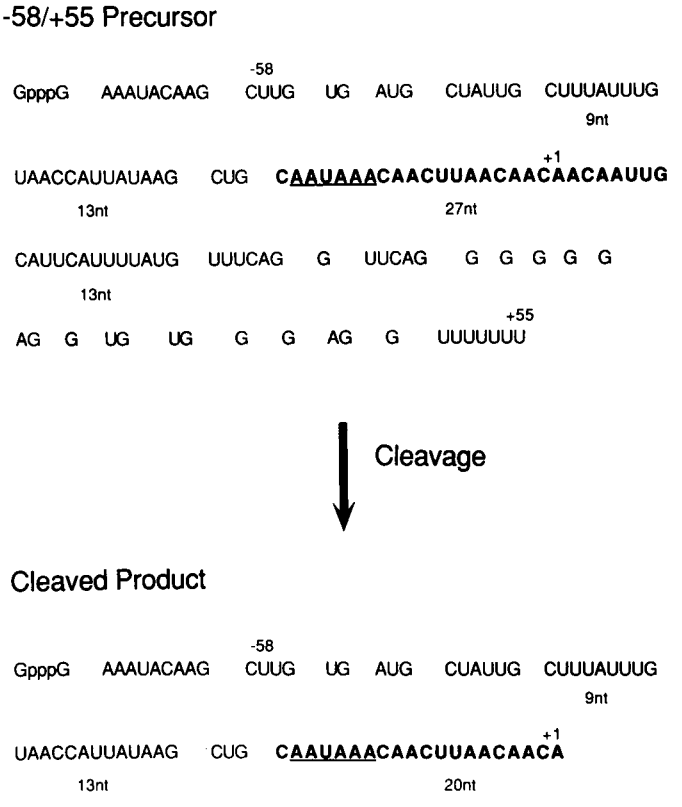

FIGURE 6. Strategy of the T1 ribonuclease assay: predicted T1 oligonucleotides before and after cleavage of −58/+55 RNA. Only the 5′ half-molecule generated by cleavage is shown ('cleaved product').

−58/+55 RNAs, each with a different point mutation in AAUAAA. Each RNA was incubated with nuclear extract and 3′dATP. This ATP analog permits cleavage, but prevents extension of the poly (A) tail. As a result, cleavage of −58/+55 AAUAAA-containing RNA generates a 71 nucleotide product, the 5′ 'half-molecule' (Fig. 3, lanes 1 and 2). 80% of the AAUAAA containing substrate is cleaved during the 30 minute incubation. All seven point mutants tested reduce cleavage efficiency (Fig.3, lanes 3 to 16).

As with polyadenylation, cleavage is impaired to different extents by each mutation (Figs. 2 and 3). Importantly, the quantitative effects of each mutation on cleavage and on polyadenylation are very similar, though not identical (Fig. 4). From these results we infer that cleavage and polyadenylation probably share a common processing factor that interacts with AAUAAA. The interaction of this factor with substrates lacking AAUAAA is rate limiting for both reactions.

Mutations at the +1 position prevent accurate cleavage, but do not affect cleavage efficiency

The conserved nucleotide to which poly (A) is added (i.e., the +1 position) is commonly an adenosine (see Methods). To examine whether this moderately conserved adenosine is required for efficient cleavage, we compared cleavage of RNAs containing either A, C, U or G at this position. Each substrate yields the same amount of 5′ half-molecule (Fig. 5). Thus the efficiency of cleavage is not affected by the identity of the base at +1.

To assess whether mutations at the +1 position affect the accuracy of cleavage, we developed a simple assay that identifies

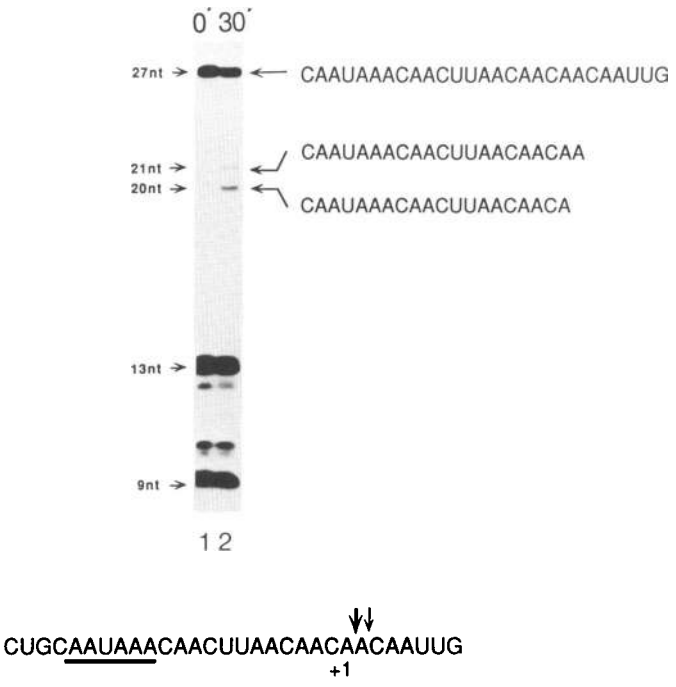

FIGURE 7. Defining the 3' ends of the −58/+55 cleavage products using the T1 ribonuclease assay. Labeled −58/55 RNA was incubated in extract containing 1mM EDTA for the times indicated above each lane. The products were incubated with RNase T1 and then analyzed by electrophoresis through a 15% polyacrylamide gel. The oligonucleotides 20 and 21 nucleotides in length (lane 2) correspond to cleavage after the +1A and the +2A respectively (see Fig. 6). The arrows above the sequence at the bottom of the figure indicate the deduced locations of the 3' termini of the 5' half-molecules. The darkness of the arrow is proportional to the abundance of that 5' half-molecule.

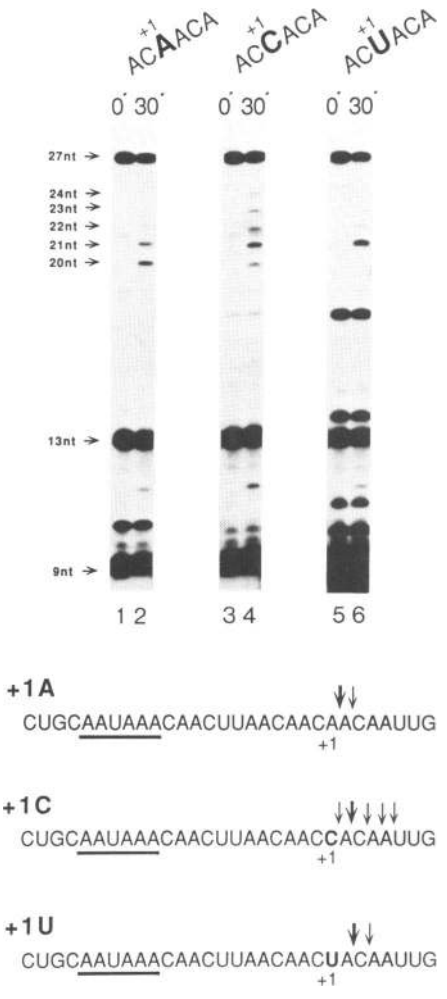

FIGURE 8. Point mutations at the poly (A) addition site shift 3' termini of 5' half-molecules. Labeled −58/+55 RNA was incubated in extract containing 1mM EDTA for the times indicated above each lane. The products were incubated with RNase T1 and then analyzed by electrophoresis through a 15% polyacrylamide gel. Lanes 1 and 2, +1A-containing RNA (the natural sequence); lanes 3 and 4, +1C-containing RNA; lanes 5 and 6, +1U-containing RNA. The arrows above the sequence at the bottom of the figure indicate the deduced sites of cleavage. The darkness of the arrow is proportional to the abundance of that 5' half-molecule. The bands found in both lanes 5 and 6 approximately 10, 14 and 18 nucleotides in length are due to a contaminating non SV40 RNA present in the starting material.

the 3' terminus of the 5' half-molecule at single nucleotide resolution. This assay is depicted in Figs. 6 and 7. Uniformly [^{32}P]UTP labeled RNA is incubated in nuclear extract containing 1 mM EDTA. EDTA permits cleavage, but prevents polyadenylation (6,18,21). After incubation, the total RNA is recovered and treated with RNase T1. The entire mixture is then analyzed by gel electrophoresis. The G normally present at the −9 position of the substrate has been changed to a C. As a result, the RNase T1 oligonucleotide containing the cleavage site is longer (20 nucleotides) than all other T1 oligonucleotides in the cleaved RNA, and so is well separated from all other products. It is short enough, however, that its length can be determined precisely. The results of such an assay are depicted in Fig. 7, in which we have used an RNA containing adenosine at the poly (A) addition site (i.e. the natural sequence). RNase T1 treatment of the RNA incubated in extract (Fig. 7, lane 2) generates two new oligonucleotides not found in the precursor (Fig. 7, lane 1). These new oligonucleotides are 20 and 21 nucleotides in length, and so correspond to 3' termini at +1 and +2. These termini are identical to the those identified by extensive fingerprint analysis (21), thereby validating the assay method.

The assay was used to position precisely the 3' termini generated by cleavage of RNAs with either U or C at the +1 position (Fig. 8). As a control, an RNA containing an A at +1 was analyzed in parallel. RNA containing a C at +1 generates 3' termini at +2A and, to lesser extents, at +1C, +3C, +4A and +5A. RNA containing a U at +1 generates 3' termini almost exclusively at +2A. As expected, the wild type control generates 3' termini after +1A and, to a lesser extent, +2A. We conclude that the identity of the base at +1 influences the precise position of the 3' ends of 5' half-molecules, but does not influence the efficiency of the reaction.

A double mutation replacing the two adjacent adenosines at the poly (A) addition site does not prevent cleavage

Converting the A at +1 to either U or C shifted the terminus of the 5' half-molecule to the adjacent adenosine downstream (Fig. 8). Thus, although an adenosine at +1 is not essential, cleavage might still require an adenosine near that position. If this were true, then simultaneously changing the A's both at +1 and +2 might reduce the efficiency of cleavage. We therefore prepared RNA in which both the +1A and +2A were converted to U's. As seen in Fig. 9, the same amount of cleaved product was generated from this substrate as from wild type RNA. The 3' termini generated from the double mutant substrate are shifted

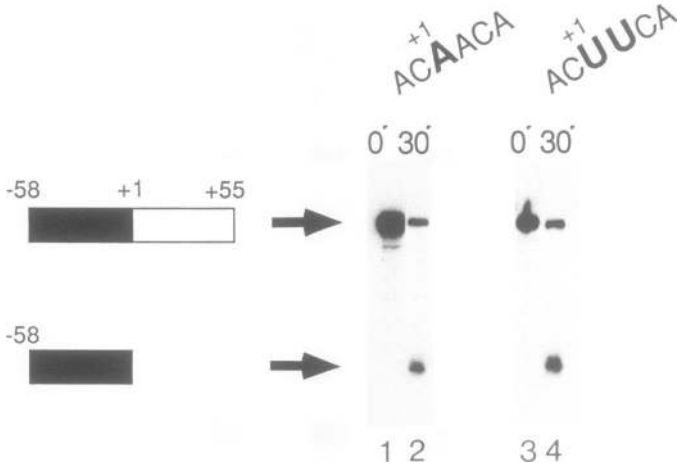

FIGURE 9. A double mutation replacing the two adjacent adenosines at the poly (A) addition site does not prevent cleavage. Labeled −58/+55 RNA was incubated in extract containing 3′dATP for the times indicated above each lane. The products were analyzed by electrophoresis through a 6% polyacrylamide gel. Lanes 1 and 2, +1A,+2A-containing RNA (the natural sequence); lanes 3 and 4, +1U,+2U-containing RNA.

to +3C, and to a lesser extent, +4A and +5A (Fig. 10). We conclude the adenosines at +1 and +2 are required for accurate 3′ end formation, but not for efficient cleavage.

DISCUSSION

The data presented here demonstrate that each base in AAUAAA is required for polyadenylation and are consistent with a recent analysis of the effects of AAUAAA mutations on cleavage (31). The entire AAUAAA sequence is necessary for cleavage and for polyadenylation. Furthermore, AAUAAA is virtually sufficient for polyadenylation: a 14 nucleotide RNA in which the first six nucleotides are AAUAAA efficiently receives poly (A) (32). Efficient cleavage requires a downstream element as well (1−4).

Although AAUAAA is very highly conserved, natural variants do occur. A consensus sequence, derived by the comparison of 269 vertebrate mRNA sequences (see Methods), is as follows.

$$A_{98}A_{86}U_{98}A_{98}A_{95}A_{96}$$
$$U_{12}$$

As demonstrated in this report, mutations in AAUAAA decrease the rate of cleavage to different extents. The mildest mutation, AUUAAA, corresponds to the most common natural variant. The mutation to AGUAAA also is processed rather efficiently (approximately 30% as well as AAUAAA), but is not commonly found in natural mRNAs. All other AAUAAA mutations severely impair polyadenylation and cleavage, and are very rare among natural mRNAs.

In light of our results, the fact that these other natural variants occur raises the question of how they are tolerated *in vivo*. One obvious possibility is that cleavage and polyadenylation may not be rate-limiting for expression of those genes, even though it is decreased 20-fold or more in absolute rate (i.e., scissions per unit time). However, for several experimentally manipulated genes, mutations in AAUAAA do dramatically reduce the amount of mRNA produced *in vivo* (11−14), demonstrating that quantitative defects in cleavage rate due to AAUAAA mutations can indeed make the reaction rate-limiting for mRNA expression

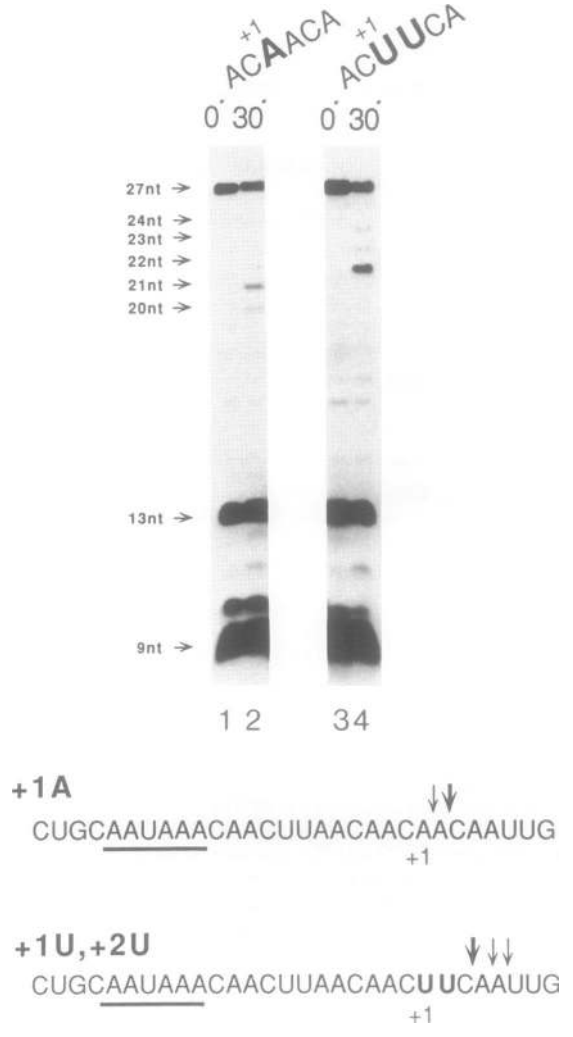

FIGURE 10. A double mutation replacing the two adjacent adenosines at the poly (A) addition site shifts 3′ termini of 5′ half-molecules. Labeled −58/+55 RNA was incubated in extract containing 1mM EDTA for the times indicated above each lane. The products were incubated with RNase T1 and analyzed by electrophoresis through a 15% polyacrylamide gel. Lanes 1 and 2, +1A,+2A-containing RNA (the natural sequence); lanes 3 and 4, +1U,+2U-containing RNA. The arrows above the sequence at the bottom of the figure indicate the deduced sites of cleavage. The darkness of the arrow is proportional to the abundance of that C5′ half-molecule.

in vivo. A second possibility is that natural variants of AAUAAA might be tolerable because sequences elsewhere in the precursor compensate for the defect in AAUAAA. Certain natural variations in AAUAAA co-vary with other nucleotides. For example, AAUAAG most commonly is followed by an A, rather than a C as in the SV40 late mRNAs tested here. However, at least in the SV40 late mRNA context, converting AAUAAGC to AAUAAGA does not enhance polyadenylation efficiency (not shown). Further tests using natural precursors that contain AAUAAA variants may reveal whether the context within which an AAUAAA variant is found can enhance its use.

Our data strongly suggest that the same factor is involved in AAUAAA recognition in cleavage and in polyadenylation, since mutations in AAUAAA effect both reactions to a similar extent (Fig. 4). This conclusion is supported by several other lines of evidence. For example, cleavage is inhibited by an excess of RNA that ends at the poly (A) site, as long as that RNA contains AAUAAA (33). In addition, biochemical fractionation of the

nuclear extract strongly suggests that the same component provides AAUAAA-recognition activity for both cleavage and the polyadenylation reactions (34–38).

The nucleotide to which poly (A) is added—that is, the 3′ terminus of the 5′ half-molecule—is an adenosine in approximately 70% of vertebrate mRNAs (1,8; Methods). Each of the other three bases do appear at this position in certain mRNAs, albeit less frequently (see Methods). In our experiments, alterations at the +1 adenosine had no effect on the efficiency of cleavage, but did alter the 3′ end of the resulting 5′ half-molecule. These effects would not be expected to reduce levels of mRNA due to inefficient polyadenylation, since the identity of the terminal nucleotide has little effect on poly (A) addition (32).

The biochemical basis of the effects of the +1 and +2 mutations on the position of 3′ termini can not yet be determined, because it is not known precisely how the poly (A) addition site is formed from even a wild type precursor RNA. *In vitro*, cleavage generates two half-molecules (19–21). The 5′ half-molecule ends at the normal poly (A) addition site (+1) and carries a 3′ hydroxyl group. A family of 3′ half-molecules are produced, with 5′ termini scattered over a range of 8 nucleotides. The longest of these has a 5′ terminus at +2. Each member of this family ends in a 5′ phosphate group. Thus, endonucleolytic cleavage could occur at the phosphate between +1 and +2. The RNAs downstream of the cleavage site would then be formed by a 5′ to 3′ exonuclease. In this model, mutations at +1 would exert their effects by shifting the phosphodiester bond that is broken. In an alternative view, endonucleolytic cleavage would occur downstream of +1, at each of the sites represented in the family of 3′ half-molecules. The unique 3′ terminus of the 5′ half-molecule would then result from a 3′ to 5′ exonuclease that encounters a barrier at +1. In this model, mutations at +1 would affect the position of the barrier, not the sites of endonucleolytic cleavages. To distinguish between these explanations will require identifying the precise sites of endonucleolytic scission. Regardless, the data presented here demonstrate that mutations at the moderately conserved, natural poly (A) addition site do not detectably reduce cleavage efficiency, but do disturb the accuracy of the process.

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