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Sequences capable of restoring poly(A) site function define two distinct downstream elements

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Several recent studies have shown that a functional poly(A) site consists of both an AAUAAA element as well as sequences downstream of the cleavage site. Two downstream regions were analyzed in an attempt to accurately locate and define the critical sequences. Chemically synthesized oligonucleotides of sequence from the early SV40 and the adenovirus E2A poly(A) sites were able to restore efficient cleavage to a deleted SV40 poly(A) site. Inversion of the sequence completely abolished poly(A) site function. A series of base substitution mutants were generated in each downstream sequence. Certain single base changes drastically altered poly(A) site function. Thus, it is concluded that a defined downstream sequence of limited complexity is important for efficient processing of the primary transcript at the poly(A) site. The position of the downstream elements relative to the AAUAAA and cleavage site was found to be critical since moving either the E2 element or the SV40 element an additional 40 nucleotides downstream abolished function. There were differences, however, in the effect of spacing on the function of the two elements. This observation, along with the fact that the two sequences are clearly different, indicates that there are at least two distinct genetic elements that direct efficient cleavage at the poly(A) site.

Key words: poly(A) site cleavage/downstream elements

Introduction

The expression of a gene can be regulated at many steps in the process of mRNA biogenesis including the formation of the poly(A) addition site (Nevins, 1982, 1983; Birnsteil *et al.*, 1985). In several cases it has been shown that poly(A) site selection can regulate the final output from a transcription unit (Nevins and Wilson, 1981; Amara *et al.*, 1984). In light of this, it is essential to develop an understanding of the actual mechanism for poly(A) site formation, including a definition of sequences critical for poly(A) site formation, delineation of those sequences necessary for regulated expression, and identification of factors that recognize these sequences. This latter goal now seems feasible given the development of cell-free systems that accurately process RNAs at the poly(A) site (Moore and Sharp, 1985) and that recognize essential sequences (Hart *et al.*, 1985b; Zarkower *et al.*, 1986).

We have previously demonstrated the presence of sequences in the early SV40 and adenovirus E2A poly(A) sites that, in addition to the AAUAAA, were critical for poly(A) site formation (McDevitt *et al.*, 1984; Hart *et al.*, 1985a). Similar studies have been carried out in a number of other gene systems with the same general result (Simonsen and Levinson, 1983; Sadofsky and Alwine, 1984; Gil and Proudfoot, 1984; Woychik *et al.*, 1984; Cole and Stacy, 1985; McLauchlan *et al.*, 1985; Conway and Wickens, 1985). In all of this previous work the presence of such critical sequences was inferred by deletion analysis and subsequent loss of function. We have further analyzed the early SV40 and adenovirus E2A poly(A) sites to identify specific downstream sequences as the functional elements by virtue of the ability of short oligonucleotides to restore efficient cleavage activity. This approach has allowed us to define two such elements that differ in sequence and functional properties, that could efficiently restore cleavage activity.

Results

Restoration of poly(A) site cleavage with oligonucleotides

To simplify the analysis of poly(A) site function, we have made use of a single deleted early SV40 poly(A) site, that retains a cleavage site but a minimal amount of additional sequence, as an assay system for sequences that can restore activity. The sequence of the early SV40 poly(A) site and the non-functional deletion mutant pEC+5 are shown in Figure 1. The plasmid pEC+5 derives from the plasmid pEC (Imperiale *et al.*, 1985) and contains an adenovirus E2 promoter, the chloramphenicol acetyl transferase gene, an SV40 splice site and the early SV40 poly(A) site deleted to within five nucleotides of the cleavage site (Hart *et al.*, 1985a). A comparison of the activity of this poly(A) site to either the wild-type early SV40 poly(A) site or one that retains 18 nucleotides of downstream sequences demonstrates that the pEC+5 is less than 1% as efficient as the pEC+18 (Hart *et al.*, 1985a) (see also Figure 2A). We have therefore made use of this vector, which retains an AATAAA element and a cleavage site, as an assay system for sequences necessary for restoration of efficient cleavage at the SV40 poly(A) site.

Based on our previous experiments that roughly defined the SV40 sequences necessary for cleavage, we constructed an oligonucleotide as shown in Figure 1B that includes a portion of the sequences removed by the pEC+5 deletion. The oligonucleotide was synthesized with a *Bam*HI site on one end and a *Sau*IIIa site on the other and then inserted into the *Bam*HI site of pEC+5. As shown in Figure 2A, the SV40 oligonucleotide could indeed restore efficient poly(A) site cleavage as determined by an RNase protection assay using an SP6 RNA probe. The band indicated by the arrow in this and subsequent experiments is the RNase product resulting from hybridization to SV40 RNA correctly cleaved at the poly(A) site. The slower migrating band results from hybridization to uncleaved transcripts and the faster migrating bands result from hybridization to RNA cleaved at minor sites (Hart *et al.*, 1985a). In multiple assays, the efficiency of poly(A) site function was increased at least 30-fold and was ~35% the efficiency of the pEC+18 plasmid. The less than complete restoration of activity may be in part due to the construction, which displaces the sequence seven nucleotides downstream

(see Figure 1D), as well as to a small contribution from residues between +14 and +18. This contribution is minimal, however, since an oligonucleotide of the full sequence between +6 and +18 was only about 2-fold more active (data not shown). If the oligonucleotide was inserted in the opposite orientation, there was no restoration of cleavage function, consistent with our recent observations that the downstream sequence is recognized in the RNA (Hart *et al.*, 1985b). It seems unlikely that the five nucleotides remaining beyond the poly(A) site in pEC+5 (nucleotides +1 to +5) also play a role since this sequence and the oligonucleotide are interrupted by the linker. Therefore, these results allow us to conclude that poly(A) site function can be efficiently restored by a small DNA element of limited complexity.

Mutagenesis of the SV40 downstream element

To begin to define sequences in the downstream element important for function, we have generated a series of single base substitutions. We made use of a procedure described by Matteucci and Heyneker (1983) that involves oligonucleotide synthesis with mixed nucleotides at each position. One strand was synthesized as the wild-type sequence and the other was synthesized with mixtures to favor single base substitutions (see Materials

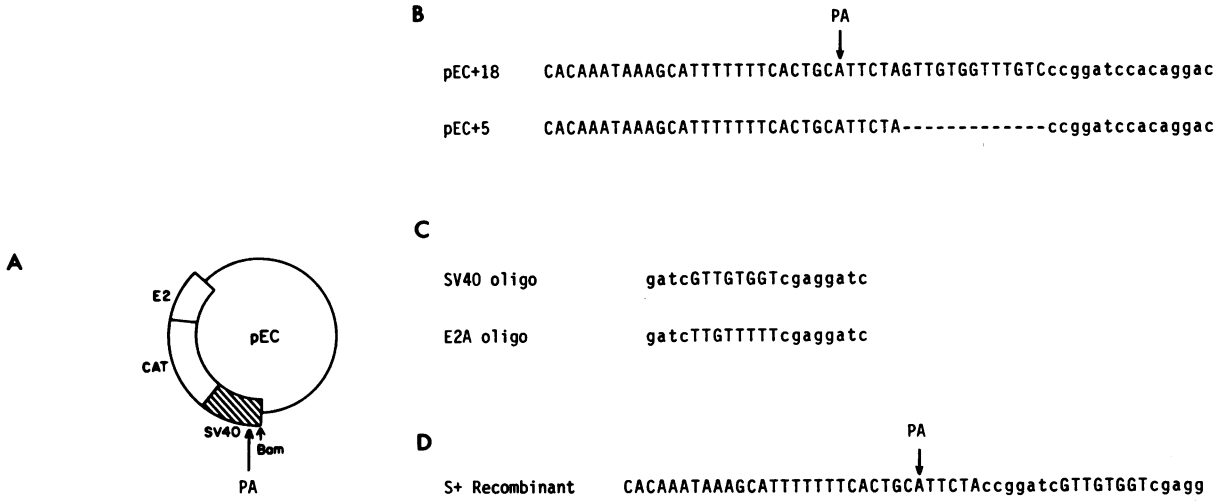

Fig. 1. Sequences of the SV40 early poly(A) site, oligonucleotides and reconstructed poly(A) sites. **(A)** Map of the plasmid pEC (Imperiale *et al.*, 1985). The plasmid contains an adenovirus E2 promoter, the chloramphenicol acetyl-transferase (CAT) gene, and the early SV40 poly(A) site including 53 nucleotides of SV40 specific sequence downstream of the cleavage site. **(B)** Nucleotide sequence of the early SV40 poly(A) site including the 18 nucleotides of downstream sequence retained in the pEC+18 plasmid and the nature of the deletion in the pEC+5 plasmid. The sequence in lower case is the vector sequence. **(C)** Nucleotide sequence of the synthetic oligonucleotides used for restoration of poly(A) site activity. The lower-case nucleotides are linker sequences that allowed re-insertion into the pEC+5 vector. **(D)** Nucleotide sequence of the recombinant resulting from insertion of the SV40 oligonucleotide into the pEC+5 vector.

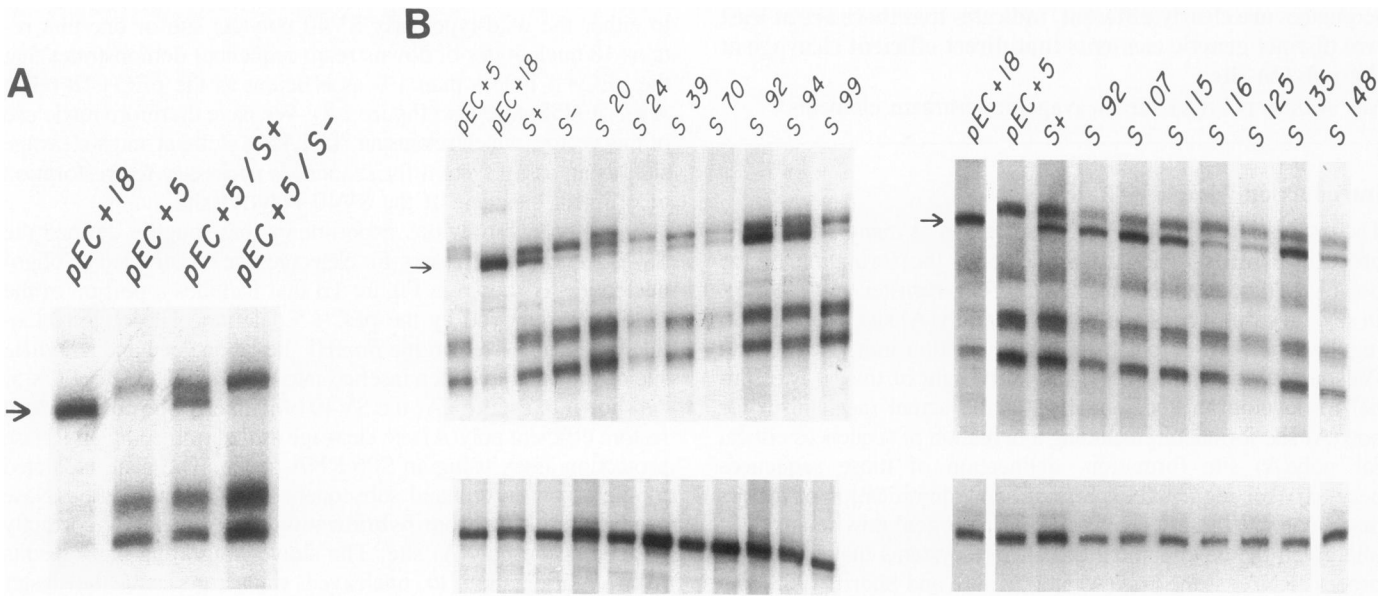

Fig. 2. Activity of the early SV40 oligonucleotide and mutants in restoring poly(A) site function. **(A)** The pEC+18 and the pEC+5 plasmids were assayed by transfection into 293 cells followed by 3′ end analysis with an SP6 probe. In adddition, assays of the pEC+5 vector with the SV40 oligonucleotide inserted in the correct orientation (S+) or the reverse orientation (S−) are shown. **(B)** SP6 assays of the various base substitution mutants of the early SV40 downstream sequence. The arrow indicates the RNA protection product of correctly cleaved transcript. The nature of the base changes in the plasmids are given in Table I. Shown in the bottom panel are assays of internal controls from each transfection. A constant amount of the plasmid pE2 wsa included in each transfection and then assayed for 3′ end formation using an E2-specific SP6 probe (Hart *et al.*, 1985b).

and methods). These were annealed, ligated to pEC+5 and used to transform. Colonies were screened by hybridization with the oligonucleotide of wild-type sequence followed by washes at increasing stringency. Screening was effective in isolating a number of desired mutants.

An analysis of the function of the set of early SV40 substitution mutants is shown in Figure 2B and is summarized in Table I. In each case, the activity was controlled by employing multiple assays as well as an internal control. As can be seen, certain point mutations, for example S-94, S-70, S-148, S-125, had little if any effect on the function of the oligonucleotide. Others, however, had significant effects on the ability of the element to restore function. Surprisingly, several single base changes increased the activity of the element to restore cleavage, in some cases as much as 2.9-fold (S-107, S-92). Other mutations (S-39, S-20) clearly had an adverse effect on function of the downstream element with more than a 10-fold differential between the most efficient (S-107) and the least efficient (S-20). In addition, two double mutants exhibited additive effects. The mutants S-39 and S-20, each harboring a single base change, were reduced in activity by factors of three and four respectively. A combination of these two mutations in S-24 resulted in an additional 8-fold drop in activity, virtually eliminating any cleavage function. Another combination with the S-39 mutation had the opposite effect. The S-107 mutation increased cleavage function. When combined with the S-39 mutation (S-94) there was also an increase in the activity. These results clearly show the importance of these residues in the cleavage function.

From these results we draw two conclusions. First, the mutation analysis indicates that there are indeed critical residues in the oligonucleotide since single base changes either significantly increase or decrease activity. This is most dramatically illustrated by the S-24 double mutant. The RNA produced from this plasmid is identical, except for two nucleotides, to that from the S+ plasmid yet the cleavage efficiency is reduced 30-fold. Second, the SV40 downstream element does not appear to function at full capacity since single base substitutions can significantly increase cleavage activity.

Table I. Relative activity of SV40 base substitution mutants

Plasmid	Sequence	Activity[a]
S(+)	G T T G T G G T	100
S-107	– – – – – – T –	289
S-92	– – – – – T – –	286
S-135	T – – – – – – –	169
S-115	– – – – – – – – T	169
S-146	– – – – – – – –	160
S-148	– – – – – – C –	91
S-125	– – – A – – – –	91
S-70	– – – – – – A –	86
S-94	– – – – A – T –	77
S-116	– – – – C – – –	49
S-99	– – – – – A – –	43
S-39	– – – – A – – –	29
S-20	– – – T – – – –	23
S-24	– – – T A – – –	3
S(–)	A C C A C A A C	7

[a]Activity is the level of RNA produced with the correct 3′ end, as assayed with an SP6 probe and corrected by the internal controls, relative to the level produced with the wild-type sequence which was given a value of 100. The data was obtained by densitometric scanning of films such as shown in Figure 2 and is the average of at least two independent experiments.

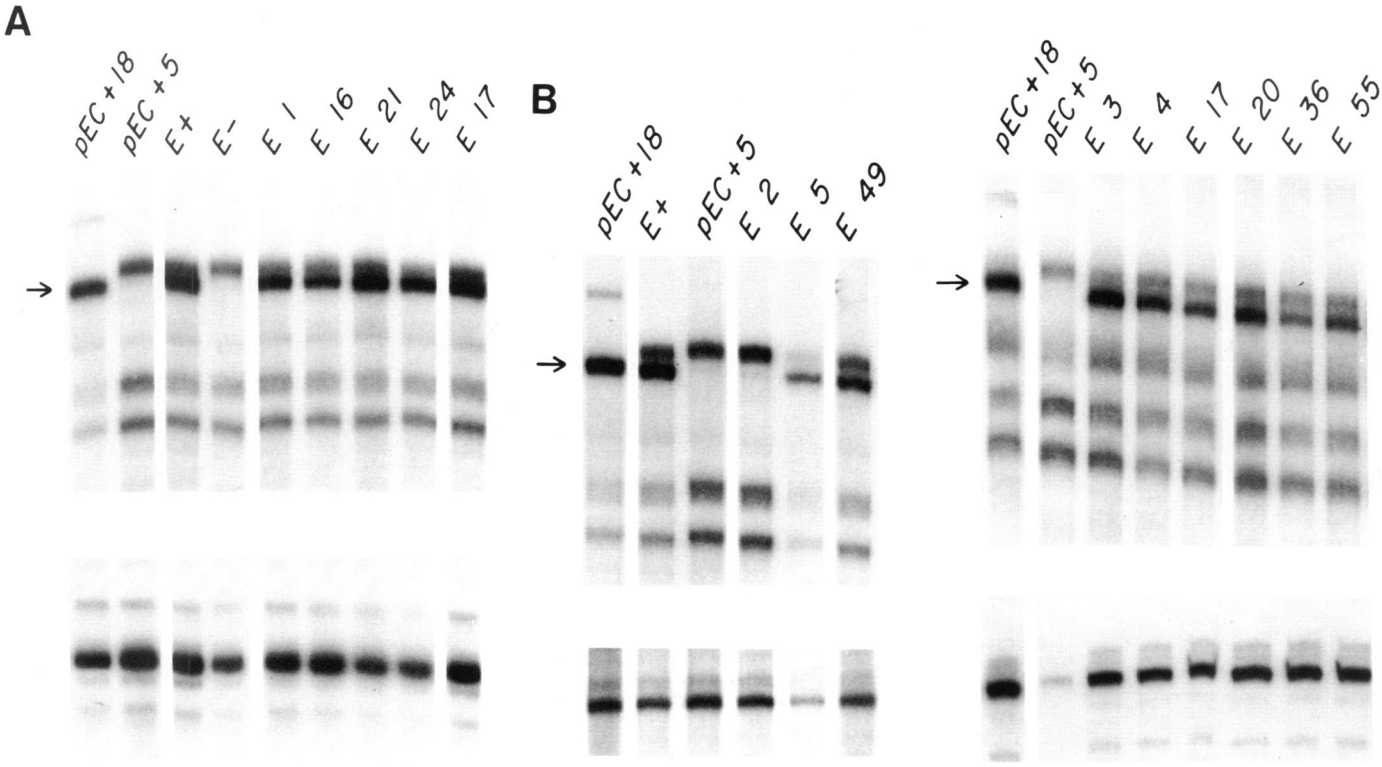

Fig. 3. Activity of the adenovirus E2A oligonucleotide and mutants in restoring poly(A) site function. **(A)** Assays as described in Figure 2 are shown for pEC+18, pEC+5 and the E2A oligonucleotide recombinant inserted in the correct orientation (E+) and the reverse orientation (E–). Also shown are assays of several E2A base substitution mutants. Assays of internal control are shown below. **(B)** Assays of additional E2A base substitutions. See Table II for the nature of the changes.

Analysis of an adenovirus E2A downstream element

We have previously defined sequences in the adenovirus E2A poly(A) site, downstream of the cleavage site, that were critical for function (McDevitt *et al.*, 1984). More recent experiments had suggested that the sequence TT(A/G)TTTTT might be the critical element (Hart *et al.*, 1985a). We have employed the procedures described above for the SV40 element to determine the critical domain in the E2A downstream region. The sequence TTGTTTTT was synthesized, as well as base substitution variants of this sequence, and inserted into the pEC+5 assay vector. We have already shown that the natural E2A downstream sequence can restore cleavage activity to the SV40 +5 deletion (Hart *et al.*, 1985a). As shown in Figure 3A, the E2A oligonucleotide also restores function to the pEC+5 plasmid. In fact, this sequence functioned better than the SV40 oligonucleotide and even better than the natural SV40 (pEC+18) poly(A) site (data not shown). In addition, as with the SV40 element, the E2A sequence inserted in the opposite orientation was not active.

Table II. Activity of E2A base substitution mutants

Plasmid	Sequence	Activity[a]
E(+)	T T G T T T T T	100
E-21	– – A – – – – –	132
E-4	– – T – – – – –	112
E-24	– – – A – – – –	101
E-3	– – – – – A – –	99
E-5	– – – – – – G –	96
E-16	– – – – G – – –	95
E-20	– – – C – – – –	86
E-49	– – – – – G – –	83
E-1	– – – – – – A –	67
E-17	– – – – – C – –	66
E-55	– – – – A – – –	62
E-36	– – C – – – – –	61
E-63	– – A G – – – –	50
E-2	A A A G A C A A	3
E(−)	A A A A A C A A	2

[a]See legend to Table I.

A similar analysis of the set of adenovirus E2A base substitution mutants is shown in Figure 3B and summarized in Table II. One of the 'mutants' isolated (E-21) was a G to A change yielding TTATTTTT, the other form of the suspected essential sequence. Indeed, this sequence was better at restoring function than the TTGTTTTT. In addition, a G to T change, resulting in a TTTTTTTT sequence (E-4), was also more effective than the original sequence. Several changes did have a down effect on activity, although never to less than 38% of wild-type activity (activity of the TTATTTTT element). These results would suggest once again that we have defined a rather small sequence capable of restoring poly(A) site function and that the essential sequence may simply be a run of T residues (U residues in the RNA). This contrasts with the results of mutating the SV40 sequence when a G to T alteration, in a run of T residues, effec-

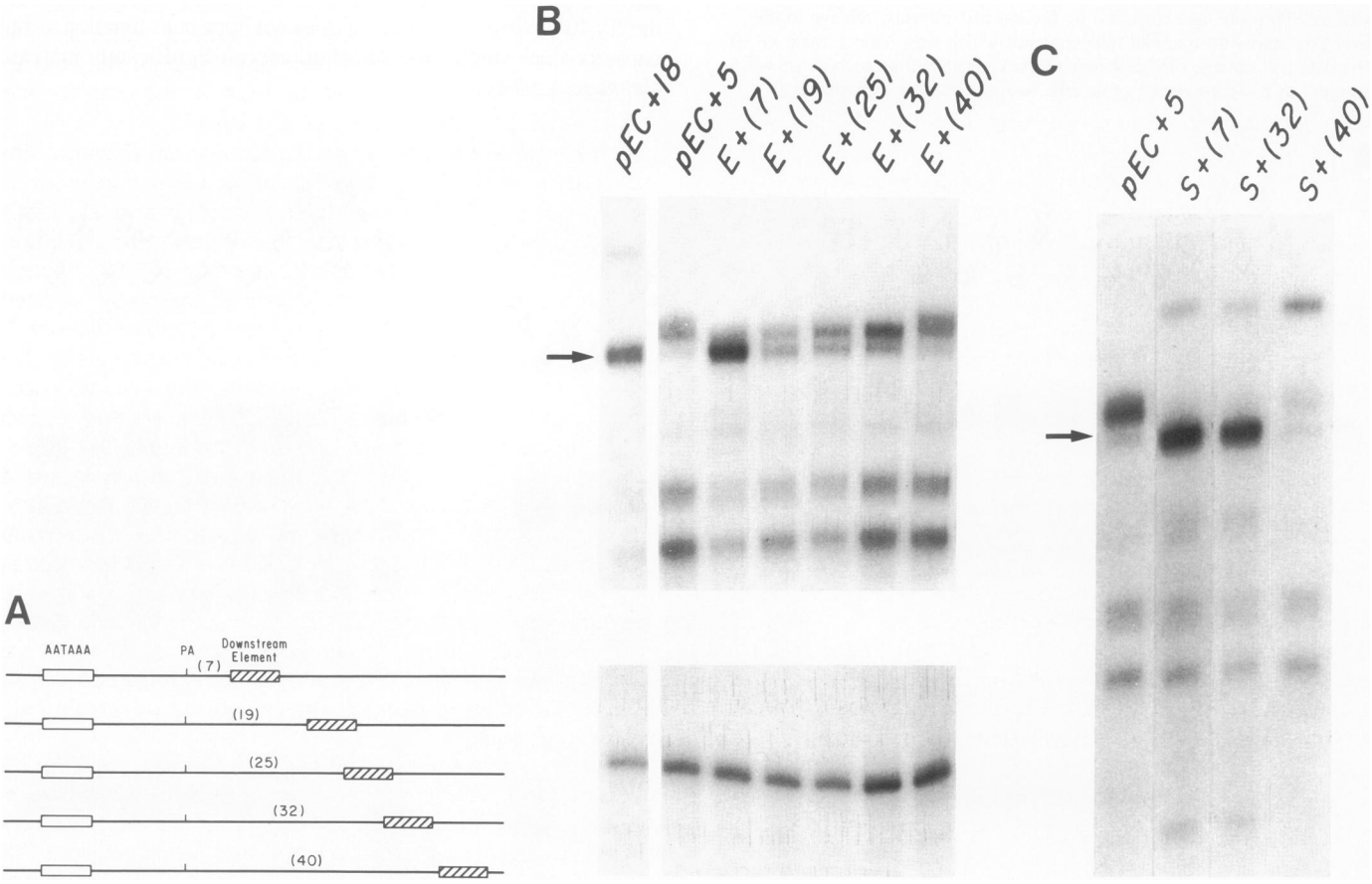

Fig. 4. Activity of the downstream elements dependent on spacing relative to the AAUAAA/cleavage site. **(A)** Schematic of the structure of the poly(A) sites in which the downstream element has been displaced relative to the cleavage site. The numbers in parenthesis refer to the number of nucleotides of polylinker DNA that have been inserted before the E2A or SV40 oligonucleotide, spacing it further downstream from the poly(A) site (see Materials and methods). **(B)** and **(C)** SP6 assays, as described in Figure 2, for 3′ end formation from the various plasmids containing either an E2A downstream element **(B)** or an SV40 downstream element **(C)**.

Table III. Effects of spacing of the downstream element on the relative activity of oligonucleotide-restored poly(A) sites

Oligonucleotide	Spacing	Activity
SV40	7	100
	32	90
	40	7.5
E2	7	100
	19	42
	27	32
	32	16
	40	5

SP6 3′ end assays, as shown in Figure 4, were scanned with a densitometer. The value for the construction inserted into the +5 position of pEC, which places the element seven nucleotides downstream (see Figure 1D), was set at 100.

tively abolished activity (mutant S-20). In addition, the results appear to indicate that the activity of the E2A sequence is relatively immune to single base changes and in some cases even double base changes (E-63). Clearly, a gross change of sequence abolishes activity (e.g. the inversion) but the limited changes do not. Possibly double base changes at critical residues would eliminate activity as was found for the SV40 sequence.

Effects of relative spacing of the downstream elements

We next determined if the position of the downstream element, relative to the cleavage site and AAUAAA, was important for function of the poly(A) site. This was accomplished by first cloning the oligonucleotide into the polylinker of the pSP64 or pSP65 plasmid, excising with different restriction digestions, and then inserting the excised fragments into pEC+5. The SV40 oligonucleotide used in these studies contained the SV40 sequence GTTGTGGTTTGT, residues +5 to +18. Varying amounts of stuffer sequence, derived from the polylinker, was placed between the oligonucleotide and the cleavage site displacing the downstream element relative to the cleavage site (shown schematically in Figure 4A). Control plasmids containing only the M13 sequences without the oligonucleotides inserted were constructed and showed only low levels of expression (data not shown). As shown in Figure 4B and summarized in Table III, there were indeed severe effects on expression dependent upon the position of the downstream element. For both the SV40 element and the E2A element, a displacement of 40 nucleotides relative to the normal position of the SV40 element reduced the activity nearly 20-fold. For the E2A sequence, there was a gradual decline as the distance from the AAUAAA/cleavage site increased. Even a move of 12 nucleotides reduced activity by 50%. Although we have less data for the SV40 element, it does appear that there are differences between the two elements. Displacing the SV40 sequence 32 nucleotides beyond the normal position had little or no effect on its activity, whereas this position nearly abolished the ability of the E2 element to restore activity. From these results we therefore conclude that the position of the downstream sequence relative to the cleavage site and the AAUAAA is critical for activity. Furthermore, the effect of spacing can vary depending upon the sequence involved.

Discussion

Through an oligonucleotide-mediated 'reversion' of a defective poly(A) site, we have established the presence of a genetic element, downstream of the poly(A) addition site, that is critical for cleavage of the RNA precursor to generate the mature mRNA 3′ terminus [poly(A) site formation]. The physiological significance of the reversion was indicated by the observation that the restored poly(A) site was nearly as functional as the wild-type poly(A) site and the reversion required the oligonucleotide to be inserted in the correct orientation. Previous work has shown that the AAUAAA element was required for poly(A) site function (Fitzgerald and Shenk, 1981; Montell *et al.*, 1983; Wickens and Stephenson, 1984), that deletions downstream of the cleavage site impaired function (McDevitt *et al.*, 1984; Simonsen and Levinson, 1983; Sadofsky and Alwine, 1984; Gil and Proudfoot, 1984; Cole and Stacy, 1985; McLauchlan *et al.*, 1985; Conway and Wickens, 1985), and that the actual cleavage site was not critical (Fitzgerald and Shenk, 1981). These observations, together with the results presented here in which a small downstream element was found to be sufficient for restoration of function, argues that a functional poly(A) site may consist only of the AAUAAA element and a downstream element of limited complexity. The fact that the two functional downstream elements defined here differ in sequence and certain other properties suggests that there can be variation in the nature of the downstream element. In contrast, the AAUAAA element is highly conserved (Proudfoot and Brownlee, 1976). In some respects the make-up of the functional elements of the poly(A) site resembles the eukaryotic polymerase II promoter in which there is a highly conserved TATA element, that is probably not a regulatory element, and a variety of upstream elements that mediate the regulatory interactions that influence the rate of promoter utilization (Nevins, 1983). We might speculate that in a like manner, the downstream element of the poly(A) site may be recognized by factors that determine efficiency of poly(A) site utilization or possibly tissue-specific recognition events such as may be the case for the calcitonin/CGRP locus (Amara *et al.*, 1984).

Our conclusion concerning specific downstream elements contrasts with a recent discussion of sequence requirements for 3′ end formation of the *Xenopus* globin gene (Mason *et al.*, 1986) in which these authors suggest that there was no specific requirement for downstream sequences. They observed that although large deletions of downstream sequences adversely affected poly(A) site function, small changes, that maintained the overall sequence context, did not impair function and thus suggested that the large deletions were bringing in sequences that caused a non-specific negative effect. Our results are clearly not consistent with such a hypothesis since point mutations had significant effects on poly(A) site function. Of course, if downstream elements were reiterated then alteration of any one element might not eliminate activity. In fact, this appears to be true for the E2A downstream region where there is a duplication of the T-rich sequence. In this case, an extensive deletion removing both T-rich sequence elements was necessary to abolish function (McDevitt *et al.*, 1984; Hart *et al.*, 1985a). The experiments we report here were successful in defining an element by point mutagenesis due to the fact that an assay plasmid was engineered to contain only one such element.

Since the sequence of the SV40 and E2A elements are distinctly different, we conclude that there are at least two downstream elements that affect the efficiency of poly(A) site utilization. Indeed, an examination of many poly(A) sites further supports this notion as the majority of the downstream sequences appear to fall into these two groupings. Previously, other workers have examined a large number of downstream sequences and from this analysis derived a consensus sequence of YGTGTTYY (McLauchlan *et al.*, 1985). Our results as well as others (Cole

and Stacy, 1985) only partially support such a consensus element. The early SV40 sequence generally conforms to this sequence although certainly not precisely. However, the adenovirus E2A sequence does not fit. Indeed, of the downstream sequences examined by McLauchlan *et al.* (1985) that did not contain the consensus (~30% of the total) most did in fact contain a T-rich sequence. One of these is the late SV40 poly(A) site and recent experiments suggest that this T-rich element is critical for poly(A) site function (Sadofsky *et al.*, 1985; Conway and Wickens, 1985) as is the case for the E2A poly(A) site. All of these results support the suggestion that there are at least two distinct sequence elements downstream of poly(A) sites, a GT element and a T-rich element. Furthermore, the results indicate that these two sequences may be the majority.

Two additional considerations support the suggestion that there are two distinct elements. First, there were clear differences in the effect of spacing on the function of the two downstream elements. Both downstream sequences were able to restore cleavage activity when displaced seven nucleotides downstream of the normal SV40 position and both were inactive when displaced 40 nucleotides downstream. However, the SV40 element retained activity when displaced 32 nucleotides downstream of the normal position of the SV40 element whereas the E2A element did not function in this position. Presumably, this reflects distinct recognition events that differ somewhat in their ability to effectively utilize these sequences. Furthermore, the E2A sequence appears to be somewhat stronger than the early SV40, again suggesting a different recognition. The quantitative difference in the two sites is small but is reproducible. And, given the fact that the E2A oligonucleotide can restore function to a higher level than the natural SV40 poly(A) site (pEC+18), clearly suggests it is a stronger element. This result is also consistent with recent *in vitro* experiments that indicated that an RNA precursor containing the E2A poly(A) site was more efficiently processed than one containing the early SV40 poly(A) site (Hashimoto and Steitz, 1986; McDevitt and Nevins, unpublished data).

We previously suggested that the downstream element might base pair with the AAUAAA element since there was an extensive degree of homology between the two regions in the adenovirus E2A poly(A) site (McDevitt *et al.*, 1984). However, based on the results of the mutagenesis described here, we conclude that there is likely to be no such interaction. The E2A mutant E-4 that changes a G to a T residue would eliminate base pairing to the U residue within the AAUAAA element yet this mutant is as functional, if not more so, than the 'wild-type' sequence. Therefore, the downstream element may not be part of an overall structure but rather may be a recognition site for specific factors. It is possible that the relative strength of the two sites (E2A and SV40) is the result of one factor with different affinities but this seems unlikely in view of the differences in the spacing effects as described above. Rather it seems more likely that there are multiple factors that recognize downstream elements and that the E2A-specific factor might be more abundant (in 293 cells) than the SV40-specific factor.

Obviously, at this stage we cannot decide between the above possibilities. We can only say that there are apparent differences between the two poly(A) sites. To distinguish between the underlying mechanisms, the recognition factor must be isolated and defined. Are there tissue-specific factors that recognize poly(A) sites such as the calcitonin and CGRP? Possibly the recently devèloped *in vitro* poly(A) site processing systems (Moore and Sharp, 1985), at least one of which recognizes the downstream element (Hart *et al.*, 1985b), will allow such a definition.

Materials and methods

Cells

The human transformed 293 cell line (Graham *et al.*, 1977) was used throughout. Cells were grown in Dulbecco's modified Eagle medium (DME) containing 10% FCS.

Plasmid constructions

The construction of the plasmids pEC+18 and pEC+5 have been described previously (Hart *et al.*, 1985a) (see also Figure 1). For insertion of the oligonucleotides, the pEC+5 plasmid was linearized with *Bam*HI and then ligated with the double strand oligonucleotide (see below).

Spacing mutants were constructed by cloning the E2A oligonucleotide (gatcTTGTTTTTcgaggatc) or the SV40 oligonucleotide (gatcGTTGTGGTTTGTcgaggatc) into the *Bam*HI site of pSP64 and pSP65. These plasmids as well as control SP64 and SP65 plasmids were digested with enzymes in separate reactions to yield fragments of varying size containing the oligonucleotide sequence. pSP64 was digested with *Hin*dIII, *Xba*I or *Sal*I and pSP65 was digested with *Eco*RI. The ends were filled in with Klenow and then ligated with *Bcl*I linkers. Fragments were then digested with *Bcl*I and *Bam*HI. This resulted in fragments with 40 nucleotides (from the *Hin*dIII digest), 32 nucleotides (*Eco*RI digest), 25 nucleotides (*Sal*I digest), or 19 nucleotides (*Xba*I digest) of polylinker DNA sequence between the *Bcl*I end and the oligonucleotide sequence. The *Bcl*I – *Bam*HI fragments were then cloned into the +5 deletion mutant. Correct orientation clones were identified by *Taq*I digestion and sequenced.

Transfection assays

Calcium phosphate transfections and RNA expression (3′ end analysis) assays using SP6 RNA probes were as described previously (Hart *et al.*, 1985a).

Oligonucleotides

The 'wild-type' sequence for SV40 and adenovirus E2A oligonucleotides are depicted in Figure 1. This sequence is referred to as the plus strand. For the production of base substitutions, the synthesis of the opposite strand was carried out with nucleotide mixtures at the positions where substitutions were desired. These mixtures consisted of 75% wild-type nucleotide and 8% of each of the other three nucleotides. Such ratios yield on average an oligonucleotide product with one base change relative to the wild-type sequence (Matteucci and Heynecker, 1983). The oligonucleotides were purified by chromatography on a Sephadex G-50 column, the wild-type plus strand and mutant minus strand were annealed, and the mixture was then ligated to pEC+5 vector that had been cleaved with *Bam*HI. The ligated DNA was used to transform *Escherichia coli* MM 294. Colony lifts were prepared and screened by hybridization with the wild-type plus strand oligonucleotide. After hybridization, filters were washed at increasing temperature (usually 4, 24, and 45°C) to identify colonies containing no inserts, single or double base changes, or wild-type sequence. Appropriate colonies were picked, mini-preps were prepared, and dideoxy sequencing was performed. A secondary transformation of MM 294 cells followed by large-scale plasmid preps, purification on CsCl and secondary dideoxy sequencing to confirm the identify of the mutants preceded transfection.

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