IN VITRO STUDIES ON mRNA 3' PROCESSING USING A HERPES SIMPLEX VIRUS POLY A SITE

by

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in

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To Evelyn,
and my parents.

SUMMARY

An *in vitro* mRNA 3' processing system was established. Nuclear extracts capable of supporting 3' processing in this system were prepared from both HeLa cells and K562 cells; this is the first report of the use of K562 nuclear extracts for *in vitro* 3' processing. While the processing activities of the K562 extracts appeared to be identical to those of HeLa extracts, their overall levels of activity were significantly lower.

HeLa nuclear extracts were used to investigate the properties of a 67bp synthetic poly A site composed of sequences from the HSV-2 equivalent of the HSV-1 UL38 gene. It was established that the synthetic poly A site contained sequences necessary and sufficient to direct 3' processing *in vitro* in a sequence-specific manner. The efficiency of *in vitro* 3' processing at the synthetic poly A site was comparable to that obtained with the HSV-2 IE-5 gene poly A site.

Experiments using variants of the synthetic poly A site, in which the spatial relationship of the essential sequence elements was altered, indicated that reducing or increasing the spacing between the poly A signal (AAUAAA) and the downstream, GU-rich sequence element caused a diminution of 3' processing activity. The quantitative and qualitative effects on 3' processing of the sequences between the poly A signal and the downstream sequence element were also investigated. Processing of a synthetic poly A site variant, in which the sequence between the poly A signal and the downstream sequence element were inverted, occurred at a different location from that normally observed and also with a slightly higher efficiency. This result indicated that the poly A signal and the downstream signal element are not solely responsible for defining the efficiency and accuracy of cleavage.

Gel mobility retardation assays were used to investigate the interactions between nuclear processing factors and precursor RNAs derived from the synthetic poly A site and its variants. The synthetic poly A site formed the same processing-specific complexes as the HSV-2 IE-5 gene poly A site. The variants, in which processing efficiency was lower than that of the synthetic poly A site, did not efficiently form the processing-specific complexes, with one exception. In the latter case, the spacing between the poly A signal and the downstream sequence element was reduced to such an extent that binding of the complex components to the RNA was possible but processing was not.

A transient CAT gene expression assay was used to analyse the efficiency of polyadenylation directed by the synthetic poly A site and certain of its variants *in vivo*. The relative CAT activities observed confirmed the results obtained using the *in vitro* 3' processing system.

The interactions between processing factors and the synthetic poly A site were investigated using complementary RNAs and sense-strand DNA oligonucleotides. By annealing complementary RNAs to the precursor RNA prior to incubation with nuclear extract, it was shown that the presence of double-stranded RNA at either the poly A signal, the cleavage site or the downstream element prevented specific complex assembly and 3' processing. This indicates that processing factors interact with all three elements of the poly A site. Pre-incubation of nuclear extract with the oligonucleotide GGTGTGTT, which corresponds to the GU-rich downstream sequence, inhibited specific complex assembly and 3' processing. This suggested that this oligonucleotide interacted with a processing factor, perhaps that which binds to the downstream sequence element. The implications of such an interaction for 3' processing *in vivo* are discussed. Other DNA oligonucleotides, corresponding to the poly A signal, the cleavage site and the vector-derived 3' sequence, had no effect on 3' processing *in vitro*.

ABBREVIATIONS

A	- adenine
Ad2	- adenovirus 2
ADP	- adenosine diphosphate
ADP(CH_2)P	- α,β - methylene ATP
ALV	- avian leukosis virus
AMP	- adenosine monophosphate
AMP(CH_2)P_2	- β, Γ - methylene ATP
ARE	- AU-rich element
ATP	- adenosine triphosphate
bp	- base pair(s)
BSA	- bovine serum albumin
^{14}C	- radiolabelled carbon
C	- cytosine
CaMV	- cauliflower mosaic virus
CAT	- chloramphenicol acetyl transferase
CF	- cleavage factor
CGRP	- calcitonin gene-related peptide
cm	- centimetre
CPE	- cytoplasmic polyadenylation element
cpm	- counts per minute
CTP	- cytidine triphosphate
cRNA	- complementary RNA
CstF	- cleavage stimulating factor
dATP	- deoxyadenosine triphosphate
DCP	- downstream cleavage product
dCTP	- deoxycytidine triphosphate
ddATP	- dideoxyadenosine triphosphate
ddCTP	- dideoxycytidine triphosphate
ddGTP	- dideoxyguanosine triphosphate
ddTTP	- dideoxythymidine triphosphate
dGTP	- deoxyguanosine triphosphate
dNTP(s)	- deoxyribonucleoside triphosphate(s)
E	- early
E coli	- Escherichia coli
EDTA	- sodium ethylenediamine tetra-acetic acid
G	- guanine
GM-CSF	- granulocyte macrophage colony stimulating factor

GTP	- guanosine triphosphate
h	- hour(s)
HEPES	- N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
hGH	- human growth hormone
HIV	- human immunodeficiency virus
hnRNP	- heterogeneous nuclear ribonucleoprotein particle
HPV	- human papillomavirus
HSV	- herpes simplex virus
HTLV	- human T-cell leukaemia virus
IE	- immediate early
kb	- kilobase (pairs)
kD	- kilodalton
L	- late
LNPAGE	- low-concentration, non-denaturing PAGE
LPF	- late polyadenylation factor
LTR	- long terminal repeat
M	- molar
mg	- milligram(s)
min	- minute(s)
ml	- millilitre(s)
MLP	- major late promoter
MLTU	- major late transcription unit
mM	- millimolar
mRNA	- messenger RNA
mRNP	- messenger ribonucleoprotein particle
nM	- nanomolar
NPC	- nuclear pore complex
N-terminal	- amino terminal
nu	- nucleotide(s)
^{32}P	- radiolabelled phosphate
PABP	- poly A binding protein
PAGE	- polyacrylamide gel electrophoresis
PAP	- poly A polymerase
PEG	- polyethylene glycol
pmol	- picomole(s)
poly A	- polyadenylic acid
pre-mRNA	- precursor messenger RNA
pre-tRNA	- precursor transfer RNA

RexRE - Rex response element
RNase - ribonuclease
rpm - revolutions per minute
R region - repeated region (of retrovirus LTR)
rRNA - ribosomal RNA
RSV - Rous sarcoma virus
S - svedberg
S cerevisiae - Saccharomyces cerevisiae
SDS - sodium dodecyl sulphate
sec - second(s)
SF - specificity factor
snRNA - small nuclear RNA
snRNP - small nuclear ribonucleoprotein particle
SNV - spleen necrosis virus
SV40 - simian virus 40
T - thymine
TEMED - N, N, N', N'-tetramethylethylene diamine
tk - thymidine kinase
TLC - thin layer chromatography
tRNA - transfer RNA
TTP - thymidine triphosphate
U - uracil
UCP - upstream cleavage product
UTP - uridine triphosphate
UTR - untranslated region
UV - ultraviolet
U3 region - 3' unique region (of retrovirus LTR)
U5 region - 5' unique region (of retrovirus LTR)
V - volt(s)
v/v - volume/volume
WCE - whole cell extract
w/v - weight/volume
X laevis - Xenopus laevis
Y - pyrimidine moiety
2'-O-CH_3 - 2'-methoxy
3'dATP - 3' deoxyadenosine
µCi - microcurie
µg - microgram
µl - microlitre

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INTRODUCTION

In vertebrates, expression of the majority of mRNAs involves post-transcriptional (or co-transcriptional) processing of the primary transcript: capping of the 5' terminus (Salditt-Georgieff *et al*, 1980b), removal of introns via the process of splicing (reviewed by Green, 1986; Padgett *et al*, 1986; Jacquier, 1990), formation of the 3' terminus and addition of the poly A tail (reviewed by Birnstiel *et al*, 1985; Wickens, 1990) occur before the mature mRNAs are transported from the nucleus to the cytoplasm. There is evidence of interactions between the processes of transcriptional termination, 3' processing, splicing and transport (Chang and Sharp, 1989; Cullen *et al*, 1984; Niwa *et al*, 1990).

This Introduction is intended to provide a background to the mRNA 3' processing reaction, the RNA sequences and nuclear factors involved, their interactions with other elements of mRNA biochemistry and the importance of the functions of the poly A tail.

In most cases, the 3' ends of mature eukaryotic mRNAs are formed from elongated primary transcripts via two sequential reactions (reviewed by Darnell, 1982; Nevins, 1983; Birnstiel *et al*, 1985; Humphrey and Proudfoot, 1988; Manley, 1988). The pre-mRNAs undergo endonucleolytic cleavage at a specific phosphodiester bond, leaving an exposed 3'-hydroxyl group (Moore *et al*, 1986; Sheets *et al*, 1987). Poly A tails are added one nucleotide at a time to the novel 3' termini; the tails are limited in length to approximately 200-250 residues (Moore and Sharp, 1985; Moore *et al*, 1986; Sheets *et al*, 1987). In the cytoplasm, mRNA poly A tails are shortened to produce a range of tail lengths between 50 and 70 nucleotides (nu) in the steady state (Sheiness and Darnell, 1973; Jeffrey and Brawerman, 1974; Brawerman and Diez, 1975; Brawerman, 1976; Adams and Jeffrey, 1978; Palatnik *et al*, 1980).

In vivo, cleavage and polyadenylation are tightly coupled, although the two processes can be uncoupled *in vitro* (Manley *et al*, 1985; Zarkower *et al*, 1986; Moore *et al*, 1986). Thus, the requirements for each reaction can be established in the absence of the other.

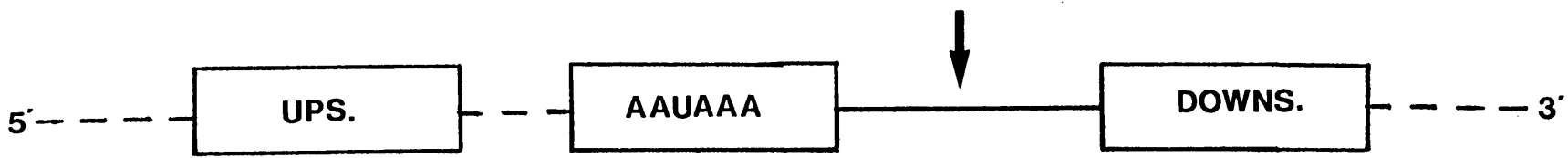
RNA sequence elements required for mRNA 3' processing

The poly A signal

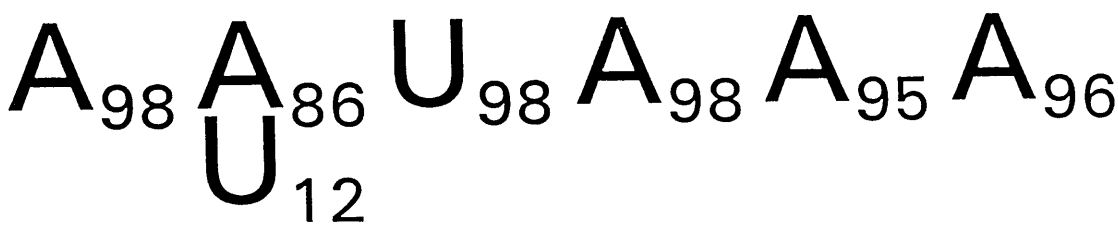
A polyadenylation site is defined by a number of sequence elements (Fig 1). The hexanucleotide, AAUAAA, or a derivative thereof, is found 10-30 nu upstream of most poly A addition sites; this sequence, known as the poly A signal, is highly conserved and is essential for polyadenylation *in vivo*, and for both cleavage and polyadenylation *in vitro* (Proudfoot and Brownlee, 1976; Fitzgerald and Shenk, 1981; Montell *et al*, 1983; Higgs *et al*, 1983; Wickens and Stephenson, 1984; Manley *et al*, 1985; Orkin *et al*, 1985; Zarkower *et al*, 1986; Kessler *et al*, 1986). Mutations within AAUAAA disrupt mRNA synthesis *in vivo*, resulting in unstable, and often elongated, mRNAs (Higgs *et al*, 1983).

Figure 1. Schematic representation of the sequence elements which comprise a vertebrate poly A site (not to scale).

The relative positions of the upstream (UPS.) and the downstream (DOWNS.) sequence elements, the poly A signal (AAUAAA) and the cleavage site (arrow) are indicated.


5′
UPS.
AAUAAA
DOWNS.
3′

Comparison of the sequences of 269 vertebrate cDNAs has produced the following consensus sequence for the poly A signal:



(Wickens, 1990; the figures denote the percentage frequency of occurrence of the nucleotide shown at that position).

The only common naturally occurring variant (AUUAAA; 12%) functions almost as efficiently as AAUAAA *in vivo* (Hagenbuchle *et al*, 1980; Jung *et al*, 1980), while the variants AAGAAA, AAUGAA and AAUAAG all reduced or abolished cleavage/polyadenylation activity (Higgs *et al*, 1983; Montell *et al*, 1983; Whitelaw and Proudfoot, 1983). However, the hexanucleotide AAUACA can function as an efficient poly A signal (Mason *et al*, 1985). Although mutation of AAUAAA to AAGAAA in the adenovirus E1A poly A site virtually abolishes cleavage at that site *in vivo*, those RNA molecules which are cleaved are also correctly polyadenylated, implying greater flexibility in the sequence requirements for the polyadenylation reaction (Montell *et al*, 1983). However, polyadenylation of pre-cleaved RNAs (ie, synthetic RNAs with 3' ends located precisely at the normal cleavage site) was eliminated by mutation of AAUAAA to AACAAA (Zarkower *et al*, 1986).

Pre-cleaved RNAs are efficiently polyadenylated *in vitro* (Zarkower *et al*, 1986), the reaction requiring the AAUAAA hexanucleotide (Manley *et al*, 1985; Skolnik-David *et al*, 1987; Zarkower *et al*, 1986). However, there appears to be no requirement for a specific sequence between AAUAAA and the 3' end of a pre-cleaved RNA (Conway and Wickens, 1987; Wigley *et al*, 1990; Ryner *et al*, 1989). The minimum length of AAUAAA-containing RNA which is efficiently polyadenylated is 11 nu: eight or more nu downstream of the poly A signal are required for maximum efficiency (Wigley *et al*, 1990).

It appears that recognition of AAUAAA by the processing machinery is not confined solely to interactions with the bases but also requires contact with certain of the sugar residues of the RNA backbone. Methylation of single riboses within AAUAAA inhibits poly A addition to a pre-cleaved substrate RNA (Bardwell *et al*, 1991).

Additional sequences required for cleavage of the pre-mRNA

The poly A signal, AAUAAA, alone is not sufficient to direct efficient cleavage/polyadenylation: there are many examples of intragenic AAUAAA hexanucleotides, which, despite being transcribed, do not direct processing of the transcript (eg, Aho *et al*, 1983; Fiers *et al*, 1978; Fitzgerald and Shenk, 1981; Perricaudet *et al*, 1980; Reddy *et al*, 1979; Tosi *et al*, 1981). Therefore, some additional, regulatory sequence element(s) must be required to define a functional poly A site.

Sequences which enhance the efficiency of polyadenylation have been identified at locations up to 50nu downstream from the site of poly A addition (Fig 1) (Bhat and Wold, 1985; Cole and Stacy, 1985; Conway and Wickens, 1985; Danner and Leder, 1985; Gil and Proudfoot, 1984, 1987; Hales *et al*, 1988; Hart *et al*, 1985a, 1985b; Kessler *et al*, 1986; Mason *et al*, 1986; Moore and Sharp, 1984; McDevitt

et al, 1984; McLauchlan *et al*, 1985; Sadofsky *et al*, 1985; Simonsen and Levinson, 1983; Skolnik-David *et al*, 1987; Woychik *et al*, 1984; Zarkower and Wickens, 1987b, 1988; Zhang and Cole, 1987; Zhang *et al*, 1986). For example, comparison of co-linear sequences from HSV types 1 and 2 revealed striking conservation of a GU-rich sequence element about 30 nu downstream from the poly A signal (McLauchlan and Clements, 1983; Whitton and Clements, 1984). Previously, similar downstream sequence elements had been noted as potential 3' processing signals (Taya *et al*, 1982). Deletion of the GU-rich element from a HSV poly A site considerably reduces mRNA 3' processing, both *in vivo* and *in vitro* (McLauchlan *et al*, 1985, 1988). Although GT- or T-rich motifs are prevalent, a consensus sequence for the downstream element is unclear (Benoist *et al*, 1980; Berget, 1984; Hart *et al*, 1985a, b; McLauchlan *et al*, 1985).

The sequences downstream of the adenovirus E2A poly A site and the SV40 early (SV40 E) poly A site have been shown to comprise two functionally equivalent elements of distinct sequence - U-rich and GU-rich, respectively - which are required for efficient cleavage/polyadenylation in transformed human kidney cells (McDevitt *et al*, 1986). The observation that the U-rich downstream sequence element, $U(A/G)U_5$, required for cleavage/polyadenylation at the adenovirus E2A site, is an almost perfect complement to the poly A signal, AAUAAA, led to the suggestion that these two sequences might interact by means of base-pairing to form a stem-loop structure (McDevitt *et al*, 1984). However, subsequent experiments, in which single base substitutions were made within the U-rich element, indicate that sequences which would form less stable stem-loops direct cleavage/polyadenylation more efficiently than the natural sequence (McDevitt *et al*, 1986).

The rabbit β-globin gene poly A site contains both a U-rich and a GU-rich downstream element. The two downstream elements function synergistically to produce maximum

cleavage efficiency *in vivo*; deletion of one of the elements or an increase in the distance between them led to reduced efficiency of processing (Gil and Proudfoot, 1987).

The sequences downstream of the *Xenopus* β-1 globin gene major poly A addition site have no apparent effect on the efficiency of cleavage/polyadenylation in oocytes but do influence the precise location at which cleavage occurred (Mason *et al*, 1986). Deletion analysis of these sequences suggests that the reduced efficiency of cleavage/ polyadenylation observed at the mutated sites is due to inhibitory sequences being brought closer to the poly A signal, rather than removal of positive regulatory elements (Mason *et al*, 1986). However, there have been no further reports of similar inhibitory elements in this or any other gene.

The spatial arrangement of poly A site sequences is critical for function

Alteration of the distance between the poly A signal, AAUAAA, and the U- or GU-rich downstream element reduces the efficiency of cleavage/polyadenylation (Gil and Proudfoot, 1987; Mason *et al*, 1986; McDevitt *et al*, 1984). However, in one of these cases, short deletions which remove part of the GU-rich element have no significant effect on the efficiency of cleavage/polyadenylation, perhaps because the deletions introduce a cryptic U-rich element which may functionally replace the deleted GU-rich motif (Mason *et al*, 1986; McDevitt *et al*, 1986).

The efficiency of use of a poly A site is seriously reduced when the spacing between the AAUAAA motif and the downstream sequence element is increased by the insertion of non-functional sequences (Gil and Proudfoot, 1987; Heath *et al*, 1990; McDevitt *et al*, 1986; Weiss *et al*, 1991). However, the downstream element still exerts an influence on the efficiency of 3'-end formation, even when it is placed a

considerable distance downstream from the AAUAAA motif: in this case, the poly A site is used more efficiently than one from which the downstream element has been deleted (Cole and Stacy, 1985; Heath *et al*, 1990; Levitt *et al*, 1989; McDevitt *et al*, 1986). The critical importance of the position of the downstream element relative to the AAUAAA motif implies an important interaction between these sequence elements, or factors which bind to them. Increasing the spacing between the AAUAAA motif and the downstream element reduces the stability of the committed processing complex *in vitro* (Weiss *et al*, 1991).

The type 1 human T cell leukaemia virus (HTLV-1) poly A site is remarkable in that its cleavage site and downstream GU-rich are separated from the AAUAAA hexanucleotide by more than 250 nu (Temin, 1981). The intervening sequence forms a stable stem-loop structure which brings the AAUAAA and downstream sequence elements into positions only 15nu apart (Seiki *et al*, 1983). The integrity of the stem-loop is required for formation of a stable processing complex and for cleavage of the precursor *in vitro* and efficient 3' end formation *in vivo* (Ahmed *et al*, 1991). Insertion of DNA fragments containing potential stem-loop structures between the AAUAAA hexanucleotide and the cleavage site does not significantly affect the efficiency of processing, but shifts the cleavage site downstream by the length of the insert (Brown *et al*, 1991).

Other sequence requirements for poly A site function

Chemical modification of single nucleotides in precursor RNAs containing the SV40 late (SV40 L) poly A site reveal that no sequence other than the poly A signal (AAUAAA) is required for cleavage *in vitro*, while modification of any of the nucleotides in the AAUAAA motif or the nucleotide immediately upstream from the cleavage site to which poly A is added interfere with polyadenylation

in vitro (Conway and Wickens, 1987). The SV40 L poly A site downstream region contains either diffuse or redundant sequence elements.

In the region between the AAUAAA hexamer and the downstream sequence element, which contains the site of RNA cleavage and poly A addition, there appears to be no specific sequence required for efficient 3' end processing, although poly A site efficiency is subtly affected by different sequences at this location (Sadofsky and Alwine, 1984; Wickens and Stephenson, 1984; McDevitt *et al*, 1986; Levitt *et al*, 1989; Heath *et al*, 1990). While not producing substantial quantitative effects on 3' processing, different sequences between AAUAAA and the downstream element cause minor variations in the precise location of the cleavage site (Fitzgerald and Shenk, 1981; Woychik *et al*, 1984; Mason *et al*, 1986; Levitt *et al*, 1989). There is an apparent preference for an A residue as the nucleotide after which cleavage occurs and to which poly A is added (Birnstiel *et al*, 1985; McLauchlan *et al*, 1985; Moore *et al*, 1986; Mason *et al*, 1986). However, efficient cleavage occurs downstream of residues other than As at a number of poly A sites (eg, McLauchlan *et al*, 1985; Sadofsky and Alwine, 1984).

While no specific nucleotides between AAUAAA and the poly A addition site appear to be essential for 3' processing, there is evidence of protein/RNA interactions involving the ribose moieties of the RNA backbone in this region: for example, 2'-O-methylation of the 3'-terminal ribose of a pre-cleaved substrate RNA strongly inhibits polyadenylation when additional 2'-O-CH_3 groups are present on riboses in the region between AAUAAA and the 3' end (Bardwell *et al*, 1991). Methylation of riboses immediately upstream from AAUAAA has no effect on polyadenylation of the pre-cleaved RNA, indicating that this region is probably not involved in interactions with the polyadenylation machinery (Bardwell *et al*, 1991).

Sequences upstream from the poly A signal

In a number of transcription units, the influence of sequence elements located upstream from the AAUAAA hexamer on 3' end processing has been reported.

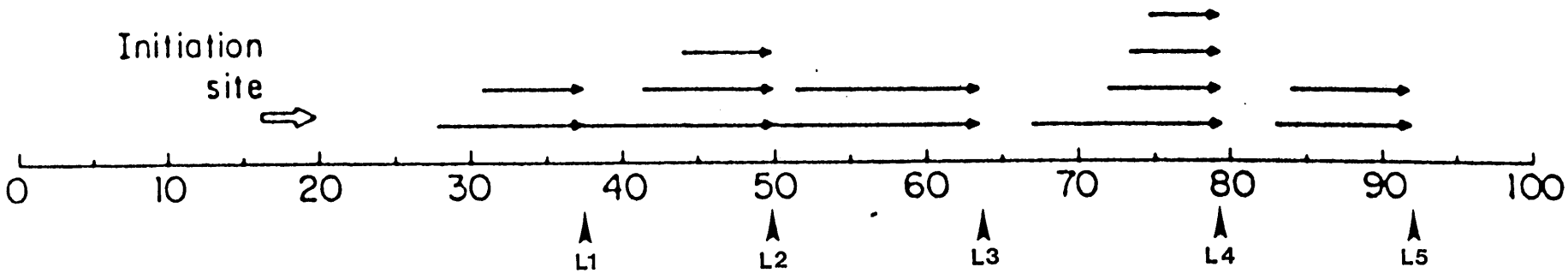
The production of the five colinear mRNA families of the adenovirus major late transcription unit (MLTU) which are defined by five different poly A sites (Fig 2), is temporally regulated (Le Moullec *et al*, 1983; McGrogan and Heschel, 1978; Nevins and Darnell, 1978b). The adenovirus L1 poly A site in the MLTU is utilised predominantly relative to alternative downstream sites at early times of infection (Nevins and Wilson, 1981). Predominant use of the L1 poly A site in mini-MLTUs containing two poly A sites is mediated by sequences upstream of the L1 poly A signal: the so-called selector element defined by deletion is not required for efficient 3' processing in simple transcription units and has no effect on mRNA stability (Dezazzo and Imperiale, 1989). Since all five late poly A sites are used equally efficiently late in infection, the affinities of the promoter-distal sites for processing factors may be higher than those of the promoter-proximal sites (Nevins and Darnell, 1978a). Thus, the L1 selector element may be required early in infection to increase the affinity of the L1 site for processing factors (Dezazzo and Imperiale, 1989).

The influence of upstream sequences on poly A site use has been observed in the case of hepatitis B virus, a retroid element. The structure and replication cycle of retroid elements is such that their genomic transcripts contain two copies of an identical poly A site, one close to the mRNA 5' end, which is not utilised, the other specifying the 3' terminus. The hepatitis B virus poly A site contains a non-canonical poly A signal (UAUAAA) which is not recognised by the processing machinery unless an upstream,

Figure 2. Schematic representation of the adenovirus major late transcripts.

The locations on the adenovirus genome of the common leader exons (open arrow), the variable late exons (thin arrows) and the late poly A sites (L1-L5) are indicated.

(After Nevins and Darnell, 1978).


Initiation
site
0
10
20
30
40
50
60
70
80
90
100
L1
L2
L3
L4
L5

positive regulatory element, which is located 5' of the transcriptional cap site and therefore is present upstream of the 3' poly A site only, is present (Russnak and Ganem, 1990). Presumably, the upstream element acts to potentiate recognition of the otherwise inefficient poly A signal via interaction(s) with a processing factor(s).

During the late phase of infection, HSV-infected cells contain a factor, late polyadenylation factor or LPF, which selectively promotes use of an HSV late gene poly A site *in vitro* (McLauchlan *et al*, 1989). LPF seems to act at sequences upstream from AAUAAA (R Purves, personal communication) and may function in a similar fashion to the adenovirus L1 selector element.

While processing of the SV40 L poly A site *in vivo* and *in vitro* requires sequences downstream of the cleavage site, additional sequences between 48 and 29nu upstream of the SV40 L poly A site appear to constitute an element of the poly A site, since their deletion dramatically reduces late mRNA levels, without affecting the stability of the mRNA or its precursor (Carswell and Alwine, 1989). Presumably, these sequences increase the affinity of the poly A site for processing factors.

Poly A sites in retroid elements

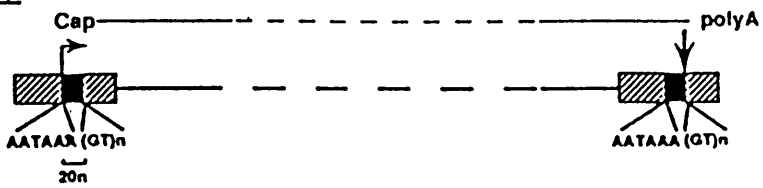
Retroid elements, such as retroviruses, hepatitis B viruses, caulimoviruses and some families of transposable elements, replicate via reverse transcription of a terminally redundant RNA. The terminal redundancy in retroviruses, for example, is generated by transcribing the R region of the viral LTR twice (see Fig 3). The 5' end of the R region corresponds to the transcription cap site while the 3' end of R represents the site of 3' cleavage and polyadenylation. Since the 3' end of the 5' R is identical to that of the 3' R but is not recognised as a poly A site, retroid elements must have evolved some means of allowing

Figure 3. Pararetrovirus and retrovirus poly A signals (not to scale).

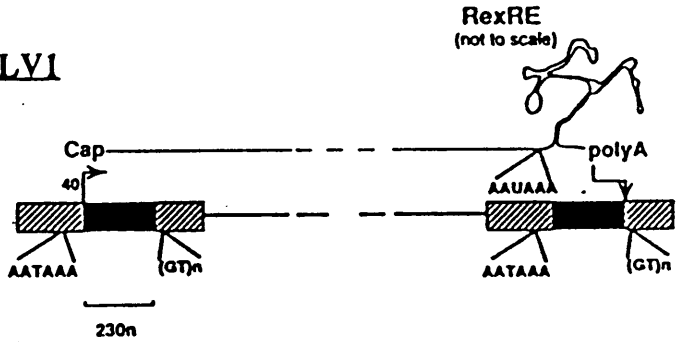
Boxes denote LTR sequences; the central filled-in portion defines the R region, the length of which is indicated in nucleotides (n), with the U3 and U5 regions to its left and right, respectively. The positions of the cap site and the poly A site are indicated by arrows, as is the upstream processing sequence (PS) for CaMV. In parts (C) and (D), the plus sign denotes the active poly A site, while minus denotes the inactive or occluded poly A site. The asterisk in part (D) indicates the GT-rich signal of HIV-1 implicated as an occlusion-specific element.

(From Proudfoot, 1991).

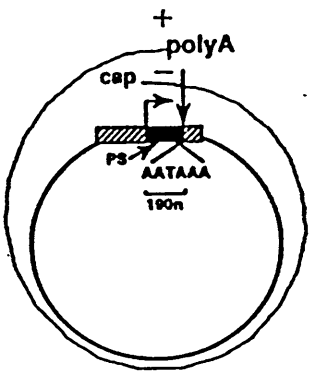
A. RSV



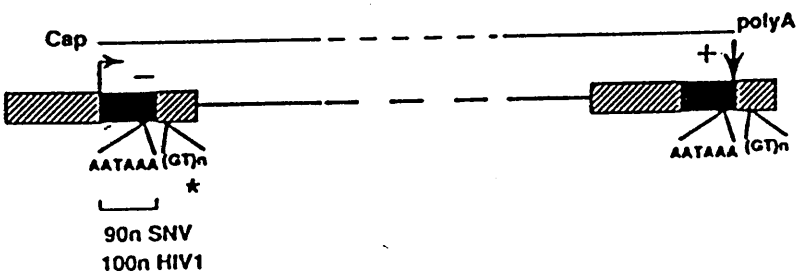
B. HTLV1



C. CaMV



D. SNV, HIV1



the processing machinery to ignore the 5' poly A site, while still being able to recognise the 3' poly A site. Different retroid elements utilise different means to overcome this apparent paradox.

Since retroviral RNA 3' end formation requires the same sequence elements as the cellular process (ie the AAUAAA hexamer and a downstream, less highly conserved sequence element), it seems likely that retroid elements utilise the cellular cleavage/polyadenylation machinery. (Bohnlein *et al*, 1989). In some cases, the processing machinery appears to be altered by the retrovirus, whereas in others the retroviral sequence has evolved so as to exclude the possibility of RNA 3' processing at the 5' R/U5 junction.

In Rous sarcoma virus (RSV) and in HTLV-1, the AAUAAA hexanucleotide is located entirely within the U3 region of the LTR, upstream of the transcription initiation site, so that the complete poly A site can be transcribed from the 3' end of the viral genome only (Fig 3A and B) (Ju and Cullen, 1985; Seiki *et al*, 1983). In the case of RSV, this arrangement means that the efficiency of viral replication may be compromised by the exceptional shortness of the R region.

In stark contrast, HTLV-1's R region is considerably longer than those found in other retroviruses (Temin, 1981; Seiki *et al*, 1983). As a result, the 3' cleavage site and its associated GU-rich sequence element is located more than 250nu downstream from the AAUAAA hexamer (Fig 3B), instead of the usual 10-30 nu (McLauchlan *et al*, 1985; Proudfoot and Brownlee, 1976). Normally, any increase in the spacing between the AAUAAA motif and the downstream sequence element results in a significant reduction in the efficiency of poly A site use (Gil and Proudfoot, 1987; Mason *et al*, 1986; McDevitt *et al*, 1986; Heath *et al*, 1990). Indeed, a poly A site whose downstream element was removed from the AAUAAA

hexamer to the extent found in HTLV-1 would be expected to be non-functional. However, the U3-R region of the HTLV-1 LTR contains a cis-acting RNA sequence element, the Rex response element or RexRE, which can form a stable RNA stem-loop secondary structure (Hanly *et al*, 1989; Seiki *et al*, 1983; Seiki *et al*, 1988). When the RNA of the RexRE is in the stable stem-loop configuration, the AAUAAA hexamer and the 3' cleavage site are located only 15nu apart on either side of the base of the primary stem (Seiki *et al*, 1983). The integrity of the RexRE stem-loop structure is essential for *in vivo* 3' processing and *in vitro* cleavage of viral transcripts, and for the formation of the stable processing complex containing SF and CstF (Ahmed *et al*, 1991), although the precise sequence of the RNA contained in the stem-loop appears to be irrelevant. The pre-mRNA 3' processing machinery must be sufficiently flexible to accommodate the large stem-loop structure formed by the RexRE.

The structure of many retroid elements is such that the entire poly A site must be transcribed twice. Such elements include murine leukaemia virus, murine mammary tumour virus, spleen neurosis virus (SNV), type 1 human immunodeficiency virus (HIV-1) and the Ty retrotransposons in yeast (Boeke *et al*, 1985; Clare and Farabaugh, 1985; Elder *et al*, 1983; Weiss, 1985). In these retroid elements, the R region of the LTR contains the AAUAAA hexamer, while the downstream element is located in U5, the cleavage site being, as usual, at the R-U5 junction (Fig 3D) (Bohnlein *et al*, 1989; Weiss, 1985). It was suggested that sequences within the U3 region might mediate functional differentiation between the 5' and 3' poly A sites, since these sequences would be present only upstream from the 3' site (Benz *et al*, 1980; Dougherty and Temin, 1987). However, it was subsequently demonstrated that the U3 region of SNV is dispensible for 3' end formation (Iwasaki and Temin, 1990a). Instead, the SNV poly A site is used efficiently only when placed more than 1400nu downstream from the RNA cap site, unlike the HSV thymidine kinase (tk)

and SV40 L poly A sites which function efficiently even at a distance which almost completely inactivate the SNV site (Iwasaki and Temin, 1990b). Since in all of the constructs used in these experiments transcription was driven by the SNV LTR promoter, it may be that the inactivation of the SNV poly A site is promoter specific. Obviously, further experiments using different promoters in conjunction with the SNV poly A site will clarify this point.

Inactivation of the upstream poly A site due to proximity of the promoter may represent a common mechanism in retroid elements. Both the cauliflower mosaic virus (CaMV) LTR poly A site and the HIV-1 poly A site display similar effects (Sanfaçon and Hohn, 1990).

Since non-retroviral poly A sites are efficiently utilised at distances downstream of promoters which completely inactivate the SNV and HIV-1 poly A sites (Iwasaki and Temin, 1990b; Weichs an der Glon *et al*, 1991), the (non-identical) downstream sequence elements may be differentially recognised by the 3' processing machinery. Indeed, in HIV-1 the occlusion of the 5' poly A site seems to be a function of the downstream GU-rich sequence element (Fig 3D) (Weichs an der Glon *et al*, 1991). Occlusion of the HIV-1 poly A site is not HIV-1 promoter-specific and occurs when the poly A site is placed a short distance downstream from the α-globin promoter (Weichs an der Glon *et al*, 1991). It is unclear whether the poly A site occlusion mechanism requires a novel recognition or processing factor, or involves interaction of the normal polyadenylation machinery with the mRNA 5' cap structure in a downstream sequence-dependent manner.

Vaccinia virus mRNA 3' end formation

Vaccinia virus (a poxvirus) has been extensively studied as a model for vertebrate transcription (reviewed by Moss, 1990). The 3' ends of vaccinia virus early mRNAs are

formed by transcriptional termination downstream from a U-rich regulatory sequence element. Poly A tails are added directly to the 3' termini without further processing of the transcripts. In general, AAUAAA is not present close to the 3' ends of poxvirus mRNAs and is not required for vaccinia virus mRNA polyadenylation. The enzymes and other factors required for vaccinia virus early mRNA transcription and polyadenylation are encoded by the viral genome, so it is not surprising that the mechanism for viral mRNA 3' end formation differs considerably from that of the host cell.

Poly A sites in plants

In plants the mechanism of mRNA 3' end formation, or at least the sequences required to mediate the process, seems to differ from that in vertebrates, since mammalian poly A sites are inefficiently processed in plants (Hunt *et al*, 1987). Computer analysis of poly A sites and their flanking regions have revealed that only 40% of plant poly A sites contain a perfect AAUAAA hexamer and that, although a highly conserved GU-rich downstream sequence element has been observed, its position 3' of the cleavage site varies considerably (Joshi, 1987). However, deletion of either the AAUAAA hexamer or a GU-rich downstream element reduces gene activity, in some cases (An *et al*, 1989; Ingelbrecht *et al*, 1989), suggesting that these elements are required for polyadenylation. These findings suggest that the plant 3' processing machinery may be significantly more flexible than its mammalian counterpart.

The CaMV poly A site has been subjected to detailed analysis. CaMV is a plant pararetrovirus which produces two major mRNA species whose 3' termini are formed at the same poly A site (Corey *et al*, 1981; Guilley *et al*, 1982). Like the SNV poly A site in vertebrates, the CaMV poly A site is utilised inefficiently when placed close to the promoter (Iwasaki and Temin, 1990b; Sanfaçon and Hohn, 1990). Efficient cleavage at the CaMV poly A site requires an

intact AAUAAA hexamer, although the point mutation AAUAAA to AAGAAA in the CaMV poly A site has a less marked effect on processing efficiency than does the same mutation in vertebrate poly A sites (Montell *et al*, 1983; Sanfacon *et al*, 1991). The precision of cleavage, but not its efficiency, is affected by sequences downstream from the CaMV poly A site (Sanfaçon *et al*, 1991). In vertebrate systems, minor mutations in the downstream sequence elements produce similar precision effects (Mason *et al*, 1986; Woychik *et al*, 1984). Deletion of sequences upstream from the CaMV AAUAAA hexamer reveal a GU-rich element between 53 and 32nu 5' of the poly A signal which is essential for efficient processing at the CaMV poly A site (Fig 3C): indeed, an oligonucleotide containing these sequences, when placed upstream of a normally silent, exogenous AAUAAA motif induces efficient 3' processing downstream from the AAUAAA signal (Sanfaçon *et al*, 1991).

It may be wise to bear in mind that, in vertebrates, the poly A sites of retroviruses are not entirely typical of those of their hosts' genes. It remains to be seen whether the features of the CaMV poly A site typify plant poly A sites in general.

Non-polyadenylated RNA polymerase II transcripts and their 3' processing

Polyadenylated mRNAs are the major species transcribed by RNA polymerase II. In addition, RNA polymerase II also transcribes histone mRNAs and most small nuclear RNAs (snRNAs). Neither of these RNA types is polyadenylated; the 3' ends of histone mRNAs and snRNAs are formed by distinct and unique mechanisms.

Small nuclear RNAs

With the exception of U6 snRNA [which is transcribed by RNA polymerase III (Kunkel *et al*, 1986; Reddy *et al*, 1987)], the major snRNA genes are transcribed by RNA polymerase II (eg, Frederiksen *et al*, 1978; Gram-Jensen *et al*, 1979). In contrast to the majority of RNA polymerase II-transcribed genes, snRNA genes lack both the 5' TATA box and the polyadenylation signal, AAUAAA: snRNAs are not polyadenylated. Rather, snRNA genes from several different species have been found to contain highly conserved 5' and 3' flanking sequences (reviewed by Birnstiel *et al*, 1985). A 3' consensus box is required for the accurate formation of the 3' ends of the U1 and U2 transcripts (Hernandez, 1985; Yuo *et al*, 1985).

Unlike the primary transcripts produced from polyadenylated mRNA transcription units, which often have very long 3' extensions, snRNA precursors are only 1 to 12 nu longer than the mature snRNA (reviewed by Birstiel *et al*, 1985). In the snRNA genes U1 to U5, the site of RNA 3' end formation is signalled by a short, conserved block of sequence, the 3' box, located 9-19 nucleotides downstream of the mature 3' end of the RNAs and conserved among different species (Hernandez, 1985; Yuo *et al*, 1985; Neuman de Vegvar *et al*, 1986; Ciliberto *et al*, 1986; Ach and Weiner, 1987), which probably directs termination of transcription. U1 RNA 3' end formation at the 3' box also requires an element(s) located in the U1 5' flanking sequences, so that if the U1 promoter is replaced by an mRNA promoter, normal U1 3' end formation is inhibited and most of the RNAs produced extend to a polyadenylation site inserted downstream of the U1 gene (Hernandez and Weiner, 1986; Neuman de Vegvar *et al*, 1986). The observation that the U2 promoter can substitute for the U1 promoter without deleterious effect on U1 3' end formation indicates that 3' end formation is coupled to transcription and probably arises by transcription termination.

The promoters of the vertebrate U1 and U2 genes have a similar organisation. Two sequence elements can be distinguished. A proximal element is required to position the transcriptional start site and to ensure efficient transcription (Skuzeski *et al*, 1984; Ares *et al*, 1985; Ciliberto *et al*, 1985; Mattaj *et al*, 1985; Mangin *et al*, 1986; Murphy *et al*, 1987), while a distal element enhances transcription (Skuzeski *et al*, 1984) relatively independently of orientation and position (Ares *et al*, 1985; Mattaj *et al*, 1985; Mangin *et al*, 1986; Murphy *et al*, 1987). Mutational analysis of the human U2 promoter has not identified sequence elements involved solely in 3' end formation (Hernandez and Lucito, 1988), although replacement of the U2 distal element by SV40-derived synthetic enhancers caused strong inhibition of 3' end formation, but not of initiation. Thus, elements required specifically for 3' end formation cannot readily be dissociated from elements whose function is concerned with initiation of transcription, suggesting the U2 promoter directs formation of a specialised transcription complex, containing RNA polymerase II and U RNA-specific termination factor(s), which is distinct from mRNA transcription complexes and which recognises the 3' box as a termination signal.

Processing at the 3' end of the primary snRNA transcript occurs in the cytoplasm. Short-lived snRNA precursors, which carry a 2, 2, 7-trimethylguanosine cap and are associated with snRNPs, are found in cytoplasmic extracts (Ellicieri, 1974; Madore *et al*, 1984a, b, c; Wieben *et al*, 1985; Zieve and Penman, 1976). Both cap hypermethylation of snRNAs and snRNP assembly occur in anucleate frog oocytes (Mattaj, 1986), demonstrating that these are cytoplasmic events. HeLa cell cytoplasmic extracts possess an activity that can process endogenous U2 RNA precursors (Wieben *et al*, 1985). This activity, which sediments at 15 S, has been partially purified (Kleinschmidt and Pederson, 1987) and appears to effect 3' processing via an exonucleolytic mechanism.

Histone mRNAs

The 3' ends of mature histone mRNAs are formed by endonucleolytic cleavage of an extended precursor molecule. Histone mRNAs are not polyadenylated. All known cell-cycle-regulated histone mRNAs of all eukaryotic species, except yeast, have a virtually identical palindrome near the 3' end of the mature mRNA which is followed a few nucleotides downstream by a highly conserved spacer sequence (Birnstiel *et al*, 1985; Wells, 1986; Turner *et al*, 1983).

The 3' processing of histone pre-mRNAs requires the presence of trans-acting small RNPs, in particular U7 snRNP (Galli *et al*, 1983; Strub *et al*, 1984; Gick *et al*, 1986). In the sea urchin, U7 snRNA base-pairs with the highly conserved palindromic and spacer sequences (Strub *et al*, 1984). Both of these sequence elements are essential for 3' processing of sea urchin histone precursor RNAs (Birchmeier *et al*, 1983, 1984; Georgiev and Birnstiel, 1985). A point mutation in the spacer sequence of the sea urchin H3 pre-mRNA that renders 3' processing of the precursor defective is suppressed by a compensatory change in U7 snRNA which restores the base-pairing potential of U7 and precursor RNA (Schaufele *et al*, 1986). Similarly, the 5' sequence of murine U7 snRNA exhibits extensive sequence complementarity to a conserved motif in the downstream spacer element of mammalian histone precursor RNA; oligonucleotide-directed RNaseH cleavage of this portion of murine U7 snRNA inhibits *in vitro* processing of histone pre-mRNAs (Cotten *et al*, 1988).

Alterations of the number of nucleotides between the palindromic and spacer sequences causes strong inhibition of 3' processing (Georgiev and Birnstiel, 1985). In the sea urchin histone pre-mRNA, the processing rate was reduced by up to 50% in *Xenopus* oocyte injection experiments as a result of the addition of only two intervening nucleotides.

Termination of transcription of the sea urchin H2A histone gene is unaffected by the removal of the palindrome and spacer sequence elements, which are required for 3' processing (Johnson *et al*, 1986; Birchmeier *et al*, 1984). Three separate sequence elements, one within the coding sequence, the others in the 3' flanking region, are required for termination of histone pre-mRNAs, which occurs efficiently even when transcription is driven by a non-histone RNA polymerase II promoter (Johnson *et al*, 1986).

Termination of mRNA transcription and pre-mRNA 3' processing

The mRNA-encoding genes of higher eukaryotes appear to contain no single consensus termination element. Rather, different genes display characteristics of several possible mechanisms for transcription termination.

Termination of transcription by RNA polymerase II is defined as the cessation of RNA chain elongation followed by disengagement of the polymerase from the DNA template and release of the new RNA molecule (Holmes *et al*, 1983) and usually occurs some distance downstream from the polyadenylation site in the mRNA-encoding genes of higher eukaryotes (reviewed by Birnstiel *et al*, 1985; Proudfoot, 1989).

Because 3' end formation and polyadenylation occur very rapidly after the transcription complex passes through the polyadenylation site, very few terminated and unprocessed primary transcripts are present in the nucleus, hampering efforts to accurately define RNA polymerase termination sites (Nevins and Darnell, 1978a; Acheson, 1984; Salditt-Georgieff *et al*, 1980a; Darnell, 1982; Montell *et al*, 1983; McDevitt *et al*, 1984).

Dissociation of RNA polymerase II from eukaryotic templates apparently occurs, with some exceptions, over large regions of DNA, spanning hundreds of base pairs which

may contain weak, reiterated termination signals (Citron *et al*, 1984; Frayne *et al*, 1984; Hagenbuchle *et al*, 1984; Le Meur *et al*, 1984). In some cases, a functional poly A site has been shown to be required for efficient termination of transcription by RNA polymerase II (Whitelaw and Proudfoot, 1986; Logan *et al*, 1987; Connelly and Manley, 1988; Lanoix and Acheson, 1988; Citron *et al*, 1984) and in certain instances, a further signal, in addition to the poly A site, is essential for efficient termination (Logan *et al*, 1987; Connelly and Manley, 1989a). A transcriptional pause element, located between the human α2 and α1 globin genes causes transcriptional termination when it is placed downstream from a strong poly A site (Enriquez-Harris *et al*, 1991). In contrast, the activity of a strong transcriptional termination element near the 3' end of the adenovirus genome apparently does not require the presence of a functional upstream poly A site (Dressler and Fraser, 1987).

Transfection experiments using chimeric episomal virus constructs indicate that, in the absence of normal polyadenyation, concomitant failure of transcriptional termination results in the accumulation of giant nuclear RNAs, produced by several rounds of continuous transcription of the complete plasmid (Connelly and Manley, 1988). Similar giant RNAs are also produced by wild-type polyoma virus (Acheson, 1984). Replacement of the intrinsically weak polyoma virus poly A site with the much stronger rabbit β-globin poly A site causes efficient termination downstream from the poly A site (Lanoix and Acheson, 1988; Gil and Proudfoot, 1987).

Several models have been proposed to explain the requirement for polyadenylation for the process of termination. Firstly, transcription across a poly A site may cause the release of an elongation factor from the polymerase, resulting in an increased rate of non-specific termination downstream of the poly A site (Logan *et al*,

1987). Alternatively, following 3' processing, the newly exposed 5' end downstream from the processing site may be degraded by a 5'-3' exonuclease; the combined action of the nuclease and a DNA:RNA helicase may then degrade all of the nascent RNA, resulting in the release of the polymerase: this mechanism has been proposed by Proudfoot and Whitelaw (1988) and Connelly and Manley (1988). Since genes with weak poly A sites will produce longer nascent transcripts before 3' processing occurs, it may be suggested that termination of transcription of these genes will occur further downstream of the poly A site than is the case for genes with efficient poly A sites. This seems to be the case in polyoma virus, for example (Lanoix and Acheson, 1988).

Indirect support for the nuclease/helicase model comes from the observation that the bacterial termination factor, Rho, possesses an ATP-dependant RNA:DNA helicase (Brennan *et al*, 1987); however, no mammalian RNA:DNA helicase has been described. The mechanism of Rho-dependent transcription termination involves interactions between DNA, RNA polymerase, nucleoside triphosphates, the nascent RNA and Rho itself (reviewed by Platt, 1986): details of the mode of action of Rho are still unclear.

Termination of transcription may involve an interaction between the transcription complex and a factor bound to a terminator element on the template DNA. In vertebrates, termination of transcription by RNA polymerase I is mediated by the binding of TTF-1, a specific termination factor, to an 18bp DNA consensus sequence (Grummt *et al*, 1985, 1986a, b; Henderson and Sollner-Webb, 1986; McStay and Reeder, 1986; Bartsch *et al*, 1988). In a number of RNA polymerase II-transcribed genes, the binding of a protein to DNA has been implicated in termination. In the μ-δ immunoglobulin gene, cell-type specific poly A site-dependent termination appears to be mediated by protein binding at a site downstream of the μ_m poly A site

(Law *et al*, 1987). The CCAAT box within the adenovirus major late promoter (MLP) elicits termination of transcription of pol II via specific protein binding (Connelly and Manley, 1989a, b). The MLP CCAAT box is bound by CP1, a protein purified from HeLa cells (Jones *et al*, 1987).

Although the relationship between transcriptional termination and mRNA 3' end formation/polyadenylation *in vivo* is still unclear, termination may be important in preventing transcriptional interference between neighbouring genes (Cullen *et al*, 1984; Proudfoot, 1986). Readthrough from one gene into an adjacent promoter can inhibit initiation of transcription of the second gene: in the retroviruses avian leukosis virus (ALV) and HIV-1, the promoter in the 3' LTR is directly inactivated by transcription initiated at the 5' LTR promoter (Cullen *et al*, 1984; Weichs an der Glon *et al*, 1991). A similar instance of transcriptional interference has been demonstrated for RNA polymerase I-transcribed genes where formation of a transcription initiation complex can be inhibited by transcription from an upstream promoter (Bateman and Paule, 1988; Henderson *et al*, 1989). Inactivation of the ALV 3' promoter can be overcome by insertion of a strong poly A site between the LTRs (Cullen *et al*, 1984).

In some cases, there is no evidence for the involvement of protein binding to DNA in termination. For example, the SV40 promoter/origin region contains several well-characterised protein binding sites which do not mediate termination; rather, termination in the region appears to be related to potential hairpin/loop structures (Grass *et al*, 1987; Connelly and Manley, 1989b). Similarly, a very stable potential hairpin/loop structure lies close to the chicken β^H-globin gene termination site (Pribyl and Martinson, 1988). A number of potential secondary structure elements have been tested for their involvement in

termination of RNA polymerase II transcription *in vitro* but have been found not to be required (Kessler *et al*, 1989; Resnekov *et al*, 1988; Wiest and Hawley, 1990). Bacterial Rho-dependent terminators contain potential hairpin/loop structures which are believed to mediate a pause in transcription which is followed by release of RNA polymerase at an adjacent oligo U motif (Platt, 1986).

Several eukaryotic genes exhibit premature transcriptional termination within a few hundred nucleotides of the cap site, resulting in the production of unstable, attenuated transcripts (Hay *et al*, 1982; Kao *et al*, 1987; Bentley and Groudine, 1988; Proudfoot and Whitelaw, 1988; Rougvie and Lis, 1988; Skarnes *et al*, 1988). Such premature termination may represent a means of regulating synthesis of a number of gene products (Evans *et al*, 1979; Hay *et al*, 1982; Maderious and Chen-Kiang, 1984; Bender *et al*, 1987; Fort *et al*, 1987; Kao *et al*, 1987; Rougvie and Lis, 1988; Skarnes *et al*, 1988; Chinsky *et al*, 1989; Lattier *et al*, 1989; Resnekov and Aloni, 1989). In the case of the HIV LTR promoter, the viral Tat polypeptide possesses an activity which overcomes premature termination of the retroviral transcripts, ie Tat acts as a transcriptional antiterminator (Kao *et al*, 1987). *In vitro* experiments have demonstrated that Tat increases the abundance of a more-processive form of transcription complex initiated from the HIV promoter, but has no effect on a less-processive form of transcription complex (Marciniak and Sharp, 1991). Since retroviruses have their origins as "escaped" cellular genes, there is likely to be a homologous cellular antiterminator activity.

The poly A addition site of the C2 gene and the cap site of the Factor B gene in the human major histo-compatibility complex class III region are separated by only 421 nu: these genes are expressed simultaneously in the adult liver (Wu *et al*, 1987). Transcriptional termination occurs within about 100 nu of the C2 poly A site and is, in part, due to the binding of a protein to a specific DNA

element: the same, or a closely related, protein binds to a homologous sequence element in the murine c-myc gene, which is required for attenuation of c-myc transcription (Ashfield *et al*, 1991).

The α globin pause signal and the termination sequences defined in the C2 gene, histone H2A, SV40 and Rho-independent prokaryotic terminators are all A-, T- or AT-rich (Johnson *et al*, 1986; Platt, 1986; Hsieh and Griffith, 1988; Briggs *et al*, 1989; Ashfield *et al*, 1991; Enriquez-Harris *et al*, 1991), which can lead to reduced affinity between the nascent RNA and the template DNA, in prokaryotes at least. It seems likely that a combination of DNA sequence, secondary structure and/or protein binding may be required to constitute a termination signal.

Transfection of chimeric genes containing polyadenylation sites linked to RNA polymerase I or RNA polymerase III promoters leads to correct polyadenylation of the RNAs transcribed (Smale and Tjian, 1985; Lewis and Manley, 1986). In addition, *in vitro* experiments have demonstrated accurate and efficient polyadenylation of exogenous pre-mRNA substrates in the absence of transcription (eg, Moore and Sharp, 1985). Thus, RNA polymerase II transcription (and termination) is not required for specific mRNA 3' processing.

Poly A binding proteins, mRNA stability and translational activation

In eukaryotic cells, mRNA is associated with specific proteins in complexes known as messenger ribonucleoprotein particles (mRNPs) (Spirin *et al*, 1965). Poly A binding proteins (PABPs) are constituents of mRNPs and in yeast and mammals appear to bind poly A in a "beads-on-a-string" configuration, with a stoichiometry of 1 PABP/25-27 A residues (Baer and Kornberg, 1980, 1983; Sachs *et al*, 1987). The N-terminal domain of PABP is highly

conserved in yeast and mammals and contains four segments which share homology not only with each other but also with other RNA-binding proteins (Adam *et al*, 1986; Grange *et al*, 1987; Sachs *et al*, 1986). In yeast, PAB, the gene which encodes PABP, is essential for viability, although a 66 amino acid polypeptide containing a single RNA binding segment is sufficient for binding poly A and for maintenance of cell viability (Sachs *et al*, 1987).

Nuclear PABPs in yeast and mammalian cells are derived from cytoplasmic PABPs by cleavage of a portion of the C-terminus and fail to confer the "beads-on-a-string" configuration on poly A tracts (Sachs and Kornberg, 1985; Sachs *et al*, 1986). An important role(s) for PABPs in the metabolism and/or activity of poly A^+ mRNAs seems likely given their evolutionary conservation and their high affinity for poly A (Blobel, 1973; Adam *et al*, 1986; Manrow and Jacobson, 1987; Sieliwanowicz, 1987; Swanson and Dreyfuss, 1988a).

Almost all eukaryotic mRNAs possess a poly A tail (Kates, 1970; Lim and Canellakis, 1970; Darnell *et al*, 1971a; Lee *et al*, 1971; Edmonds *et al*, 1971). The ubiquity of this feature suggests that it fulfils an important role in gene expression. It has been proposed that the poly A tail may play crucial roles in mRNA transport, translation and stability.

The poly A tail and mRNA stability

Exposed 5' and 3' termini seem to pre-dispose RNAs to instability and degradation. *In vivo*, RNA is rapidly digested by cellular exonucleases after it has been destabilised (Ross, 1988). The stabilities of circular RNAs on injection into *Xenopus* embryos are considerably greater than those of their linear counterparts (Harland and Misher, 1988). Both natural and synthetic mRNAs which contained a 5' cap structure are protected from degradation by 5'-to-3'

exonucleases *in vivo* (Furuichi *et al*, 1977; Green *et al*, 1983). The poly A tail appears to play an important role in protecting mRNAs from degradation by 3'-to-5' exonucleases. The binding of PABPs to poly A tails provides resistance to the activities of 3'-to-5' exonucleases (Bergman and Brawermann, 1977; Bernstein *et al*, 1989). The stability of *in vitro*-synthesised mRNA is increased, following injection into *Xenopus* oocytes and embryos, by enzymatic addition of a poly A tail (Drummond *et al*, 1985; Harland and Misher, 1988). However, the half-lifes of some mRNAs seem to be independent of their degree of polyadenylation. In the cases of both α_2μ-globulin and interferon mRNAs, the stabilities of the poly A^+ and poly A^- RNAs are similar in microinjected oocytes (Deshpande *et al*, 1979; Soreq *et al*, 1981). These mRNA species may contain another sequence or structural element which mediates their stability independently of poly A.

Several lines of evidence support the hypothesis that the poly A tail acts to increase the stability of mRNA. In certain cases, the degradation of an mRNA follows a shortening of its poly A tail (Brewer and Ross, 1988; Colot and Rosbash, 1982; Green and Dove, 1988; Hyman and Wormington, 1988; Krowczynska *et al*, 1985; Mercer and Wake, 1985; Nakai *et al*, 1982; Restifo and Guild, 1986; Swartout and Kinniburgh, 1989; Wilson and Treisman, 1988; Wilson *et al*, 1978). The stabilities of a number of mRNAs have been shown to vary in response to intra- or extra-cellular events or signals (Casey *et al*, 1988; Hereford and Osley, 1981; Mullner and Kuhn, 1988; Yen *et al*, 1988). Specific stabilisation of mRNAs accompanies activation of transcription by prolactin and steroid hormones (Brock and Shapiro, 1983; Guyette *et al*, 1979). Stabilisation of the hGH mRNA in response to glucocorticoids is accompanied by an increase in its poly A tail length (Paek and Axel, 1987).

In *Xenopus* oocytes, the stability of histone mRNA is increased by the addition of a poly A tail *in vitro*, prior

to microinjection (Huez *et al*, 1978). Messenger RNAs whose poly A tails contain 32 or more residues are at least one order of magnitude more stable in *Xenopus* oocytes than mRNAs which possess tails of only 16 residues (Nudel *et al*, 1976). It has been suggested that the interaction of PABP with an mRNA's poly A tail may mediate the increase in stability. Since a single PABP molecule binds to approximately 27 A residues (Baer and Kornberg, 1980, 1983; Sachs *et al*, 1987), it may be that poly A tails shorter than 27 residues are unable to bind PABP and, therefore, are susceptible to attack by nucleases.

An important role for PABP in mediating mRNA stability via binding to the poly A tail is further suggested by the observations that: (i) *in vitro*, in the presence of PABP, the stability of poly A^+ mRNA is decreased by the addition of competitor poly A; and (ii) while having no effect on the stability of histone mRNA or deadenylated mRNA, purified PABP increases the stability of poly A^+ mRNA *in vitro* (Bernstein *et al*, 1989).

Determinants of mRNA stability

In both prokaryotes and eukaryotes, mRNA degradation plays an important role in controlling gene expression (reviewed by Brawerman, 1987; Belasco and Higgins, 1988; Bernstein and Ross, 1989). The transient expression of some oncogene and lymphokine gene products results from the rapid disappearance of their inherently unstable mRNAs after cessation of transcription (Shaw and Kamen, 1986). One example of a transiently induced gene is c-fos. A wide range of mammalian cell types can be induced to transiently express c-fos mRNA (Curran and Morgan, 1985, 1986; Greenberg and Ziff, 1984; Greenberg *et al*, 1985, 1986b). Controlled transient expression of c-fos appears to be critical for normal cell function since deregulated expression can lead to oncogenic transformation (Miller *et al*, 1984; Meijlink *et al*, 1985; Jenuwein and Muller, 1987). Following

transport of newly-synthesised c-fos mRNA to the cytoplasm, it is rapidly degraded, allowing only a short period of translation (Kruijer *et al*, 1984; Muller *et al*, 1984). Indeed, c-fos mRNA is one of the least stable mRNAs in mammalian cells (Greenberg and Ziff, 1984).

The c-fos mRNA is specifically targetted for rapid decay by two functionally distinct cis-acting sequence elements, an AU-rich element (ARE) located in the 3' untranslated region (3'UTR) and a structurally distinct element within the coding region (Fort *et al*, 1987; Kabnick and Housman, 1988; Rahmsdorf *et al*, 1987; Shyu *et al*, 1989; Wilson and Treisman, 1988). Treatment of the cells with transcription inhibitors increased the stability of c-fos mRNA (Greenberg *et al*, 1986a). Indeed, de novo synthesis of a labile gene product, whose activity is lost within 3 h after transcription is blocked, is required for the ARE-dependent degradation pathway (Shyu *et al*, 1989). In contrast, the alternative c-fos mRNA degradation pathway is not dependent on RNA synthesis (Shyu *et al*, 1989). The apparent "belt-and-braces" approach to c-fos mRNA degradation may reflect the importance to the cell of the rapid destruction of this message. After its export from the nucleus to the cytoplasm, the poly A tail of c-fos mRNA undergoes significant shortening, suggesting that this event may be responsible for destabilising the message (Wilson and Treisman, 1988). Indeed, both pathways of c-fos mRNA degradation involve deadenylation of the message as an initial, destabilising event (Shyu *et al*, 1991).

It has been suggested that a group of genes whose 3' UTRs contain AREs may utilise a common ARE-dependent destabilising and degradation mechanism (Caput *et al*, 1986; Shaw and Kamen, 1986). A number of other mammalian mRNAs contain sequence elements which mediate mRNA instability (Casey *et al*, 1988; Levine *et al*, 1987; Mullner and Kuhn, 1988; Shaw and Kamen, 1986). In addition, the 3' UTRs of both type 1 bovine papillomavirus and type 16 human

papillomavirus (HPV16) late mRNAs contain sequence elements which mediate a negative effect on gene expression (Baker, 1990; Kennedy *et al*, 1990). Poly A$^+$ mRNAs containing the HPV16 negative regulatory element were significantly less stable *in vitro* than poly A$^+$ mRNAs in which the element was not present (Kennedy *et al*, 1991). Late mRNA instability may explain why the majority of cell types are unable to support papillomavirus late gene expression: presumably, permissive cells express a factor which stabilises late mRNAs.

Otherwise stable mRNAs can be rendered unstable by the inclusion of cis-acting destabilising sequence elements (reviewed by Ross, 1988). It has been suggested that the destabilising sequences may act by reducing the capacity of poly A to bind PABP (Bernstein and Ross, 1989). Alternatively, they may mediate removal of the poly A tail (which would, of course, completely prevent PABP-binding) leading to destabilisation of the mRNA.

Mutations in either AAUAAA or the U-rich elements which prevent poly A addition in maturing oocytes also cause poly A to be removed from the RNA (Fox and Wickens, 1990). Therefore, the elements which cause poly A addition during oocyte maturation are responsible for preventing removal of the poly A tail, which is the normal default response. In contrast, the inherent instability of GM-CSF mRNA in somatic cells is due to a AU-rich sequence, which is essential for its deadenylation (Wilson and Treisman, 1988). This suggests that the mechanisms by which the stability of poly A tails in oocytes and in somatic cells is regulated are considerably different.

In *S cerevisae*, it appears that deadenylation does not destabilise stable mRNAs, since the poly A metabolisms of stable and unstable mRNAs are not significantly different (Herrick *et al*, 1990). Yeast contains PABPs whose RNA binding domains are highly conserved relative to those of

mammalian PABPs, so why is there such an apparent difference in their functions? It may be that the length of poly A tail is only one of a number of factors which determine mRNA stability, although there is no incontrovertible evidence.

The stability of their mRNAs is, in part, instrumental in achieving the high levels of expression of the globin genes (Aviv *et al*, 1976; Lowenhaupt and Lingrel, 1978; Volloch and Hausman, 1981). Conversely, the transient expression of some oncogenes and lymphokines results from the rapid disappearance of their inherently unstable mRNAs after cessation of transcription (Shaw and Kamen, 1986). Therefore, post-transcriptional control of mRNA stability and decay can play a significant role in the regulation of gene expression.

The influence of translation on mRNA stability

In vivo translation seems to influence mRNA stability. Some translated mRNAs are apparently protected from degradation. Mutations in the otherwise stable β-globin, immunoglobulin μ-heavy chain and triose phosphate isomerase mRNAs that cause premature termination of translation destabilise the transcripts (Bauman *et al*, 1985; Daar and Maquat, 1988; Maquat *et al*, 1981). Conversely, the stabilities of some mRNAs is adversely affected by active translation. Degradation of tubulin, histone, transferrin receptor and c-myc mRNAs is linked to translation, so that translation inhibitors increase these mRNAs' half-lives (Graves *et al*, 1987; Mullner and Kuhn, 1988; Pachter *et al*, 1987). In addition, untranslatable mutant tubulin, histone and transferrin receptor mRNAs are more stable than their wild-type counterparts (Graves *et al*, 1987; Mullner and Kuhn, 1988; Pachter *et al*, 1987). In general, mRNAs are stabilised when translation is inhibited (reviewed in Brawermann, 1989; Ross, 1988; Shaw and Kamen, 1986).

The influence of the poly A tail on translation

The poly A tail apparently enhances initiation of translation (Sachs and Davis, 1989; Munroe and Jacobson, 1990). It has been suggested that the interaction of cytoplasmic PABP with a critical minimum length of poly A may facilitate the initiation of translation (Jacobson and Favreau, 1983; Palatnik *et al*, 1984; Manrow and Jacobson, 1986, 1987; Shapiro *et al*, 1988). This hypothesis receives indirect support from the suggested secondary structures of rabbit α-globin and murine β-globin mRNAs which show the 5' and 3' domains to be in close proximity (Heindell *et al*, 1978; Lockard *et al*, 1986). Furthermore, circular or 'folded-back' polysomes have been observed in electron micrographs (Warner *et al*, 1962; Dubochet, 1973; Hsu and Coca-Prodos, 1979; Ladhoff *et al*, 1981; Christensen *et al*, 1987).

In addition, correlations have been noted between specific changes in mRNA poly A tail length and translation efficiency (Rosenthal *et al*, 1983; Palatnik *et al*, 1984; Restifo and Guild, 1986; Huarte *et al*, 1987; Hyman and Wormington, 1988; Strickland *et al*, 1988; Carrazana *et al*, 1988; Robinson *et al*, 1988; Zingg *et al*, 1988; Vassalli *et al*, 1989; Paek and Axel, 1987; Robinson *et al*, 1988) and between the abundance and stability of PABPs and the rate of translation initiation (Manrow and Jacobson, 1986, 1987). *In vitro*, poly A^+ mRNAs are more efficiently translated than poly A^- mRNAs (Doel and Carey, 1976; Hruby and Roberts, 1977; Deshpande *et al*, 1979; Galili *et al*, 1988; Hyman and Wormington, 1988; Rubin and Halim, 1987; Gallie *et al*, 1989; Munroe and Jacobson, 1990), while exogenous poly A is a potent and specific inhibitor of the *in vitro* translation of poly A^+ but not poly A^- mRNAs (Jacobson and Favreau, 1983; Bablianan and Banerjee, 1986; Lemay and Milward, 1986; Sieliwanowicz, 1987; Grossi de Sa *et al*, 1988). Furthermore, purified PABP has been shown to stimulate translation *in vitro* (Sieliwanowicz, 1987). In yeast,

extragenic suppressors of PABP deletion include mutations in the 60S ribosomal protein, L46, and an RNA helicase which seems to be involved in the assembly of 60S subunits (Sachs and Davis, 1989, 1990). The reduced translational capacity of poly A^- mRNAs *in vitro* is due to a reduction in their efficiency of recruitment into polysomes: poly A^- mRNAs have a reduced ability to form 80S initiation complexes (Munroe and Jacobson, 1990). Thus, the presence of a 3' poly A tail to which PABP binds may facilitate binding of an initiation factor or ribosomal subunit at the mRNA 5' end.

Efficient translation may require a certain PABP/adenosine residue ratio on the poly A tail, predicting that poly A^+ mRNAs compete for limiting amounts of PABPs (Jacobson and Favreau, 1983; Sieliwanowicz, 1987; Grossi de Sa *et al*, 1988). PABPs may also prevent degradation of poly A^+ mRNAs by binding to the poly A tail (Bernstein *et al*, 1989). Translation and stability of poly A^+ mRNAs *in vitro* are influenced by PABPs (Benrstein *et al*, 1989; Jacobson and Favreau, 1983; Sieliwanowicz, 1987; Grossi de Sa *et al*, 1988). The fact that, in sea urchins at least, there is a vast excess of PABPs suggests that the role(s) of PABPs *in vivo* may not reflect those observed *in vitro* (Drawbridge *et al*, 1990). Indeed, in the sea urchin, both translated and non-translated poly A^+ mRNAs are bound by PABP (Drawbridge *et al*, 1990).

In summary, the binding of PABP to their poly A tails appears to be essential for recruitment of mRNAs into polysomes. PABP must interact with a site on the 60S ribosomal subunit (in yeast) to permit initiation of translation.

Cytoplasmic polyadenylation and translational activation

In mature *Xenopus* oocytes, the total poly A content is reduced by about 40% as a result of the deadenylation of a considerable number of maternal mRNAs (Sagata *et al*,

1980). One class of mRNAs which are actively translated in sea urchin eggs or *Xenopus* oocytes are deadenylated and dissociate from polysomes during fertilisation or maturation (Hyman and Wormington, 1988; Paynton *et al*, 1988; Rosenthal and Ruderman, 1987; Rosenthal *et al*, 1983). Another class of translationally dormant mRNAs undergo lengthening of their poly A tails and are recruited into polysomes during fertilisation or maturation (Dworkin *et al*, 1985; Huarte *et al*, 1987; McGrew *et al*, 1989; Paynton *et al*, 1988; Rosenthal and Ruderman, 1987; Rosenthal *et al*, 1983). Transcriptional activation of a third class of maternal mRNAs during oocyte maturation is accompanied by no change in poly A tail length or even by a reduction (eg Ruderman *et al*, 1979). Oocyte maturation has no effect on the translational activity of a further class of maternal mRNAs (Woodland *et al*, 1979; Lee *et al*, 1984; Stick and Hausen, 1985; Taylor *et al*, 1986). Deadenylation of maternal mRNAs during oocyte maturation generally correlates with markedly reduced translational activity (Sturgess *et al*, 1980; Hyman and Wormington, 1988; Wormington, 1989).

Two discrete cis-acting sequence elements, the AAUAAA hexanucleotide and a U-rich cytoplasmic polyadenylation element (CPE), both located in the 3' UTR of the mRNA, are required for cytoplasmic polyadenylation and translational activation during oocyte maturation (Fox *et al*, 1989; McGrew *et al*, 1989; McGrew and Richter, 1990; Paris and Richter, 1990). A default pathway through which transcripts lacking a CPE undergo poly A removal is responsible for deadenylation and transcriptional inactivation of maternal mRNAs during *Xenopus* oocyte maturation (Varnum and Wormington, 1990; Fox and Wickens, 1990). The AAUAAA hexanucleotide is also required to enable an RNA to escape the deadenylation mechanism (Fox and Wickens, 1990).

Removal of the poly A tail during maturation or after fertilisation appears to have no effect on the

stability of the mRNA since deadenylated transcripts persist in non-polysomal form (Fox and Wickens, 1990; Dworkin and Dworkin-Rastl, 1985; Dworkin *et al*, 1985; Hyman and Wormington, 1988; Duval *et al*, 1990; Ruiz i Altalba *et al*, 1987; Paris and Philippe, 1990). In contrast, in somatic cells, some but not all mRNAs are rapidly degraded following deadenylation (reviewed by Bernstein and Ross, 1989; Brawerman, 1981).

Comparison of the sequences of CPEs from a number of genes shows that an active CPE usually comprises at least 4U residues closely followed by an invariant AU dinucleotide (McGrew and Richter, 1990; Fox *et al*, 1989; McGrew *et al*, 1989; Sagata *et al*, 1988; Paris and Richter, 1990; Paynton *et al*, 1988). Cytoplasmic polyadenylation does not require the AU dinucleotide (McGrew and Richter, 1990). There is a considerable degree of flexibility in the location of CPE since it can function either 5' or 3' of the AAUAAA hexanucleotide and up to 26nu away (McGrew and Richter, 1990; Paris and Richter, 1990).

Some diversity within the process of maturation-specific polyadenylation and translational activation has been observed with different RNA substrates. The B4, G10 and D7 RNAs are polyadenylated and recruited into polysomes during *Xenopus* oocyte maturation (McGrew *et al*, 1989; Maniatis *et al*, 1982; Fox *et al*, 1989). As well as the invariant ubiquitous AAUAAA hexanucleotide, cytoplasmic poly A elongation of G10 and B4 mRNAs requires a CPE: the G10 CPE has the sequence U_6AUA_3G, whereas that in the B4 RNA is U_5A_2U (McGrew *et al*, 1989; Paris and Richter, 1990). Despite similarities in the sequences of their CPEs, different RNAs are subject to differential temporal regulation of cytoplasmic polyadenylation, B4 and G10 being polyadenylated early and D7 at a later stage during oocyte maturation (Fox *et al*, 1989; McGrew and Richter, 1989; Paris and Richter, 1990).

There are also apparent differences between the mechanisms by which the mRNAs undergo translational activation. Active polyadenylation of G10 RNA, rather than a specific or minimum length of poly A tail, is required for its recruitment into polysomes (McGrew *et al*, 1989). In contrast, B4 RNA acquires approximately 150 A residues, in a regulated manner; translational activation of this RNA requires a poly A tail 150nu in length (Paris and Richter, 1990). Possibly translational activation of other RNAs occurs by a similar mechanism (Drummond *et al*, 1985; Galili *et al*, 1988). The differences between B4 and G10 RNA polyadenylation and translational activation are almost certain to be the result of differential recognition of their CPEs (Paris and Richter, 1990).

Maturation-specific cytoplasmic polyadenylation has been reproduced *in vitro* using cell free *Xenopus* egg extracts (Paris and Richter, 1990). Polyadenylation complexes, whose assembly require an intact CPE and the AAUAAA hexanucleotide, but not active extension of the poly A tail, can be detected using gel-mobility shift assays (Paris and Richter, 1990). Competition assays showed that both an intact CPE and the AAUAAA hexanucleotide are required to compete for the polyadenylation apparatus and that polyadenylation of two different RNAs, B4 and G10, which display different modes of activation, requires at least one common factor, which has a greater affinity for B4 RNA (Paris and Richter, 1990).

The mechanism by which CPE-containing mRNAs are protected from deadenylation is unclear. Extracts of *Xenopus* eggs but not oocytes contain an 82kD CPE-binding protein which may be involved (McGrew and Richter, 1990). The cytoplasmic poly A tail may contain modified A residues which inhibit the nuclease responsible for deadenylation. *Xenopus* eggs have been shown to contain an RNA helicase activity which, additionally, can convert adenosine residues to inosine (Bass and Weintraub, 1988).

After fertilisation of oocytes, during the first several cleavage divisions, certain mRNAs undergo changes in the lengths of their poly A tails (Dworkin and Dworkin-Rastl, 1985; Paris *et al*, 1988). Cytoplasmic poly A addition during oocyte maturation requires signals located within the 3' UTRs of substrate mRNAs (Huarte *et al*, 1987; Strickland *et al*, 1988; Vassalli *et al*, 1989; McGrew *et al*, 1989; Fox *et al*, 1989). Removal of the 3' UTR abolishes polyadenylation (Strickland *et al*, 1988; McGrew *et al*, 1989). Ligation of a 3' UTR to a usually non-polyadenylated mRNA results in a chimeric RNA which is a substrate for cytoplasmic polyadenylation (Huarte *et al*, 1987; McGrew *et al*, 1989). When injected into oocytes, an appropriate 3' UTR is polyadenylated (Vassalli *et al*, 1989; Fox *et al*, 1989). Therefore, the 3' UTR is necessary and sufficient for cytoplasmic polyadenylation during oocyte maturation.

In *Xenopus*, both AAUAAA and a U-rich motif (U_5AU or a closely related sequence), which has been identified just upstream of AAUAAA in a number of mRNAs which are polyadenylated during maturation, are required for polyadenylation during maturation (McGrew *et al*, 1989; Fox *et al*, 1989). Comparison of the sequences of a number of mRNAs has shown that the precise sequence of the U-rich element can vary considerably (Smith *et al*, 1988a, 1988b; McGrew *et al*, 1989; Wickens, 1990). In addition to the poly A signal and the U-rich element, the intervening sequence also influences the efficiency of polyadenylation (Fox *et al*, 1989).

Natural mRNAs possess a short poly A tail before the onset of oocyte maturation: these oligo A tails probably result from AAUAAA-specific polyadenylation, either in the nucleus or in the cytoplasm (Richter, 1987; Rosenthal and Wilt, 1987). Injection of pre-cleaved RNAs into the oocyte cytoplasm results in their acquisition of short, presumably oligo A, tails (Fox *et al*, 1989). The cytoplasmic

polyadenylation apparatus appears to recognise these oligo A tails as substitutes for AAUAAA. The second phase of nuclear poly A addition also requires oligo A, which obviates the requirement for AAUAAA (Sheets and Wickens, 1989).

Export of mRNAs from the nucleus

In order to be translated, mature mRNAs must be exported from the nucleus to the cytoplasm following their release from the transcription and processing machinery. All macro-molecules which cross the nuclear membrane seem to do so via nuclear pore complexes (NPCs). Although non-specific RNA export via NPCs has been demonstrated, there is some evidence of selection of transcripts for transport to the cytoplasm (reviewed by Goldfarb and Michaud, 1991). For example, excess free m^7GpppG oligonucleotide cap analogue inhibits cap-dependent export of snRNA primary transcripts, suggesting an essential interaction between the m^7GpppN cap of the snRNAs and a specific intranuclear receptor (Hamm and Mattaj, 1990). However, no such receptor has been identified to date, nor has the mechanism of NPC dilation and RNA export been elucidated.

There is evidence of links between pre-mRNA processing and the subsequent export of the mature transcript to the cytoplasm. It seems that assembly of pre-splicing complexes on an intron-containing pre-mRNA can anchor the transcript within the nucleus. In yeast, mutations in the introns of the pre-mRNAs or in the components of pre-splicing complexes cause an increase in the export of unspliced pre-mRNAs to the cytoplasm (Legrain and Rosbash, 1989). These results demonstrate that the presence of introns and splice junctions in itself does not interfere with the mRNA export process. Similarly, in a mammalian system, a mutant β-globin pre-mRNA which possesses inactive 5' and 3' splice junctions is efficiently exported, while transcripts in which only one or other splice junction

is mutated fails to leave the nucleus (Chang and Sharp, 1990), indicating that the formation of pre-splicing complexes is sufficient to prevent export of the transcript.

Control of the export of mRNAs can be exerted via regulation of their release from pre-splicing complexes. The Rev protein of HIV stimulates the release of intron-containing mRNAs from pre-splicing complexes, or prevents their formation, resulting in an alteration of the cytoplasmic ratio of spliced and unspliced viral mRNAs (Chang and Sharp, 1990). Possibly such control is not mediated via the interaction of the mRNA with the export machinery.

Nonsense codons placed within the exon downstream from the last intron have no effect on the levels of either dihydrofolate reductase or triosephosphate isomerase mRNAs, while nonsense codons in exons close to the 5' ends of these mRNAs reduce the steady-state levels of these transcripts in the cytoplasm, and in the latter case in the nucleus (Urlaub *et al*, 1989; Cheng *et al*, 1990). Models postulated to explain these results suggest that the 5'-proximal nonsense mutations are detected either in the cytoplasm, where they cause premature termination of translation, or alternatively in the nucleus, by a novel nuclear scanning activity. Detection would cause failure of pre-mRNA processing further downstream on the same transcript, or otherwise lead to degradation of the RNA. The first model requires that the 5' end of the transcript be processed, released and transported to the cytoplasm, while the 3' portion remained bound to the processing and transcription machinery in the nucleus: a single RNA molecule traversing a NPC would need to be available for simultaneous translation and processing. The second model predicts the existence of a nuclear activity which can detect in-frame nonsense mutations and target transcripts bearing them for degradation in the nucleus. No evidence for either of these requirements has been presented.

In vitro 3' processing systems

In order to obtain sufficient RNA for analysis, most *in vivo* pre-mRNA processing experiments involved the use of virus-infected cells, plasmid-transfected cells or microinjected *Xenopus* oocytes (eg, Berk and Sharp, 1978; Conway and Wickens, 1985; Ford and Hsu, 1978; Higgs *et al*, 1983; Nevins and Darnell, 1978a; Reddy *et al*, 1979; Simonsen and Levinson, 1983; Wickens and Gurdon, 1983; Wickens and Stephenson, 1984). Because the cleavage and polyadenylation reactions could not be uncoupled *in vivo*, the development of a cell-free RNA processing system was highly desirable. Such an *in vitro* system would permit investigation of the cleavage and polyadenylation reactions in isolation, and would facilitate biochemical fractionation of the activities involved in the processing reactions.

Soluble lysates of mammalian cells which are capable of supporting accurate initiation by RNA polymerase II have been prepared by a number of groups (Cepko *et al*, 1981; Dignam *et al*, 1983; Handa *et al*, 1981; Kole and Weissman, 1982; Manley *et al*, 1980; Weil *et al*, 1979; Weingartner and Keller, 1981). In none of these cases have significant levels of splicing or cleavage/polyadenylation been detected.

Whole cell extracts prepared from HeLa cells have been found to be capable of supporting polyadenylation of pre-mRNAs *in vitro*: while these experiments accurately demonstrate a number of aspects of the polyadenylation process, several features of the *in vivo* reaction are not seen (Manley, 1983; Moore and Sharp, 1984). In the former case, pre-mRNAs transcribed *in vitro* using a HeLa cell whole cell extract are purified and then incubated under polyadenylation conditions with fresh whole cell extract: between 150 and 300 adenylate residues are added to precursor RNAs whose 3' termini are located at or slightly

downstream from the *in vivo* 3' end (Manley, 1983). In the latter system, the precursor RNA is transcribed and cleaved and polyadenylated in a single incubation: exogenous RNAs are not substrates for cleavage but are end-polyadenylated (Moore and Sharp, 1984).

Nuclear extracts capable of accurate and specific cleavage and polyadenylation of exogenous, *in vitro*-transcribed precursor RNAs have been prepared from HeLa cells (Moore and Sharp, 1985). In this system, as *in vivo*, polyadenylation occurs rapidly after cleavage of the precursor RNA. The reaction conditions required for cleavage and polyadenylation have been investigated. Free Mg^{2+} is not required for cleavage, nor for polyadenylation, although chelation of Mg^{2+} by excess EDTA results in a reduction in the extent of processing, a reduction of the rate of polyadenylation and production of a shorter poly A tail. In the absence of ATP, both cleavage and polyadenylation are inhibited, suggesting that ATP is required as a co-factor in reaction steps other than poly A synthesis. At suboptimal ATP concentrations, tails containing fewer than 33 A residues are not observed, suggesting that the ATP concentration required for initiation is greater than that required for elongation of the poly A tail.

The beta-gamma-methylene analogue of ATP does not affect processing of precursor RNA, although shorter poly A tails are observed in its presence. Both 3' dATP and α-β-methylene ATP inhibit poly A synthesis, but not cleavage at the poly A addition site. *In vivo*, the appearance of mature cytoplasmic mRNAs is almost completely inhibited in the presence of 3'dATP, while only slight inhibition of hnRNP synthesis has been observed (Darnell *et al*, 1971a; Mendecki *et al*, 1972). *In vitro*, RNA polymerase II is considerably less sensitive than purified poly A polymerase to inhibition by 3'dATP (reviewed by Edmonds, 1982).

In all but one case (Hart *et al*, 1985a; Gilmartin *et al*, 1988), the mRNA cap structure appears to exert no influence on 3' processing *in vitro* (Moore and Sharp, 1985; Conway and Wickens, 1987). These conflicting results may reflect different features of the substrate RNAs or processing extracts used.

The polyadenylation reaction

In vivo studies of the kinetics of poly A addition have revealed a two-stage process; a rapid initial phase of poly A synthesis is followed by slower elongation (Brawerman and Diez, 1975; Sawicki *et al*, 1977). Similarly, *in vitro*, the first 130 residues of the poly A tail are added rapidly; further elongation occurs more slowly, in a Mg^{2+}-dependent manner (Moore and Sharp, 1985). *In vitro* polyadenylation of adenovirus L3 mRNA yields a number of short, presumably oligoadenylated molecules, suggesting that, at least in this case, polyadenylation is biphasic (Skolnik-David *et al*, 1987). Highly purified vaccinia virus poly A polymerase (PAP) mediates polyadenylation via short poly A tracts containing 10-16 residues which are subsequently converted into full-length poly A tails (Shuman and Moss, 1988). Similarly, two distinct phases of the addition of poly A tails to pre-cleaved RNAs *in vitro* have been observed: in the first phase, poly A addition occurs slowly, the addition of each adenylate residue requiring an intact poly A signal; the second phase, which commences with the addition of the tenth A residue is characterised by rapid polymerisation and results in a poly A tail whose length is regulated to 200nu; the oligo A tract, but not AAUAAA, is essential for the second phase (Sheets and Wickens, 1989).

Nuclear factors in 3' processing

Experiments using the *in vitro* 3' processing system identified large, specific complexes which form in association with the substrate RNA during 3' end formation

and polyadenylation: the complexes have been identified by a number of means including gel retardation assays, velocity centrifugation and gel-filtration chromatography (Humphrey *et al*, 1987; Moore *et al*, 1988a; McLauchlan *et al*, 1988; Skolnik-David *et al*, 1987; Zarkower and Wickens, 1987a; Zhang and Cole, 1987). Formation of the specific complexes requires the AAUAAA sequence and the downsteam element (Humphrey *et al*, 1987; Skolnik-David *et al*, 1987; Zarkower and Wickens, 1987b, 1988; Zhang and Cole, 1987). These complexes may contain snRNAs since trimethyl-cap-specific antibodies have been shown to precipitate substrate RNA present in a polyadenylation-specific complex (Moore *et al*, 1988b).

RNAs which are substrates for cleavage and polyadenylation *in vitro* are specifically assembled into a heparin-resistant 25S complex which contains a factor bound to the AAUAAA motif (Stefano and Adams, 1988). The requirements for complex assembly are identical to those for cleavage and polyadenylation *in vitro*. In the assembled complex, the AAUAAA motif and a downstream U-rich motif are resistant to RNaseH digestion, but the cleavage site is accessible, although to a lesser extent than in naked RNA, suggesting that a specific processing factor or secondary structure element may be located nearby. Binding of the AAUAAA-specific factor requires neither ATP nor the downstream sequence element, suggesting that AAUAAA recognition may be an initial, uncommitted event in complex assembly.

Pre-cleavage complexes, formation of which requires the AAUAAA hexanucleotide and the downstream sequence element, and in which both of these sequences are specifically protected from RNase T1 and RNase H digestion, form on pre-mRNAs (Humphrey *et al*, 1987; Skolnik-David *et al*, 1987; Stefano and Adams, 1988; Zarkower and Wickens, 1987a). Pre-cleaved RNAs also form AAUAAA-dependent complexes with the consequent RNase protection of the poly A

signal (Zarkower and Wickens, 1987a). The formation of these specific complexes is prevented by chemical modifications of the RNA substrate or by hybridisation of DNA oligonucleotides to AAUAAA, both of which also block 3' processing (Conway and Wickens, 1987; Zarkower and Wickens, 1987b). The involvement of a common AAUAAA-specific factor in both cleavage and polyadenylation reactions seems likely since formation of the pre-cleavage complex is competitively inhibited by an AAUAAA-containing pre-cleaved RNA (Zarkower and Wickens, 1987a).

The pre-cleavage complex has a relative molecular weight of 500-1000 kD (Manley *et al*, 1989). Alteration of the electrophoretic mobility of the complex following cleavage suggests the release of one or all of the components which are required for cleavage, but not for polyadenylation (Zarkower and Wickens, 1987b).

Identification of nuclear processing factors

An activity has been detected in nuclear extracts which interacts with the AAUAAA hexanucleotide to block hybridisation of a complementary oligonucleotide (Zarkower and Wickens, 1987a). A 64/68kD polypeptide is specifically crosslinked to precursor RNA undergoing cleavage/ polyadenylation *in vitro* in an AAUAAA-dependent manner (Moore *et al*, 1988a; Wilusz and Shenk, 1988). Biochemical fractionation of the processing extracts indicate that the 64kD protein is not PAP (Wilusz and Shenk, 1988). Rather, it may represent a cleavage or specificity factor involved in 3' end formation (Takagaki *et al*, 1988). Similarly, a protein of molecular weight 155kD present in nuclear extracts, which possesses an Sm epitope, has been shown to exhibit AAUAAA-dependent RNA-binding activity (Moore *et al*, 1988a).

Biochemical fractionation of nuclear extracts has revealed that the cleavage and polyadenylation reactions

require different combinations of separable factors. Reconstitution of the cleavage reaction at the SV40 L poly A site requires four factors: an AAUAAA-specificity factor (SF), a cleavage stimulation factor (CstF) and two cleavage factors (CFI and CFII) (Gilmartin and Nevins, 1989; Takagaki *et al*, 1989). Cleavage of all other poly A sites tested to date requires PAP in addition to the four factors described above (Bardwell *et al*, 1990; Christofori and Keller, 1988, 1989; Ryner *et al*, 1989; Takagaki *et al*, 1988, 1989; Terns and Jacob, 1989). Polyadenylation of pre-cleaved pre-mRNAs requires only two factors, namely SF and PAP (Gilmartin and Nevins, 1989; Sheets and Wickens, 1989).

Recognition of the SV40 L poly A site involves co-operative binding of SF and CstF to the pre-mRNA in an AAUAAA- and downstream element-dependent manner, to form a stable ternary complex (Gilmartin and Nevins, 1989). The stability of this "committed" ternary complex determines the efficiency of processing at a particular poly A site, and is dependent on the nature of the downstream element (Weiss *et al*, 1991). Following formation of the committed complex, cleavage at the poly A addition site occurs when CFI and CFII join the complex (Gilmartin and Nevins, 1989; Takagaki *et al*, 1990).

The properties of specificity factor and cleavage stimulating factor

Both SF and CstF are multicomponent complexes, comprising some five and three distinct polypeptides, respectively. SF- and CstF-containing, partially purified fractions are sufficient to reconstitute AAUAAA-dependent cross-linking of the 64kD polypeptide to precursor RNA, suggesting that this polypeptide is a component of one of these factors (Wilusz *et al*, 1990; Gilmartin and Nevins, 1991).

SF appears to consist of polypeptides of molecular weights 170kD, 130kD, 100kD, 74kD and 42kD. These polypeptides co-sediment with SF activity during purification (Gilmartin and Nevins, 1989) and are co-precipitated by anti-Sm antibodies from a SF-containing, purified nuclear fraction (Gilmartin and Nevins, 1991). Anti-Sm antibodies deplete an essential processing activity from a SF-containing crude fraction and block cleavage at the poly A site *in vitro* (Gilmartin and Nevins, 1989), as well as retarding complexes containing SF in electrophoresis assays (Gilmartin and Nevins, 1991). A factor possessing an Sm-epitope associates with precursor RNA in an AAUAAA-specific manner (Hashimoto and Steitz, 1986). Since the 170kD polypeptide can be UV cross-linked to RNA in an AAUAAA-dependent manner, it seems likely that this protein is identical to the 155kD polypeptide which was identified, using anti-Sm antibodies, in a previous study (Moore *et al*, 1988a).

Experiments using pre-cleaved RNA substrates containing either single base substitutions or single 2'-O-methylated nucleotides in AAUAAA confirm that RNA/protein interactions involving contacts between SF and both ribose and base moieties at different nucleotide positions are required for recognition of substrate RNA by SF (Bardwell *et al*, 1991).

The constituents of CstF have been defined as polypeptides of molecular weights 76-77kD, 64kD and 48-50kD (Gilmartin and Nevins, 1991; Takagaki *et al*, 1990). Using monoclonal antibodies directed against either a 64kD or a 50kD polypeptide, both of which co-purified with CstF, it was shown that these two molecules, together with another polypeptide of 77kD, constitute CstF, the subunits being present in a ratio of 1:1:1 (Takagaki *et al*, 1990). The combined estimated molecular weights of the three subunits

(191kD) is in reasonable agreement with the previous estimate of the molecular weight of CstF (200kD) (Takagaki *et al*, 1989).

The 64kD component of CstF can be UV cross-linked to pre-mRNA in a downstream element-dependent fashion, reflecting what appears to be an unstable interaction (Gilmartin and Nevins, 1991). The addition of SF stimulates cross-linking of the 64kD polypeptide by more than 12-fold, suggesting co-operative interactions between SF and CstF (Gilmartin and Nevins, 1991). Further evidence of conformation changes induced by interactions between SF and CstF is provided by the observation that the 130kD component of SF can be cross-linked to RNA only when CstF is present (Gilmartin and Nevins, 1991).

The dramatic reduction of the efficiency of use of a poly A site caused by altering the spacing between the poly A signal and the downstream element may be explained by a requirement for direct physical interaction between SF and CstF for the stability of the precursor RNA/SF/CstF ternary complex (McDevitt *et al*, 1988; Gil and Proudfoot, 1987; Gilmartin and Nevins, 1989). The stability of the committed ternary complex, comprising substrate RNA, SF and CstF, is a determinant of the efficiency of processing of a particular poly A site and is dependent on the nature of the downstream element (Weiss *et al*, 1991).

hnRNP C proteins are not required for 3' processing

A strong interaction by hnRNP C polypeptides with a number of poly A sites has been detected by UV-cross-linking (Moore *et al*, 1988a; Stolow and Berget, 1990; Wilusz *et al*, 1988). Deletion of the U-rich sequence element 30-55nu downstream of the SV40 L poly A addition site abolishes hnRNP C cross-linking (Wilusz *et al*, 1988). In some cases, though not all, the efficiency of cleavage/polyadenylation is reduced by deletion of the U-rich downstream sequence

element (Cole and Stacey, 1985; Conway and Wickens, 1985; Gil and Proudfoot, 1984; Hart *et al*, 1985b; Sadofsky and Alwine, 1984; Simonsen and Levinson, 1983). Cross-linking of hnRNP C to the SV40 L poly A sites is abolished by point mutations within the U-rich tract which do not affect the efficiency of cleavage/polyadenylation (Wilusz *et al*, 1988).

Depletion of the hnRNP C polypeptides inhibits formation of the spliceosome and splicing *in vitro* is inhibited by anti-hnRNP C antibodies (Choi *et al*, 1986). The hnRNP C polypeptides have been shown to interact with the polypyrimidine tract at the 3' end of the intron (Garcia-Blanco *et al*, 1989; Kumar *et al*, 1987; Swanson and Dreyfuss, 1988a, b). However, in chimeric RNAs containing both splicing signals and the SV40 L poly A site, hnRNP C cross-linking is reduced more than 20-fold, while mutation of either the splicing or polyadenylation consensus, or the use of reaction conditions which inhibit polyadenylation, restores hnRNP C binding to the levels observed for polyadenylation or splicing sequences in isolation (Stolow and Berget, 1990). Furthermore, in the presence of competing poly U, cross-linking of hnRNP C to precursors containing only the SV40 L poly A site is eliminated, although polyadenylation is unaffected (Stolow and Berget, 1990). An important role for hnRNP C in either the cleavage/polyadenylation or splicing reactions seems most unlikely.

U_{11} snRNA is not required for mRNA 3' processing

The suggestion that an snRNP, perhaps that containing U11 snRNA, may comprise an element of specificity factor (Christofori and Keller, 1988; Montzka and Steitz, 1988) is the subject of some controversy. The most abundant RNA species in partially purified specificity fractions is U11 snRNA (Montska and Steitz, 1988; Christofori and Keller, 1988; Takagaki *et al*, 1989). However, U11 and SF do not precisely cofractionate (Takagaki *et al*, 1989). Since U11

snRNA cannot be inactivated by the usual methods, it has not been possible to prove its involvement in SF beyond doubt (Montzka and Steitz, 1988). Furthermore, the requirement for the 2'-hydroxyl group of U in AAUAAA for SF binding (Wigley *et al*, 1990) and the inhibition of SF binding to AAUAAA by 2'-O-methylation of riboses within AAUAAA (Bardwell *et al*, 1991) suggests that at least one contact with AAUAAA is made by protein, rather than by an RNA. In any case, none of the known cellular snRNAs, including U11, contains a sequence complementary to AAUAAA, which might be expected to be involved in an RNA-RNA recognition mechanism (Montzka and Steitz, 1988). The results of Wigley *et al* (1990) and Bardwell *et al* (1991) do not exclude the possibility that an snRNA is a component of SF, only that it is not involved in recognition of AAUAAA.

Nuclear factors required for polyadenylation

At least two distinct activities, PAP and an AAUAAA-specificity factor (SF), are required for accurate polyadenylation of cleaved pre-mRNAs (Takagaki *et al*, 1988, 1989; Christofori and Keller, 1988, 1989; Ryner *et al*, 1989; Terns and Jacob, 1989; Bardwell *et al*, 1990). PAP alone is not sufficient for accurate and efficient polyadenylation.

PAP is usually defined as an activity which adds adenylate residues to the 3' termini of an RNA primer, has been isolated from many different sources and has been shown to consist of a single polypeptide (reviewed by Edmonds, 1989). Since the normal definition of PAP activity does not include AAUAAA-specificity, it can not be stated unequivocally that an activity identified in this way is responsible for polyadenylation of mRNA precursors.

Partially purified PAP lacks sequence specificity, is unable to perform site-directed cleavage of a pre-mRNA, exhibits a low rate of polymerisation, and fails to size the adenylate tail correctly (reviewed by Edmonds, 1989). A PAP

which functions in an AAUAAA-dependent manner in the presence of Mg^{2+} and a complementary nuclear extract fraction, but which shows no specificity when assayed alone in the presence of Mn^{2+}, has been partially purified from HeLa cell nuclear extracts (Christofori and Keller, 1989; McDevitt *et al*, 1988; Takagaki *et al*, 1988). The PAP-containing fraction is essential for both cleavage and polyadenylation of most precursors (Christofori and Keller, 1989; Takagaki *et al*, 1989).

However, PAP is not required for efficient cleavage of the SV40 L poly A site (Takagaki *et al*, 1989). The PAP described by the groups above displays properties similar to those of the previously described non-specific activity (Christofori and Keller, 1989; Gilmartin and Nevins, 1989; Nevins and Joklik, 1977; Rose and Jacob, 1976; Rose *et al*, 1977; Ryner *et al*, 1989; Terns and Jacob, 1989). When mixed with a specificity fracton of a HeLa nuclear extract, a classical, non-specific PAP purified from calf thymus acquires AAUAAA-specificity, strongly suggesting that the classical enzyme is responsible for mRNA polyadenylation (Bardwell *et al*, 1990). Finally, the inhibition of endonucleolytic cleavage and both AAUAAA-specific and non-specific polyadenylation by specific anti-PAP antibodies *in vitro* demonstrates unequivocally that the classical PAP is the enzyme responsible for the addition of the poly A tract at the cleavage site and explains the coupling of the cleavage and polyadenylation reactions in terms of the requirement of PAP for the former, as well as the latter (Terns and Jacob, 1989).

Both initiation and extension of poly A tails requires SF (Gilmartin and Nevins, 1989; Sheets and Wickens, 1989). In addition to PAP, another factor - possibly either SF or PABP - seems to be required for the second phase of polyadenylation, since partially purified PAP shows no marked preference for oligo A as a substrate (Winters and Edmonds, 1973).

In crude nuclear extracts, poly A synthesis is accurately terminated. Using either yeast or HeLa cell nuclear extracts, poly A tails which closely correspond to the *in vivo* length are produced (Butler and Platt, 1988; Butler *et al*, 1990; Moore and Sharp, 1985). In HeLa extracts, extension of the poly A tail beyond the normal *in vivo* length of 200-250 A residues did occur, albeit relatively slowly (Moore and Sharp, 1985).

HeLa cell nuclear extracts appear to contain an activity which discriminates the length of an RNA's poly A tail: poly A tails of less than 250 residues are extended after addition to nuclear extract, whereas poly A tails longer than 250nu are not (Sheets and Wickens, 1989). Such an activity is not a property of purified PAP.

Regulation of 3' processing

Messenger RNA 3' end formation and polyadenylation is one of several post-transcriptional RNA processing events which can contribute to the regulation of gene expression. For example, utilisation of alternative poly A sites within a single transcription unit, perhaps as a result of modulation of their relative processing efficiencies, can result in the synthesis of more than one mRNA species, from which different polypeptides may be expressed (reviewed by Nevins, 1983; Leff and Rosenfeld, 1986).

The quality or "strength" of a polyadenylation site can profoundly influence the level of expression of stable mRNA from a gene (Whitelaw and Proudfoot, 1986). The efficiency of utilisation of a number of poly A sites *in vivo* has been shown to encompass a range of strengths (Mason *et al*, 1986; Norbury and Fried, 1987; Sasavage *et al*, 1982; Simonsen and Levinson, 1983; Weidemann and Perry, 1984; Woychik *et al*, 1984). Similarly, *in vitro*, cleavage of a number of viral poly A sites occurs with different efficiencies (Gilmartin *et al*, 1988; Ryner *et al*, 1989).

Differential efficiencies of utilisation of poly A sites must reflect differential efficiencies of RNA/processing factor interactions, resulting from the presence of different sequences in pre-mRNAs. Since the poly A signal, AAUAAA, is almost invariant, the relative efficiencies of the majority of poly A sites must be determined by other sequence elements, either upstream or downstream of AAUAAA (Gilmartin and Nevins, 1989). Although similar, the downstream GU- or U-rich sequence elements required for efficient 3' end formation *in vitro* are far from identical at all poly A sites (Hales *et al*, 1988; Zarkower and Wickens, 1988). Since the nature of the downstream element determines the stability of the interaction between the pre-mRNA, SF and CstF, and therefore the strength of the poly A site, it follows that the efficiency of use of a particular poly A site depends crucially on its downstream sequence element(s) (Weiss *et al*, 1991). Exquisite fine-tuning of poly A site efficiency is possible because it is influenced not only by the sequence of the downstream elements but also by their locations relative to the poly A signal.

Regulation of the relative efficiencies of different poly A sites in response to the cell-cycle, differentiation or intra- or extracellular signals or events may be achieved by transient or stable expression of one or more auxiliary processing factors or alternative CstF species, resulting in altered affinities for different downstream elements. That some poly A sites require additional processing factors has already been established. For example, while the SV40 L poly A site is efficiently cleaved *in vitro* in its absence, PAP is required for cleavage at all the other sites tested so far (Bardwell *et al*, 1990; Christofori and Keller, 1988, 1989; Ryner *et al*, 1989; Takagaki *et al*, 1988, 1989; Terns and Jacob, 1989).

In some complex transcription units, changes in poly A site choice may determine which mRNA and protein species are expressed. In most of the complex transcription units investigated in detail there is no unequivocal evidence that differential poly A site utilisation is the single or major determining factor in switching between alternative mRNA processing patterns. However, a processing factor (LPF) which is not present in extracts from uninfected HeLa cells and which mediates a processing switch from early to late HSV gene poly A sites *in vitro* has been identified in extracts prepared from HSV-infected HeLa cells (McLauchlan *et al*, 1989). LPF appears to be a cellular factor which is induced at late times during HSV infection (J McLauchlan, personal communication). Such a factor could function in the normal processing of a subset of cellular poly A sites.

The role of 3'processing in complex transcription units

In complex transcription units containing multiple poly A sites, regulation of cleavage and polyadenylation can contribute to control of differential gene expression (reviewed by Leff and Rosenfeld, 1986; Breitbart *et al*, 1987). Extensive studies of a number of complex transcription units, in which choices between splicing and polyadenylation sites are made, have been undertaken. The genes which have been studied in greatest detail have provided examples of distinct mechanisms which control the choices between alternative splicing patterns and poly A sites.

In general, polyadenylation seems to precede splicing of primary transcripts *in vivo* (Darnell, 1982; Lai *et al*, 1978; Nevins *et al*, 1978a). Therefore, in complex transcription units, the choice between alternative poly A sites, which would dictate subsequent splicing patterns, might seem the most obvious point at which regulation of differential mRNA expression might occur. However, there is

evidence that some nascent mRNAs can be spliced before cleavage/polyadenylation occurs. This seems to be the most likely explanation for the failure to observe a full-length transcript from the immunoglobulin δ locus (Mather *et al*, 1984). In addition, a spliced poly A$^-$ RNA transcribed from adenovirus has been reported (Berget and Sharp, 1979).

Further support for the possibility of splicing of nascent RNAs comes from electron micrographs of transcriptionally active chromatin. hnRNP particles are seen to assemble at splice junctions on nascent transcripts, with the formation of loops between the particles, the loops being lost from more mature transcripts via a process whose characteristics resemble RNA splicing (Beyer and Osheim, 1988; Beyer *et al*, 1981; Osheim *et al*, 1985). The hnRNP particles may represent spliceosomes since they are bound by anti-snRNP antibodies (Brody and Abelson, 1985; Fakan *et al*, 1986).

Polyadenylation does not appear to be a pre-requisite for RNA splicing. An *in vitro*-synthesised, poly A$^-$ globin mRNA is efficiently spliced following microinjection in *Xenopus* oocyte nuclei (Green *et al*, 1983). Furthermore, cordycepin, which blocks polyadenylation, does not inhibit splicing *in vivo* (Zeevi *et al*, 1981).

It has been suggested that the pre-mRNA splicing process may occur only in specific compartments within the nucleus (Fu and Maniatis, 1990), following the observations that immunofluorescent staining for several of the factors involved in the splicing machinery reveals discrete speckles within the nucleus (Fu and Maniatis, 1990; Lerner *et al*, 1981; Nyman *et al*, 1986). The distribution of the mRNA 3'-end processing factor, CstF, is found to be distinct from that of the splicing factors, although it does also display a granular pattern suggesting that splicing and 3'-end formation occur in separate sub-nuclear compartments (Takagaki *et al*, 1990).

The immunoglobulin μ heavy chain locus

Primary transcripts from the immunoglobulin μ heavy chain transcription unit are differentially processed to produce two distinct mRNAs in a developmentally-regulated, cell-specific manner. The μ_s and μ_m mRNAs, which encode secreted and membrane-bound forms of the μ heavy chain respectively, differ only in their 3' terminal sequences, which are specified in distinct exons with distinct poly A sites (Fig 4) (Alt *et al*, 1980; Early *et al*, 1980; Rogers *et al*, 1980). The μ_m-specific 3' terminal exons lie downstream of the μ_s 3' terminal exon.

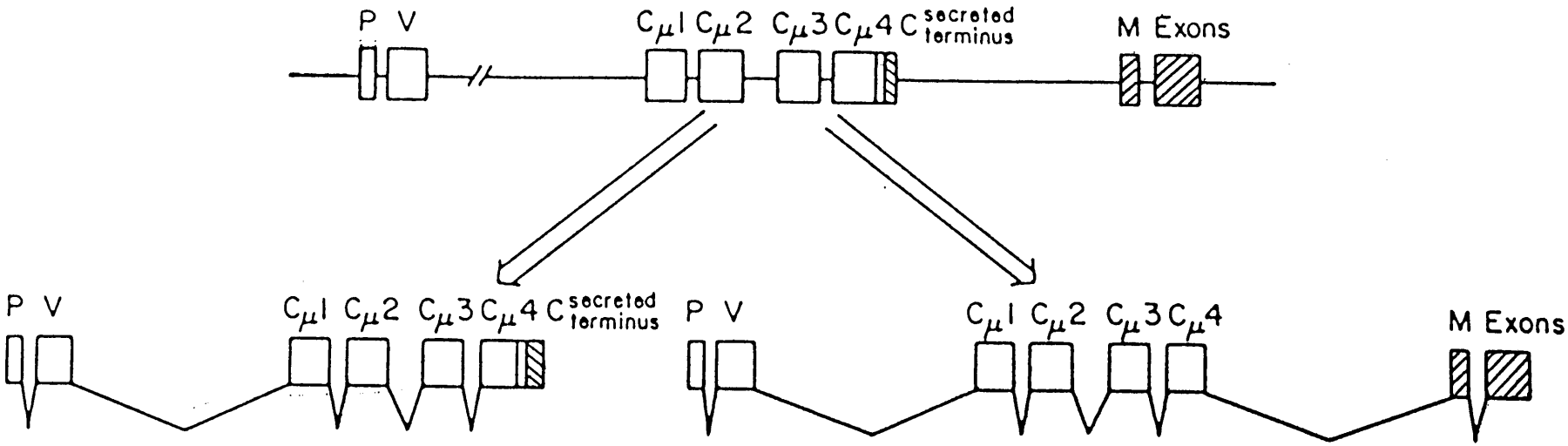
In plasma cells (and their transformed equivalents, plasmacytomas), the μ_s mRNA predominates, while in B cells (and lymphomas), approximately equal levels of μ_s and μ_m mRNAs are produced (Alt *et al*, 1980; Early *et al*, 1980; Rogers *et al*, 1980). Differential expression of two colinear RNAs may reflect the RNAs' differing stabilities. In the case of μ_s and μ_m mRNA, transient co-expression in lymphoid cell lines of recombinant μ genes which could express only one μ mRNA results in the accummulation of approximately equal amounts of each mRNA, suggesting that, during B cell maturation, the relative stability of μ_s and μ_m is virtually invariant, and indicating that linkage of μ_m and μ_s sequences may be required for normally regulated expression (Peterson and Perry, 1986).

Experimental results have led to suggestions that the cell-specific expression of μ_s and μ_m mRNA may be variously controlled through developmentally regulated alterations of transcription termination, of utilisation of alternative splice junctions or in the relative rates of cleavage/polyadenylation at the μ_s and μ_m poly A sites (Danner and Leder, 1985; Galli *et al*, 1987; Kobrin *et al*, 1986; Peterson and Perry, 1986). In some plasmacytomas, the $\mu_s >> \mu_m$ phenotype results, at least in part, through

Figure 4. Schematic representation of splicing patterns for immunoglobulin μ_m and μ_s mRNAs.

Raised boxes indicate exons. 3' untranslated sequences are shaded. P denotes the signal peptide and V the rearranged V_H exon. Bent lines indicate RNA splicing between exons.

(From Early *et al*, 1980).



secreted mu mRNA

membrane mu mRNA

transcription termination upstream from the μ_m poly A site, but, since this is not exclusively the case, the primary determinant of the regulatory mechanism cannot be transcription termination (Danner and Leder, 1985; Galli *et al*, 1987; Guise *et al*, 1988; Kelley and Perry, 1986; Law *et al*, 1987; Reuther *et al*, 1986; Yuan and Tucker, 1984).

A mechanism involving competition between the μ_s and μ_m poly A sites for a limiting processing factor has been suggested to account for the equal use of the μ_s and μ_m sites in B cells; in plasma cells, it is implied, the processing factor is not limiting and the intrinsically weaker, proximal μ_s site is utilised predominantly (Galli *et al*, 1987, 1988). An initial attempt to reproduce regulated μ_s and μ_m poly A site use *in vitro*, using nuclear extracts from murine B cells and plasmacytoma cells, has proved unsuccessful; the μ_s poly A site is not preferentially processed in plasmacytoma extracts compared with B cell extracts (Virtanen and Sharp, 1988).

An alternative model involving competition between cleavage/ polyadenylation at the μ_s poly A site and the $C_4\mu$-to-M1 splicing reaction has been suggested (Peterson and Perry, 1986; Tsurushita *et al*, 1987, 1988). Unequivocal evidence in favour of this model has been provided by mutating the suboptimal sequence at the 5' splice junction of the $C_4\mu$-to-M1 exon to increase its identity with the consensus sequence (Shapiro and Senapathy, 1987) and consequently its complementarity to U1 snRNA, resulting in exclusive μ_m mRNA production, instead of predominantly μ_s mRNA production, in plasmacytomas (Peterson and Perry, 1989). Splice junctions which are more similar to the consensus sequence and which can therefore base pair more strongly with U1 snRNA have been shown to be stronger, more efficient splice junctions (Zhuang *et al*, 1987).

The calcitonin/CGRP gene

The pattern of alternative processing of calcitonin/CGRP gene transcripts is subject to cell-specific regulation. In thyroid C cells, 98% of the primary transcript is processed to produce calcitonin mRNA, which contains only the first four exons and which is polyadenylated at the proximal poly A site (Fig 5) (Amara *et al*, 1982; Sabate *et al*, 1985). In the brain and peripheral nervous system, use of alternative splice sites and the distal polyadenylation site results in the production of predominantly CGRP mRNA, which contains the first three exons spliced to exons 5 and 6 (Fig 5) (Amara *et al*, 1982). Irrespective of cell-type, a single transcriptional initiation site is utilised and transcription terminates more than one kilobase downstream of the distal poly A site (Amara *et al*, 1984). Since CGRP mRNA is efficiently expressed only in the neural tissues of transgenic mice containing a metallothionein-CGRP fusion gene, it seems likely that neurons alone possess the specific regulatory machinery required for the processing of CGRP mRNA from calcitonin-CGRP primary transcripts (Crenshaw *et al*, 1987).

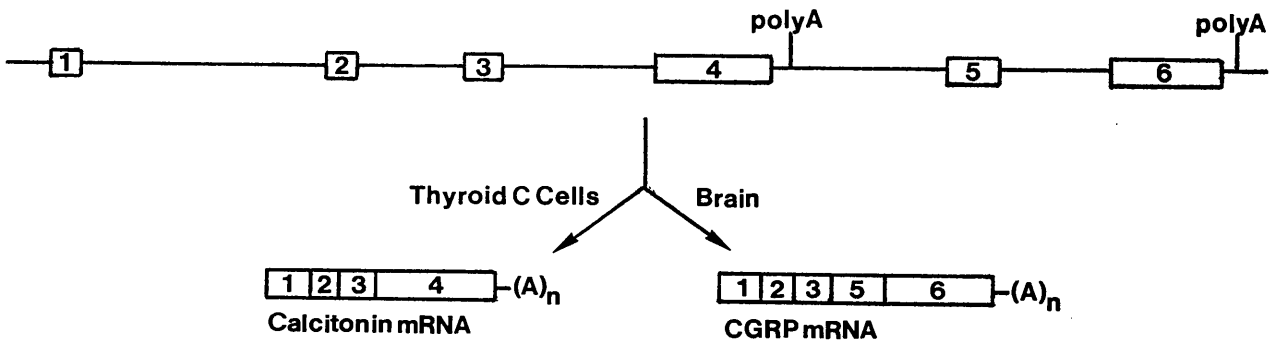
Expression experiments using the wild-type and a series of mutated calcitonin/CGRP genes demonstrated that selection of the poly A site is not regulated but that commitment to cell-specific RNA processing is determined by the choice of either the exon 4 or the exon 5 splice acceptor site (Leff *et al*, 1987). It was suggested that production of CGRP may require a neuron-specific activity, which favours use of the exon 5 splice acceptor, and that commitment to the use of this splice acceptor concomitantly inhibits utilisation of the proximal (calcitonin) poly A site (Leff *et al*, 1987).

Figure 5. Splicing patterns for calcitonin and CGRP mRNAs.

Boxes denote the calcitonin and CGRP exons. The poly A sites are indicated.

(From Crenshaw *et al*, 1987).

CALCITONIN/CGRP GENE



The adenovirus E3/L4 transcription unit

Transcription of the adenovirus MLTU is driven by a single promoter and generates at least 20 different mRNA species via differential RNA processing involving five poly A sites (L1 - L5) and multiple splicing events (reviewed by Nevins and Chen-Kiang, 1981). The MLTU also contains an early transcription unit, E3, which overlaps part of the L4 sequences including the L4 poly A site. The L4 poly A site is located within the first intron of E3 (Fig 6). Production of mRNA from the L4/E3 transcription units is subject to regulated differential processing. Early in infection, E3 primary transcripts are processed at the E3 splice sites but not at the L4 poly A site, while late in infection the L4 poly A site, but not the E3 splice sites, is utilised (Bhat and Wold, 1986).

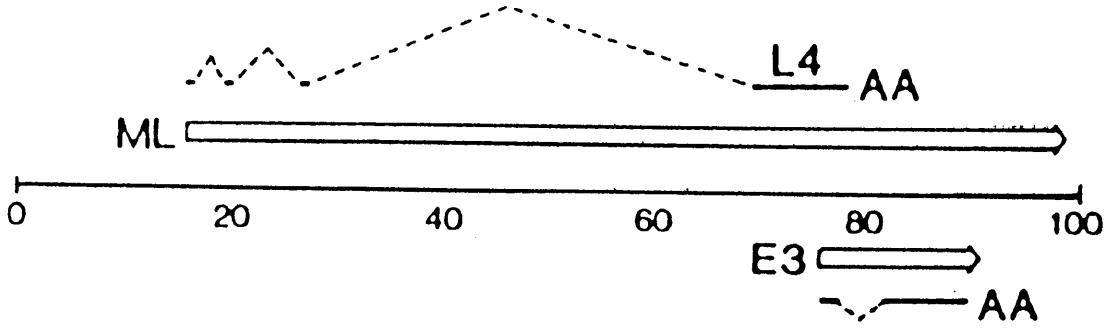
Transfection experiments using an intact E3 transcription unit produced the following results: (i) in the absence of upstream, MLTU splicing signals, the L4 poly A site is inactive, even in late infected cells; (ii) the block on L4 poly A site use is not overcome by driving E3 transcription unit from the major late promoter; and (iii) deletion of either the E3 splice donor or splice acceptor site permits utilisation of the L4 poly A site (Adami and Nevins, 1988). These results indicate that, in adenovirus complex transcription units, poly A site choice is subordinate to splice site selection in the control of differential mRNA processing.

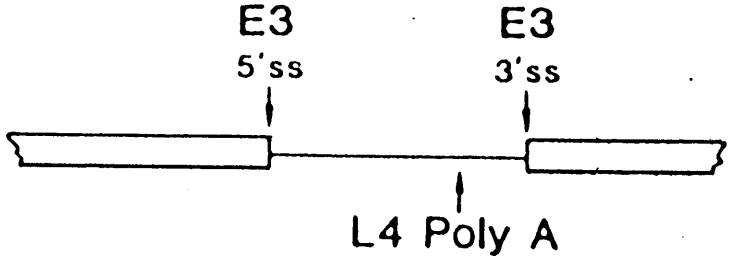
Newly synthesised major late poly A^+ RNA is largely unspliced (Nevins and Darnell, 1978a; Nevins, 1979); here, selection and commitment of the appropriate splice sites must precede polyadenylation.

Figure 6. Schematic representation of the adenovirus genome.

The major late transcription unit (ML), L4 coding sequences and the E3 transcription unit are indicated. The splicing patterns have been simplified for clarity. The enlarged view below shows the large E3 intron: the E3 exons are represented as open boxes with the introns as a line. The L4 poly A site is indicated.

(From Adami and Nevins, 1988).


L4
AA
ML
0
20
40
60
80
100
E3
AA


E3
5'ss
E3
3'ss
L4 Poly A

Other complex transcription units

In situations where a poly A site choice exists, but there is no apparent differential regulation of polyadenylation, the proximal poly A site is utilised more frequently (Gerlinger *et al*, 1982; Mason *et al*, 1985; Nishikura and Vuocolo, 1984; Tosi *et al*, 1981). In transfection experiments investigating the relationship between tandem poly A sites, the distance between the cap site and the first poly A site has no influence on poly A site use (Denome and Cole, 1988). However, in the case of the retrovirus, SNV, the efficiency of poly A site use depends on the distance between the 5' end of the RNA and the poly A site (Iwasaki and Temin, 1990b). In general, the relative frequencies of use of tandem poly A sites depends on the distance between them: the greater the distance, the more frequent the utilisation of the proximal site (Denome and Cole, 1988; Galli *et al*, 1987; Peterson and Perry, 1986; Tsurushita and Korn, 1987).

In the HSV genome, there are few intron-containing genes, so that, in the majority of HSV genes, 3' processing occurs in the absence of concomitant splicing (reviewed by Wagner, 1985). A number of 5' co-terminal HSV transcripts which utilise more than one poly A site, some in an infection-time-dependent manner, have been described (reviewed by Wagner, 1985). *In vitro*, differential stimulation of HSV late poly A site use is mediated by an HSV-induced processing factor, LPF (McLauchlan *et al*, 1989). Thus, regulation of poly A site choice represents a potential mechanism for the control of HSV gene expression.

AIMS OF THE PROJECT

The aims of this project were as follows:

(1) to institute *in vitro* processing reactions for the cleavage and polyadenylation of precursor RNAs;

(2) to investigate the 3' processing activities present in nuclear extracts from different cell types;

(3) to identify the sequence requirements for *in vitro* 3' processing using sequences based on a HSV-2 poly A site;

(4) to investigate the interactions between precursor RNAs and nuclear processing factors, using a gel retardation assay;

(5) to confirm, by means of transient gene expression assays, the *in vivo* significance of RNA sequences identified *in vitro*; and

(6) using DNA oligonucleotides and complementary RNAs, to define the interactions between precursor RNAs and processing factors.

MATERIALS AND METHODS

The majority of the methods used in this work were essentially as described by Maniatis *et al* (1982).

Bacteria

Transformation of bacteria

Competent *E Coli* DH5α (subcloning efficiency) were obtained from GIBCO-BRL. Transformation of the bacterial cells was performed according to the supplier's instructions.

5µl of ice-cold DNA solution was gently mixed with 50µl of ice-cold bacterial suspension in a 1.5ml Eppendorf centrifuge tube and placed on ice for 30 min. Following incubation at 37°C for 20 sec, the mixtures were placed on ice for 2 min. 950µl of L-broth was added and the cells were pelleted by centrifugation at 14,000rpm for 20 sec and the supernatants removed. The pellets were resuspended in 30µl L-broth and diluted, if necessary. The suspension was spread on L-agar plates containing ampicillin (50µg/ml) and allowed to dry at room temperature. The plates were inverted and incubated at 37°C for 16 h.

Culture of recombinant E coli

Individual ampicillin-resistant colonies were picked using sterile tooth-picks and inoculated into 2 ml aliquots of L-broth containing ampicillin (50µg/ml) in 20 ml Universal. These cultures were used to perform small-scale DNA preparations (see below). Cultures of transformed bacteria for large-scale DNA preparations were produced by inoculating 100µl of a saturated, small-scale overnight culture into 200 ml of L-broth containing ampicillin

(50 µg/ml) in a 1 litre flask. Both small- and large-scale cultures were incubated at 37°C with shaking (225rpm) for 16 h.

Storage of recombinant bacteria

Transformed bacteria were stored as frozen glycerol stocks. 0.85 ml of saturated bacterial overnight culture was mixed with 0.15 ml of sterile glycerol in a screw-cap tube. The glycerol stocks were stored at -20°C.

Identification of recombinant bacterial colonies by in situ hybridisation

A nitrocellulose filter was placed onto an agar plate containing ampicillin (50µg/ml). Bacterial colonies were transferred first to the filter and then to a duplicate agar plate, also containing ampicillin, using sterile toothpicks. Both plates were inverted and incubated at 37°C for 24 h. The duplicate plate was stored at 4°C during the hybridisation procedure.

The filter was placed sequentially, colony-side up on three pieces of Whatman 3MM paper saturated with 10% SDS, denaturing solution and neutralising solution, for 3 min, 5 min and 5 min, respectively. Finally, the filter was placed on a dry sheet of 3MM paper and allowed to dry in air at room temperature for 60 min. The filter was baked at 80°C for 2 h in a vacuum oven.

The baked filter was floated on 6 x SSC until thoroughly wetted and then submerged for 5 min. The filter was transferred to a beaker containing 10 ml of prewashing solution and incubated with gentle shaking at 42°C for 2 h. The prewashing solution was removed and 10 ml of prehybridisation solution was added. The filter was incubated with gentle shaking at 42°C for 5 h. Denatured, ^{32}P-labelled oligonucleotide probe was added to the

prehybridisation solution covering the filter, the top of the beaker tightly sealed with cling film and incubation continued with gentle shaking at 42°C for 16 h.

The filter was washed four times for 10 min each wash with 200 ml 2 x SSC and 0.1% SDS at room temperature, then twice for 60 min each wash with 1 x SSC and 0.1% SDS at 68°C. The filter was dried in air at room temperature on a sheet of 3MM paper. The dry filter was covered with cling film and an autoradiograph established.

After developing the autoradiograph, DNA was prepared from the duplicates of colonies which had hybridised to the probe (small-scale plasmid DNA preparation) and subjected to restriction endonuclease analysis.

Nucleic acids

Small-scale plasmid DNA preparation

1 ml of saturated bacterial overnight culture was placed in a 1.5 ml Eppendorf centrifuge tube and the cells harvested by centrifugation at 14,000 rpm for 20 sec. The supernatant was discarded and the pellet resuspended in 200 µl of STET solution. 8µl of lysozyme solution (10mg/ml) was added and the tube was placed in a boiling water bath for 50 sec. The lysate was cleared by centrifugation at 14,000rpm for 5 min and the supernatant transferred to a fresh Eppendorf tube. The DNA was recovered by isopropanol precipitation (see below).

Large-scale plasmid DNA preparation

The cells were harvested from 200ml of saturated, bacterial overnight cultures by centrifugation at 6,000rpm for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in 25ml of STET solution. The suspension

was transferred to a 100ml conical flask. 3ml of lysozyme solution (10mg/ml) was added. The mixture was brought to the boil over a bunsen flame and then incubated at 100°C for a further 50 sec. Following centrifugation at 18,000rpm for 40 min at 15°C, the supernatant was transferred to a 50ml Falcon tube and the DNA recovered by isopropanol precipitation (see below). The pellet was resuspended in 10ml of distilled water. 11g of caesium chloride were added and dissolved, followed by 1ml of ethidium bromide solution (5mg/ml). The mixture was incubated at 0°C for 5 min and then centrifuged at 4,000rpm for 5 min at 4°C. The supernatant was transferred to ultracentrifuge tubes and centrifuged at 45,000rpm for 64 h at 10°C. The covalently-closed circular plasmid DNA band was removed from the caesium chloride gradient using a hypodermic needle and syringe and transferred to a 15ml Falcon tube. The ethidium bromide was removed from the solution by repeated extraction with iso-butanol and the DNA recovered by ethanol precipitation (see below).

Precipitation of DNA and RNA with iso-propanol

To ten volumes of DNA or RNA solution were added one volume of 5M ammonium acetate solution, ten volumes of iso-propanol and, if necessary, 2µl of yeast tRNA solution (10mg/ml). The mixture was incubated at -20°C for 30 min. The precipitate was collected by centrifugation, either at 14,000rpm for 10 min in 1.5ml Eppendorf tubes or at 4,000rpm for 20 min in 50ml Falcon tubes. The pellet was rinsed with 70% ethanol (v/v), air-dried and resuspended in distilled water.

Precipitation of DNA and RNA with ethanol

To ten volumes of DNA or RNA were added one volume of 5M ammonium acetate solution, 25 volumes of 100% ethanol and, if necessary, 2µl of yeast tRNA solution (10mg/ml).

The mixture was placed on dry ice for 10 min and the precipitate collected and processed as described for iso-propanol precipitation (above).

Purification of DNA and RNA by extraction with phenol/ chloroform

One volume of DNA or RNA solution containing contaminating proteins, etc. was vigorously mixed with one volume of phenol chloroform (1:1, v/v) in a 1.5ml Eppendorf tube. The mixture was centrifuged at 14,000rpm for 5 min. The aqueous phase was transferred to a fresh Eppendorf tube and vigorously mixed with one volume of chloroform. Again, the mixture was centrifuged at 14,000rpm for 5 min. The aqueous phase was transferred to a fresh Eppendorf tube and the DNA or RNA recovered by ethanol precipitation (see above).

Manipulation of DNA *in vitro*

Restriction endonucleases

Restriction endonuclease digests were performed under the conditions specified and using the buffers supplied by the manufacturers. DNA restriction fragments for use in ligation reactions were usually purified by gel electrophoresis.

Klenow fragment of E Coli *DNA polymerase I*

Recessed 3' ends generated by restriction endonuclease digestion were converted to blunt ends, if required, by adding 1µl of dNTP solution (containing all four dNTPs, each at a concentration of 2.5mM) to newly-digested DNA in restriction buffer and incubating the mixture at 37°C for 30 min. The DNA was then purified by gel electrophoresis.

T4 polynucleotide kinase

The 5' termini of newly-synthesised oligonucleotides were radioactively labelled for use as probes, using T4 polynucleotide kinase to catalyse the transfer of the labelled phosphate moiety from (gamma-^{32}P) ATP to the 5'-OH group of the oligonucleotide. Reaction mixtures containing 30-40 pmol of DNA, 50 pmol of (gamma-^{32}P) ATP (150µCi) and 10 units of T4 polynucleotide kinase in 1 x kinase buffer were incubated at 37°C for 30 min. 2µl of 0.5M EDTA (pH 8.0) was added and the DNA purified by phenol/chloroform extraction and ethanol precipitation.

T4 DNA ligase

All ligation reactions contained 5 units of T4 DNA ligase, 1mM ATP and 0.02-0.2µg of DNA in 10µl of 1 x ligation buffer. The reactions were incubated at 16°C for 16 h.

Preparation of ^{32}P-labelled DNA molecular weight markers

To HpaII-digested pBR322 DNA in 1 x restriction buffer was added 2µCi of (α-^{32}P)dCTP and one unit of Klenow fragment of *E Coli* DNA polymerase I. The mixture was incubated at room temperature for 10 min. An equal volume of denaturing dye mixture was added.

Aliquots of the ^{32}P-labelled DNA fragments were used as molecular weight markers in denaturing polyacrylamide gel electrophoresis.

Oligonucleotide synthesis

Oligonucleotides were prepared on an Applied Biosystems automated DNA synthesiser using phosphoramidite chemistry.

In vitro transcription

To produce radiolabelled precursor RNA for *in vitro* polyadenylation reactions, transcription was performed using 50µCi (α-^{32}P)UTP (5µM), 1µg of EcoRI-digested plasmid DNA and 10 units of SP6 RNA polymerase in 40µM Tris-Cl (pH 7.9), 6mM $MgCl_2$, 10mM DTT, 2mM spermidine, 1 unit/µl RNasin, 0.1µg/ml BSA, 0.5mM m^7G(5')ppp(5')G, 0.1mM ATP, GTP, CTP and UTP, at 37°C for 60 min.

The same conditions were employed to produce unlabelled complementary RNA, except that (α-^{32}P)UTP was omitted and 1µg of HindIII-digested plasmid DNA and 0.5mM ATP, GTP, CTP and UTP were used.

2.5 units of RQ1 RNase-free DNase were added and incubation continued at 37°C for 10 min. The RNA was purified by phenol/chloroform extraction iso-propanol precipitation and, finally, ethanol precipitation.

Electrophoresis of nucleic acids

Agarose gel electrophoresis

Agarose was dissolved by boiling in 1 x TBE buffer to a final concentration of 0.7-1.2%, as required. Ethidium bromide was added to a final concentration of 50ng/ml and the gel cast on a horizontal glass plate. Electrophoresis was performed with the gel submerged in 1 x TBE at 5V/cm. Following electrophoresis, the gel was removed from the glass plate, placed on an ultraviolet transilluminator and photographed.

Standard, non-denaturing polyacrylamide gel electrophoresis (SNPAGE)

Gels were prepared from a stock solution containing acrylamide and bisacrylamide (29% and 1% w/v, respectively)

by mixing with water and 20 x TBE to a final composition of 1 x TBE and 4-12% acrylamide, as required, in a total volume of 60ml. Polymerisation was initiated by the addition of 0.1ml ammonium persulphate (10% w/v) and 10µl TEMED. The gel was cast between two glass plates (gel dimensions: 20 x 12 x 0.2 cm). To facilitate removal one of the glass plates used to cast all polyacrylamide gels was treated with Repelcote. Electrophoresis was performed vertically with 1 x TBE as the tank buffer, at 5-10V/cm.

Following electrophoresis, gels were removed from the glass plates, stained in ethidium bromide solution (1µg/ml) for 15 min, placed on an ultraviolet transilluminator and photographed.

Samples for agarose gel electrophoresis and SNPAGE were prepared by mixing one volume of loading dye mixture containing 50% sucrose, 0.25% bromophenol blue and 0.25% xylene cyanol in 5 x TBE with four volumes of DNA solution.

Low-concentration, non-denaturing polyacrylamide gel electrophoresis (LNPAGE)

This technique was utilised to investigate the interactions between pre-mRNAs and nuclear factors involved in 3'-end processing (McLauchlan *et al*, 1988).

Low-concentration, non-denaturing polyacrylamide gels were prepared in ½ x TBE buffer and contained acrylamide (final concentration 4%) and bisacrylamide (final concentration 0.05%). Polymerisation of 60ml of gel mix was initiated by addition of 0.5ml of ammonium sulphate solution (10% W/v) and 0.1ml of TEMED. The dimensions of these gels were the same as those of the standard, non-denaturing polyacrylamide gels.

The nature of the samples applied to these gels was such (they were of a sufficiently high density) that it was

unnecessary to add glucose solution to them. Marker dyes (bromophenol blue and xylene cyanol) were applied to a separate well in ½ x TBE containing glucose (10% w/v).

Electrophoresis was performed as for SNPAGE, except that ½ x TBE was used as the tank buffer.

Following electrophoresis, the glass plates were removed and the gel transferred to paper and dried under vacuum. An autoradiograph was produced by exposing X-ray film to the dried gel.

Denaturing polyacrylamide gel electrophoresis

Denaturing polyacrylamide gels contained 7M urea and 8% acrylamide and 0.27% bis-acrylamide in 1 x TBE. 60ml of gel mixture was polymerised by the addition of 0.1ml of ammonium sulphate solution (10% m/v) and 10µl of TEMED. 1 x TBE was used as the tank buffer and electrophoresis was performed at a constant power of 30W.

Two different types of glass plates and gel spacers were used. The first (gel dimensions, 20 x 12 x 0.2 cm) were used for the purification of oligonucleotides. The second (gel dimensions, 45 x 12 x 0.05 cm) were used for the separation of the components of DNA sequencing reactions or RNA processing reactions.

Before assembling the gel cast, both plates were thoroughly washed with distilled water, dried, rinsed with 70% ethanol and dried again. One plate was treated with Repelcote. 5-10ml of Replecote was spread evenly over the plate using a tissue and allowed to dry. The plate was then rinsed with 70% ethanol polished with a fresh tissue and the gel cast assembled.

Samples were prepared for denaturing PAGE, as follows. Oligonucleotides were dissolved in 90% formamide.

RNA precipitates were redissolved in denaturing dye mixture (90% formamide, 10mM EDTA (pH8.0), 0.1% bromophenol blue, 0.1% xylene cyanol). DNA sequencing reactions were mixed with an equal volume of denaturing dye solution. Samples were incubated at 90°C for 5 min before being loaded onto the gel. An aliquot of denaturing dye solution was loaded in a well adjacent to oligonucleotide samples.

Following the electrophoresis of oligonucleotides, the DNA was recovered from the gel, as described below.

One of the glass plates was removed from gels containing radiolabeled DNA or RNA (from DNA sequencing reactions or RNA processing reactions, respectively), the gel covered with cling film and an autoradiograph established.

Purification of DNA fragments by gel electrophoresis

Newly-synthesised single-stranded DNA oligonucleotides were purified by electrophoresis on denaturing polyacrylamide gels. The position of the desired product was ascertained by means of ultraviolet shadowing using a phosphorescent screen. The section of the gel containing the oligonucleotide was excised and placed in a 1.5ml Eppendorf tube. The DNA was eluted by adding sufficient distilled water to cover the gel slice and shaking the tube at 37°C for 16 h. The supernatant containing the eluted DNA was then transferred to a fresh Eppendorf tube and phenol/chloroform extracted and ethanol precipitated.

DNA restriction fragments were separated either by SNPAGE (fragments smaller than 600bp) or by agarose gel electrophoresis (fragments larger than 600bp). A section of the gel containing the desired fragment was excised and placed in a 1.5ml Eppendorf tube. DNA fragments were eluted from non-denaturing polyacrylamide gels by the same method as described for denaturing polyacrylamide gels (above).

DNA-containing sections of agarose gels were frozen (on dry ice, 10 min) and thawed (37°C, 10 min) three times. The gel section was transferred to a Costar Spin-X filter and centrifuged at 14,000rpm for 15 min. The filtrate was transferred to a fresh Eppendorf tube and phenol/chloroform extracted and ethanol precipitated.

Cell culture

HeLa and K562 cells were maintained as monolayers in Dulbecco's MEM containing 2.5% calf serum and 2.5% fetal calf serum, and in suspension in S-MEM containing 5% calf serum, both at 37°C. In suspension, the cells were maintained between 2 x 10^5 and 6 x 10^5 cells/ml.

Short term gene expression assay

DNA transfection by calcium phosphate precipitation

10µg of covalently-closed circular plasmid DNA dissolved in 140µl of TE was mixed with 20µl of 2M calcium chloride solution. This mixture was added to an equal volume of 2 x HBS and incubated at room temperature for 30 min. The suspension of calcium-DNA precipitate was transferred to a 50mm culture dish containing 5 x 10^4 HeLa cells and 4ml medium, and incubated at 37°C for 24 h. The medium was replaced and incubation continued at 37°C for 24 h.

Preparation of cell extract

The medium was removed and the cells washed with PBS. 1ml of TEN was added to the dish and left to stand for 2 min. The cells were scraped off the dish using a rubber policeman and the suspension transferred to a 1.5ml Eppendorf tube. The cells were pelleted by centrifugation at 6500rpm for 1 min and the supernatant removed. The cells were resuspended in 50µl of 250mM Tris (pH 7.8) and lysed by

freezing and thawing three times (on dry ice and at 37°C for 10 min, respectively). The cell debris was pelleted by centrifugation at 14,000rpm for 5 min and the supernatant transferred to a fresh tube.

Chloramphenicol acetyl transferase (CAT) assay

A mixture containing 50% cell extract, 1µCi ^{14}C-chloramphenicol (20nM) and 1mM acetyl co-enzyme A in 50µl of 150mM Tris (pH 7.8) was prepared and incubated at 37°C for 20 min.

200µl of ethyl acetate was added and thoroughly mixed. The mixture was centrifuged at 14,000rpm for 2 min. The ethyl acetate fraction, containing the chloramphenicol and its acetyl derivatives, was transferred to a fresh tube and evaporated to dryness.

The residue was redissolved in 15µl ethyl acetate and applied to a thin-layer chromatography (TLC) plate. Ascending chromatography was performed using a solvent consisting of 95% chloroform and 5% methanol (v/v). The TLC plate was air-dried and an autoradiograph established.

Preparation of nuclear extracts

A method adapted from that of Dignam *et al* (1983) was used to prepare nuclear extracts (McLauchlan *et al*, 1989). All of the procedures were performed at 4°C 2-15 x 10^9 HeLa or K562 cells in suspension were pelleted by centrifugation at 2,000rpm for 10 min and the supernatant discarded. The cells were resuspended in five volumes of PBS, and pelleted by centrifugation at 2,000rpm for 10 min and the supernatant discarded. The pellet was resuspended in 5 packed cell volumes (PCVs) of buffer A and incubated at 4°C for 10 min. The cells were pelleted by centrifugation at 2,000rpm for 10 min and the supernatant discarded. The swollen cells were resuspended in 2 PCVs of buffer A and

lysed in a Dounce homogeniser by 10 strokes with a type B pestle. Unlysed cells and large debris were pelleted by centrifugation at 2,000rpm for 10 min. The supernatant was transferred to a fresh tube and the nuclei pelleted by centrifugation at 14,500rpm for 20 min. The supernatant was discarded and 3ml of buffer C/10^9 cells added. The nuclei were resuspended and lysed in a Dounce homogeniser by 10 strokes with a type B pestle. The debris was pelleted by centrifugation at 14,500rpm for 30 min. The supernatant was aliquotted and frozen in liquid nitrogen and subsequently stored at -70°C.

In vitro polyadenylation reactions

Reactions containing 1-5 x 10^4cpm RNA were performed in 1.5mM ATP or 1mM 3' dATP, 5mM creatine phosphate, 264mM KCl, 0.7mM $MgCl_2$, 8.8% glycerol, 8.8mM HEPES (pH7.6), 0.1mM EDTA, 0.2mM DTT, 2.5% PEG (total volume 25µl, of which nuclear extract 11µl) at 30°C for 2 h, unless otherwise stated (Moore and Sharp, 1985; McLauchlan *et al*, 1988).

RNA was isolated for analysis, as follows. 100µl of 2 x PK buffer, 50µg of proteinase K and 40µg of yeast tRNA were added and the total volume adjusted to 200µl with distilled water. Proteinase K digestion was performed at 30°C for 15 min. The RNA was purified by phenol/chloroform extraction and ethanol precipitation, and analysed by denaturing polyacrylamide gel electrophoresis.

RNAase T_2 protection assay

The structure of polyadenylated RNAs produced in ATP-containing reactions was analysed by means of an RNAase end-protection assay, in order to distinguish between polyadenylated and cleaved-and-polyadenylated RNA.

An aliquot of *in vitro* polyadenylated RNA was hybridised to 20µg complementary RNA in 750mM NaCl, 50mM

HEPES (pH6.95), 1mM EDTA (total volume, 15µl) by denaturing at 80°C for 10 min, then incubating at 50°C for 16 h. The hybridised RNA was placed on ice. 200µl of HNE and 20 units of RNAase T_2 were added. RNAase digestion was performed at 30°C for 30 min. 50µg of proteinase K and 40µg of yeast tRNA were added and incubation continued at 30°C for 30 min. The RNA was purified by phenol/chloroform extraction and ethanol precipitation, and analysed by denaturing polyacrylamide gel electrophoresis (Moore and Sharp, 1985; McLauchlan *et al*, 1988).

Analysis of RNA interactions with nuclear factors

In vitro polyadenylation reactions containing ATP were set up as described above and incubated at 30°C for between 10 min and 2 h. After the required time of incubation, heparin was added to a final concentration of 5µg/ml, the samples incubated at 0°C for 10 min and then loaded directly onto low-concentration, non-denaturing polyacrylamide gels (McLauchlan *et al*, 1988). Zero time samples were prepared by adding heparin immediately after the addition of nuclear extract. Electrophoresis was performed as described above.

DNA sequencing

DNA sequencing was performed using denatured, double-stranded plasmid DNA templates, according to the method of Hattori and Sakaki (1986).

Preparation of plasmid DNA

The alkaline lysis method was used to prepare plasmid DNA (Maniatis *et al*, 1983). 1ml of saturated bacterial overnight culture was placed in a 1.5ml Eppendorf tube and the cells pelleted by centrifugation at 14,000rpm for 20 sec. The pellet was gently resuspended in 100µl of solution I and incubated at room temperature for 5 min.

200µl of solution II was added and gently mixed. The mixture was incubated at 0°C for 5 min. 150µl of solution III was added and gently mixed. Incubation was continued at 0°C for 5 min. The lysate was cleared by centrifugation at 14,000rpm for 10 min. The supernatant was transferred to a fresh tube and extracted with phenol/chloroform. The DNA was then precipitated with ethanol. The pellet was redissolved in 50µl TE, 0.5µg of RNaseA added and the mixture incubated at 37°C for 30 min. 30µl of 20% polyethylene glycol 6,000-2.5M NaCl was added and mixed well. The mixture was incubated at 0°C for 60 min and then centrifuged at 14,000rpm for 5 min. The DNA pellet was rinsed with 70% ethanol, dried and redissolved in 50µl TE.

Alkaline denaturation of plasmid DNA

18µl of DNA solution (3-5µg) was mixed with 2µl of 2M NaOH and incubated at room temperature for 5 min. The DNA was ethanol precipitated and the pellet redissolved in 10µl of distilled water.

Sequencing reactions

A mixture containing 1.5-2.5µg of DNA and 0.5pmol of primer in 1.25 x Klenow buffer was prepared and incubated at 60°C for 15 min and then at room temperature for 15 min. 20µCi of (α-^{32}P)dCTP and 2 units of Klenow fragment of *E Coli* DNA polymerase were added and mixed well. 3µl aliquots of this mixture were added to 2µl of each of the G-, A-, T- and C-specific deoxy-dideoxynucleotide mixtures and incubated at 42°C for 15 min. 1µl of chase solution was added and incubation continued at 42°C for 15 min.

The components of the sequencing reactions were separated by denaturing polyacrylamide gel electrophoresis.

Autoradiography

Autoradiography was performed at -70°C using Kodak X-Omat S XS-1 X-ray film and a Du Pont Cronex Lightning Plus intensifying screen. Exposed films were processed in a Kodak X-Omat automatic processor.

Standard solutions

The compositions of the standard solutions used in this work were as follows:

Buffer A	10mM HEPES (pH 7.9) 1.5mM magnesium chloride 10mM potassium chloride 0.5mM dithiothreitol
Buffer C	20mM HEPES (pH 7.9) 25% glycerol 0.6M potassium chloride 1.5mM magnesium chloride 0.2mM EDTA 0.5mM dithiothreitol
Chase solution	1mM dATP 1mM dCTP 1mM dGTP 1mM TTP 10mM Tris-Cl (pH 7.5)
Denaturing dye mixture	90% formamide 10mM EDTA (pH 8.0) 0.1% bromophenol blue 0.1% xylene cyanol FF
Denaturing solution	0.5M sodium hydroxide 1.5M sodium chloride
50x Denhardt's solution	1% Ficoll 1% polyvinylpyrrolidone 1% bovine serum albumin

ddATP-dNTP mixture	83.3µM dGTP 6.25µM dATP 83.3µM TTP 125µM ddATP 10mM Tris-Cl (pH 7.5) 0.1mM EDTA
ddCTP-dNTP mixture	62.5µM dGTP 62.5µM dATP 62.5µM TTP 125µM ddCTP 10mM Tris-Cl (pH 7.5) 0.1mM EDTA
ddGTP-dNTP mixture	4.2µM dGTP 83.3µM dATP 83.3µM TTP 83.3µM ddGTP 10mM Tris-Cl (pH 7.5) 0.1 mM EDTA
ddTTP-dNTP mixture	83.3µM dGTP 83.3µM dATP 6.25µM TTP 250µM ddTTP 10mM Tris-Cl (pH 7.5) 0.1mM EDTA
2 x HBS	274mM sodium chloride 1mM potassium chloride 1.5mM disodium hydrogenphosphate 11mM dextrose 42mM HEPES (pH 7.05)
HNE	20mM HEPES (pH 7.9) 200mM sodium chloride 1mM EDTA

10 x kinase buffer	0.5M Tris-Cl (pH 7.6) 0.1M magnesium chloride 50mM dithiothreitol 1mM spermidine 1mM EDTA
10 x Klenow buffer	70mM Tris-Cl (pH 7.5) 200mM sodium chloride 70mM magnesium chloride 1mM EDTA
L-agar	1% tryptone 0.5% yeast extract 1% sodium chloride 1.5% agar
L-broth	1% tryptone 0.5% yeast extract 1% sodium chloride
10 x ligation buffer	0.66M Tris-Cl (pH 7.6) 50mM magnesium chloride 50mM dithiothreitol
Loading dye mixture	50% sucrose 0.25% bromophenol blue 0.25% xylene cyanol FF 445mM Tris 10mM EDTA 445mM boric acid
Neutralising solution	1.5M sodium chloride 0.5M Tris-Cl (pH 8.0)
PBS	137mM sodium chloride 2.7mM potassium chloride 8mM disodium hydrogenphosphate 1.8mM potassium dihydrogenphosphate (pH 7.4)

2 x PK buffer	0.2M Tris-Cl (pH 7.5) 25mM EDTA 0.3M sodium chloride 2% SDS
Prehybridisation solution	50% formamide 5 x Denhardt's solution 5 x SSPE 0.1% SDS 0.1mg/ml denatured, salmon sperm DNA
Prewashing solution	50mM Tris-Cl (pH 8.0) 1M sodium chloride 1mM EDTA 0.1% SDS
Proteinase K	20mg/ml in H_2O
RNase A solution	10mg/ml RNase A 10mM Tris-Cl (pH 7.5) 15mM sodium chloride
Solution I	0.4% lysozyme 50mM glucose 25mM Tris-Cl (pH 8.0) 10mM EDTA
Solution II	0.2M sodium hydroxide 1% SDS
Solution III	3M potassium acetate 2M acetic acid
20 x SSC	3M sodium chloride 0.3M sodium citrate (pH 7.0)
20 x SSPE	3M sodium chloride 0.2M sodium dihydrogenphosphate (pH 7.4) 20mM EDTA

STET	0.1M sodium chloride 10mM Tris-Cl (pH 8.0) 1mM EDTA 5% Triton X-100
10 x TBE	890mM Tris 20mM EDTA 890mM boric acid
TE	10mM Tris-Cl (pH 8.0) 1mM EDTA
TEN	10mM Tris-Cl (pH 8.0) 1mM EDTA 0.1M sodium chloride

Reagent suppliers

Reagents were obtained from the following suppliers:

(gamma-^{32}P)ATP, (α-^{32}P)UTP and (α-^{32}P)dCTP (Amersham).

Ampicillin, bovine serum, albumin, caesium chloride, lysozyme, pBR322 DNA, proteinase K, Tris, ATP, CTP, GTP and UTP (Boehringer, Mannheim).

RNAase T_2 (Calbiochem).

Spin-X centrifuge filter units (Costar).

Oligonucleotide synthesis reagents (Cruachem).

Agarose, competent *E Coli* DH5α, restriction endonucleases, Klenow fragment of *E Coli* DNA polymerase, T4 DNA ligase, T4 polynucleotide kinase, dATP, dCTP, dGTP, TTP, ddATP, ddCTP, ddGTP, ddTTP, tissue culture plastics, medium and supplements, calf serum and fetal calf serum (Gibco-BRL).

Ficoll and salmon sperm DNA (Pharmacia).

RNasin, RQ1 RNase-free DNase, SP6 RNA polymerase and T7 RNA polymerase (Promega).

Nitrocellulose filters and thin-layer chromatography plates (Schleicher and Schuell); and

Acetyl co-enzyme A, cordycepin 5' triphosphate, creatine phosphate, dextrose, heparin, spermidine, xylene cyanol FF and yeast transfer RNA (Sigma).

All other reagents were obtained from BDH.

RESULTS AND DISCUSSION

This thesis presents an analysis of the process of mRNA 3' end formation and polyadenylation using an *in vitro* processing system. With this *in vitro* system, exogenously synthesised precursor RNAs containing sequences which span a mRNA poly A site are accurately and efficiently cleaved and polyadenylated at the mature mRNA 3' end by soluble factors extracted from mammalian cell nuclei. The components of the *in vitro* processing system, including precursor RNA, nuclear extracts and biochemical co-factors, are further described below.

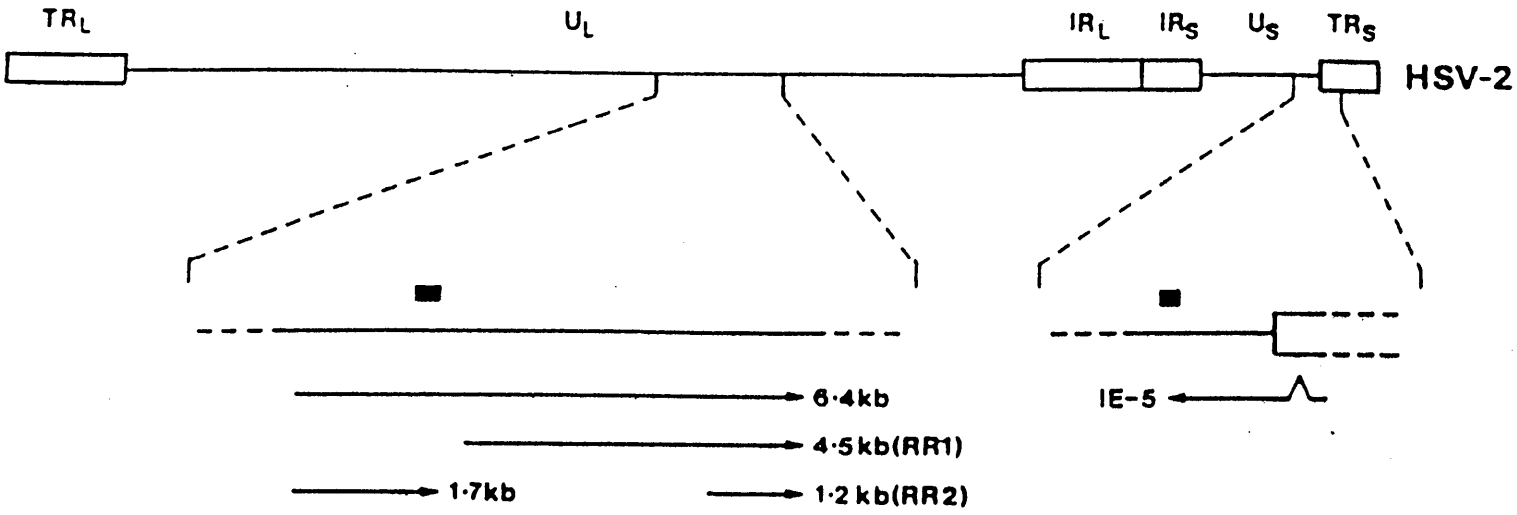
SECTION 1: RESULTS

Poly A site sequences used

Automated oligonucleotide synthesis facilitates the production of DNA molecules of precise sequence, containing convenient restriction endonuclease recognition sites, if desired: the poly A site studied here was produced by synthesising DNA oligonucleotides. The sequences used were based on the poly A site located at 0.563 map units on the HSV-2 genome which defines the 3' terminus of a 1.7kb late mRNA, as shown in Fig 7 (Anderson *et al*, 1981; McLauchlan, 1986; Yei *et al*, 1990): this mRNA contains the open reading frame equivalent to UL38 in HSV-1 (reviewed by McGeoch, 1989). The 1.7kb mRNA forms part of a complex transcription unit that also contains the open reading frames which encode the large and small subunits of the viral ribonucleotide reductase enzyme, which are translated from mRNAs of 4.5kb and 1.2kb in length, respectively (Fig 7) (McLauchlan and Clements, 1983; Nikas *et al*, 1986). The late 1.7kb mRNA poly A site was chosen as the basis for the synthetic poly A site as it is the subject of other studies in this laboratory, and because its sequence required very few nucleotide changes in order to introduce strategically located restriction sites.

Figure 7. Locations on the HSV-2 genome of the 1.7kb mRNA poly A site and the IE-5 mRNA poly A site.

Transcripts specified by HSV-2 between 0.55 and 0.60 map units and 0.94 and 0.97 map units. The filled boxes indicate regions of DNA specifying the 1.7kb and IE-5 mRNA poly A sites. R1 and R2 denote transcripts encoding the R1 and R2 proteins of the viral ribonucleotide reductase.


TR_L
U_L
IR_L
IR_S
U_S
TR_S
HSV-2
6·4kb
4·5kb(RR1)
1·7kb
1·2 kb(RR2)
IE-5

At the DNA level, the HSV-2 1.7kb mRNA polyadenylation site (Fig 8) contains the conserved hexanucleotide, AATAAA (the poly A signal), which is essential for mRNA 3' end formation, and a downstream motif, TGTGTTTG, which conforms almost exactly (seven out of eight positions) to the consensus, YGTGTTYY, derived by comparison of the sequences of more than one hundred mammalian and viral poly A sites (McLauchlan *et al*, 1985). Another GT-rich element, the hexamer, GTGTGT, is found immediately downstream of the consensus element. Deletion of such GT-rich elements results in substantial reduction of the efficiency of *in vivo* and *in vitro* 3' end formation. The 3' end of the 1.7kb mRNA synthesised during lytic infection was mapped to a location between the poly A signal and the downstream GT-rich elements (nucleotide 26 in Fig 8; McLauchlan, 1986).

The 1.7kb late mRNA poly A site sequences were considered most suitable for modification since few nucleotides needed to be inserted or altered in order to provide several internal restriction endonuclease recognition sites. Two complementary 67 base oligonucleotides [67A (sense strand) and 67B (anti-sense strand)] were synthesised which comprised sequences from the 1.7kb mRNA poly A site except for the modifications described below (Fig 8).

The T and C residues at positions 19 and 34 respectively within oligonucleotide 67A represent single nucleotide insertions into the natural sequence, while the GA dinucleotide (positions 21 and 22) represents an inversion of the AG dinucleotide at the equivalent location in the natural sequence; these sequence alterations introduce a Xho I site (^{18}CTCGAG^{23}) and a Sal I site (^{32}GTCGAC^{37}) which were utilised to manipulate the poly A site sequences *in vitro*. The alteration of sequences between the AATAAA hexamer and the downstream sequence elements are predicted to have little or no effect on poly A site function.

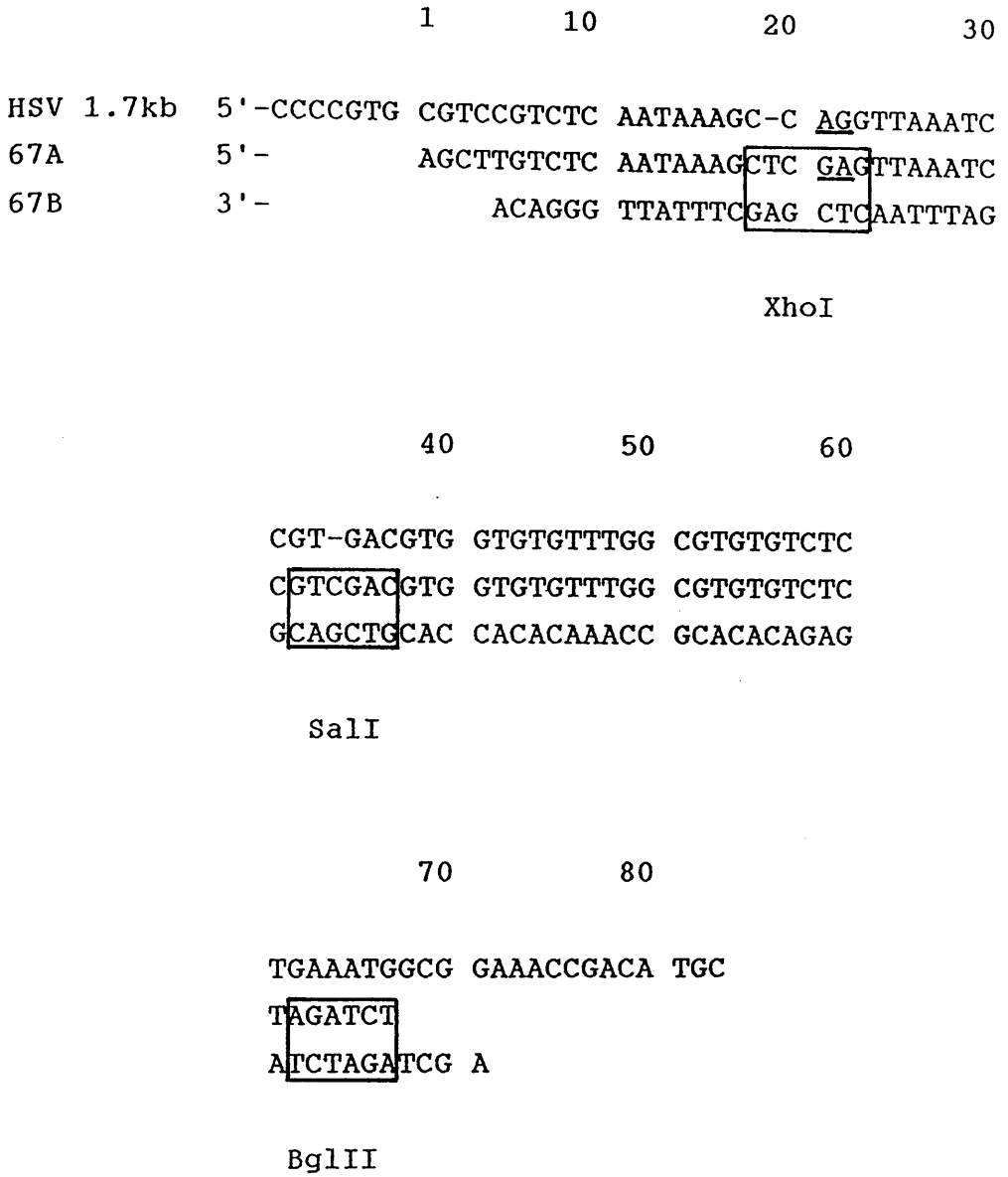


Figure 8. Nucleotide sequences of the 1.7kb mRNA poly A site and the synthetic oligonucleotides 67A and 67B.

The sequences are aligned to show the substitutions (underlined) and insertions (-) introduced to produce oligonucleotides 67A and 67B. Restriction endonuclease recognition sites are denoted by boxes.

Oligonucleotides 67A and 67B were synthesised such that on annealing 5' overhanging sticky ends compatible with those of a Hind III site were produced. When inserted into a Hind III-digested vector, the restriction site at the 5' end of oligonucleotide 67A would be retained while that at the 3' end would be lost. Inclusion of a novel BglII recognition site at the 3' end of oligonucleotide 67A would permit subsequent excision of the cloned oligonucleotide sequences as a HindIII/BglII fragment.

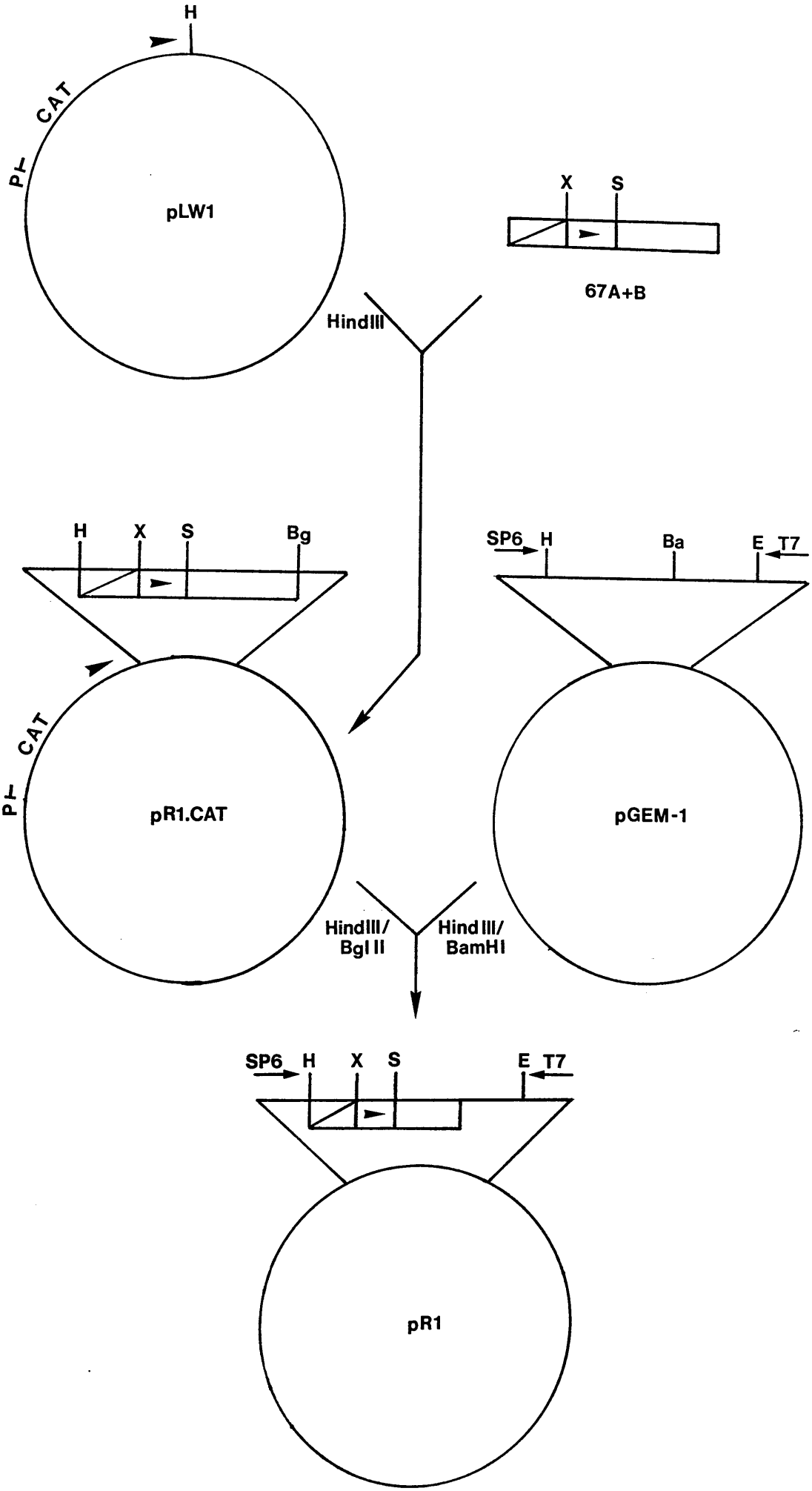
The oligonucleotides contained all of the 3' processing signals, defined either by functional analysis or sequence comparison, which were present in the natural 1.7kb mRNA poly A site [ie AATAAA and GT-rich elements], as indicated in Fig 8. However, subsequent experiments have revealed that the natural 1.7kb mRNA poly A site contains a sequence element upstream from AATAAA which is required for recognition of the poly A site by the HSV-induced processing factor, LPF (McLauchlan *et al*, 1989; R Purves, unpublished). This sequence is not present in the synthetic poly A site.

Cloning of the synthetic poly A site

Oligonucleotides 67A and 67B were annealed and ligated into the unique HindIII site which lies downstream of the chloramphenicol acetyl transferase (CAT) gene in plasmid pLW1 (Fig 9). In order that a single copy of the synthetic poly A site was inserted, the oligonucleotides were not phosphorylated prior to cloning. For this reason, a low cloning efficiency was expected. Therefore, recombinants were initially identified by colony hybridisation (see Materials and Methods). From eighty colonies picked, only one was found to hybridise strongly to 5'-labelled oligonucleotide 67A (Fig 10, g5). Restriction analysis of DNA from this colony confirmed that the synthetic poly A site had ligated in the correct orientation (Fig 9). This plasmid was designated pR1.CAT and was utilised in transient expression assays (see Section 5).

Figure 9. Construction of plasmids pR1.CAT and pR1 (not to scale).

The positions of the SP6 and T7 promoters (⟶), the chloramphenicol acetyl transferase gene coding region (⊢ CAT ⟶), the HSV-2 IE4/5 gene promoter (P) and relevant restriction endonuclease recognition sites (H = HindIII, X = XhoI, S = SalI, Bg = BglII, Ba = BamHI and E = EcoRI) are indicated.


H
CAT
PL
pLW1
X
S
67A+B
HindIII
H
X
S
Bg
SP6
H
Ba
E
T7
CAT
PL
pR1.CAT
pGEM-1
HindIII/
BglII
HindIII/
BamHI
SP6
H
X
S
E
T7
pR1

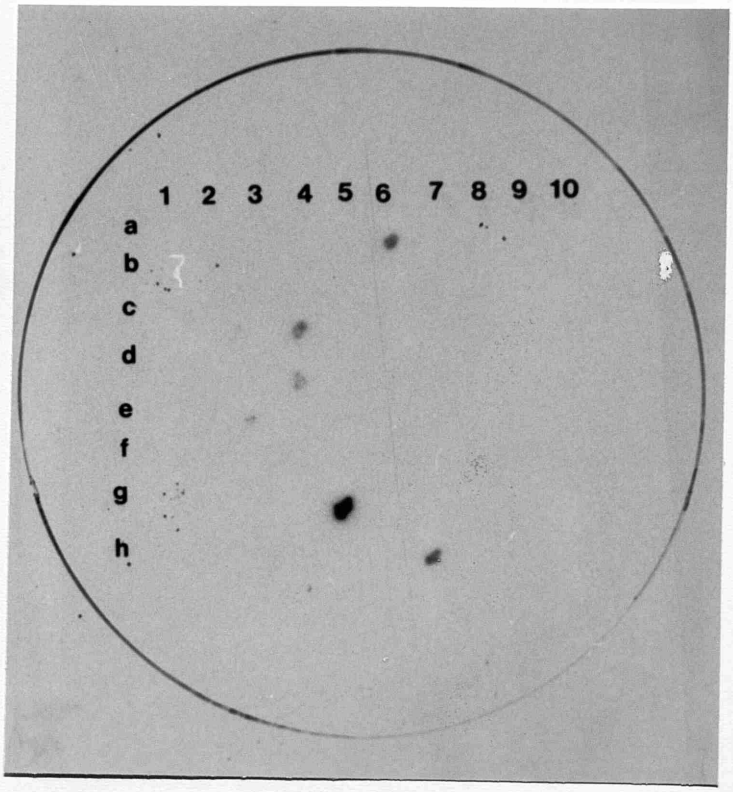


Figure 10. Detection of oligonucleotide-containing recombinant bacterial colonies by *in situ* hybridisation.

The large quantities of pure RNA which are required as precursors in the *in vitro* RNA processing system can be produced from appropriate plasmids using purified bacterial RNA polymerases such as those from bacteriophages T7 and SP6 (Butler and Chamberlain, 1982; Davanloo *et al*, 1984; Melton *et al*, 1984). These polymerases initiate transcription only at specific promoters; the sequences of the SP6 and T7 RNA polymerase promoters are shown in Fig 11A. Plasmid pGEM-1 contains the SP6 and T7 promoters in opposite orientations on either side of a multiple cloning site, which permits transcription of DNA inserts with either SP6 or T7 RNA polymerase (Fig 11A).

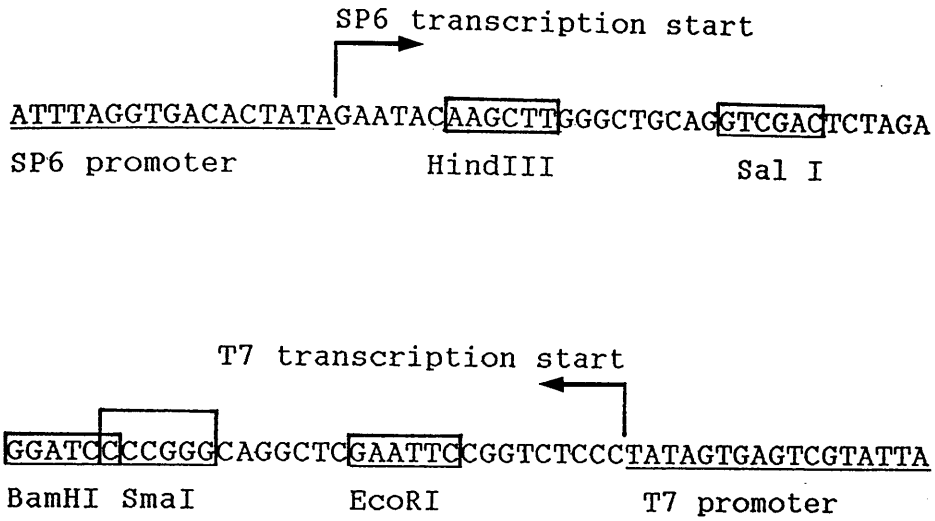
To produce a construct from which precursor RNA containing the synthetic poly A site sequences could be transcribed, the HindIII/BglII fragment from plasmid pR1.CAT was cloned into the HindIII/BamHI fragment of pGEM-1. The resultant plasmid was designated pR1 (Fig 9). Plasmid pR1 and its derivatives (see Sections 3 and 4) were sequenced using the dideoxy method described by Hattori and Sakaki (1986) using denatured plasmid templates and both SP6 and T7 promoter primers (Fig 11B). In all cases, the sequences obtained were identical to those predicted.

In vitro synthesis of precursor RNA

Termination of transcription by SP6 and T7 RNA polymerases in plasmid pGEM-1 is effected when the enzymes reach the 5' end of the DNA template strand, producing a run-off transcript. For SP6 transcription of pR1 and its derivatives, DNA was normally digested with EcoRI whereas T7 transcription of the same plasmids was performed on HindIII-digested DNA.

The dinucleotide cap analogue m^7GpppG was included in *in vitro* transcription reactions. The presence of the dinucleotide at the 5' terminus increases the stability of transcripts in the presence of nuclear extract, probably by

A



B

SP6 promoter primer 5'-GATTTAGGTGACACTATAG

T7 promoter primer 5'-TAATACGACTCACTATAGGG

Figure 11

A. Nucleotide sequence of the SP6 and T7 promoters and the multiple cloning site of plasmid pGEM-1. The SP6 and T7 polymerase initiation sites are indicated (arrows). Selected restriction endonuclease recognition sites are denoted by boxes.

B. Nucleotide sequences of the SP6 and T7 promoter DNA sequencing primers

protecting the precursor RNA from 5'→3' exoribonucleases. Furthermore, m^7GpppG produces a 1-9-fold increase in RNA yield from T7 polymerase transcription, perhaps through enhancement of fidelity of initiation (Nielsen and Shapiro, 1986). Both SP6 and T7 RNA polymerases efficiently incorporate m^7GpppG as the first residue at the 5' end of transcripts.

Transcription of EcoRI-digested pR1 DNA with SP6 RNA polymerase resulted in the production of essentially a single radiolabelled RNA species (R1 precursor RNA) 92 nucleotides in length (Fig 12, lane 2). Several minor contaminants of shorter length were also generated, which may have been produced by premature termination of transcription (perhaps due to the condition of the DNA template) or by endonucleolytic cleavage of full-length precursor.

There is an apparent discrepancy between the sizes of the pBR322/HpaII DNA markers (Fig 12, lane 1) and the predicted lengths of the RNA molecules. The RNA molecules migrated at the same rate as DNA molecules approximately 10% shorter than themselves due, at least in part, to the difference in molecular weight between ribo- and deoxyribonucleotides.

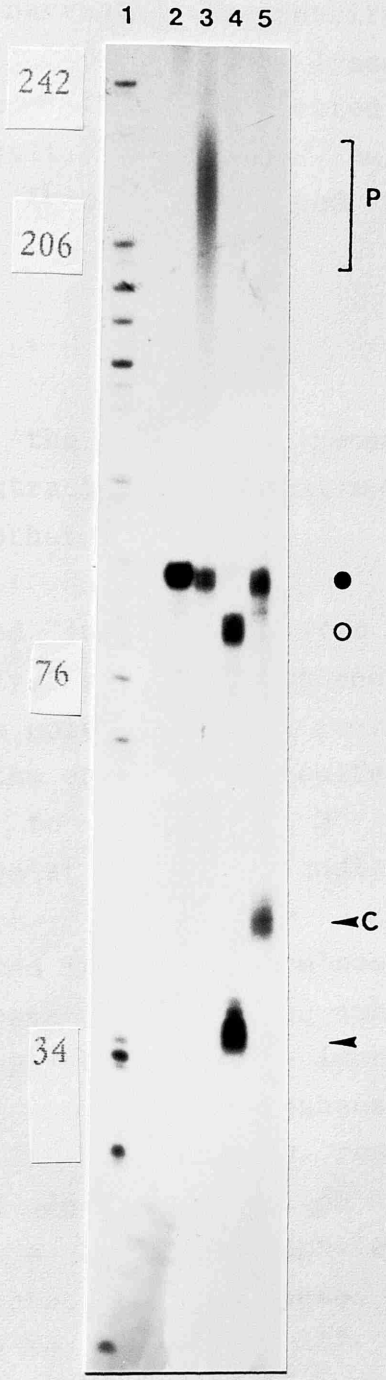
T7 RNA polymerase transcription was used to produce unlabelled complementary RNA (cRNA) probes which were used to analyse reaction products of the *in vitro* RNA processing system (see below).

Preparation of nuclear extracts

Nuclear extracts were required for the *in vitro* 3' processing system to provide the diffusible trans-acting factors which interact with the RNA substrate to catalyse the specific cleavage and polyadenylation reactions.

Figure 12. R1 RNA processing products.

In vitro 3' processing reactions were performed for two hours and the RNAs analysed by denaturing polyacrylamide gel electrophoresis as described in Materials and Methods. Lane 1, pBR322 DNA, HpaII fragments; lane 2, unprocessed R1 precursor RNA; lane 3, R1 RNA, ATP reaction; lane 4, R1 RNA, ATP reaction, RNAase T_2 protection assay; lane 5, R1 RNA, 3' dATP reaction. The positions of unprocessed precursor RNA (●), RNAase T_2-protected, unprocessed RNA (o), RNAaseT_2-protected, cleaved RNA (◄), cleaved RNA (◄C) and poly A^+ RNA (P) are indicated.


1 2 3 4 5
242
206
76
34
P
C

Extracts containing soluble nuclear components were prepared from 3-5 litre suspension cultures of HeLa or K562 cells containing 1-2 x 10^9 cells.

The cells were harvested by centrifugation, washed, swollen in hypotonic buffer and lysed by douncing. Following centrifugation, the pelletted nuclei were resuspended in high-salt extraction buffer containing 0.6M KCl. The soluble fraction obtained was used without dialysis.

Exogenous co-factors

In addition to the *in vitro* transcribed substrate RNA and the nuclear extract, the *in vitro* reaction mixture contained a number of other components.

ATP was included in the *in vitro* reaction mixture for two reasons: firstly, as a source of energy and secondly as a substrate for the polyadenylation reaction. Formation of the poly A tail by the enzyme PAP results in the addition of 150-250 A residues to the novel 3' end exposed by endonucleolytic cleavage at the poly A addition site.

The energy stored in ATP is released by hydrolysis of the alpha-beta or beta-gamma phosphoanhydride bonds to produce AMP + pyrophosphate or ADP + inorganic phosphate, respectively. In order to prevent exhaustion of the ATP during the *in vitro* 3' processing reaction, creatine phosphate was provided in the reaction mixture. Since creatine phosphate has a higher phosphate group transfer potential than ATP, creatine phosphate can transfer a phosphoryl group to ADP to regenerate ATP. This process is catalysed by endogenous creatine phosphokinase present in the nuclear extract (Moore and Sharp, 1985).

Inclusion of polyethylene glycol (PEG) in *in vitro* processing reactions greatly potentiated the efficiency with

which substrate RNA was cleaved (J McLauchlan, personal communication). In some circumstances, inclusion of polyvinyl alcohol has a similar effect on the efficiency of the *in vitro* 3' processing reaction (Zarkower *et al*, 1986). This effect may be due to concentration of trans-acting factors, since both PEG and polyvinyl alcohol effectively reduce the volume of the reaction mixture.

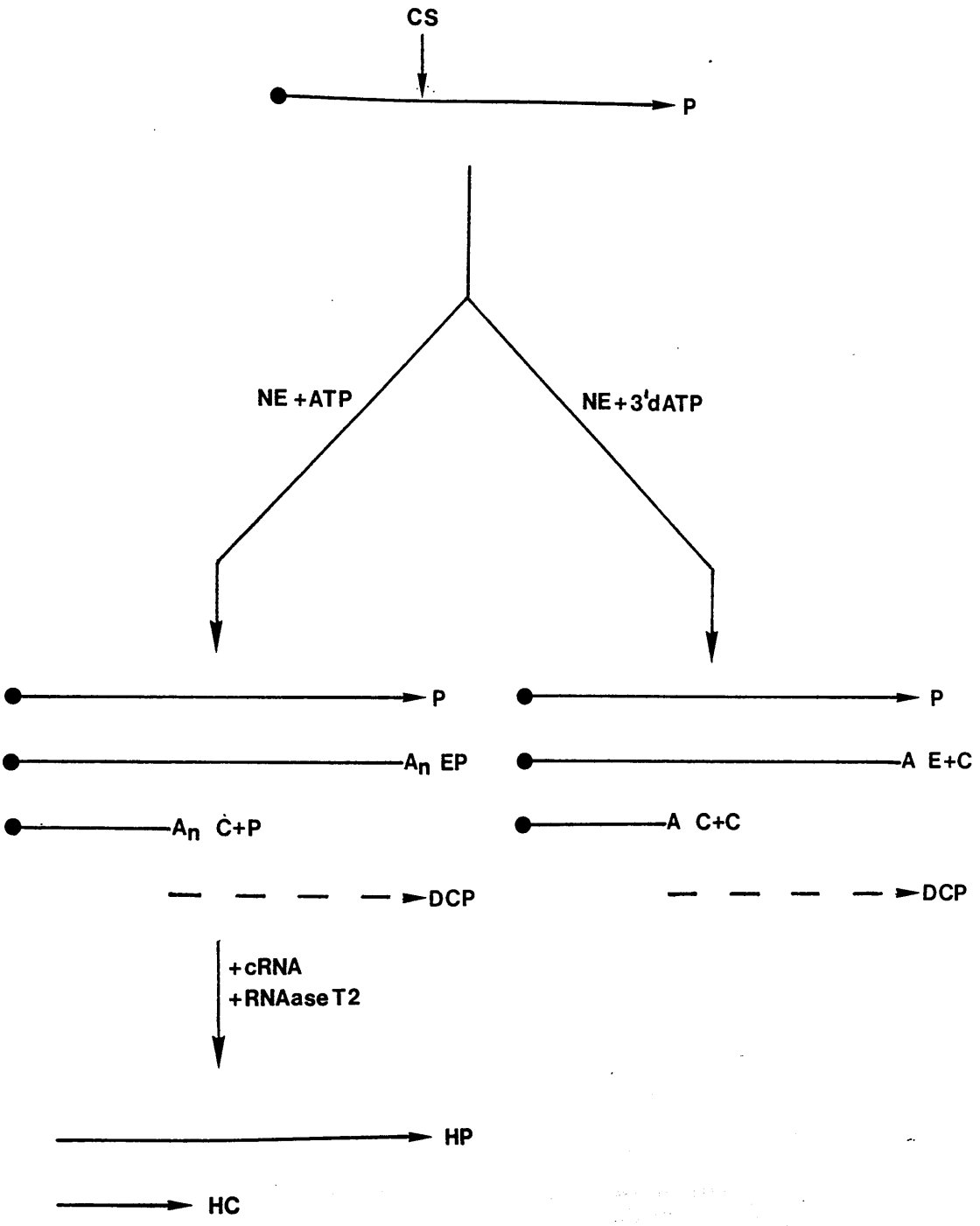
Results of a typical 3' processing reaction

The products from a typical *in vitro* 3' processing reaction using R1 precursor RNA are shown in Fig 12. In the presence of ATP, two reaction products were generated, a single band representing unreacted precursor and a smear of RNA of between 150 and 250 nucleotides in length (Fig 12, lane 3). The smear consists of polyadenylated RNA which was produced either by cleavage of the precursor RNA at the poly A site followed by polyadenylation or by polyadenylation at the 3' end of uncleaved precursor RNA (Fig 13); the latter process has been termed end-polyadenylation. The heterogeneous length of the poly A tails, consisting of up to 200 A residues, may have been generated by PAP which is known to produce poly A tails of variable length *in vivo* (Nevins and Darnell, 1978a). Alternatively, RNase H, a ribonuclease which cleaves stretches of A residues in RNA and which is present in nuclear extracts, may have partially degraded the poly A tail.

Due to the heterogeneous length of the poly A tail, it was not possible to distinguish directly between cleaved, polyadenylated RNA and end-polyadenylated precursor RNA. The amount of precusor RNA cleaved during the ATP-containing reaction was determined by hybridising the reaction products to cRNA transcribed with T7 RNA polymerase from plasmid pR1 which had been digested with HindIII. Following hybridisation for 16h, single-stranded RNA regions were removed by treatment with RNAase T_2 (see Fig 13). Two

Figure 13. Structures of products of RNA processing reactions.

A schematic representation of the fate of precursor RNAs in processing reactions. In reactions containing ATP, unprocessed RNA (P) may undergo polyadenylation without cleavage to produce end-polyadenylated precursor (EP), may be cleaved and polyadenylated (C+P) or may remain unreacted (P). The position of the cleavage site (CS) is indicated. Because of the heterogenous nature of poly A tail lengths, EP and C+P cannot be distinguished by denaturing polyacrylamide gel electrophoresis. Hybridisation to complementary RNA (cRNA), followed by digestion with RNAase T_2 produces two species of RNA. HP is produced from either unreacted precursor (P) or end-polyadenylated RNA (EP), while HC is produced from cleaved, polyadenylated RNA (C+P). HP and HC are easily distinguished by denaturing polyacrylamide electrophoresis. In reactions containing 3' dATP, precursor RNA may remain unaltered (P), may receive a single 3' dATP residue (E+C) in analogy to end-polyadenylation or may undergo cleavage and subsequently receive a single 3' dATP (C+C) in analogy to cleavage and polyadenylation. In the presence of either ATP or 3' dATP, cleavage of the precursor RNA also gives rise to an unstable species, the downstream cleavage product (DCP).


CS
P
NE +ATP
NE+3'dATP
P
An EP
An C+P
DCP
P
A E+C
A C+C
DCP
+cRNA
+RNAaseT2
HP
HC

protected species were detected following electrophoresis on a 6% denaturing polyacrylamide gel, the longer of which represented precursor RNA (Fig 12, lane 4). The shorter products of 26-30 nu in length were the upstream portions of R1 precursor RNA generated by cleavage at the poly A site; these RNAs are referred to as the upstream cleavage products (UCPs, Fig 12, lane 4). The length of both the unreacted precursor RNA and the UCPs was reduced by eight nucleotides in RNAase protection assays, because the cRNA used for the hybridisations extended only to the HindIII site in pR1. Thus, the cap dinucleotide and six nucleotides of the 5' leader sequence of R1 precursor RNA were not protected in hybridisations and were consequently removed by RNAase T_2 digestion. The RNA/RNA hybrid formed by end-polyadenylated RNA was the same length as that formed with unreacted precursor RNA (see Fig 13).

The lengths of the UCPs would place the major cleavage sites at three consecutive A residues 34-36 nucleotides downstream from the RNA 5' end. Minor cleavage sites were located at the U and C residues (37 and 38, respectively) immediately downstream of the triplet of A residues (Fig 14).

Cleavage of R1 transcripts at these sites also generated products which extended from the cleavage site to the 3' end of the precursor; these fragments are referred to as downstream cleavage products (DCPs). The predicted sizes of these DCPs would be between 55 and 59 nucleotides. However, no bands corresponding to these sizes were detected in *in vitro* reactions. DCPs do not possess a cap structure at their 5' termini and as a result are susceptible to degradation by 5'→3' exonucleases present in the nuclear extract.

Direct study of cleavage at poly A sites *in vitro* is possible by uncoupling the cleavage and polyadenylation reactions. This can be achieved by replacing ATP in

reaction mixtures with an analogue, cordycepin triphosphate (3'dATP), which allows cleavage of precursor RNA but prevents polyadenylation (Moore and Sharp, 1985). Since 3' dATP lacks a hydroxyl group at the 3'-carbon, incorporation of this residue into RNA prevents elongation of the poly A tail (see Fig 13). The phosphoanhydride bonds of 3'dATP can be hydrolysed in the same way as those of ATP and therefore 3'dATP can replace ATP as a source of energy in the reaction.

The results of an *in vitro* 3' processing reaction containing 3'dATP and R1 precursor RNA are shown in Fig 12, lane 5. As in the hybridisation/RNAase T2 analysis of the ATP-containing reaction (Fig 12, lane 4), two RNA species corresponding to unreacted precursor and UCPs were produced in the 3'dATP-containing reaction; as expected, no polyadenylated RNA was detected. The lengths of the UCPs were 35-39 nucleotides. The products extended from the 5' end of the precursor RNA, including the 5' cap structure, to the 3' cleavage site and were further elongated by a single 3'dATP residue at their 3' terminus (see Fig 13). The lengths of these UCPs place the cleavage sites at the same residues as those utilised in the ATP-containing reaction. Moreover, as in the ATP-containing reaction, the frequency of cleavage at the triplet of A residues was greater than at the U and C residues. However, both here and as shown below, in the presence of 3'dATP the overall level of cleavage was reduced to approximately 50% of the available precursor, compared to approximately 70% cleavage in the ATP-containing reaction. Thus, although 3'dATP can replace ATP in the 3' cleavage reaction, it acted less efficiently as a co-factor.

Comparison with 3' processing of a wild-type poly A site

In order to establish the relative efficiency of use of the synthetic poly A site cloned in plasmid pR1, a

comparison was made with the *in vitro* cleavage efficiency of the poly A site sequences from the HSV-2 IE-5 gene (Fig 7).

It has previously been shown that a 100bp fragment containing the HSV-2 IE-5 gene polyadenylation site is sufficient to direct efficient 3' processing of a transcript *in vitro* (McLauchlan *et al*, 1988). In addition to the poly A signal, AATAAA, this DNA fragment contains two downstream sequences, TGTGTTGC and a G-rich tract (Fig 14) which, by comparative sequence analysis and deletion studies, were defined as signals required for efficient 3' end formation (McLauchlan *et al*, 1985).

Transcription of pJMC8 DNA, which contains the HSV-2 IE-5 gene poly A site sequences described above, from the SP6 promoter to the EcoRI site produced a precursor RNA of 148 nucleotides in length (Fig 15, lane 2; McLauchlan, *et al*, 1988). Cleavage of this precursor at the IE-5 poly A site *in vitro* generated a UCP of 74 nucleotides (Fig 15, lane 5; McLauchlan *et al*, 1988). For this experiment, precursor RNA approximately the same length as JMC8 RNA was prepared from pR1 by transcribing from the SP6 promoter to the unique PvuII site (Fig 20), generating transcripts of 136 nu.

From analysis of reactions with both ATP and 3'dATP, JMC8 and R1 precursors underwent polyadenylation and cleavage with approximately the same efficiency (Fig 15, lanes 3, 4, 5, 7, 8 and 9). For example, in the presence of ATP, approximately 80% of JMC8 RNA and 85% of R1 RNA was converted to UCP (Fig 15, lanes 4 and 8). In the case of JMC8 RNA, a number of bands shorter than the UCP were formed in the presence of both ATP and 3'dATP. The sizes of these species suggest that they are 5' cleavage products generated by endonucleolytic cleavage close to the poly A signal; an A-rich tract in this region (Fig 14) is likely to be a recognition sequence for cleavage by RNase H which is present in the nuclear extract.

GGGGACAGGG TGGTTGGTGT AATTAATAAT AAAATCGTGA

AAATTGAAAT CGCTTTGTGT GTTGCTGCGG GGACGGGGGC

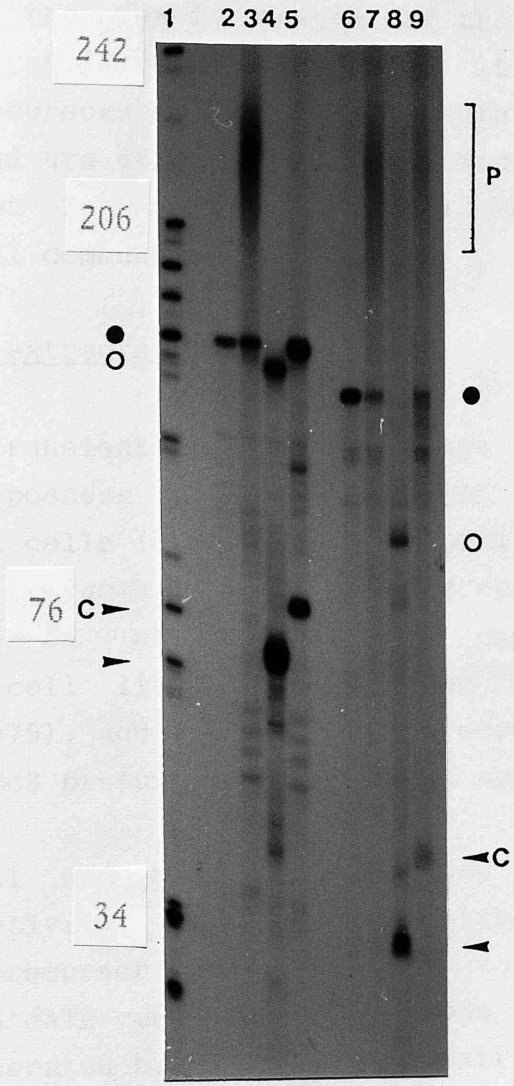
AAATGCGTCG TGACT

Figure 14. Nucleotide sequence of the HSV-2 IE-5 mRNA poly A site present in pJMC8.

The poly A signal and the GT-rich downstream sequence element are underlined. The position of the cleavage site is indicated (arrow).

Figure 15. Comparison of the processing efficiencies of the synthetic poly A site and the IE-5 mRNA poly A site.

In vitro 3' processing reactions were performed for two hours and the RNAs analysed by denaturing polyacrylamide gel electrophoresis as described in Materials and Methods. Lane 1, pBR322 DNA, HpaII fragments; lane 2, unprocessed JMC8 precursor RNA; lane 3, JMC8 RNA, ATP reaction; lane 4, JMC8 RNA, ATP reaction, RNAase T_2 protection assay; lane 5, JMC8 RNA, 3' dATP reaction; lane 6, unprocessed R1 (PvuII) precursor RNA; lane 7, R1 RNA, ATP reaction; lane 8, R1 RNA, ATP reaction, RNAase T_2 protection assay; lane 9, R1 RNA, 3' dATP reaction. The positions of unprocessed precursors (●), unprocessed, RNAase T_2-protected RNA (o), RNAase T_2-protected, cleaved RNA (◄), cleaved RNA (◄ C) and poly A+ RNA (P) are indicated.


1 2 3 4 5 6 7 8 9
242
206
76
34
C
P
C

The efficiency of processing of the extended R1/PvuII precursor (136 nu) (Fig 15, lanes 7-9) was not significantly different from that of the R1/EcoRI precursor (92nu) (Fig 12, lanes 3-5). This suggests that, provided the distance between the 5' end of the RNA and the poly A site is unchanged, the overall length of the precursor has no effect on the efficiency with which it is processed. Conversely, in precursors of the same length, poly A sites close to the 5' end are utilised more efficiently than those positioned further from the 5' end (J McLauchlan and S Simpson, personal communication).

K562 cell nuclear extracts

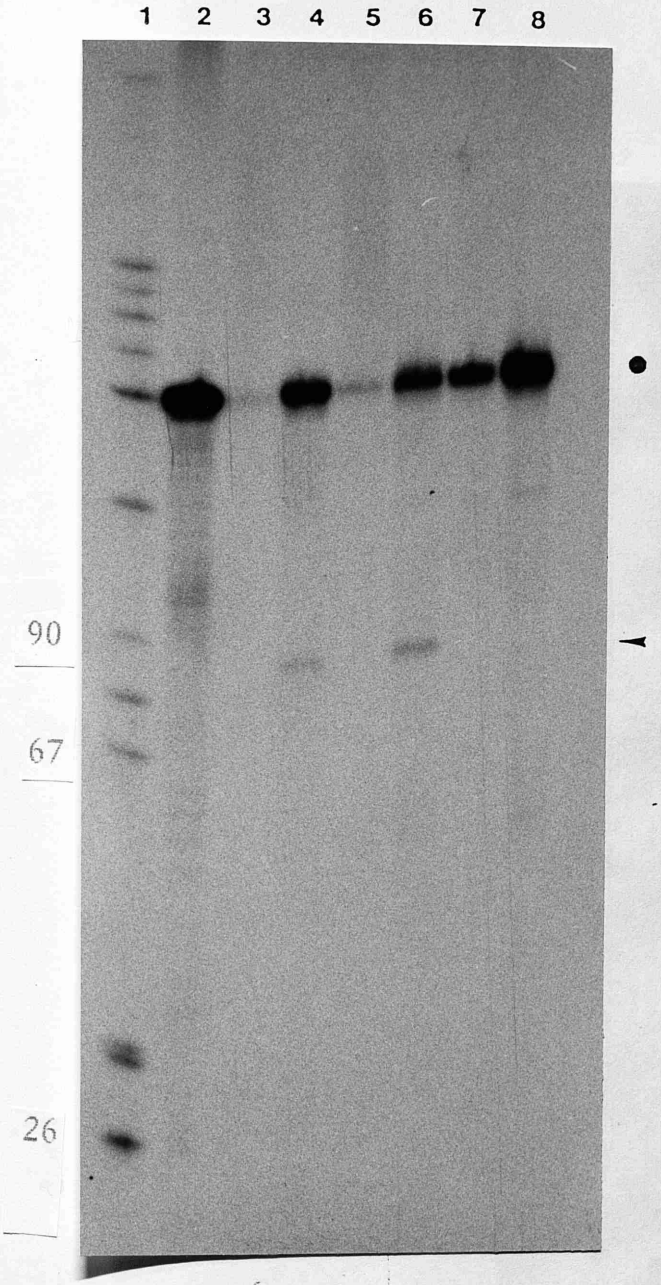
Previous transient expression assays indicated that K562 cells might possess higher levels of 3' processing activity than HeLa cells (J McLauchlan, unpublished). Three different nuclear extracts (K1-3) were prepared from K562 cells, which are an undifferentiated lymphoblastic human erythroleukaemic cell line (Lozzio and Lozzio, 1975; Anderson *et al*, 1979), and tested for 3' processing activity *in vitro*, using JMC8 precursor RNA (Fig 16A and B).

Extracts K1 and K2 both possessed cleavage and polyadenylation activity. In each case, although less than 5% of the JMC8 precursor was converted to UCP, the UCP produced in the 3'dATP-containing reactions was the same length as that generated by HeLa nuclear extracts (Fig 16A, lanes 4 and 6). Similarly, the sizes of poly A tails produced by K562 nuclear extracts were within the same size range as those observed with HeLa extracts (Fig 16B, lanes 3 and 5). Thus, although the overall level of 3' processing obtained with K562 nuclear extracts was significantly lower than with HeLa extracts, both cell types appear to possess identical 3' processing machinery. Extract K3 (Fig 16A and B, lanes 7 and 8) was found to contain neither cleavage nor polyadenylation activity.

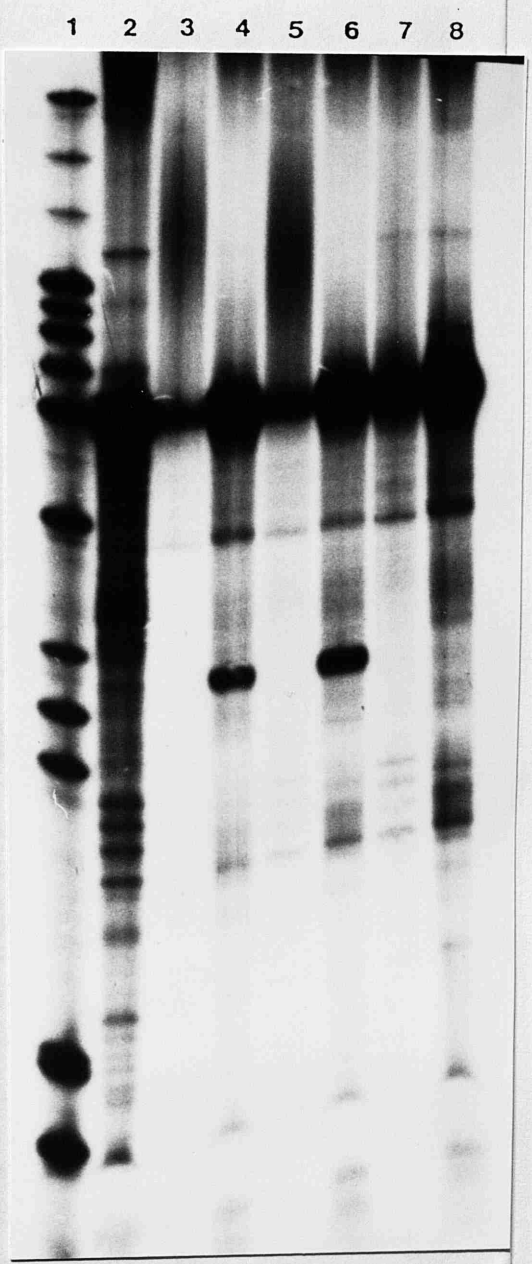
Figure 16. Comparison of the processing activities of K562 cell nuclear extracts and HeLa cell nuclear extracts.

In vitro 3' processing reactions using JMC8 precursor RNA were performed for two hours and the RNAs analysed by denaturing polyacrylamide gel electrophoresis, as described in Materials and Methods.
(A) and (B). Lane 1, pBR322 DNA, HpaII fragments; lane 2, unprocessed JMC8 precursor RNA; lane 3, nuclear extract K1, ATP reaction; lane 4, nuclear extract K1, 3' dATP reaction; lane 5, nuclear extract K2, ATP reaction; lane 6, nuclear extract K2, 3' dATP reaction; lane 7, nuclear extract K3, ATP reaction; lane 8, nuclear extract K3, 3' dATP reaction.
(C). Lane 1, unprocessed JMC8 precursor RNA; lane 2, nuclear extract H6, 3' dATP reaction; lane 3, nuclear extract H13, 3' dATP reaction; lane 4, nuclear extract H15, 3' dATP reaction.
The positions of unprocessed precursor RNA (●), cleaved RNA (◄) and poly A+ RNA (P) are indicated.

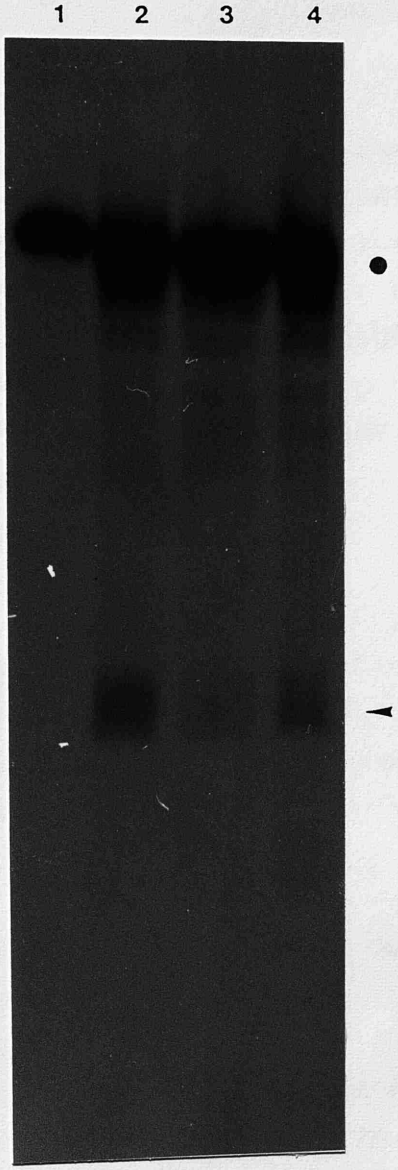
A



B



C



Variability between nuclear extracts

The finding that extract K3 possessed neither cleavage nor polyadenylation activity was not exceptional. Deficient nuclear extracts were also produced from HeLa cells. In fact, consistent production of active nuclear extracts was the major technical difficulty encountered throughout this work.

Figure 16C shows the results of cleavage reactions in the presence of 3' dATP using 3 different HeLa nuclear extracts (lanes 2-4). None of these extracts possessed a high level of cleavage activity: all of them converted less than 5% of R1 precursor RNA to UCPs, although the cleavage which did occur was accurate. Very little degradation of the precursor RNA was observed during incubation with these extracts.

SECTION 1: DISCUSSION

The results presented in this section demonstrate that nuclear extracts from HeLa and K562 cells contain soluble factors which are sufficient to support accurate 3' end formation and polyadenylation *in vitro* using suitable precursor RNAs.

With an alternative *in vitro* system, HeLa whole cell extracts (WCEs) polyadenylate the 3' termini of exogenously added precursor RNAs which contain a poly A signal close to their 3' ends; however, these RNAs are not cleaved at the poly A site. Results with this WCE system indicate that increasing the distance between the RNA 3' end and the poly A signal significantly reduces the efficiency of polyadenylation (Manley, 1983). Polyadenylation of the 3' termini of precursor RNAs also occurs with HeLa cell nuclear extracts.

By comparison with the IE-5 poly A site sequences, precursor RNA transcribed from the cloned oligonucleotides appears to contain all the sequences necessary to direct efficient cleavage at the poly A site and subsequent polyadenylation of the UCP. The precursor carrying the IE-5 poly A site sequences contained more extensive viral downstream sequences than the R1 precursor. However, there was little difference in the efficiency with which the two precursors were processed. This suggests that the viral 1.7kb mRNA poly A site sequences downstream of those present in pR1 are not required for processing at the poly A site.

Cleavage of the R1 precursor at the poly A site generated two products, an UCP which contained a 5' cap structure and a DCP which lacked a 5' cap. The major cleavage sites were located at three A residues positioned 10-12 nucleotides downstream from the poly A signal. The previously mapped location for the 1.7kb mRNA cleavage site was positioned nine nucleotides downstream from the poly A signal (McLauchlan *et al*, 1985). The synthetic poly A site contains a single nucleotide insertion between the poly A signal and the cleavage site, so the *in vitro* reaction can be considered to be accurate.

In vivo, cleavage occurs, in different poly A sites, between 10 nucleotides (eg, McLauchlan and Clements, 1982) and 29 nucleotides (eg, Gingeras *et al*, 1982) downstream of the poly A signal. Definition of the precise site of poly A addition by the RNA sequences is poorly understood. Alterations of the downstream sequences of the *Xenopus* β-1 globin poly A site have little effect on the efficiency of polyadenylation but affect the position at which cleavage occurs in oocyte microinjection experiments (Mason *et al*, 1986). In contrast, experiments using the SV40 E, Ad2 E2A and a hybrid SV40/Ad2 poly A sites in transfection assays show that the location of the poly A site is unaffected while processing activity is profoundly influenced by alterations of the downstream sequences (Hart *et al*, 1985a;

McDevitt *et al*, 1986). The 3' terminal nucleotide at which poly A addition occurs in a number of extensively studied poly A sites is an A residue [eg, SV40 E, Ad2 E2A (Hart *et al*, 1985a), Hepatitis B surface antigen (Simonsen and Levinson, 1983), rabbit β-globin (Gil and Proudfoot, 1984)]. The location at which precursor RNA containing the synthetic poly A site is cleaved may reflect the apparent preference for an A residue immediately upstream from the cleavage site.

In reactions containing ATP, cleavage at the poly A site is rapidly followed by the addition of a poly A tail to the 3' end of the UCP, so that the UCP is not detectable as a separate entity. Thus, cleavage and polyadenylation *in vitro* are tightly coupled processes. *In vivo*, unpolyadenylated pre-mRNAs which extend beyond the poly A site are detectable by labelling nascent RNAs but again polyadenylation rapidly follows cleavage of precursor and UCPs are not detected (Darnell, 1979, 1982). *In vivo*, between 150 and 200 A residues are polymerised onto the newly exposed 3' end following site-specific endonucleolytic cleavage of a precursor RNA (Nevins and Darnell, 1978a). Using the *in vitro* processing system described here, poly A tails consisting of up to 200 A residues were added to cleaved precursors. In this respect, the *in vitro* 3' processing system described here accurately replicates the activities observed *in vivo*.

Inclusion of 3'dATP instead of ATP in reactions prevented polyadenylation of R1 precursor and resulted in the production of discrete UCPs by cleavage at the same sites as in the presence of ATP, albeit with slightly reduced efficiency. The abilities of several ATP analogues to act as co-factors in 3' processing reactions in place of ATP have been tested by Moore and Sharp (1985): no 3' processing activity was observed when ATP is replaced by 2' dATP.

The methylene analogues of ATP [ADP(CH_2)P and AMP(CH_2)P_2], which contain non-hydrolysable beta-gamma and alpha-beta bonds respectively, can serve as high energy co-factors by hydrolysis of their respective alpha-beta or beta-gamma phosphoanyhdride bonds. AMP(CH_2)P_2 cannot undergo polymerisation as required for formation of poly (A) and is therefore an inhibitor of polyadenylation. However, inclusion of ADP(CH_2)P_2 in the reaction in place of ATP leads to the production of poly A tails of shorter than normal length, suggesting that this analogue is not efficiently utilised by the polymerase activity, and that phosphate from ATP is not required for polyadenylation. Since both ADP(CH_2)P_2 and AMP(CH_2)P_2 act as co-factors for the 3' cleavage reaction, the ATP requirement for this reaction must be as an energy source (Moore and Sharp, 1985).

Cordycepin triphophate (3'dATP), which inhibits polyadenylation *in vivo* and poly A polymerase *in vitro* (reviewed by Edmonds, 1982; Jacob and Rose, 1983) is found to inhibit polyadenylation, but not 3' cleavage, *in vitro* (Moore and Sharp, 1985). Both AMP(CH_2)P_2 and 3'dATP were tested as inhibitors of poly A addition in the *in vitro* processing system described here and it was found that only the latter consistently produced the expected results (J McLauchlan, unpublished). Therefore, 3'dATP was routinely used to study the 3' cleavage reaction.

The downstream cleavage product (DCP) is not detectable in the nucleus *in vivo*. Similarly, in this *in vitro* processing system, the DCP is not normally observed. This suggests that the DCP is susceptible to degradation, probably by 5'→3' exonucleases since the 5' terminus of the DCP is not protected by a cap structure. The DCP can be stabilised *in vitro* by adjusting the EDTA concentration in the reaction mixture (Moore and Sharp, 1985). Presumably, chelation of divalent cations by EDTA inhibits the activities of the nucleases responsible for degradation of the DCPs.

As discussed below, nuclear extracts contain at least three factors which are required to support the *in vitro* 3' processing reaction. The variability in processing activity observed between different nuclear extracts may be due to variations in the overall amounts of one or more of the essential factors present. Such variations may be the results of differential extraction efficiencies, depending on the state of the nuclei being extracted. Nuclear extracts which exhibited a high level of non-specific exoribonuclease activity (not shown) may have contained a paucity of RNA binding factors which normally protect the RNA from degradation by nucleases present in the extract. Alternatively, these extracts may have been contaminated with exogenous nucleases, perhaps from the cytoplasmic fraction.

Both cleavage and polyadenylation of precursor RNA in K562 nuclear extracts displayed the same specificity as the same processes mediated by HeLa nuclear extracts. Therefore, K562 and HeLa nuclei probably contain identical 3' processing factors. However, the 3' processing activities of the K562 nuclear extracts described here were less than 10% of those typically found with HeLa cell nuclear extracts. *In vitro* 3' processing-competent nuclear extracts have been prepared from several lymphoid cell lines (Virtanen and Sharp, 1988). Using immunoglobulin poly A sites, lymphoid cell nuclear extracts were found to possess two-fold less total 3' processing activity than HeLa extracts. One possible explanation is that HeLa cell nuclei contain a higher concentration of one or more essential factors. Alternatively, it may be that one or more essential factors is less efficiently extracted from nuclei other than HeLa or that such factors in non-HeLa nuclei are inherently less stable than those in HeLa nuclei.

SECTION 2: RESULTS

Formation of specific processing complexes *in vitro*

As well as analysing the products of *in vitro* 3' processing reactions on denaturing polyacrylamide gels, it is possible to study the interactions between precursor RNAs and the trans-acting factors supplied by the nuclear extract. Large complexes which form rapidly and specifically on RNAs undergoing 3' processing *in vitro* have been resolved using density gradient centrifugation (Moore *et al*, 1988b; Stefano and Adams, 1988) and native polyacrylamide gel electrophoresis (Humphrey *et al*, 1987; Zarkower and Wickens, 1987b; Zhang and Cole, 1987). This section describes the 3' processing-specific complexes formed by R1 precursor and resolved by non-denaturing polyacrylamide gel electrophoresis (see Materials and Methods).

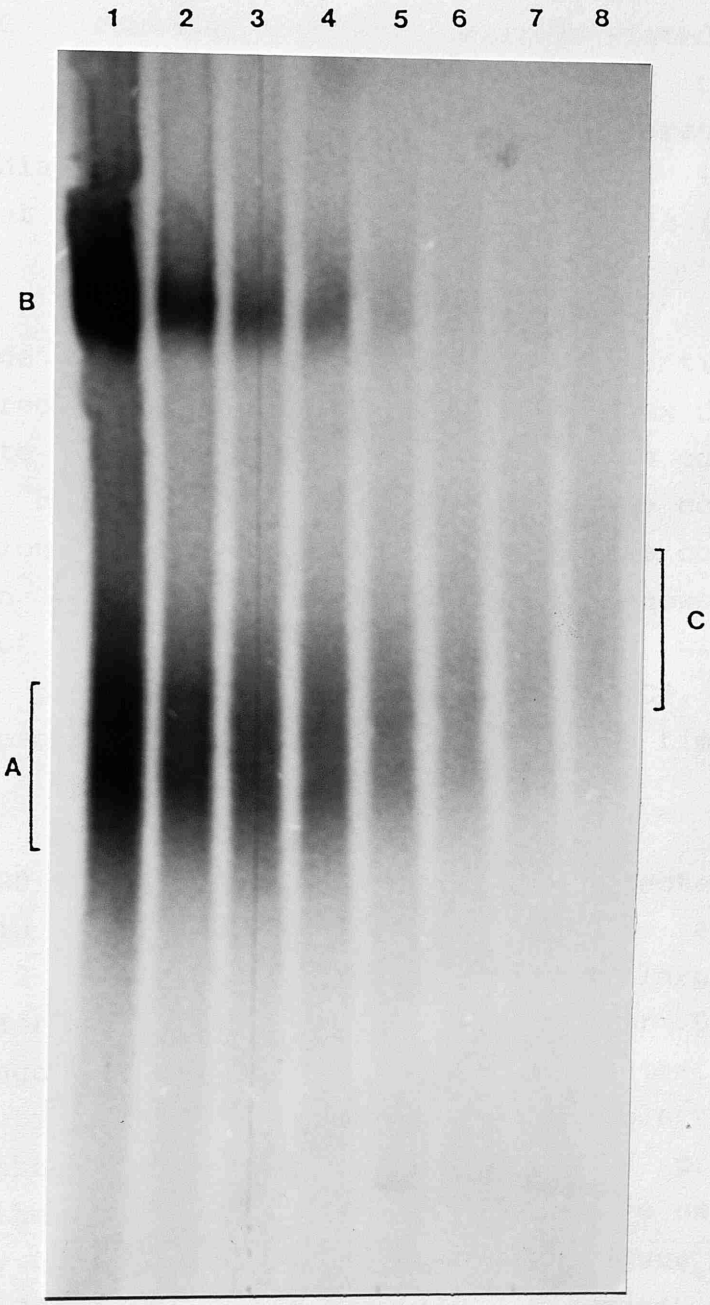
Reaction mixtures containing ATP were prepared as described in Materials and Methods and incubated at 30°C for various times before addition of heparin. Heparin-treated material was electrophoresed on 4% native polyacrylamide gels (80:1, acrylamide:bisacrylamide). A time course of complex formation using R1 precursor RNA is shown in Fig 17. Three distinct complexes were observed. Complexes A and B formed immediately after initiation of the reaction by addition of nuclear extract (Fig 17, lane 1). As the processing reaction proceeded, the proportions of complexes A and B declined and a new complex, C, which migrated more rapidly than complex B but less rapidly than complex A, appeared. In this case, the free precursor RNA has run off the bottom of the gel. The pattern of complexes A, B and C was the same as that observed by McLauchlan *et al* (1988), using JMC8 precursor RNA.

Using two-dimensional gel electrophoresis (first dimension, native polyacrylamide; second dimension,

Figure 17. Time course of processing complex formation.

In vitro 3' processing reactions containing ATP and using R1 precursor RNA were performed for the times indicated, and the RNA analysed by non-denaturing polyacrylamide gel electrophoresis, as described in Materials and Methods. Lane 1, reaction time, zero; lane 2, 10 min; lane 3, 20 min; lane 4, 30 min; lane 5, 45 min; lane 6, 60 min; lane 7, 120 min; lane 8, 180 min. The positions of complexes A, B and C are indicated.

This gel represents one of several repititions of this experiment, all of which clearly demonstrate the formation of complexes A, B and C.


1
2
3
4
5
6
7
8
B
A
C

denaturing polyacrylamide), it has been shown that complex A contains only precursor RNA, complex B contains both unreacted precursor and a small amount of poly A+ material and complex C contains only polyadenylated RNA (J McLauchlan, S Simpson and J B Clements, personal communication). This suggests that complex B represents an active intermediate in the 3' processing reaction. There is no evidence that complex A is related to complex B (eg as a precursor).

The wide smear of complex A at early times of reaction obscured the initial appearance of complex C, so it is impossible to ascertain the time point at which complex C began to form. However, comparison of the time course of complex formation (Fig 17) with a similar time course of polyadenylation and cleavage (Fig 18) shows that accumulation of complex C was concomitant with the conversion of precursor to polyadenylated UCP. Thus, complex C was probably first formed at an early time point (eg 20 min).

After 20 min and 40 min, a pair of unexpected bands were observed in the polyadenylation and cleavage reactions (Fig 18, lanes 2-5 (U)). The novel bands were larger than UCPs but smaller than the expected sizes for DCPs and appeared unchanged both before and after hybridisation and RNAase T2 digestion, suggesting that these RNA molecules did not contain the extreme 5' portion of the precursor (Fig 13). Furthermore, since these molecules were unstable, it seems likely that they lacked a 5' cap structure and may have been produced by site-specific cleavage of the precursor by an unidentified nuclease, and subsequently degraded.

As in the previous section, the properties of R1 precursor RNA were compared with those of JMC8 precursor RNA. Complex B, as formed by JMC8 and R1 precursors RNAs co-migrated on a native polyacrylamide gel (Fig 19). In

Figure 18. Time course of RNA 3' processing.

In vitro 3' processing reactions containing ATP and using R1 precursor RNA were performed for the times indicated and the RNAs analysed by denaturing polyacrylamide gel electrophoresis, as described in Materials and Methods. Lane 1, unprocessed R1 precursor RNA; lanes 2, 4, 6, 8 and 10, ATP reactions; lanes 3, 5, 7, 9 and 11, ATP reactions, RNAase T_2 protection assays; lanes 2 and 3, 20 min; lanes 4 and 5, 40 min; lanes 6 and 7, 60 min; lanes 8 and 9, 120 min; lanes 10 and 11, 180 min. The positions of unprocessed precursors (●), unprocessed RNAase T_2-protected RNA (O), RNAase T_2-protected cleaved RNA (►), poly A+ RNA (P) and unidentified bands (U) are indicated.

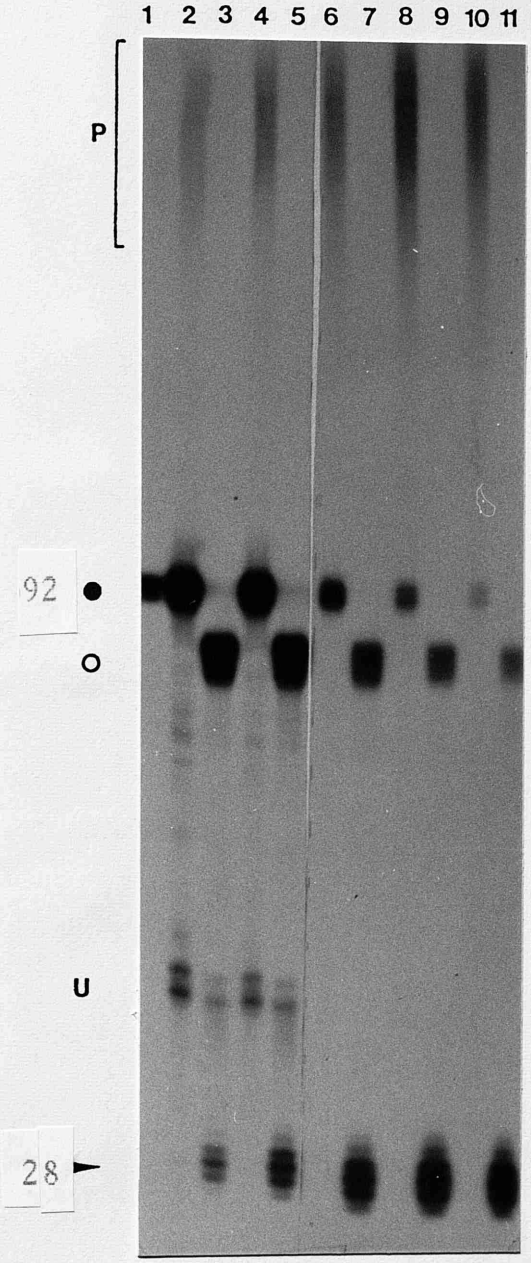
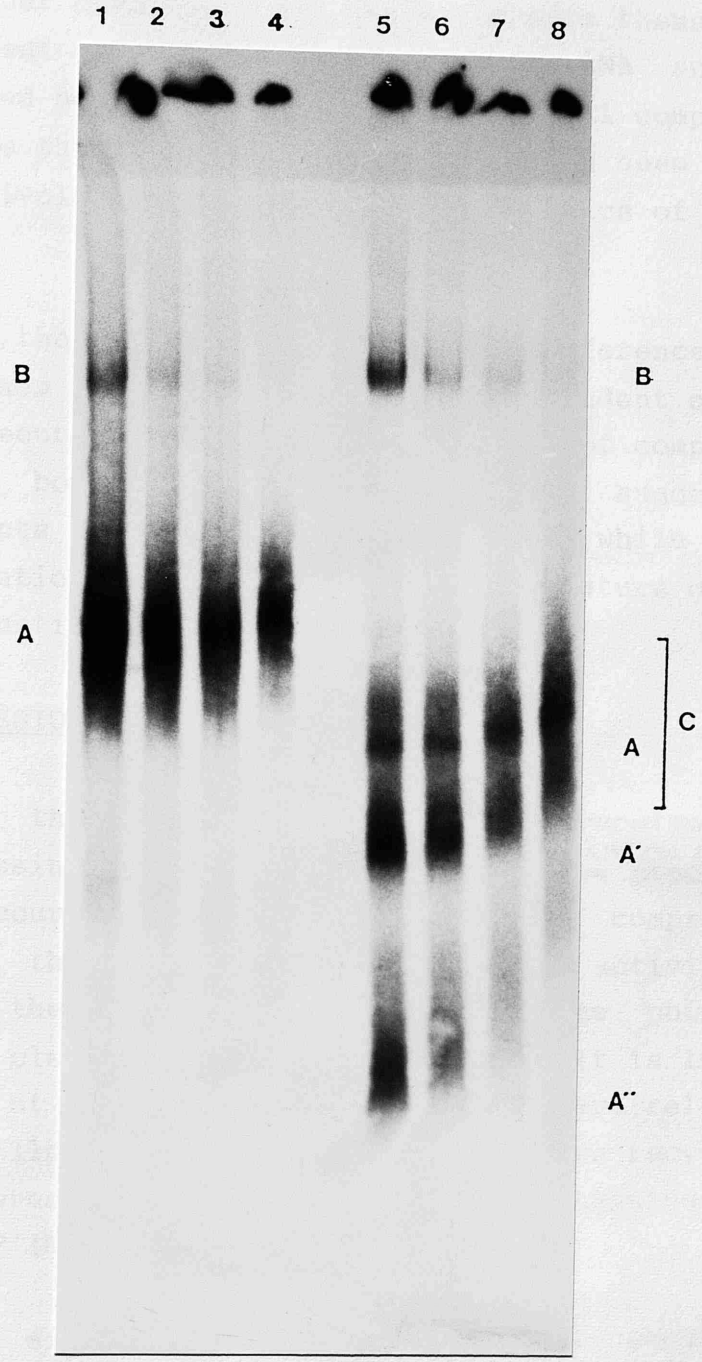

1 2 3 4 5 6 7 8 9 10 11
P
92
O
U
28

Figure 19. Comparison of complex formation on R1 and JMC8 RNAs.

In vitro 3' processing reactions containing ATP were performed for the times indicated and the RNA analysed by non-denaturing polyacrylamide gel electrophoresis, as described in Materials and Methods. Lanes 1-4, JMC8 RNA; lanes 5-8, R1 (EcoRI) RNA; lanes 1 and 5, reaction time, zero; lanes 2 and 6, 20 min; lanes 3 and 7, 60 min; lanes 4 and 8, 120 min. The positions of complexes A, A', A", B and C are indicated.


1
2
3
4
5
6
7
8
B
A
B
A
C
A'
A''

contrast, the other complexes and the precursors themselves exhibited different mobilities: R1 precursor RNA and its complexes migrated more rapidly. In this case, R1 complex A was resolved into three species (A, A', A") which seem to be functionally equivalent, in terms of the kinetics of their disappearance.

Although the migration rates (and, by inference, the sizes) of complexes A and C appeared to be dependent on the length of the precursor RNA, the migration rate of complex B was the same for both R1 and JMC8 precursors, suggesting that the components of complex B are constant while there may be some variation in the number or the nature of the factors which constitute complexes A and C.

SECTION 2: DISCUSSION

At least three nuclear factors are required to support 3' processing *in vitro*. (Gilmartin and Nevins, 1989; Takagaki *et al* 1988). Processing of the precursor RNA probably occurs in a transient complex comprising complex B plus the appropriate enzymatic activities: complex B serves the purpose of specifying the point at which the RNA is cleaved and polyadenylated. It is likely that some or all of the components of complex B are released from the RNA following 3' processing and are recycled. Complex B may represent the stable complex containing pre-mRNA, SF and CstF (Gilmartin and Nevins, 1989).

Since the migration rate of complex B is constant, irrespective of the precursor RNA on which it is assembled, it must contain only a limited set of nuclear factors which constrain the RNA such that no further factors can bind to the RNA in a heparin-resistant manner; the RNA may be held in a secondary structure or sequestered within complex B, such that additional factor binding is sterically hindered.

In complex C, the poly A tail is likely to be bound by PABPs.

SECTION 3: RESULTS

RNA sequence requirements for 3' processing and complex formation *in vitro*

Deletion of either the polyadenylation signal or the downstream element abolishes or significantly reduces 3' processing of mRNAs *in vivo* and *in vitro*. The oligonucleotides used to produce the 3' processing site for this study contain a number of restriction sites which enable deletion of each of the functional sequence elements.

Plasmids pR2 and pR3, from which the DNA fragments containing the poly A signal and the (G+T)-rich element, respectively, were deleted, were constructed as shown in Fig 20. The 107 bp XhoI/PvuII fragment of pR1 was isolated from a 5% native polyacrylamide gel, and ligated to the 2.8 kb SalI/PvuII fragment of pGEM-1, which was isolated from a 1% agarose gel, to form plasmid pR2. Plasmid pR1 was digested with SalI and SmaI, treated with Klenow fragment and dNTPs to produce blunt ends, and then religated to produce plasmid pR3.

In vitro transcription of plasmids pR2 and pR3, digested with EcoRI, with SP6 RNA polymerase produced precursor RNAs 89 and 59nu in length, respectively (Fig 21A, lane 5; Fig 21B, lane 5). The nucleotide sequences of these transcripts are shown in Figure 22.

Figure 21A shows typical products of *in vitro* 3' processing reactions containing either ATP or 3' dATP, using R1 and R2 precursor RNAs. R2 precursor RNA was neither polyadenylated (lane 6) nor cleaved (lanes 7 and 8). The short bands observed in lane 8 are also present in the untreated precursor (lane 5); these minor contaminants were not protected during hybridisation and RNAase T2 digestion, indicating that the sequences they contain do not originate

Figure 20. Construction of plasmids pR2 and pR3 (not to scale).

The positions of the SP6 and T7 promoters (⟶) and relevant restriction endonuclease recognition sites (H = HindIII, Sa = SalI, E = EcoRI, P = PvuII and Sm = SmaI) are indicated.

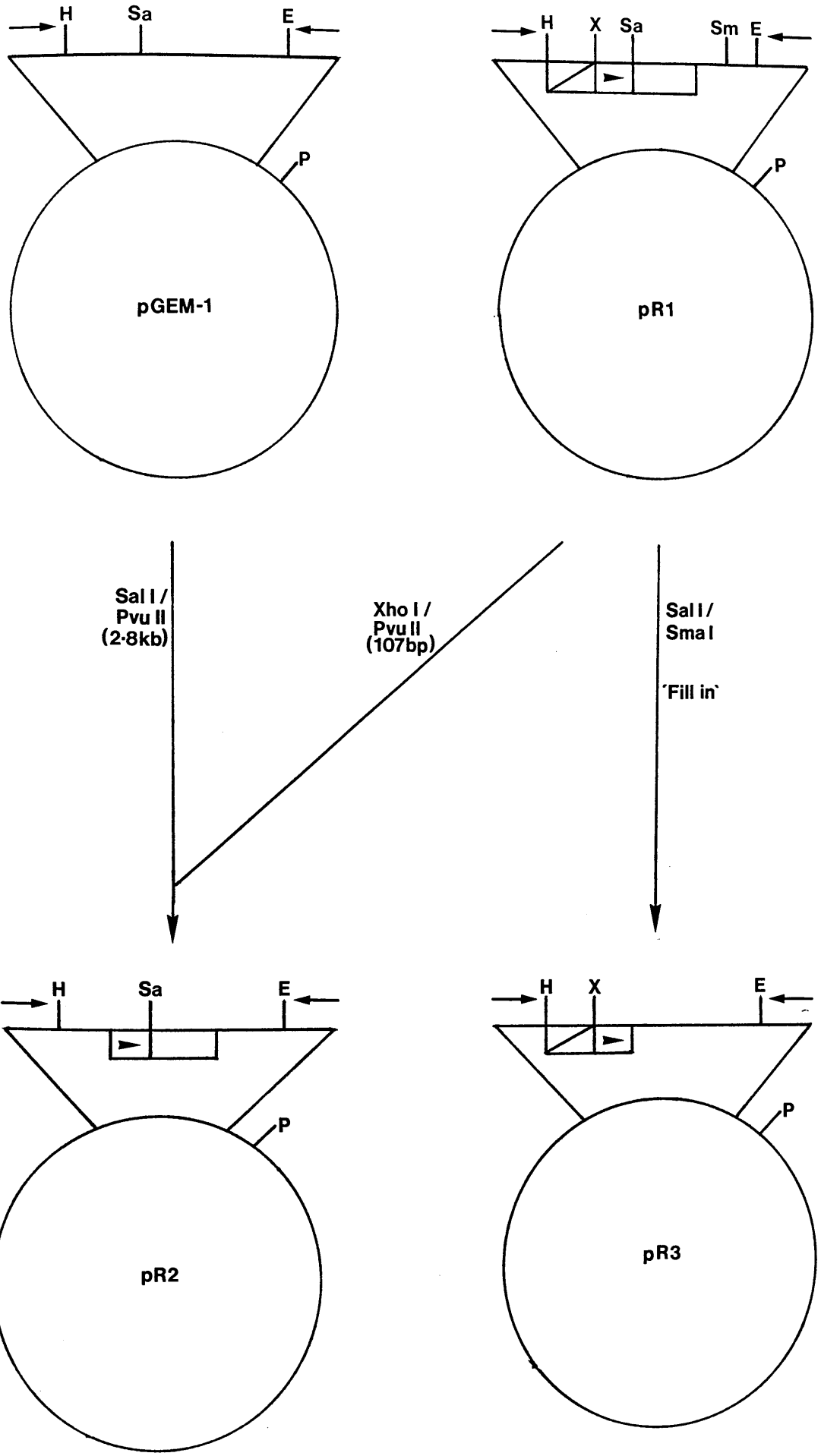
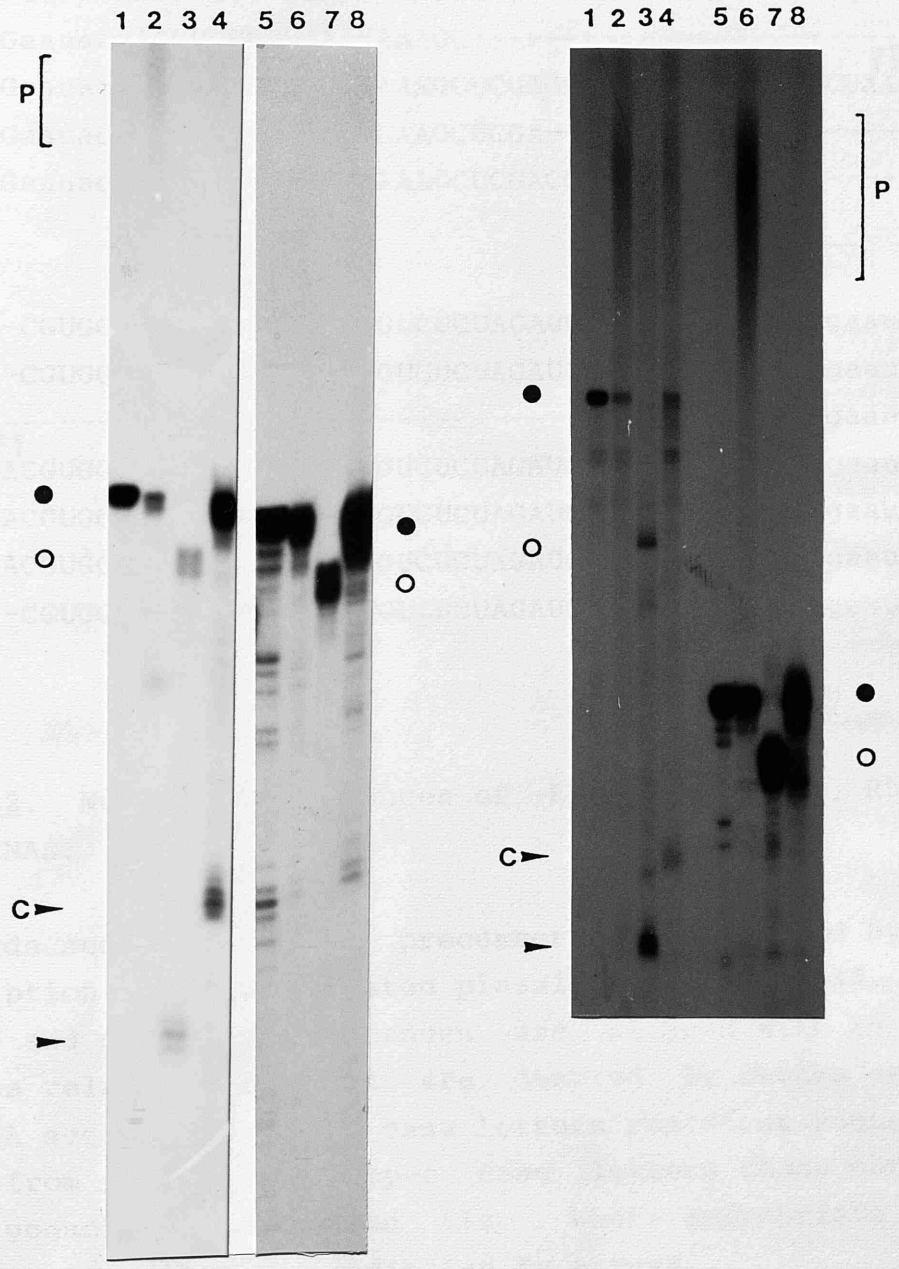

H
Sa
E
P
pGEM-1
H
X
Sa
Sm
E
P
pR1
Sal I /
Pvu II
(2·8kb)
Xho I /
Pvu II
(107bp)
Sal I /
Sma I
'Fill in'
H
Sa
E
P
pR2
H
X
E
P
pR3

Figure 21. Comparison of the processing efficiencies of R1, R2 and R3 RNAs.

In vitro 3' processing reactions were performed for two hours and the RNAs analysed by denaturing polyacrylamide gel electrophoresis, as described in Materials and Methods.
(A). Lanes 1-4, R1 (EcoRI) RNA; lanes 5-8, R2 RNA; lanes 1 and 5, unprocessed precursor RNA; lanes 2 and 6, ATP reaction; lanes 3 and 7, ATP reaction, RNAase T_2 protection assay; lanes 4 and 8, 3' dATP reaction.
(B). Lanes 1-4, R1 (PvuII) RNA; lanes 5-8, R3 RNA; lanes 1 and 5, unprocessed precursor RNA; lanes 2 and 6, ATP reaction; lanes 3 and 7, ATP reaction, RNAase T_2 protection assay; lanes 4 and 8, 3' dATP reaction.
The positions of unprocessed precursors (●), unprocessed RNAase T_2-protected RNA (O), RNAase T_2-protected cleaved RNA (►), cleaved RNA (C►) and poly A+ RNA (P) are indicated.


A
B
1 2 3 4 5 6 7 8
P
C
1 2 3 4 5 6 7 8
P
C

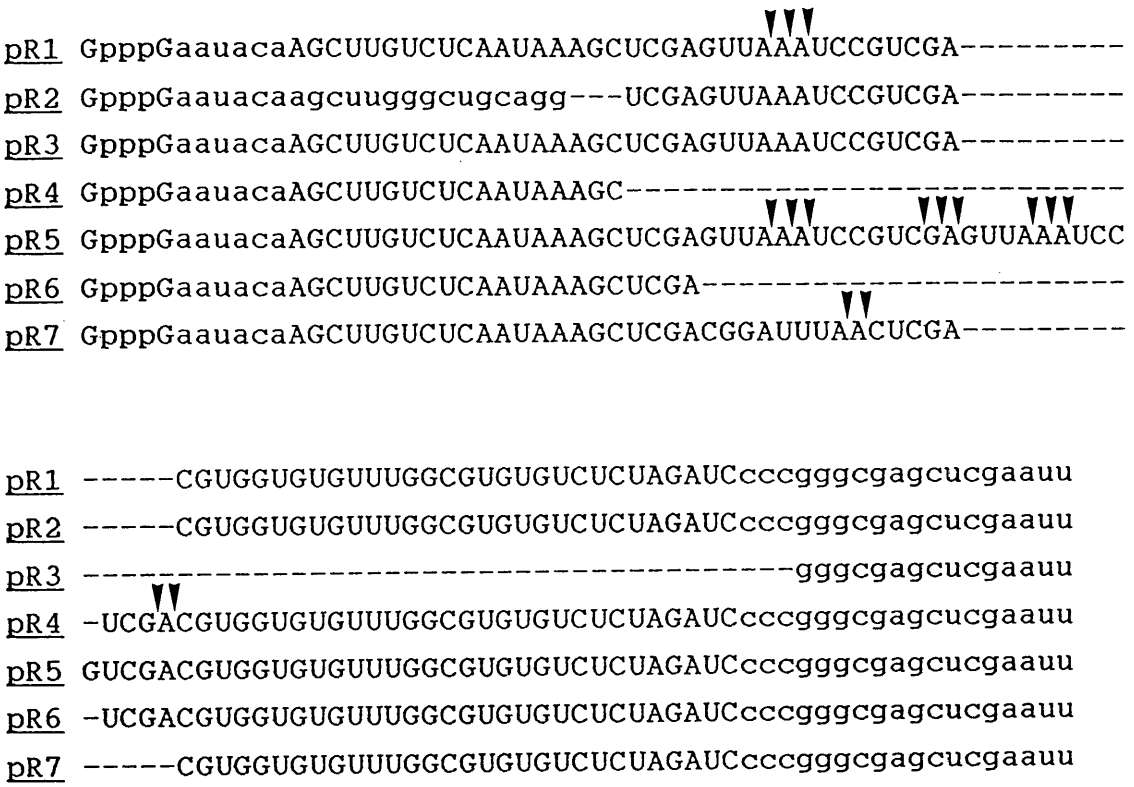


Figure 22. Nucleotide sequences of R1, R2, R3, R4, R5, R6 and R7 RNAs.

Nucleotide sequences of the precursor RNAs produced by SP6 transcription of EcoRI-digested plasmids pR1, pR2, pR3, pR4, pR5, pR6 and pR7. The sequences are aligned with R5 RNA. Deletions relative to R5 RNA are denoted by dashes in the other RNA sequences. Lower case letters represent sequences derived from pGEM-1 and upper case letters those derived from oligonucleotides 67A and 67B. Where appropriate, the major cleavage sites are indicated by arrows.

from the multiple cloning site or the insert of plasmid pR2. Rather, these RNA species may have been produced by non-specific initiation and termination of SP6 transcription elsewhere on the plasmid template.

While R3 precursor RNA was efficiently polyadenylated (Fig 21B, lane 6), it was not cleaved in the presence of ATP (lane 7) or 3' dATP (lane 8). Therefore, all of the poly A+ RNA must have been produced by end-polyadenylation. The R3 precursor RNA fulfils the requirement for end-polyadenylation since it contains a poly A signal relatively close to its 3' terminus.

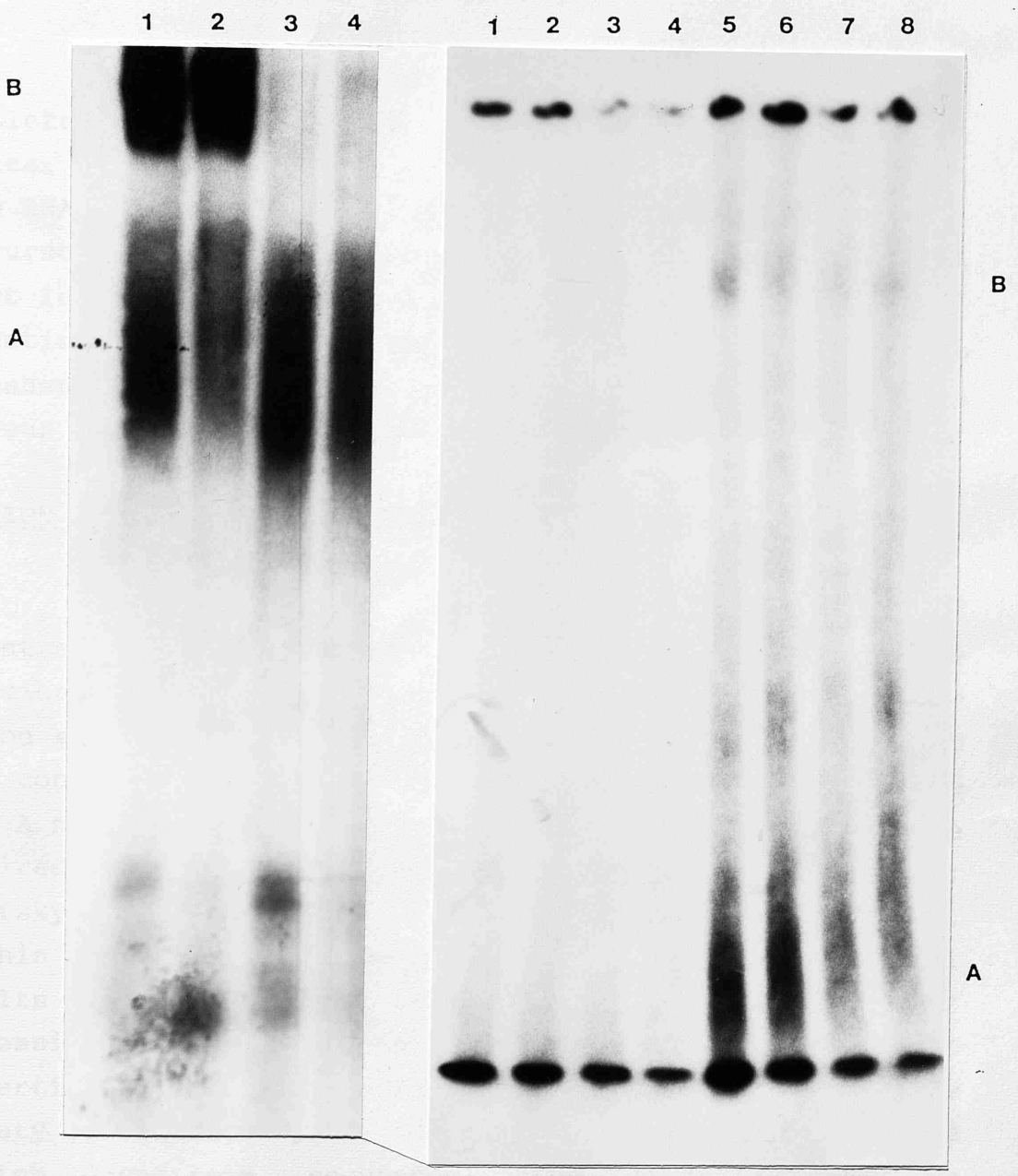
These results indicate that both the AAUAAA hexanucleotide and the downstream sequence element are essential for cleavage of the synthetic poly A site.

R2 and R3 precursor RNAs were also tested for their abilities to form complex B when incubated with nuclear extract in the presence of ATP. The complexes resolved when reactions containing R1, R2 and R3 precursor RNAs were electrophoresed on non-denaturing polyacrylamide gels are shown in Fig 23. As described in the previous section, a large proportion of R1 precursor was rapidly sequestered into complex B (while lesser amounts were assembled into the faster-migrating complex A). In contrast, substantially smaller fractions of R2 and R3 precursors were assembled into complex B, reflecting the observation that neither represents a substrate for efficient 3' processing. These results further substantiate the assumption that complex B represents an active intermediate in the 3' processing reaction. It has been shown that the stability of a complex containing SF and CstF determines the efficiency of cleavage (Weiss *et al*, 1991).

Precursor RNA transcribed from plasmid pR2 formed complex A in approximately the same proportion as R1 precursor, although R2 complex A migrated slightly more

Figure 23. Comparison of complex formation on R1, R2 and R3 RNAs.

In vitro 3' processing reactions containing ATP were performed for the times indicated and the RNAs analysed by non-denaturing polyacrylamide gel electrophoresis, as described in Materials and Methods.
(A). Lanes 1 and 2, R1 RNA; lanes 3 and 4, R2 RNA; lanes 1 and 3, reaction time, zero; lanes 2 and 4, 20 min.
(B). Lanes 1-4, R3 RNA; lanes 5-8, R1 RNA; lanes 1 and 5, reaction time, zero; lanes 2 and 6, 20 min; lanes 3 and 7, 60 min; lanes 4 and 8, 120 min.
The positions of complexes A and B are indicated.


A.
B.
1
2
3
4
1
2
3
4
5
6
7
8
B
A
B
A

rapidly than R1 complex A, perhaps reflecting the difference in length of the two RNA molecules (see Section 2).

In contrast to R1 precursor, R3 RNA almost completely failed to form complexes when incubated with nuclear extract. More than 95% of R3 precursor appeared as free RNA at the bottom of the gel, compared with 60% of R1 precursor in the parallel control experiment (Fig 22B). The short length of R3 precursor RNA may preclude efficient formation of complexes. Alternatively, the RNA may take up an unusual secondary structure which fails to interact with nuclear factors.

SECTION 3: DISCUSSION

In the *X laevis* β-globin poly A site, deletion of downstream sequence elements has a minimal effect on the efficiency of cleavage at the poly A addition site (Mason *et al*, 1986). In contrast, the results presented here confirm the widely reported findings that both the poly A signal (AAUAAA) and a downstream U- or (G+U)-rich are required for efficient 3' processing (eg, Zhang *et al*, 1986; Sadofsky and Alwine, 1984; Wickens and Stephenson, 1984). In this case, however, deletion of the downstream element results in abolition rather than diminution of 3' processing. Thus, there is wide variability between the properties of different poly A sites, reflecting the wide variety of sequences present at such sites. Although U- and GU-rich downstream sequences have been defined as constituent elements of poly A sites, unlike the highly conserved AAUAAA hexanucleotide, the downstream elements differ considerably from each other in both their sequence compositions and precise locations relative to the poly A signal and the poly A addition site.

The finding that complex B assembled efficiently only on R1 precursor, which contains an effective poly A site, and not on R2 and R3 precursors, which were not

substrates for 3' processing, strongly suggests that complex B represents an active 3' processing intermediate. Formation of complex B must require either binding of one factor to both the poly A signal and the downstream element or interaction of two (or more) separate factors with the two sequence elements and with each other.

Complex A was efficiently assembled on R2 precursor which lacks the essential poly A signal. Since R2 RNA cannot undergo 3' processing, it is unlikely that complex A plays any part in the process of pre-mRNA 3' end maturation. Nothing is known about the components of complex A.

SECTION 4: RESULTS

The spatial arrangement of the poly A site sequence elements influences the cleavage reaction

A further set of variants of plasmid pR1 was constructed in order to test the effect of altering the relative positions of the poly A signal and the downstream element on the frequency of 3' processing. Plasmids pR4, pR5 and pR7 were all produced by digestion of pR1 DNA with XhoI and SalI, followed by re-ligation (Fig 24). Deletion of the 14bp XhoI/SalI fragment by intramolecular ligation of the plasmid backbone gave rise to the plasmid which was designated pR4. Plasmid pR5 was produced by insertion of two copies of the 14bp XhoI/SalI fragment into the backbone. Restriction analysis showed that both copies of the XhoI/SalI fragment had inserted in the same orientation as in pR1; subsequent DNA sequencing of the construct confirmed this arrangement. Insertion of a single copy of the 14bp XhoI/SalI fragment in the opposite orientation to pR1 produced the plasmid which was designated pR7. Plasmid pR6 was constructed by intramolecular ligation of the blunt-ended backbone produced by treatment of XhoI/SalI-digested pR1 DNA with Klenow enzyme and dNTPs.

The precursor RNAs transcribed from EcoRI-digested pR4, pR5, pR6 and pR7 with SP6 RNA polymerase were 78, 106, 82 and 92nu in length, respectively (Fig 25A, lane 5; Fig 25B, lane 1; Fig 25C, lanes 1 and 9).

The structures of plasmids pR1, pR4, pR5 and pR6 are such that in each construct the spacing between the poly A signal and the downstream element is different, while the sequence elements themselves are unaltered (Fig 22). Taking plasmid pR1 as the prototype, in pR5 the spacing is increased by 14nu, while in pR4 and pR6 the spacing is decreased by 14 and 10nu, respectively. In plasmid pR7, the spacing between the poly A signal and the downstream element

Figure 24. Construction of plasmids pR4, pR5, pR6 and pR7 (not to scale).

The positions of the SP6 and T7 promoters (⟶) and relevant restriction endonuclease recognition sites (H = HindIII, X = XhoI, S = SalI, E = EcoRI and P = PvuI) are indicated.

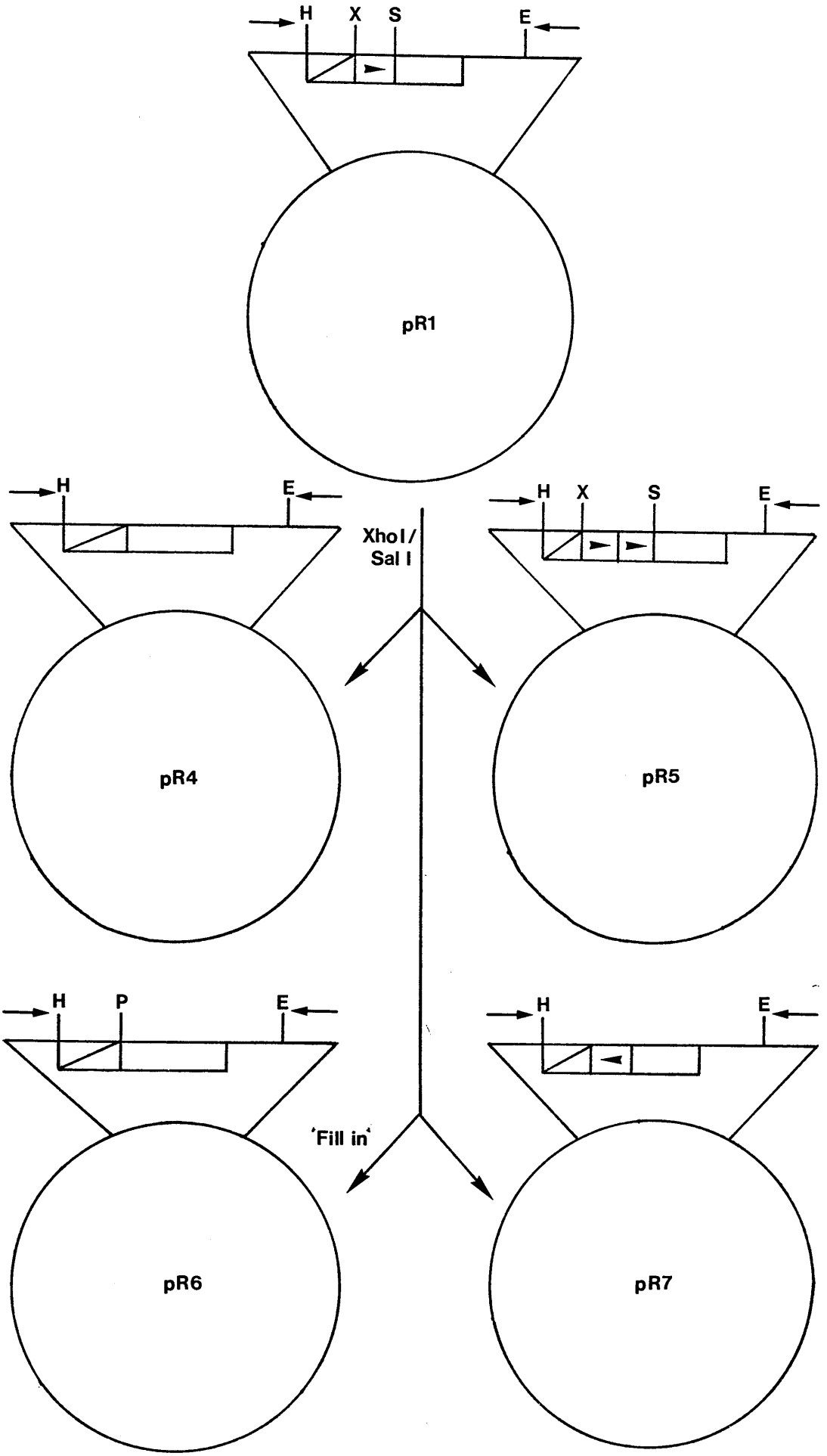
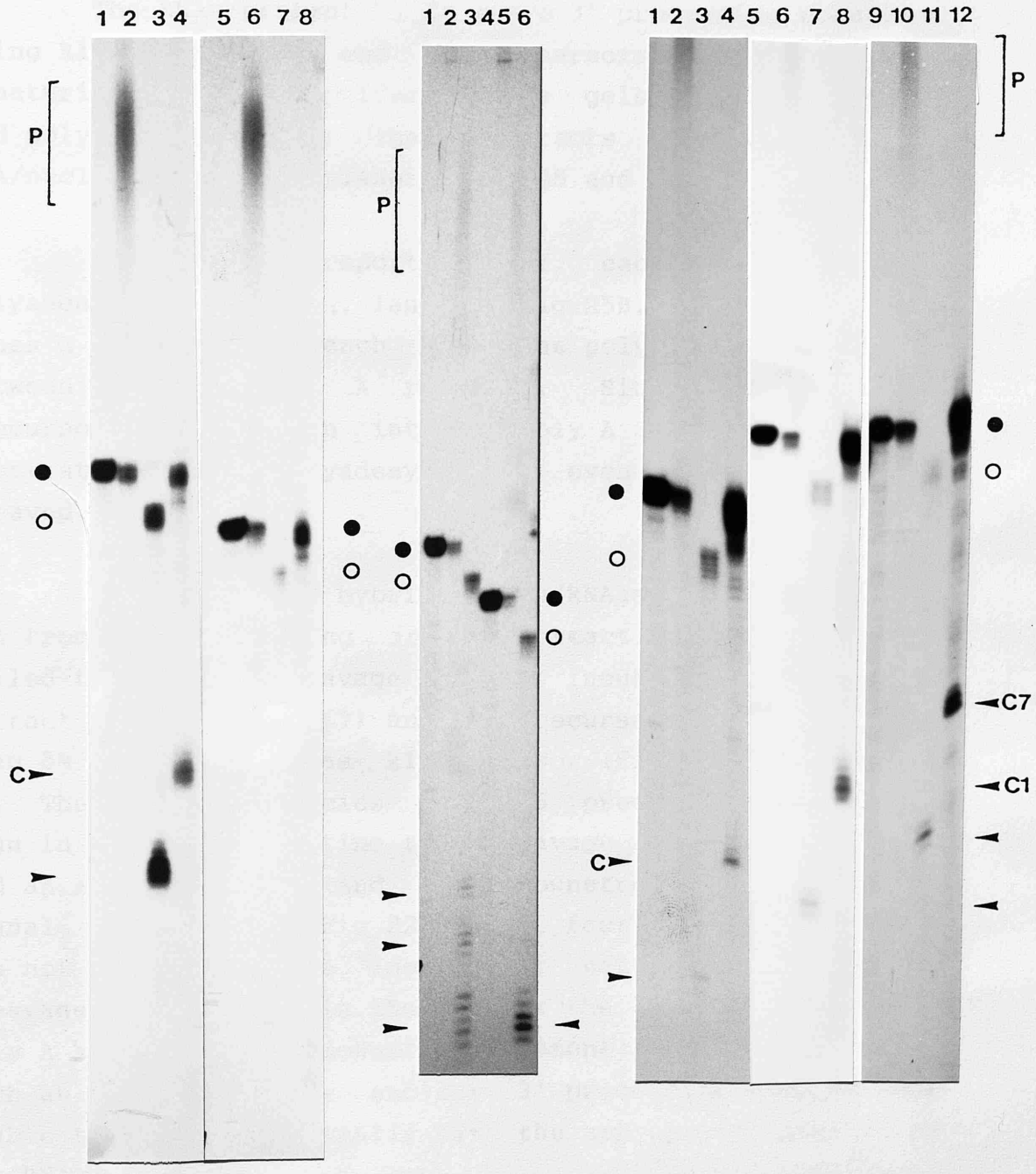

H
X
S
E
pR1
XhoI/
Sal I
pR4
pR5
P
'Fill in'
pR6
pR7

Figure 25. Comparison of processing efficiencies of R4, R5, R6 and R7 RNAs.

In vitro 3' processing reactions were performed for two hours and the RNAs analysed by denaturing polyacrylamide gel electrophoresis, as described in Materials and Methods.
(A). Lanes 1-4, R1 RNA; lanes 5-8, R4 RNA; lanes 1 and 5, unprocessed precursor RNA; lanes 2 and 6, ATP reaction; lanes 3 and 7, ATP reaction, RNAase T_2 protection assay; lanes 4 and 8, 3' dATP reaction.
(B). Lanes 1-3, R5 RNA; lanes 4-6, R1 RNA; lanes 1 and 4, unprocessed precursor RNA; lanes 2 and 5, ATP reaction; lanes 3 and 6, ATP reaction, RNAase T_2 protection assay.
(C). Lanes 1-4, R6 RNA; lanes 5-8, R1 RNA; lanes 9-12, R7 RNA; lanes 1, 5 and 9, unprocessed precursor RNA; lanes 2, 6 and 10, ATP reaction; lanes 3, 7 and 11, ATP reaction, RNAase T_2 protection assay; lanes 4, 8 and 12, 3' dATP reaction.
The positions of unprocessed precursors (●), unprocessed RNAase T_2-protected RNA (O), RNAase T_2-protected cleaved RNA (►), cleaved RNA (C ►) and poly A+ RNA (P) are indicated.


A.
B.
C.
1 2 3 4 5 6 7 8
1 2 3 4 5 6
1 2 3 4 5 6 7 8 9 10 11 12
P
C
C7
C1

is identical to that in pR1. However, in pR7 the XhoI/SalI fragment is inverted so that the sequence around the cleavage/poly A addition site is different from that in the prototype plasmid.

The RNAs present in *in vitro* 3' processing reactions using R1, R4, R5, R6 and R7 precursors were analysed on denaturing and native polyacrylamide gels to assay cleavage and polyadenylation of the precursors and formation of RNA/nuclear factor complexes (Figs 25 and 26).

A similar proportion of each precursor was polyadenylated (Fig 25A, lane 6; Fig 25B, lane 3, Fig 25C, lanes 2 and 10). In each case, the poly A tails contained between 150 and 250 A residues. Since all of these precursors contain an intact poly A signal, they are substrates for end-polyadenylation, even if they are not cleaved.

As assayed by hybridisation/RNAase T2 digestion of RNA from ATP-containing *in vitro* reactions, R4 precursor failed to undergo cleavage during incubation with nuclear extract (Fig 25A, lane 7) and R6 precursor was cleaved less than 5% as frequently as R1 precursor (Fig 25C, lanes 3 and 7). The protected species from R6 precursor were 16 and 17nu in length, indicating that cleavage occurred 3' of a G and an A residue, 9 and 10nu downstream of the poly A signal, respectively (Fig 22). The fact that R4 precursor was not cleaved and the inefficient nature of R6 precursor cleavage suggest that in these RNAs the spacing between the poly A signal and the downstream element has been reduced to such an extent that the nuclear 3' processing factors were unable to interact optimally with the appropriate signals on the RNA.

As expected, since it fails to be cleaved, R4 precursor was not assembled into complex B, although a small proportion of the RNA was sequestered into complex A; more

than 90% of the precursor migrated as free RNA (Fig 26B, lanes 7-9). However, despite being inefficiently cleaved, a large proportion of R6 precursor formed complex B (Fig 26B, lane 10). This suggests that, although the components of complex B readily interacted with R6 precursor, cleavage of the RNA occurred infrequently because another factor(s) (eg, the endonuclease activity) interacted inefficiently with R6 complex B, perhaps because of steric hindrance due to the proximity of the poly A signal and the downstream element.

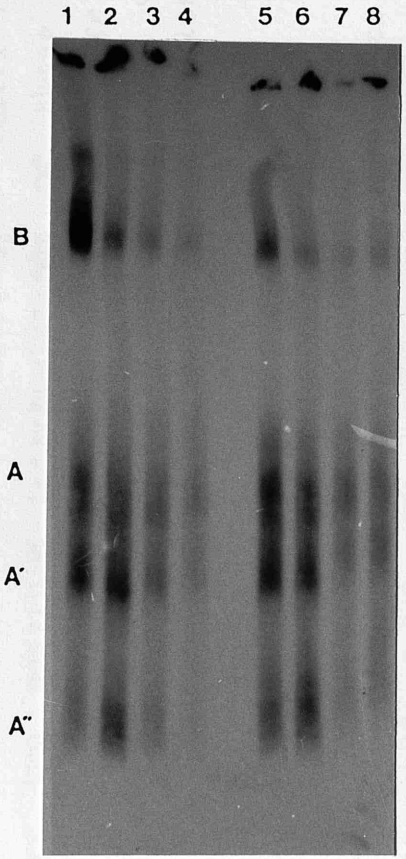
Hybridisation/RNAase T_2 digestion of R5 RNA from an ATP-containing reaction revealed three sets of UCPs (Fig 25B, lane 3). The sizes of the major protected species were 26-30, 34-36 and 40-42nu, inclusive; a lower level of cleavage occurred at intervening nucleotides, suggesting that the accuracy of processing was significantly reduced, compared to R1 precursor. The overall frequency of cleavage of R5 precursor was 30% of that for R1 precursor. The shortest and longest major UCP sets were produced by cleavage of R5 precursor at the proximal and distal copies of the normal cleavage site, while the intermediate UCPs derived from cleavage 3' to the C, G and A residues, as indicated in Figure 22. Cleavage occurred most frequently at the proximal site. R5 precursor was rapidly assembled into complexes A and B (Fig 26A, lane 5). However, the proportion of R5 RNA sequestered in complex B was smaller than that for R1 RNA (Fig 26A, lanes 1 and 5) reflecting the lower frequency of cleavage of R5 precursor compared to the prototype precursor.

Cleavage of R7 precursor in the presence of ATP, as assayed by hybridisation/RNAase T_2 digestion, produced protected species 30, 31 and 32 nucleotides in length (Fig 25C, lane 11). These correspond to UCPs of 38, 39 and 40nu, indicating that cleavage of R7 precursor occurred at the consecutive A and C residues indicated in Figure 17. The cleavage sites in R7 RNA lie four nucleotides further downstream of the poly A signal than those in the R1

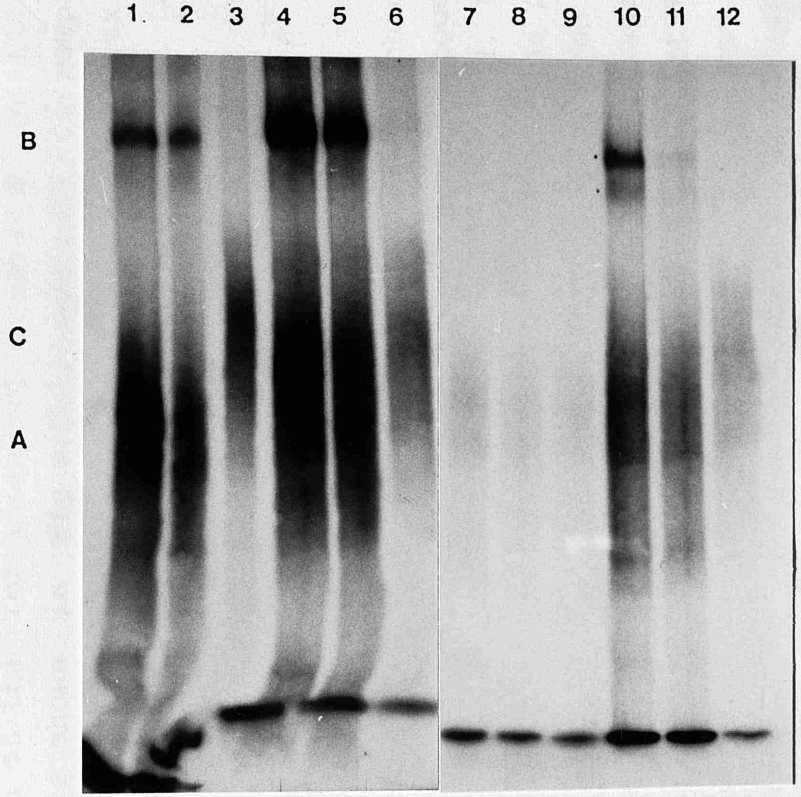
Figure 26. Comparison of complex formation on R4, R5, R6 and R7 RNAs.

In vitro 3' processing reactions containing ATP were performed for the times indicated and the RNAs analysed by non-denaturing polyacrylamide gel electrophoresis, as described in Materials and Methods.
(A). Lanes 1-4, R1 RNA; lanes 5-8, R5 RNA; lanes 1 and 5, reaction time, zero; lanes 2 and 6, 20 min; lanes 3 and 7, 60 min; lanes 4 and 8, 120 min.
(B). Lanes 1-3, R1 RNA; lanes 4-6, R7 RNA; lanes 7-9, R4 RNA; lanes 10-12, R6 RNA; lanes 1, 4, 7 and 10, reaction time, zero; lanes 2, 5, 8 and 11, 30 min; lanes 3, 6, 9 and 12, 120 min.
The positions of complexes A, B and C are indicated.

A.



B.



precursor. The R7 precursor was cleaved 20% more frequently than R1 RNA. Since these two precursors share precisely the same poly A signal/downstream element spacing, the difference between their frequencies of cleavage must arise from the sequence of the cleavage/poly A addition site. There are two possible interpretations of these data. Either the location of the cleavage site in R7 RNA relative to the poly A signal (and the downstream element), or the sequences flanking the cleavage site in R7 RNA, is closer to the optimal arrangement than that in R1 RNA. These possibilities are not mutually exclusive.

Given the results of the cleavage experiments using R7 RNA described above, it was to be expected that R7 precursor would be efficiently sequestered into complex B when incubated with nuclear extract in the presence of ATP. A comparison of the formation of complex B on R1 and R7 precursors is shown in Fig 26B (lanes 1-6). A comparable proportion of each RNA was assembled into complex B, as expected since both were efficient substrates for 3' processing *in vitro*.

SECTION 4: DISCUSSION

A number of mutants of the *X laevis* β-globin gene poly A site were constructed and assayed for 3' processing activity by microinjection into oocytes (Mason *et al*, 1986). In a number of the mutants, the spacing between the poly A signal and a downstream GU-rich element was reduced by as much as 14nu. Although the position of cleavage was altered, the efficiency of processing was at least 70% of that of the wild-type poly A for each mutant tested. The spacing alterations had minimal effects on the efficiency of processing.

In vitro experiments using the SV40 E poly A site and an SV40-Ad2 E2A composite poly A site and variants in which the downstream elements were placed at various

distances downsteam of the poly A signal showed that these poly A sites were extremely sensitive to increased spacing between the poly A signal and the downstream element; increasing the spacing by only 12nu in one case led to a reduction in the efficiency of processing of greater than 50% (McDevitt *et al*, 1986).

Using mutants of the HSV-1 tk gene poly A site in transfection experiments, it was shown that a spacing of 6nu between the poly A site and the downstream element was too short for efficient 3' processing, while a spacing of 30nu worked almost as efficiently as the wild-type spacing of 18 nu: the construct containing the 30nu spacing gave rise to an altered position of cleavage (Zhang *et al*, 1986).

Both the efficiency of processing and the position of cleavage were altered by changing the spacing between the poly A signal and the downstream GU-rich element of the synthetic poly A site described here. Reduction of the spacing by 14nu eliminates processing while a 10nu reduction diminishes cleavage to less than 5% of the prototype site. Increasing the spacing has a less radical effect but a 14nu increase still causes a 70% drop in processing efficiency. In these last two cases, novel positions of cleavage were utilised. A residues were favoured as 3' terminal nucleotides, as they were in both R1 and R7 RNAs.

A preference for cleavage of the precursor RNA 3' of A residues was also observed in the *Xenopus* β-globin gene poly A site mutants, described by Mason *et al* (1986). However, cleavage immediately downstream of an A residue is not an absolute requirement at every poly A site, eg, HSV-2 IE-5 gene pre-mRNA is cleaved 3' of a U residue (McLauchlan *et al*, 1985) and SV40 L pre-mRNA 3' of a C residue (Sadofsky and Alwine, 1984). It may be that an A residue is required only in certain sites which possess a specific downstream sequence; the synthetic poly A site used for these studies may be one such.

As previously discussed in Section 3, the ability of a precursor RNA to form complex B is indicative of its being a substrate for efficient 3' processing. All of the precursor RNAs discussed in this section adhere to this rule, except R6 precursor which, despite being inefficiently processed, formed a significant amount of complex B. It is suggested that since the poly A signal and the downstream element in R6 are 10nu closer together than in R1, the components of complex B, SF and CstF, which bind to them may be so closely apposed as to prevent interaction of the additional cleavage factors with the RNA due to steric hindrance.

The increased spacing between the poly A signal and the downstream element in R5 precursor RNA results in a significant decrease in the efficiency of cleavage compared to R1 precursor RNA. Cleavage of R5 RNA occurs at three separate sites. At each site, an A residue (or residues) is preferred as the 3' terminal nucleotide. The quantitative and qualitative alterations of 3' processing observed when the spacing between the poly A signal and the downstream element was increased are probably due to decreased stability of the interaction between the factors which bind to these sequence elements. Complex B formation on R5 precursor occurs less efficiently than on R1 RNA, suggesting that the increased spacing between the poly A signal and the downstream element produces sub-optimal interaction between the precursor RNA and the appropriate nuclear factors. Increasing the spacing reduces the stability of the RNA-SF-CstF complex (Weiss *et al*, 1991).

The altered position of cleavage of R7 precursor is accompanied by an increase in the cleavage efficiency with no apparent decrease in the ability of the RNA to form complex B. The poly A site specified in R7 RNA must be closer to the optimum than that in R1 RNA. Since both RNAs contain the same sequences apart from those around the

Complex B may represent the stable tertiary complex containing RNA, SF and CstF.

cleavage site, the increased efficiency of cleavage of R7 RNA must be due entirely to some property of that sequence. In R7 precursor, the cleavage site is located four nucleotides further downstream than in R1 RNA. This arrangement may enhance interaction of R7 RNA with one or more nuclear 3' processing factors, thereby increasing the efficiency of cleavage. Alternatively, the precise sequence at or near the cleavage site in R7 RNA, irrespective of its position relative to the poly A signal, may represent a sequence which is more efficiently recognised by the 3' processing machinery.

Although the 3' processing machinery displays considerable flexibility with regard to the locations of both the cleavage site and the downstream sequence element relative to the AAUAAA hexanucleotide, it can be assumed that certain combinations of elements and spacing will be recognised more efficiently than others. Differences in efficiency of poly A site use may be mediated either at the stage of RNA-SF-CstF tertiary complex formation or at the subsequent interaction with CFI and CFII which results in cleavage. The stability of the RNA-SF-CstF complex is dependent on the nature of the downstream element and its location relative to the AAUAAA motif. Modulation of the interaction of CFI and CFII with the stable tertiary complex may be a function of either the spacing between AAUAAA and the downstream element or the nature of the intervening sequence. Such an arrangement would permit evolutionary fine-tuning of poly A sites to produce the range of efficiencies which have been observed. The addition of cell-type or development-stage specific processing factors would permit regulation of poly A site use, such as that observed at the immunoglobulin μ heavy chain locus.

Recognition of the essential 3' processing signals (AAUAAA and the downstream sequence element) is necessary for commitment of a poly A site to processing, but may not be sufficient to determine the precise location at which

cleavage occurs. The results obtained with R1, R5 and R6 precursor RNAs suggest that the location of the downstream sequence element relative to the poly A signal influences the position of the cleavage site. However, the results obtained with R1, R5 and R7 precursors indicate that the sequence between AAUAAA and the downstream sequence element modulates the interaction between the RNA in the committed processing complex and the cleavage activity. The flexibility of the cleavage reaction is well illustrated by R5 precursor RNA which is cleaved at sites separated by as much as 16nu. Taken together, these results suggest that, while the commitment of a pre-mRNA to 3' processing by binding of SF and CstF is an essential initial step and determines the efficiency of processing, the location of the subsequent cleavage reaction involving CFI, CFII and, possibly, PAP is not entirely determined by the committed complex. The binding of SF and CstF probably defines limits within which cleavage must occur.

SECTION 5: RESULTS

Sequence requirements for pre-mRNA 3' processing *in vivo*

Transfection of plasmids containing the bacterial CAT gene under the control of appropriate eukaryotic transcription elements into mammalian cells results in the synthesis of the CAT gene product, which is easily assayed *in vitro* following lysis of the transfected cells (see Materials and Methods).

Plasmid pLW1 (Fig 9) contains the CAT gene under the control of the HSV-2 IE4/5 promoter; pLW1 contains no 3' processing signals. Plasmid pTER5 contains the CAT gene and promoter sequences as described for pLW1 plus additional sequences representing the HSV-2 IE-5 mRNA poly A site placed downstream from the 3' end of the CAT gene. Transfection of pLW1 or pTER5 into HeLa cells produces low basal levels and high levels of CAT activity, respectively (Fig 27). The increased level of CAT activity produced from pTER5 is likely due to sequence-directed 3' end formation; in pLW1, 3' end formation probably occurs at a cryptic poly A site in the bacterial plasmid sequences. Thus, pLW1 provides a background in which to test DNA sequences for their ability to direct efficient 3' processing.

As described previously, the synthetic poly A site was initially cloned into the unique HindIII site downstream of the CAT gene in plasmid pLW1, to produce plasmid pR1.CAT. The synthetic poly A site sequences in plasmid pR1.CAT were manipulated *in vitro* to produce a number of variants (Fig 28).

Plasmid pR3.CAT was produced by digesting pR1.CAT with SalI and BglII, treating the DNA with Klenow fragment and dNTPs to produce blunt ends and religating to delete the GT-rich downstream element. Digestion of pR1.CAT with XhoI and SalI, followed by religation, produced pR4.CAT and

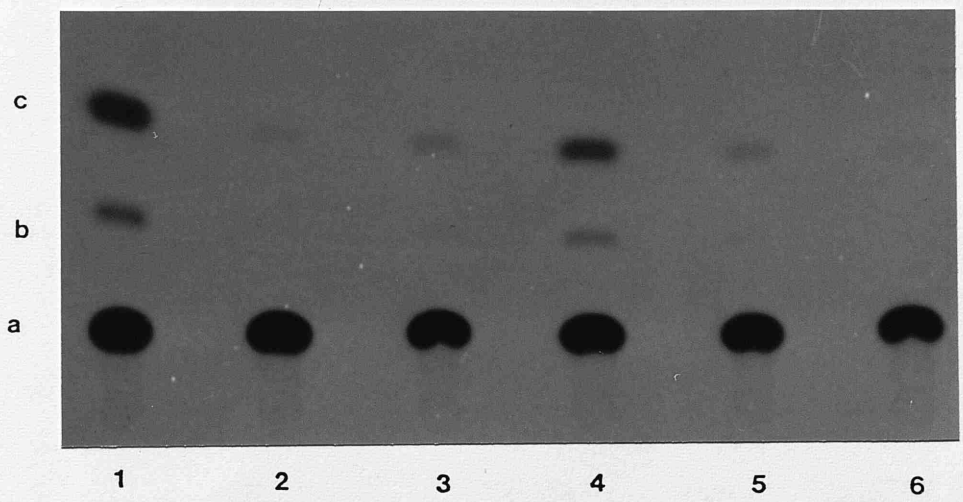
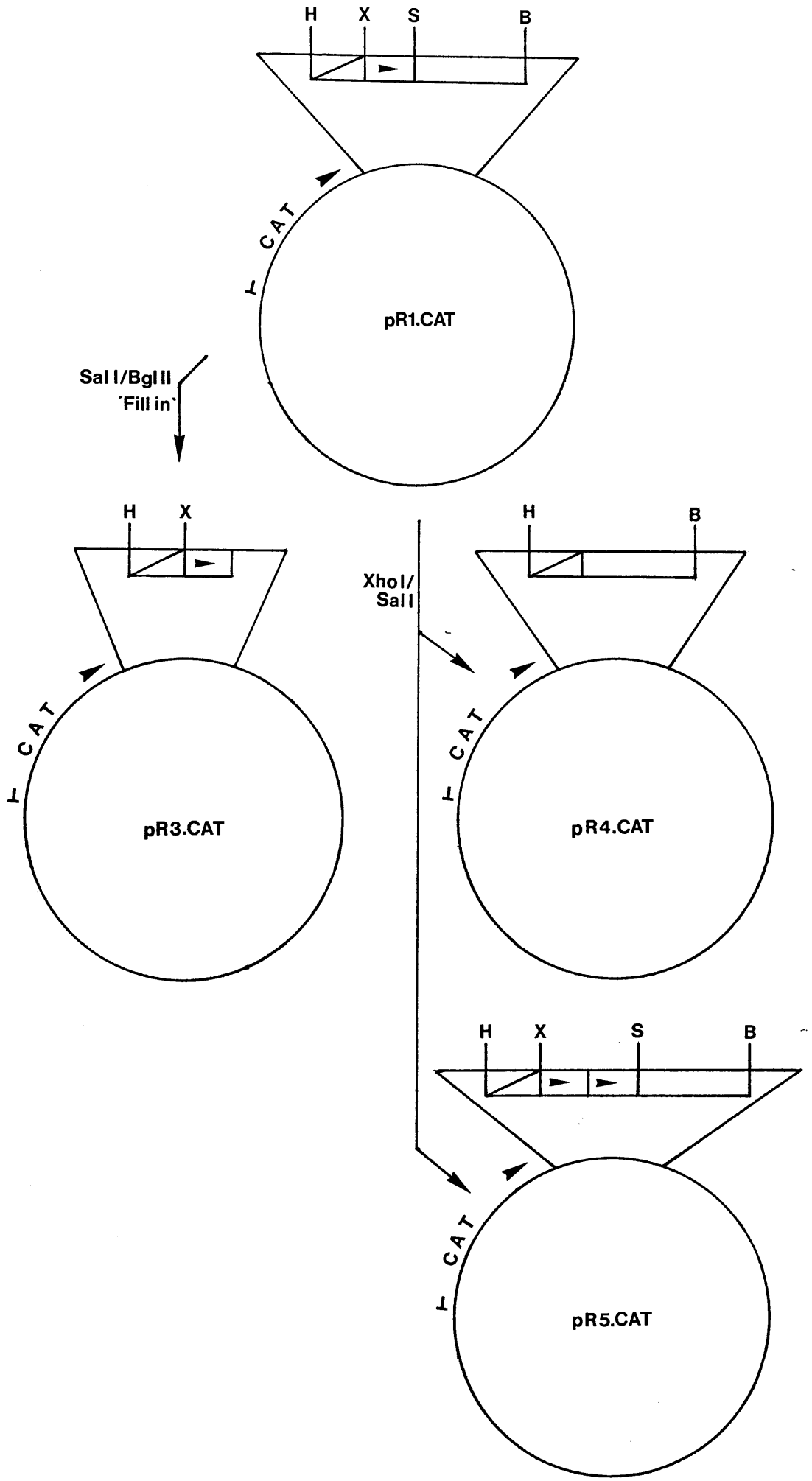


Figure 27. Comparison of CAT activities produced by plasmids pLW1, pTER5, pR1.CAT, pR3.CAT, pR4.CAT and pR5.CAT.

HeLa cells were transfected with 10µg of plasmids pLW1, pTER5, pR1.CAT, pR3.CAT, pR4.CAT or pR5.CAT. Cell extracts were prepared and CAT assays performed, as described in Materials and Methods. Lane 1, pTER5; lane 2, pLW1; lane 3, pR5.CAT; lane 4, pR1.CAT; lane 5, pR4.CAT; lane 6, pR3.CAT. The positions of unmodified chloramphenicol (a), 1-acetylchloramphenicol (b) and 3-acetylchloramphenicol (c) are indicated.

Figure 28. Construction of plasmids pR3.CAT, pR4.CAT and pR5.CAT (not to scale).

The positions of the chloramphenicol acetyl transferase gene coding region (⊢ CAT ➤) and relevant restriction endonuclease recognition sites (H = HindIII, X = XhoI, S = SalI and B = BglII) are indicated.


H
X
S
B
CAT
pR1.CAT
Sal I/Bgl II
'Fill in'
H
X
CAT
pR3.CAT
Xho I/
Sal I
H
B
CAT
pR4.CAT
H
X
S
B
CAT
pR5.CAT

pR5.CAT. In pR4.CAT, the XhoI/SalI fragment was deleted, while in pR5.CAT, two XhoI/SalI fragments were inserted, in the same orientation as in plasmid pR5. Similarly, pR1.CAT, pR3.CAT and pR4.CAT contain the same poly A site sequences as pR1, pR3 and pR4, respectively.

The results of representative CAT assays using extracts of HeLa cells transfected with equal amounts of plasmids pTER5, pLW1, pR1 CAT, pR3. CAT and pR4. CAT are shown in Fig 27.

Cells transfected with pR1.CAT produced significantly more CAT activity than those transfected with pLW1, which lacks a poly A site, but the activities were only about 20% of the CAT activity produced from pTER5, which contains the HSV IE-5 gene poly A site. This indicates that the synthetic poly A site in pR1.CAT functions relatively efficiently in HeLa cells (ie, the oligonucleotides contain sequences necessary and sufficient to direct 3' processing). Plasmid pR3.CAT, which contains a synthetic poly A site from which the GT-rich element was deleted, produced a level of CAT activity indistinguishable from that produced by pLW1, indicating that the downstream element is necessary to direct efficient 3' processing *in vivo*. Decreasing or increasing the spacing between the polyadenylation signal and the downstream element, as in plasmids pR4.CAT and pR5.CAT, resulted in significant reductions in CAT activity relative to pR1.CAT. However, both of these plasmids produced approximately two-fold higher levels of CAT activity than pLW1, indicating that 3' processing was not completely abolished.

SECTION 5: DISCUSSION

The results presented in this section are in broad agreement with those reported in sections 1, 3 and 4: (i) the synthetic oligonucleotides specify a functional polyadenylation site; (ii) the previously defined sequence

elements (the poly A signal and the downstream element) are essential for 3' processing at the synthetic poly A site; and (iii) the efficiency of the poly A site is altered by varying the spatial relationship of the poly A signal and the downstream element.

Obviously, the HeLa cell transfection system is less versatile than the cell-free *in vitro* 3' processing system. In the former, no means are currently available to investigate directly interactions between pre-mRNAs and nuclear 3' processing factors. The *in vitro* system provides opportunities for biochemical fractionation and purification of individual components of the 3' processing machinery.

SECTION 6: RESULTS

3' processing *in vitro* is inhibited by hybridising RNAs complementary to specific regions of the precursor RNA

Interaction of a complementary oligonucleotide with the poly A signal in precursor RNA, as detected by the appearance of RNase H cleavage products, is prevented when precursor RNA is incubated with nuclear extract under 3' processing conditions before addition of the oligonucleotide (J McLauchlan, personal communication). Furthermore, when an oligonucleotide complementary to the poly A signal is annealed to precursor RNA, formation of processing complexes and cleavage of the RNA are inhibited (Zarkower and Wickens, 1987a). This suggests that a nuclear factor interacts with the poly A signal during the 3' processing reaction. Use of oligonucleotides complementary to RNA regions other than the poly A signal does not interfere with mRNA 3' processing or complex formation (J McLauchlan, personal communication; Zarkower and Wickens, 1987a), suggesting that either the downstream signals required for processing can function when base-paired to DNA or the downstream oligonucleotides are actively removed during complex assembly.

The experiments described here, using stable RNA/RNA hybrids, were designed to enable detection of both stable and transient RNA/nuclear factor interactions. Unlabelled RNA molecules complementary to all or part of the R1 precursor were synthesised using T7 RNA polymerase, from appropriately digested plasmid DNA, as shown in Figure 29. The cRNAs were hybridised overnight (as described in Materials and Methods) and ethanol precipitated.

A portion of each redissolved hybrid RNA was subjected to RNAase T_2 digestion (as described in Materials and Methods) in order to confirm the integrity of the hybrids (Fig 30). Protected RNA species of 84, 66, 52 and 22nu were produced by RNAase T_2 digestion of hybrids 1,

Figure 29. Nucleotide sequences of complementary RNAs 1-6.

Nucleotide sequences of the RNAs produced by T7 transcription of the following DNAs:-

cRNA 1 - HindIII-digested pR1
cRNA 2 - XhoI-digested pR1
cRNA 3 - SalI-digested pR1
cRNA 4 - HindIII-digested pR3
cRNA 5 - XhoI-digested pR3
cRNA 6 - BamHI-digested pGEM1

The cRNAs are aligned with the sequence of R1 precursor RNA to indicate regions of complementarity. Lower case letters denote vector-derived sequences, while upper case letters denote synthetic poly A site sequences. The R1 RNA cleavage sites are indicated (arrows).

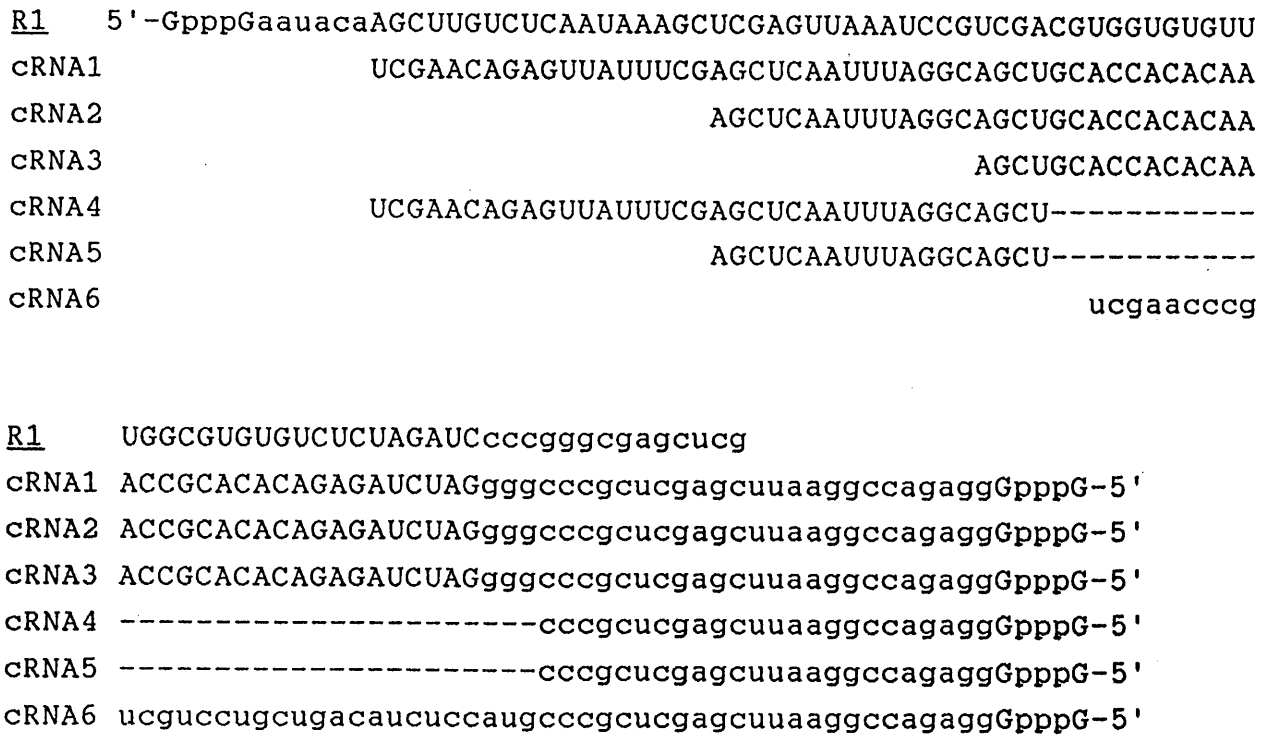
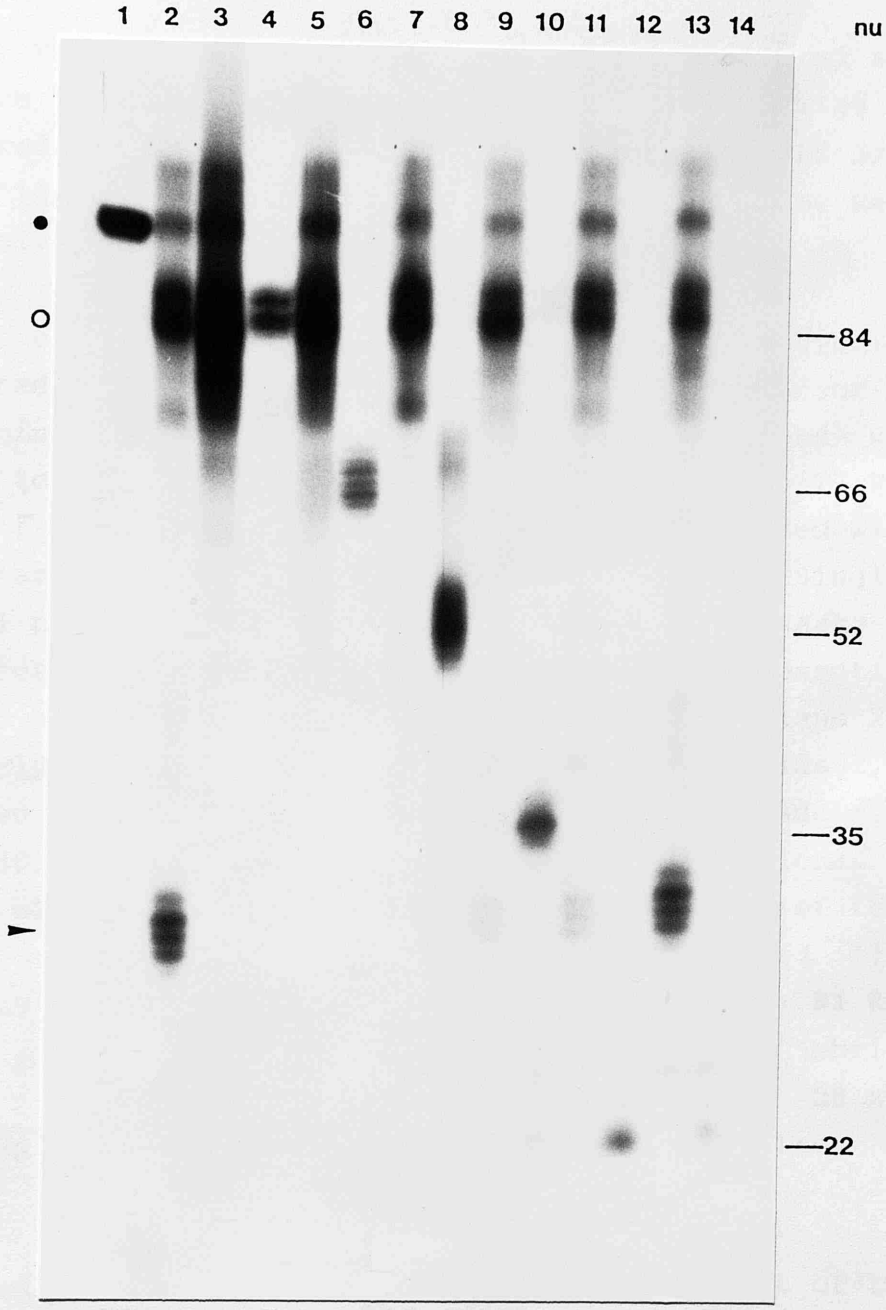

R1 5'-GpppGaauacaAGCUUGUCUCAAUAAAGCUCGAGUUAAAUCCGUCGACGUGGUGUGUU
cRNA1 UCGAACAGAGUUAUUUCGAGCUCAAUUUAGGCAGCUGCACCACACAA
cRNA2 AGCUCAAUUUAGGCAGCUGCACCACACAA
cRNA3 AGCUGCACCACACAA
cRNA4 UCGAACAGAGUUAUUUCGAGCUCAAUUUAGGCAGCU----------
cRNA5 AGCUCAAUUUAGGCAGCU----------
cRNA6 ucgaacccg
R1 UGGCGUGUGUCUCUAGAUCcccgggcgagcucg
cRNA1 ACCGCACACAGAGAUCUAGgggcccgcucgagcuuaaggccagaggGpppG-5'
cRNA2 ACCGCACACAGAGAUCUAGgggcccgcucgagcuuaaggccagaggGpppG-5'
cRNA3 ACCGCACACAGAGAUCUAGgggcccgcucgagcuuaaggccagaggGpppG-5'
cRNA4 ----------cccgcucgagcuuaaggccagaggGpppG-5'
cRNA5 ----------cccgcucgagcuuaaggccagaggGpppG-5'
cRNA6 ucguccugcugacaucuccaugcccgcucgagcuuaaggccagaggGpppG-5'

Figure 30. Comparison of processing efficiencies of hybrid RNAs 1-6 and R1 RNA.

In vitro 3' processing reactions containing ATP were performed for two hours and the RNAs analysed by denaturing polyacrylamide gel electrophoresis, as described in Materials and Methods. Hybrid RNAs containing R1 RNA and either cRNA1, cRNA2, cRNA3, cRNA4, cRNA5 or cRNA6 (hybrids 1-6) were prepared as for RNAase protection assays (see Materials and Methods). An aliquot of each hybrid RNA was subjected to RNAase T_2 protection analysis without undergoing 3' processing in order to confirm its integrity. Lane 1, unprocessed R1 precursor RNA; lane 2, R1 RNA, ATP reaction, RNAase T_2 protection assay; lanes 3 and 4, hybrid 1; lanes 5 and 6, hybrid 2; lanes 7 and 8, hybrid 3; lanes 9 and 10, hyrid 4; lanes 11 and 12, hybrid 5; lanes 13 and 14, hybrid 6; lanes 3, 5, 7, 9, 11 and 13, ATP reaction, RNAase T_2 protection assay; lanes 4, 6, 8, 10, 12 and 14, hybrid RNA, RNAase T_2 protection assay. The positions of unprocessed precursor RNA (●), unprocessed, RNAase T_2-protected RNA (O) and RNAase T_2-protected cleaved RNA (►) are indicated.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
nu
84
66
52
35
22

Addition of tRNA to processing reactions did not affect cleavage of R1 RNA (data not shown).

2, 3 and 6, respectively (Fig 30, lanes 4, 6, 8, 10, 12 and 14). RNAase T_2 digestion of hybrids 4 and 5 gave rise to two protected species in each case: these are of 35 and 22nu and 22 and 14nu, respectively. The 22 and 14nu species were poorly resolved by this gel.

The efficiencies of cleavage of the RNA/RNA hybrids were compared with that of single-stranded R1 precursor in ATP-containing reactions. RNA from these reactions was hybridised to complementary RNA transcribed with T7 RNA polymerase from HindIII-digested pR1 DNA then digested with RNAase T_2 (as described in Materials and Methods). Single-stranded R1 precursor was efficiently cleaved; RNAase T_2 produced protected species of 26, 27 and 28nu, representing the UCPs, as described in Section 1 (Fig 30, lane 2). Approximately 50% of the R1 RNA was cleaved. Hybrids 1, 2 and 3 failed completely to be cleaved at the poly A addition site (Fig 30, lanes 3, 5 and 7) while the efficiency of processing of hybrids 4 and 5 was less than 10% of that observed for R1 precursor (Fig 30, lanes 9 and 11). Hybrid 6 was cleaved approximately as efficiently as R1 RNA (Fig 30, lane 13). Cleavage of the precursor RNA in hybrids 4, 5 and 6 produced protected species of 26, 27 and 28 nu, representing the same UCPs as those produced from R1 precursor.

Thus, RNA/RNA hybrids in which all or part of the oligonucleotide-specified sequence was double-stranded were less efficient substrates for cleavage *in vitro* than single-stranded R1 precursor; in contrast, the efficiency of cleavage of a RNA/RNA hybrid, in which only vector-derived sequences were double-stranded, was unaltered relative to single-stranded R1 RNA. These results suggest that efficient cleavage of substrate RNA requires that all of the oligonucleotide-derived sequences be single-stranded, presumably to permit binding of the necessary nuclear factors to the RNA.

The abilities of the RNA/RNA hybrids to interact with the 3' processing-specific nuclear factors were investigated by testing their abilities to form complexes when incubated with nuclear extract in the presence of ATP (Fig 31). None of the hybrids, with the exception of hybrid 6 (Fig 31, lanes 13 and 14), formed amounts of complex B comparable with R1 precursor (Fig 31, lanes 1 and 2), although all were efficiently assembled into complex A. This suggests that the components of complex B are unable to interact with their target sequences in RNA when those sequences form part of a RNA/RNA duplex. Their reduced ability to form complex B (Fig 31, lanes 3-12) would explain the abolition or reduced efficiency of cleavage of hybrids 1-5 (Fig 30, lanes 3, 5, 7, 9 and 11). Formation of complex B on hybrid 6 was slightly less efficient than on R1 precursor suggesting that the presence of duplex RNA, even outside the oligonucleotide-derived sequences, may in itself be deleterious.

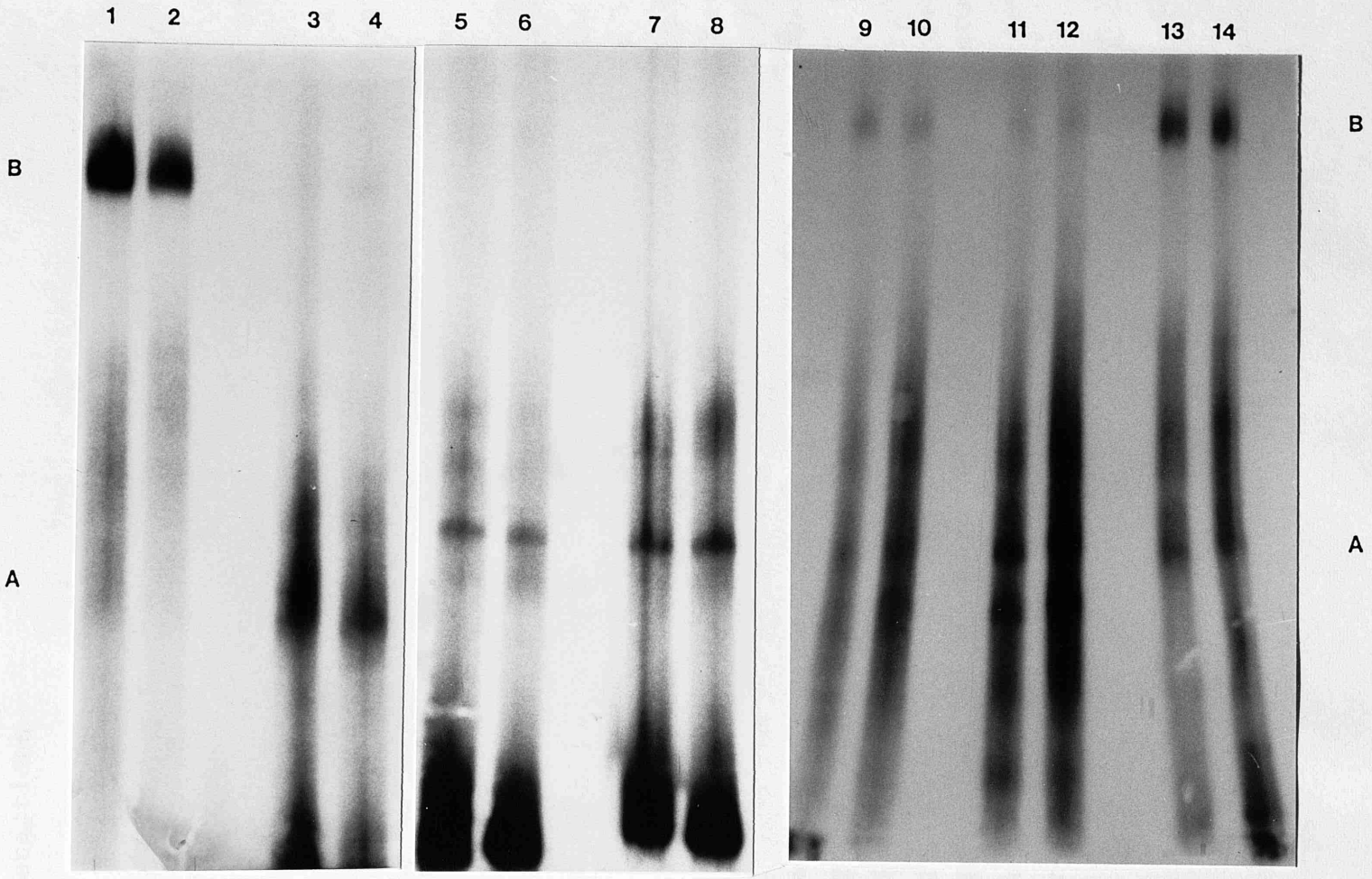
SECTION 6: DISCUSSION

Assuming that the RNA/RNA hybrids are stable when incubated with nuclear extract under processing conditions, and that processing factors are unable to interact with double-stranded RNA, inhibition of processing and complex assembly indicates a requirement for an interaction between a processing factor and all or part of the double-stranded region of RNA. Nuclear extracts might contain a RNA/RNA helicase which could disrupt double-stranded RNA but this does not appear to be the case since some of the hybrids have an effect on processing and complex formation.

Obviously, the large extents of the double-stranded regions of RNA present in hybrids 1 and 2 (see Fig 29) are sufficient to encompass, at least in part, more than one signal at which factors interact with the RNA. Therefore,

Figure 31. Comparison of complex formation on hybrid RNAs 1-6 and R1 RNA.

In vitro 3' processing reactions containing ATP were performed for the times indicated and the RNAs analysed by non-denaturing polyacrylamide gel electrophoresis, as described in Materials and Methods. Hybrid RNAs 1-6 were prepared as described for Figure 25. Lanes 1 and 2, R1 RNA; lanes 3 and 4, hybrid 1; lanes 5 and 6, hybrid 2; lanes 7 and 8, hybrid 3; lanes 9 and 10, hybrid 4; lanes 11 and 12, hybrid 5; lanes 13 and 14, hybrid 6; lanes 1, 3, 5, 7, 9, 11 and 13, reaction time, zero; lanes 2, 4, 6, 8, 10, 12 and 14, 20 min. The positions of complexes A and B are indicated.


1
2
3
4
5
6
7
8
9
10
11
12
13
14
B
A
B
A

inhibition of processing and complex formation with these hybrids cannot be said to define individual RNA/factor interactions.

Since hybrid 3, but not hybrid 6, failed to be processed and to form complex B, the presence of double-stranded RNA at the GU-rich element must prevent an essential interaction between the precursor RNA and a nuclear factor(s), a component of complex B: this factor is most likely CstF (see p 44). No such interaction was detected using complementary oligonucleotides (J McLauchlan, personal communication; Zarkower and Wickens, 1987a).

The results obtained with hybrids 4 and 5 are somewhat equivocal. Given others' results obtained using complementary oligonucleotides, it could be assumed that inhibition of complex B formation and 3' processing of hybrid 4 was entirely due to blocking of the binding of SF to the precursor RNA (see p 44). However, hybrid 5 has the same properties as hybrid 4, despite the fact that, in the former case, the poly A signal is single-stranded. The double-stranded region of hybrid 5 extends to three nucleotides downstream of the poly A signal and could overlap part of the binding site for SF. Alternatively, this double-stranded region may interfere with previously unreported binding of a factor(s) to the RNA sequence at or near the cleavage/poly A addition site.

SECTION 7: RESULTS

The DNA oligonucleotide GGTGTGTT can inhibit 3' processing *in vitro*

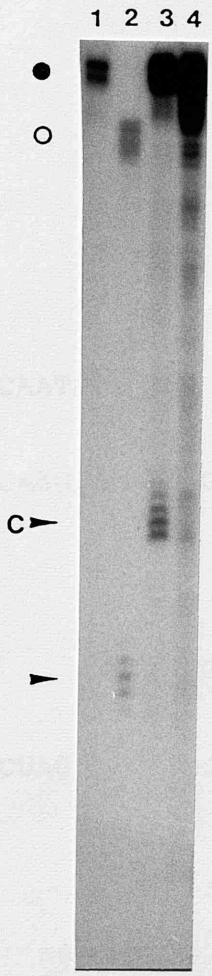
While the sequence elements of poly A sites function at the level of RNA through interactions with nuclear processing factors, there is no evidence that the same signals function at the level of DNA. However, it was observed that when nuclear extract was pre-incubated with oligonucleotide 67A prior to addition of R1 precursor RNA, the efficiency of cleavage of the precursor was significantly reduced (Fig 32, lanes 3 and 4). Oligonucleotide 67A is of the same sense as precursor RNA and contains all of the DNA sequences already shown to be necessary and sufficient, in RNA, for efficient 3' processing.

Based on the assumption that the inhibition of cleavage by oligonucleotide 67A represented an interaction of a specific nuclear factor(s) with the same target sequence in DNA as its normal target sequence in RNA, four eight residue oligonucleotides were synthesised in order to further analyse this interaction. The sequences of the 8-mers, oligonucleotides 1-4 (Fig 33), correspond to the poly A signal, the cleavage/poly A addition site, the GU-rich downstream element and a downstream plasmid sequence, all of which are represented in R1 precursor RNA.

Each of the 8-mer oligonucleotides was tested for its ability to inhibit 3' processing and complex B formation, using R1 precursor RNA (Figs 34 and 35). Pre-incubation of nuclear extract with oligonucleotide 3 produced a significant reduction in both complex B formation (Fig 35, lanes 7 and 8) and 3' processing (Fig 34, lanes 7, 8, 16 and 17) of R1 precursor, suggesting that the factor(s) which binds to the GU-rich element in the precursor RNA is also able to interact with the corresponding sequence in

Figure 32. Effect of oligonucleotide 67A on processing of R1 RNA.

In vitro 3' processing reactions were performed using R1 precursor RNA, as described in Materials and Methods, except that, where indicated, nuclear extract was pre-incubated with DNA oligonucleotide for 15 min at 30 C. Lane 1, unprocessed R1 precursor RNA; lane 2, ATP reaction, RNAase T_2 protection assay; lane 3, 3' dATP reaction; lane 4, oligonucleotide 67A (0.2µg), 3' dATP reaction. The positions of unprocessed precursor RNA (●), unprocessed RNAase T_2-protected (O), RNAase T_2-protected cleaved RNA (►) and cleaved RNA (C ►) are indicated.


1 2 3 4
C

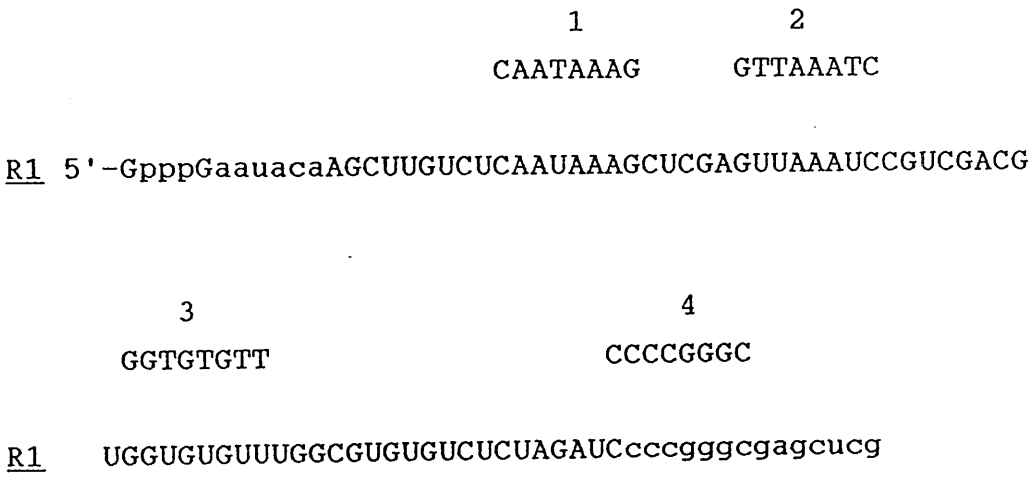


Figure 33. Nucleotide sequences and locations of oligonucleotides 1-4.

The nucleotide sequences of oligonucleotides 1-4 are shown aligned with the corresponding sequences in R1 precursor RNA.

Figure 34. Effect of oligonucleotides 1-4 on processing of R1 RNA.

In vitro 3' processing reactions were performed using R1 precursor RNA and the RNAs analysed by denaturing polyacrylamide gel electrophoresis, as described in Materials and Methods, except that, where indicated, nuclear extract was pre-incubated with DNA oligonucleotide for 15 min at 30 C. Lane 1, unprocessed R1 precursor RNA; lanes 2-10, ATP reaction; lanes 11-19, 3' dATP reaction; lanes 3 and 12, oligonucleotide 1 (0.02µg); lanes 4 and 13, oligonucleotide 1 (0.1µg); lanes 5 and 14, oligonucleotide 2 (0.02µg); lanes 6 and 15, oligonucleotide 2 (0.1µg); lanes 7 and 16, oligonucleotide 3 (0.02µg); lanes 8 and 17, oligonucleotide 3 (0.1µg); lanes 9 and 18, oligonucleotide 4 (0.02µg); lanes 10 and 19, oligonucleotide 4 (0.1µg). The positions of unprocessed precursor RNA (●), cleaved RNA (►) and poly A+ RNA (P) are indicated.

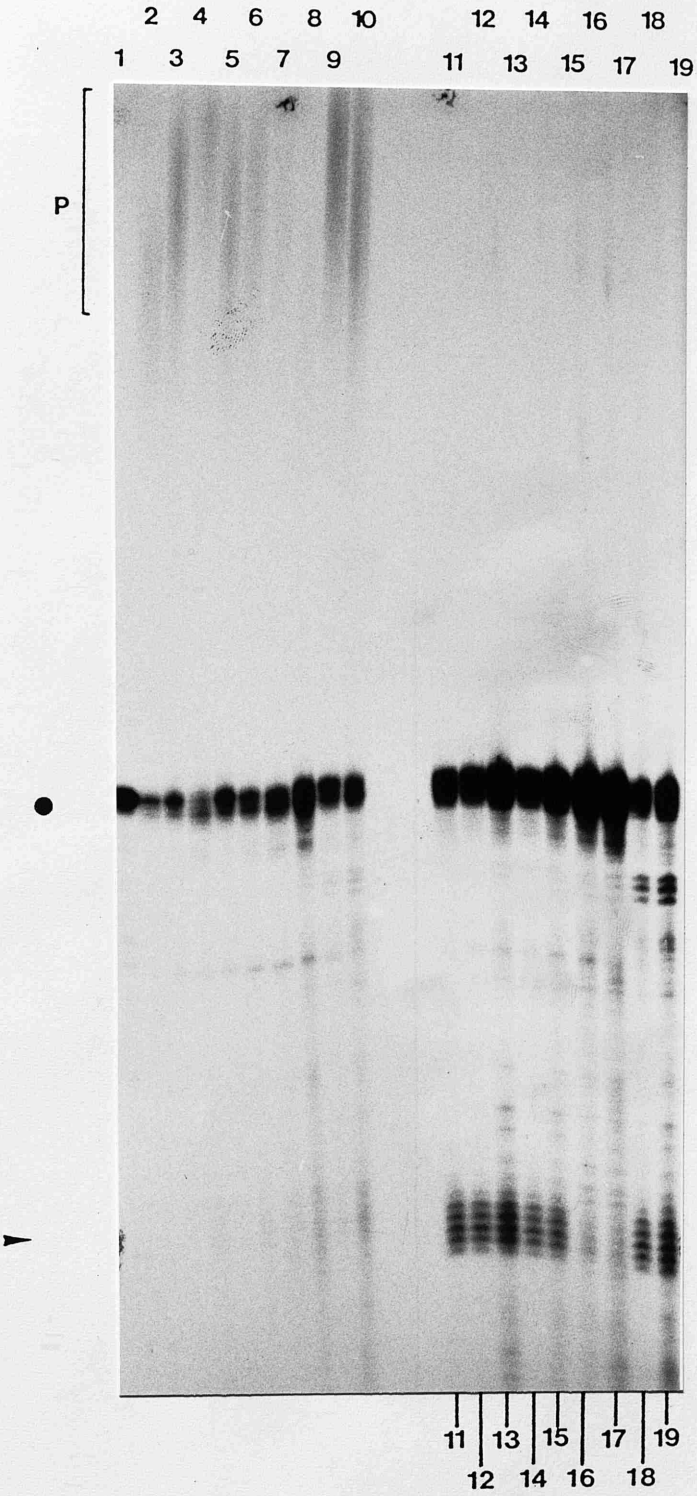
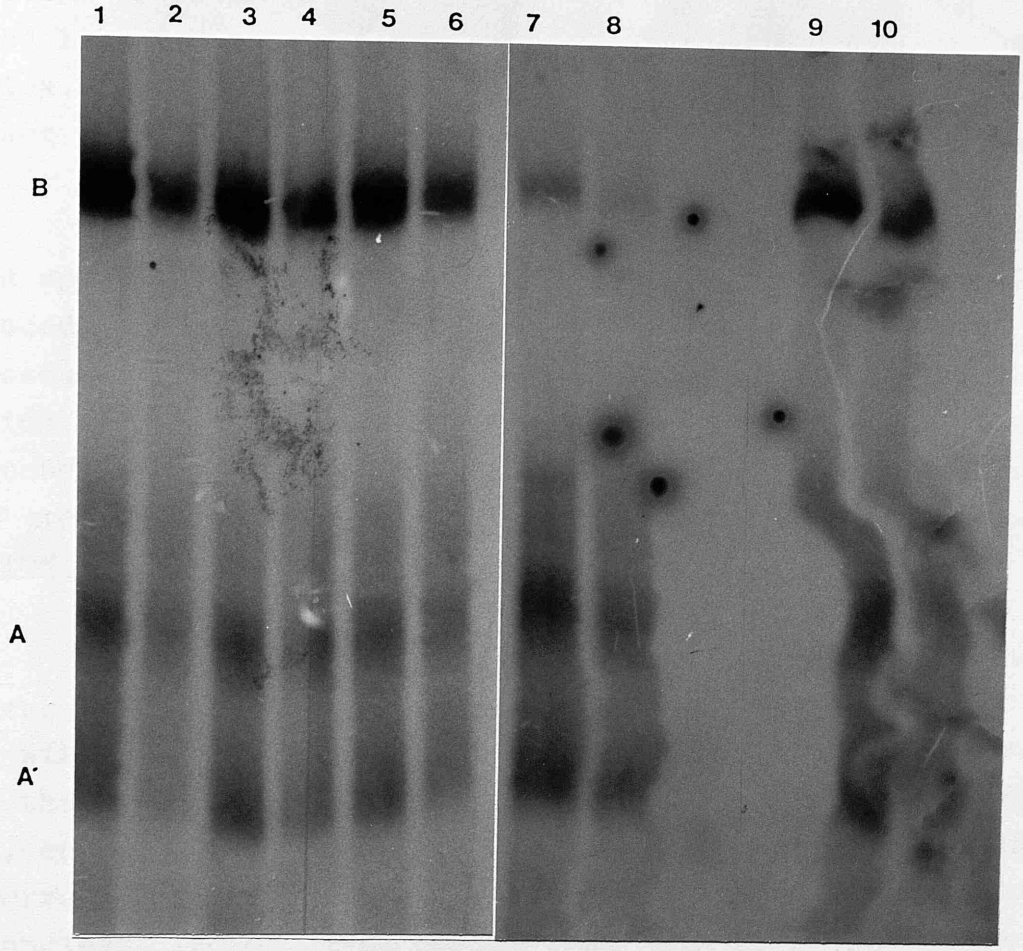

2 4 6 8 10 12 14 16 18
1 3 5 7 9 11 13 15 17 19
P
11 13 15 17 19
12 14 16 18

Figure 35. Effect of oligonucleotides 1-4 on complex formation on R1 RNA.

In vitro 3' processing reactions containing ATP were performed using R1 precursor RNA for the times indicated and the RNAs analysed by non-denaturing polyacrylamide gel electrophoresis, as described in Materials and Methods, except that, where indicated, nuclear extract was pre-incubated with DNA oligonucleotide for 15 min at 30 C. Lanes 1 and 2, R1 RNA only; lanes 3 and 4, oligonucleotide 1 (0.1µg); lanes 5 and 6, oligonucleotide 2 (0.1µg); lanes 7 and 8, oligonucleotide 3 (0.1µg); lanes 9 and 10, oligonucleotide 4 (0.1µg); lanes 1, 3, 5, 7 and 9, reaction time, zero; lanes 2, 4, 6, 8 and 10, 20 min. The positions of complexes A, A' and B are indicated.


1
2
3
4
5
6
7
8
9
10
B
A
A'

single-stranded DNA. None of the other oligonucleotides had a significant effect on the cleavage efficiency or complex B formation of R1 precursor (Figs 34, lanes 3-6, 9, 10, 12-15, 18 and 19 and Fig 35, lanes 3-6, 9 and 10). Formation of complex A was unaffected by pre-incubation of nuclear extract with any of the 8-mer oligonucleotides (Fig 35).

In reactions containing oligonucleotide 4, three bands approximately 15-20nu shorter than R1 precursor were produced (Fig 34, lanes 18 and 19). These species probably represent UCPs produced by RNase H cleavage of DNA/RNA hybrids located at the RNA sequence corresponding to oligonucleotide 4 because oligonucleotide 4 is palindromic and can anneal to the precursor RNA.

SECTION 7: DISCUSSION

These results suggest that one or more of the nuclear factors required for 3' processing can interact not only with RNA but also with DNA. In particular, it appears that the factor which interacts with the GU-rich element, CstF, can also bind to a single-stranded DNA oligonucleotide of corresponding sequence. It is unclear whether this DNA/nuclear factor interaction represents a significant activity *in vivo* or is an artefact of the *in vitro* 3' processing system.

Further experimentation will be required to establish the specificity of the DNA/factor interaction, by means of gel retardation assays and DNA footprinting, for example. Given the differences between the structures of RNA and DNA, binding of a polypeptide to the equivalent sequence in both may seem unlikely. However, a polypeptide which binds to both DNA and RNA has been identified. TF III A binds to the internal control region of the 5S ribosomal RNA (rRNA) gene (Engelke *et al*, 1980; Sakonju *et al*, 1980; Bogenhagen *et al*, 1980) and to 5S rRNA itself (Guddat *et al*, 1990) although it is not clear whether the same domains of the protein are involved in contacting both

DNA and RNA or whether TF III A binds to the equivalent sequence element in DNA and RNA.

The observation of 3' processing factor/DNA binding raises the possibility that such interactions may serve to facilitate rapid assembly of 3' processing complexes on nascent pre-mRNAs *in vivo*, perhaps by direct transfer of factors from DNA to RNA as the transcription fork traverses the poly A site. Precursor RNAs are rapidly polyadenylated following cleavage *in vivo* (Darnell, 1982; Montell *et al*, 1983; McDevitt *et al*, 1984).

The possibility that the nuclear factors which interact with the other sequence elements represented by oligonucleotides 1 and 2 (ie the poly A signal and the cleavage site, respectively) may also be able to interact with corresponding DNA sequence elements cannot be entirely ruled out. The oligonucleotides tested here may have lacked sufficiently extensive sequences to permit factor/DNA interaction.

GENERAL DISCUSSION

Poly A site sequences

The degeneracy of the downstream sequence element, while permitting "fine-tuning" of poly A site efficiency, may also serve to protect against complete loss of poly A site function due to a single nucleotide substitution or a small insertion or deletion. In this respect, the sole target for such a mutation leading to lack of function is the hexanucleotide, AAUAAA. Even in this element, some single base changes may not produce a deleterious effect. Functional poly A sites which contain hexanucleotides which differ from the canonical AAUAAA probably contain sequences which compensate for the suboptimal nature of the poly A signal. Such compensatory sequences may act to increase binding of the 170kD component of SF to the pre-mRNA. Downstream sequence elements could effect this role since binding of CstF, which is dependent on the downstream element, influences the binding of SF. Sheets *et al* (1990) have shown that some base substitutions in AAUAAA have little effect on polyadenylation.

It seems that there is an infinite variety of poly A site efficiency. Differences in the precise sequence of the downstream element and in the relative spatial arrangement of the AAUAAA hexanucleotide and the downstream element, together with the possible presence of recognition sequences for processing factors which can influence the stability of the committed processing complex containing SF, CstF and pre-mRNA, may combine to influence the efficiency of processing. In addition, the concentration of processing factors, some of them specific for particular classes of poly A sites, may vary in response to intra- or extracellular events or to differentiation. Given these variables, the efficiency of processing may vary, not only from one poly A site to another, but also between cell types.

Binding of SF to AAUAAA involves protein interaction with both base and ribose moieties at different nucleotide positions (Bardwell *et al*, 1991). It is possible that a larger contribution of protein/ribose interactions to the binding of CstF to downstream sequence elements may help to explain the lower extent of sequence conservation in these elements.

Recognition and cleavage of RNA

Several RNA processing events other than mRNA 3' end formation involve endonucleolytic cleavage of the primary transcript.

Precursor tRNA molecules (pre-tRNAs) undergo endonucleolytic cleavage catalysed by two distinct nucleases, RNAaseP and splicing endonuclease, which generate the mature tRNA 5' end and remove the introns present in some pre-tRNAs, respectively. Unlike cleavage at the poly A addition site in pre-mRNAs, recognition and cleavage specificity of pre-tRNAs by both RNAaseP and splicing endonuclease appear to be directed by secondary structure elements of the mature tRNA present in the pre-tRNA, rather than by specific sequence elements (reviewed by Mattaj, 1990). Given the highly conserved secondary structure of mature tRNAs, such an arrangement allows the recognition of a family of RNA molecules of diverse primary sequence by the same enzymes.

In contrast, the RNA sequences required for pre-mRNA cleavage and poly A addition are highly diverse in terms of both their primary and secondary structures. Although almost all poly A sites contain the hexanucleotide, AAUAAA, poly A site efficiency depends also on the much less highly conserved downstream elements and the relative positions of these elements.

A cleavage and polyadenylation mechanism, based upon a model similar to that observed for RNAaseP and splicing endonuclease, wherein cleavage to produce the poly A addition site occurs at a set distance from a common signal (AAUAAA, for example) would permit the polyadenylation of all mRNAs. However, pre-tRNAs are transcribed from simple transcription units and there is no need to regulate their maturation. The existence of a more complicated mechanism for the recognition and processing of poly A sites has permitted the evolution of complex transcription units, control of whose expression absolutely requires that the process of mRNA 3' end formation be amenable to regulation.

Primer RNA from the leading strand origin of mammalian mitochondrial DNA replication is cleaved in a site-specific manner by an endonuclease, RNase MRP. Cleavage occurs at a measured distance downstream from an essential G-rich sequence element (Karwan *et al*, 1991). Like RNaseP, RNase MRP is a ribonucleoprotein. Both complexes contain a single RNA species. The RNA components of RNaseP and RNase MRP contain limited regions of sequence homology (Gold *et al*, 1989). These sequence similarities and the common definition of the cleavage site by a distal sequence or structural element suggests that the mechanisms of substrate recognition and cleavage may be similar.

Pre-mRNA splicing also involves endonucleolytic cleavage of the primary transcript. The chemical mechanism of RNA splicing is well understood (reviewed by Cech and Bass, 1986). First, the 5' splice site undergoes endonucleolytic cleavage with concomitant ligation of the 5' end of the intron to the so-called branchpoint, between 18 and 40 nucleotides upstream from the 3' splice site, to form a lariat. Second, endonucleolytic cleavage at the 3' splice site releases the intron lariat and the exons are ligated.

Group 2 introns are capable of self-splicing, the RNA itself catalysing the reactions (reviewed by Cech and

Bass, 1986). In contrast, the splicing of pre-mRNA introns, while following the same chemical mechanism, requires a number of trans-acting nuclear factors including U snRNPs and several proteins. These factors assemble on the substrate RNA to form a large complex known as the spliceosome within which splicing occurs. It seems likely that the active spliceosome assembles only after initial recognition of the individual sequence elements by the appropriate factors and the formation thereby of pre-splicing complexes (reviewed by Mattaj, 1990).

It is not clear whether the enzymatic activities required for pre-mRNA splicing are provided by components of the spliceosome, either proteins or U snRNAs, or reside within the substrate RNA, the spliceosome being required only to provide a matrix for the correct alignment of different elements of the autocatalytic unit.

The sequences of the 3' and 5' splice sites, and of the branchpoints, are highly conserved in yeast, but to a lesser extent in plants and vertebrates. However, while these three sequence elements are sufficient for splicing in yeast, additional structural elements are required for splicing of plant and vertebrate introns (reviewed by Green, 1989; Goodall and Filipowicz, 1989). Recognition of the 5' splice site and the branchpoint involves complementary base-pairing between these sequence elements and U1 and U2 snRNAs, respectively (Green, 1989; Parker *et al*, 1987; Zhuang and Weiner, 1989; Wu and Manley, 1989).

The 3' processing complex

Unlike spliceosome assembly, which apparently can be preceeded by the formation of individual, pre-splicing complexes either at the 5' splice site, at the 3' splice site or at the branchpoint, assembly of the poly A site processing complex seems to occur in an ordered manner *in vitro*. None of the individual components is capable of

forming a stable interaction with their target sequences on the pre-mRNA, although SF binds specifically to AAUAAA in the absence of the other factors required for cleavage. However, this interaction is reversible and the binary complex of SF and pre-mRNA is unstable (Gilmartin and Nevins, 1989; Takagaki *et al*, 1989). CstF, also, exhibits RNA binding activity, but in the absence of other factors this is non-specific in nature (Wilusz *et al*, 1990).

Formation of a stable, committed pre-processing complex occurs only when SF and CstF are bound to pre-mRNA *in vitro* (Gilmartin and Nevins, 1989). The stability of the ternary complex of SF, CstF and pre-mRNA, which is dependent on the nature of the downstream sequence element, determines the efficiency of processing at a particular poly A site (Weiss *et al*, 1991). Direct protein-protein contacts between SF and CstF probably induce conformation changes in constituents of both factors leading to an increased affinity for their target sequences. The influence of different downstream sequence elements on the stability of the committed complex (and consequently on the efficiency of cleavage) is likely mediated via different conformation changes in CstF and SF. If this is the case, the tertiary structures of these factors must be exquisitely sensitive to apparently minor variations in either the location or nucleotide sequence of the downstream element.

The requirement for PAP, in addition to SF, CstF, CFI and CFII, for cleavage at poly A sites other than the SV40 late poly A site suggests that, in the absence of PAP, the components of the cleavage complex occupy an inactive configuration. In this case, binding of PAP probably induces a conformation change in one or more factors to activate the complex. It seems unlikely that PAP is directly involved in interactions with the substrate RNA in its role in the cleavage reaction, since the enzyme displays no substrate specificity in the polyadenylation reaction in the absence of SF. This implies that substrate recognition

for PAP is mediated by SF. PAP's participation in the cleavage reaction is likely to involve its binding to SF.

That the SV40 L poly A site does not require PAP for the cleavage reaction implies that the downstream sequences of this poly A site have evolved so that the processing complex which assembles on the pre-mRNA assumes a different configuration from those which form on pre-mRNAs containing other poly A sites.

The different relative 'strengths' of poly A sites may result from different relative affinities between CstF and the poly A site downstream element, combined with variations in spacing between AAUAAA and the downstream element (Gilmartin and Nevins, 1989; Lanoix and Acheson, 1988). Regulation of processing at alternative poly A sites might be affected by fluctuations in the concentration of CstF, for binding of which the sites would compete (Galli *et al*, 1987, 1988; Gilmartin and Nevins, 1989).

Poly A polymerase

While SF and CstF alone are required for recognition of the poly A site, these factors are not sufficient for cleavage. The requirement for CFI, CFII and, in most cases, PAP suggests that the endonuclease activity may reside entirely in these factors. Alternatively, the endonuclease activity may require contributions from all of the components of the cleavage complex (SF, CstF, CFI, CFII and PAP), perhaps in the form of direct contacts with the pre-mRNA or with other complex components to induce conformational changes. In this respect, it is interesting to note that three direct contacts with RNA involving constituents of SF and CstF have been detected (Gilmartin and Nevins, 1991). Two of these interactions, involving the 170kD component of SF and the 64kD component of CstF, occur in the absence of other factors, although both are unstable. However, the binding of the 130kD component of SF is

detected only when both SF and CstF are present. Assuming that only one component of SF, either the 70kD or 130kD polypeptide, binds directly to AAUAAA, it is possible that the other component may be directly involved in catalysis.

PAP is necessary, but not sufficient, for polyadenylation of pre-cleaved RNAs *in vitro*. The partially-purified enzyme lacks AAUAAA-specificity (reviewed by Edmonds, 1989) and shows no marked preference for oligo A as a substrate for polyadenylation (Winters and Edmonds, 1973). AAUAAA- and oligo A-specificity are essential features of the two phases of poly A addition *in vitro* (Sheets and Wickens, 1989).

Recognition of AAUAAA and oligo A in the context of the polyadenylation reaction seem to be a function of SF. It is, perhaps, not surprising that SF provides PAP with AAUAAA-specificity since SF is already bound to pre-mRNA in conferring AAUAAA-specificity on the cleavage reaction. In most cases, interaction between SF and PAP seems to be a prerequisite for the cleavage reaction. This interaction may place PAP in the correct position relative to the cleavage site for immediate commencement of the polyadenylation reaction following release of CstF, CFI, CFII and the downstream cleavage product from the cleavage complex. Even at the SV40 L poly A site, where it is not required for the cleavage reaction, PAP may still interact with SF in this fashion.

SF is not the only factor capable of providing PAP with substrate specificity. A novel mammalian poly A-binding protein, PAB II, can act as an additional, alternative oligo A-specificity factor for elongation of the oligo A tail during the second, rapid phase of poly A addition (Wahle, 1991). However, control of poly A tail length seems to be dependent on the presence of both AAUAAA and SF.

Although either SF or PAB II alone produce a similar efficiency of poly A tail extension, in the presence of both factors, elongation of the poly A tail is much more efficient. Presumably, during the elongation phase of poly A addition, both SF and PAB II interact, either directly or indirectly, with PAP.

The requirement for SF and PAB II for initiation and elongation of the poly A tail (Gilmartin and Nevins, 1989; Sheets and Wickens, 1989; Wahle, 1991) suggests that SF and PAB II may represent constituent of a multi-subunit, sequence-specific PAP. There is a precedent for such an enzyme. Vaccinia virus PAP consists of two non-identical subunits (Moss *et al*, 1975) and mediates poly A addition in two phases (Shuman and Moss, 1988). The PAP activity may reside in one subunit, the other contributing regulatory functions.

Cytoplasmic polyadenylation

During oocyte maturation, cytoplasmic polyadenylation of specific mRNAs resulting in their translational activation requires both the hexanucleotide, AAUAAA, and a variable, U-rich cytoplasmic polyadenylation element (CPE) (Fox *et al*, 1989; McGrew *et al*, 1989; McGrew and Richter, 1990; Paris and Richter, 1990). Polyadenylation of mRNAs containing different CPEs is differentially regulated (Fox *et al*, 1989; McGrew and Richter, 1990; Paris and Richter, 1990).

The availability of an *in vitro* system in which maturation-specific, cytoplasmic polyadenylation is reproduced (Paris and Richter, 1990) will permit purification and identification of the factors required for the process. *In vitro* competition experiments have shown that at least one common factor is required for cytoplasmic polyadenylation of two differentially processed mRNAs (Paris and Richter, 1990).

By analogy with the nuclear processing of pre-mRNAs, two distinct factors are likely to be required to recognise AAUAAA and the CPE. The PAP which mediates the cytoplasmic reaction is probably the same enzyme which polyadenylates pre-mRNAs in the nucleus. The AAUAAA-recognition factor and the PAP are likely to be common requirements for cytoplasmic polyadenylation of all substrates. Differentiation between mRNAs which are polyadenylated at different times during maturation is probably dependent on the recognition of different CPEs by distinct factors, expression of which is temporally regulated.

3' processing in complex transcription units

The immunoglobulin μ heavy chain locus and the HSV late genes are the only transcription units in which regulated poly A site use has been defined as a contributory factor in their differential expression (McLauchlan *et al*, 1989; Peterson and Perry, 1989).

During evolution of the μ locus, the relative strengths of the μ_s poly A site and the C_4μ-to-M1 splicing signals must have evolved in concert to provide the necessary balance between the two processes. Any increase in the efficiency of C_4μ-to-M1 splicing would result in increased μ_m mRNA production, whereas increased μ_s poly A site efficiency would effect an increase in μ_s mRNA accumulation.

Cell-specific regulation of μ mRNA expression may be achieved by variations in the amounts of splicing and/or 3' processing factors. A decrease in the concentration of an essential 3' processing factor, or an increase in the concentration of a factor required for C_4μ-to-M1 splicing, or a combination of the two, during B cell maturation could account for the switch in μ mRNA expression.

The HSV-induced processing factor, LPF, (McLauchlan *et al*, 1989) is probably a cellular component and may be a representative of a family of processing factors. Differential expression of such factors could effect up- and down-regulation of specific poly A site use.

In order to act in a site-specific manner, regulatory processing factors might bind to sequence elements close to the poly A site, either upstream or downstream from AAUAAA and exert their influence by increasing or decreasing the stability of the committed processing complex. Alternatively, regulatory processing factors may represent alternative components of CstF. As such, they may alter the sequence-specificity of pre-mRNA recognition. Since there exists considerable degeneracy within poly A site downstream elements, CstFs containing different regulatory factors may recognise different sequences.

Transcriptional termination and 3' processing

RNA polymerase II mediates transcription of most snRNAs, histone mRNAs and polyadenylated mRNAs. The primary transcripts of these RNA species all contain 3' extensions which are removed by post-transcriptional processing.

Pre-snRNAs contain between one and 12 nucleotides downstream from the mature 3' end. These primary transcripts are exported from the nucleus and processed in the cytoplasm, apparently via an exonucleolytic mechanism. Formation of the 3' ends of pre-snRNA molecules appears to be a transcriptional termination event. A highly-conserved 3' box is recognised as a termination signal by an snRNA promoter-specific transcription complex containing RNA polymerase II and an snRNA-specific termination factor. The RNA polymerase is apparently pre-programmed to terminate at the appropriate site by acquisition of the termination factor during initiation of transcription. Obviously, given

that they occur in separate sub-cellular compartments, termination of transcription and post-transcriptional processing of pre-snRNAs must be physically unrelated.

Termination of transcription of histone pre-mRNAs is not promoter-specific, but requires three separate sequence elements, one within the coding sequence the others downstream from the mature 3' end. The highly-conserved sequence elements which direct post-transcriptional processing of histone pre-mRNA 3' ends are not required for transcriptional termination. Therefore, termination occurs independently of 3' processing. Instead, transcriptional termination in histone genes probably involves interactions between RNA polymerase II and specific termination factors bound to the DNA template at the termination sequence elements.

Unlike transcriptional termination in snRNA and histone genes, the cessation of transcription of pre-mRNAs does not seem to occur via a single, ubiquitous mechanism. The requirement for an efficient poly A site for transcriptional termination in some, but not all, genes has been described. Several hypotheses have been advanced to account for this requirement but none has yet been proved unequivocally. In other cases, the binding of protein factors to DNA sequence elements has been implicated in transcriptional termination. In yet other cases, termination seems to occur at numerous locations over long stretches of DNA, resulting in a highly heterogenous population of 3' ends. Given the apparent importance of termination in the prevention of transcriptional interference, it is difficult to understand how such a variety of mechanisms of termination may have evolved for mRNA-encoding genes, while termination of transcription by the same RNA polymerase of snRNAs and histone mRNAs each occur by a single mechanism. The enigma of mRNA transcriptional termination may be solved through a clearer

understanding of the composition of RNA polymerase II and the functions of its constituents and associated factors.

In common with other viruses, HSV has evolved such that the genome contains a minimum of non-functional sequence. This has resulted in a complex array of closely packed and, in some cases, overlapping transcripts whose expression is temporally controlled, principally at the level of transcriptional initiation. In addition, an element of control of gene expression via differential pre-mRNA 3' processing during lytic infection has been implied by the discovery of LPF in nuclear extracts from HSV-infected cells (McLauchlan *et al*, 1989).

The importance of the 3' processing reaction for HSV gene expression may extend beyond temporal regulation of polyadenylation. Because of the arrangement of the genes in HSV, it may be important that transcription initiated at one promoter does not continue much beyond the 3' end of the mature mRNA, since this may result in transcriptional interference at downstream promoters on the same strand of the DNA or antisense RNA effects involving transcripts initiated at proximal promoter on the opposite DNA strand.

Assuming that transcriptional termination occurs in a poly A site-dependent fashion, as described for polyoma virus, then HSV poly A sites, in general, must be efficient enough to prevent extension of the nascent transcript more than a short distance beyond the 3' cleavage site. If the HSV poly A sites were not sufficiently strong, then transcription could continue into neighbouring promoter regions or complementary transcripts before 3' processing and subsequent transcriptional termination occurred.

Given the shortness of the DNA regions between the termini of neighbouring HSV transcripts, these intergenic regions are unlikely to contain specific transcription termination signals. In most cases, eukaryotic

transcription termination elements appear to span extensive regions of DNA sequence (reviewed by Birnstiel, *et al*, 1985; Proudfoot, 1989). However, transcription factors bound to their recognition sites upstream from transcriptional initiation sites may play a role in termination of upstream transcripts, thereby preventing transcriptional interference with initiation of the downstream mRNA.

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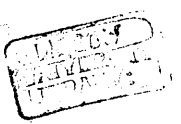

7.4.92

Application by: Robert McWILLIAM

A copy of the thesis has been sent to the Additional Examiner, who is:

Dr. D S LAtchman of University College London

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Dr. V Mautner	
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Professor J. H. Subak-Sharpe, CBE, FRSE

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JHSS/MMC

14 April 1992

Dr Vivien Mautner
Institute of Virology

Dear Vivien

This is to inform you that the oral examination of Mr Robert McWilliam's submission for the degree of PhD will take place on Thursday 28 May at 10.00 in my office.

The Special Committee will consist of Prof J H Subak-Sharpe (convener), Prof D S Latchman (external examiner), Prof J B Clements (supervisor in attendance) and yourself as internal examiner.

Yours sincerely

Professor John H Subak-Sharpe

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