Components required for *in vitro* cleavage and polyadenylation of eukaryotic mRNA

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ABSTRACT

We have studied in vitro cleavage/polyadenylation of precursor RNA containing herpes simplex virus type 2 poly A site sequences and have analysed four RNA/protein complexes which form during in vitro reactions. Two complexes, A and B, form extremely rapidly and are then progressively replaced by a third complex, C which is produced following cleavage and polyadenylation of precursor RNA. Substitution of ATP with cordycepin triphosphate prevents polyadenylation and the formation of complex C however a fourth complex, D, results which contains cleaved RNA. A precursor RNA lacking GU-rich downstream sequences required for efficient cleavage/polyadenylation fails to form complex B and produces a markedly reduced amount of complex A. As these GU-rich sequences are required for efficient cleavage, this establishes a relationship between complex B formation and cleavage/polyadenylation of precursor RNA in vitro. The components required for in vitro RNA processing have been separated by fractionation of the nuclear extract on Q-Sepharose and Biorex 70 columns. A Q-Sepharose fraction forms complex B but does not process RNA. Addition of a Biorex 70 fraction restores cleavage activity at the poly A site but this fraction does not appear to contribute to complex formation. Moreover, in the absence of polyethylene glycol, precursor RNA is not cleaved and polyadenylated, however, complexes A and B readily form. Thus, while complex B is necessary for in vitro cleavage and polyadenylation, it may not contain all the components required for this processing.

INTRODUCTION

Polyadenylation of eukaryotic mRNAs is a post-transcriptional event comprising two tightly-coupled processes (1,2). Firstly, cleavage of a precursor transcript which extends beyond the mature mRNA 3' terminus occurs at the poly A site, and this is followed by the addition of a stretch of A residues to the poly A site. The hexanucleotide sequence, AAUAAA, located upstream from the poly A site, is highly

conserved in higher order eukaryotic mRNAs and is an essential signal for polyadenylation (3,4); the level of mature polyadenylated mRNAs is markedly reduced by mutation of this sequence (5-8). Sequences downstream from the mature 3' end also play a role in polyadenylation (9-14), in particular, GU-rich and U-rich sequences located approximately 30 bases beyond the AAUAAA signal are critical for efficient mRNA 3' end formation (12,15,16).

The recent development of in vitro RNA processing systems has enabled polyadenylation to be uncoupled from transcription (17,18). With this system, exogenously-synthesised precursor RNAs are accurately cleaved and efficiently polyadenylated at the authentic poly A site by nuclear extract components (19,20). Thus, analysis of the mechanism and components required for in vitro polyadenylation should lead to a greater understanding of this process in vivo. Recent attention has focussed on the complexes formed with RNA substrates during the in vitro reaction and these complexes have been identified by sedimentation analysis on glycerol gradients (21) and electrophoresis on non-denaturing polyacrylamide gels (22-25). These studies reveal that formation of specific complexes requires the polyadenylation signal and sequences downstream from the poly A site.

Here, we present data on RNA/protein complexes generated during in vitro polyadenylation with poly A site sequences from a herpes simplex virus type 2 (HSV-2) immediate early gene (12). Complexes form extremely rapidly and, in reactions containing either ATP or cordycepin triphosphate (3'dATP), the mobility of complexes is altered by the cleavage and polyadenylation events. We show that formation of a complex which is necessary for cleavage at the poly A site requires the GU-rich sequence. In contrast to other studies, our data reveal that the formation of a second complex at the onset of the reaction is dependent on the sequences present in RNA. By fractionating the nuclear extract, we are able to reconstitute the activities required for cleavage and polyadenylation. Analysis of RNA/protein complexes produced with individual fractions shows that a fraction which forms specific complexes is unable to cleave and polyadenylate

RNA. However, RNA processing does occur following the addition of components from a second fraction which does not form any specific complexes. Moreover, extracts which require PEG for cleavage and polyadenylation form specific complexes in the absence of PEG. Thus, although formation of a specific complex is necessary for cleavage and polyadenylation at the poly A site, this complex may not contain all the components required for RNA processing.

MATERIALS AND METHODS

Plasmid Constructions

The JMC plasmid series was constructed by inserting Hind III/Pvu II fragments from the appropriate pTER plasmids (12) into the Hind III/Sma I sites of pGEMI (purchased from Promega Biotech). Details of JMC plasmids are given in Fig. 1B.

RNA Substrates

Precursor RNAs for polyadenylation reactions were synthesised using SP6 RNA polymerase (BRL) and capped by including the dinucleotide $_{HO}GpppG_{OH}$ in transcription reactions (26). For JMC8, JMC10 and JMC11 precursors, plasmids were cleaved with EcoRI and the lengths of transcripts were 148 bases for JMC8 RNA, 141 bases for JMC10 RNA and 125 bases for JMC11 RNA. L3 RNA was synthesised by cleaving plasmid pSP64L3 with Dra I generating a 267 base transcript (18) and pGEMI RNA was produced by digesting pGEMI with PvuII giving a 98 base precursor.

Complementary RNA for analysis of polyadenylation reaction products was produced by synthesis from the T7 RNA polymerase promoter using JMC plasmids which had been cleaved at a unique Sph I site. A Hind III site in plasmid JMC8 was also used for synthesis of complementary RNA; hybridisations with this RNA reduced the length of hybrids by 7 base pairs.

In Vitro Polyadenylation Reactions and RNA Analysis

Nuclear extracts from HeLa cells were prepared as described previously (18,27). Polyadenylation reactions were performed in 25ul at 30°C and consisted of 7-11µl nuclear extract, 1mM ATP, 20mM creatine phosphate, 44mM KCl, 0.7mM $MgCl_2$, 8.8% glycerol, 8.8mM HEPES (pH 7.6), 0.1mM EDTA, 0.2mM

DTT and 1-5 x 10^4 cpm RNA; reactions also contained 2.5% PEG apart from those shown in Fig. 2. In reactions containing 3'dATP, ATP was replaced with 1mM 3'dATP and the creatine phosphate concentration was reduced to 5mM. Reactions were stopped and RNA was extracted as described previously (18). RNA products of the reaction were analysed by hybridisation to complementary RNA synthesised from the T7 RNA polymerase promoter in the JMC plasmid series. Hybridisations were incubated at 52°C for a minimum of 3h. Hybridisation products were digested with 5units of RNase T2 at 30°C for 30min and thereafter, treated with proteinase K as described previously (18). Polyadenylated RNA was selected on poly(U) sepharose columns as described by Moore and Sharp (17).

Fractionation of Nuclear Extracts

For fractionation through Q-Sepharose, crude nuclear extracts were loaded on a 4cm X 1cm column containing Q-Sepharose equilibrated with 20mM HEPES (pH 7.9), 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT, 20% glycerol and 50mM KCl. Fractions were collected until no detectable protein was eluted. The KCl concentration was increased to 500mM to elute proteins bound to the column. Fractions were then pooled and stored at -70°C. Fractionation on Biorex 70 was performed as above except the column was equilibrated with buffer containing 80mM KCl.

Polyacrylamide Gel Electrophoresis

To analyse RNA/protein complex formation, polyadenylation reactions (16µl) were treated with heparin (final concentration 5µg/µl) for 10min on ice and then loaded directly on 4% non-denaturing polyacrylamide gels (acrylamide:bisacrylamide, 80:1) containing 45mM Tris-borate, 1.2mM EDTA (pH 8.3). Gels were electrophoresed and pre-electrophoresed at room temperature at approximately 10V/cm. Electrophoresis was continued until the xylene cyanol dye had migrated approximately 20cm, after which gels were dried down and autoradiographed at -70°C with an intensifying screen.

Analysis of RNA products from polyadenylation reactions and hybridisations was performed on 8% denaturing polyacrylamide gels (28).

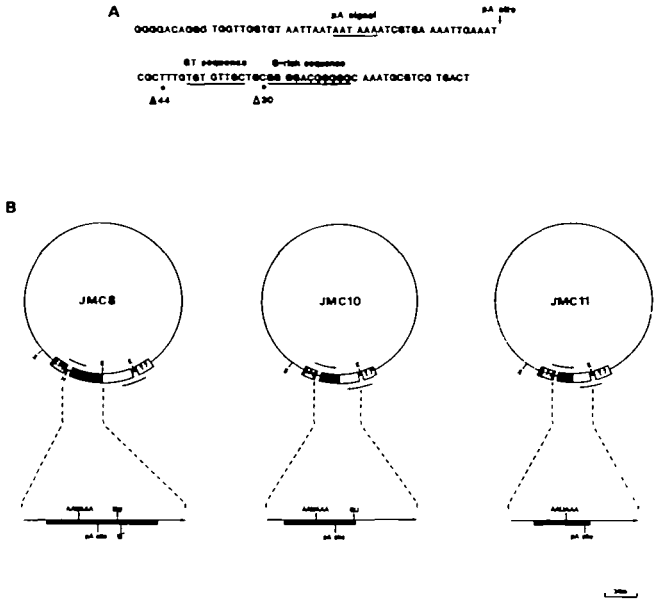

Fig. 1 (A) Nucleotide sequence and deletions at the HSV-2 immediate early 5 poly A site. Sequences involved in polyadenylation are underlined and location of the poly A site is arrowed. Also indicated are the end-points of deletions within the poly A site sequences of plasmids pTER5 Δ 30 and pTER5 Δ 44.
(B) Constructions used for synthesis of precursor RNAs. Boxed regions indicate the sequences from pTER plasmids which have been inserted into vector pGEM1; shaded boxes represent HSV-2 nucleotide sequences and open boxes denote pUC9 plasmid sequences. EcoRI (E) restriction sites used for synthesis of precursors from the SP6 promoter are shown as are Sph I (S) and Hind III (H) restriction sites which were utilised for production of RNAs from the T7 promoter. The 3' processing signals present in the precursor RNAs from these plasmids are shown and shaded boxes represent the HSV-2 sequences in each precursor.

RESULTS

Cleavage and Polyadenylation at a HSV-2 Poly A Site In Vitro

We have shown that a plasmid with a 100bp DNA fragment from the 3' end of HSV-2 immediate early gene 5 when linked to the chloramphenicol acetyltransferase (CAT) gene efficiently produced CAT activity following transfection of HeLa cells (12,29). Nuclease S1 analysis indicated that the mRNA specifying

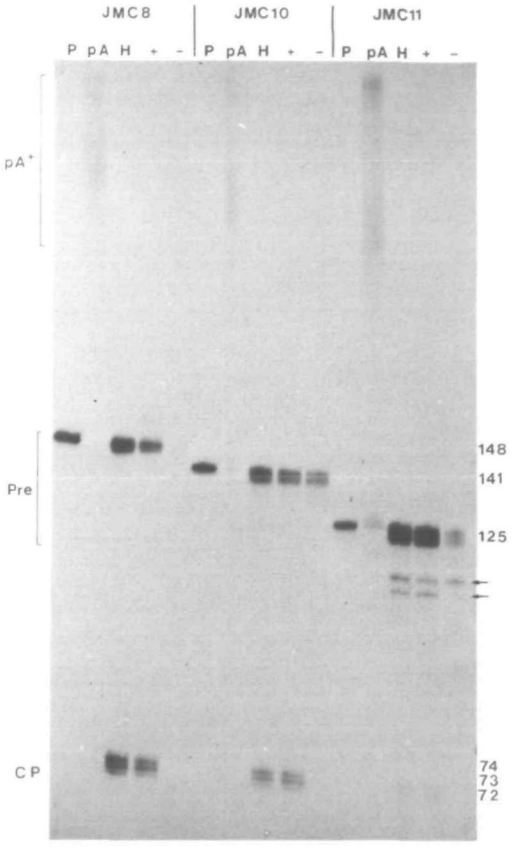

Fig. 2 Removal of downstream sequences reduces efficiency of cleavage at the poly A site in vitro. Polyadenylation reactions containing the JMC8, JMC10 and JMC11 precursors were incubated at 30°C for 2h and RNA products were examined either directly on a denaturing polyacrylamide gel (pA) or by hybridisation/RNase T2 analysis with complementary RNA (H). Poly A^+ (+) and poly A^- RNA (-) from reactions were selected by poly(U) sepharose chromatography and examined by hybridisation/RNase T2 analysis. Unreacted JMC8, JMC10 and JMC11 precursor RNAs were run in lanes marked P. The positions of polyadenylated RNA (pA^+), full-length precursor RNA (Pre) and the 5' portion of RNA cleaved at the poly A site (CP) are indicated. Two additional cleavage products generated from JMC11 RNA are arrowed. The lengths of precursor RNAs and cleavage products are given in nucleotides.

CAT activity was processed at the correct HSV-2 poly A site. From comparative sequence analysis and deletion studies, two other sequences, TGTGTTGC and a G-rich tract within this DNA

fragment were identified as 3' processing signals; these sequences were located 25 base pairs and 36 base pairs respectively downstream from the AATAAA signal (Fig. 1A).

To analyse 3' end formation at this poly A site in vitro, this HSV-2 DNA fragment was inserted into pGEM1, a plasmid containing multiple cloning sites flanked by both SP6 and T7 RNA polymerase promoters; the resultant construct was termed JMC8 (Fig. 1B). Precursor RNA for in vitro polyadenylation reactions was prepared by transcription from the SP6 promoter to the EcoR I site in JMC8, thus generating a 148 base transcript containing the HSV-2 poly A site sequences. Polyadenylation of this precursor in vitro produced polyadenylated transcripts greater than 400 bases in length (Fig. 2). To quantitate the proportion of RNA cleaved at the HSV-2 poly A site, polyadenylated RNA from this reaction was hybridised to complementary RNA synthesised using the T7 promoter in JMC8 and the hybridisation products were digested with RNase T2. Following electrophoresis on a denaturing polyacrylamide gel, full-length precursor RNA was detected; in addition, a triplet of bands ranging in size from 72-74 bases was generated and these represent the 5' portion of precursor RNA cleaved at the HSV-2 poly A site (Fig. 2). Densitometric analysis of these bands indicated that approximately 45% of the input RNA was cleaved and at the correct poly A site. Hybridisations with pA^+ and pA^- RNA produced in vitro revealed that all the RNA cleaved at the HSV-2 poly A site was polyadenylated (Fig. 2).

Sequences Downstream from the Poly A Site are Required for mRNA 3' End Formation In Vitro

Previously, we demonstrated the downstream sequence requirement in vivo using DNA fragments from two plasmids, pTER5Δ30 and pTER5Δ44 which had 30 base pairs and 44 base pairs respectively removed from the 3' end of the 100 base pair HSV-2 DNA fragment (Fig. 1A; 12). For these in vitro experiments, DNA fragments from pTER5 30 and pTER5 44 were inserted into pGEM1, generating two constructs, JMC10 and JMC11 (Fig. 1B). Plasmid JMC10, derived from pTER5Δ30, lacks the G-rich sequence frequently conserved downstream from HSV poly A sites; JMC11 is derived from pTER5Δ44 and retains the AATAAA

signal and poly A site but lacks both the downstream GT-rich and G-rich sequences (Fig. 1B). Precursors from these plasmids were polyadenylated in vitro and analysed as for JMC8 precursor RNA. Hybridisations with the RNAs produced in these reactions indicated that approximately 30% and 1% respectively of JMC10 and JMC11 precursor RNA was cleaved at the poly A site (Fig. 2). Compared with JMC8 RNA, JMC10 RNA was cleaved with an efficiency of some 70% while cleavage of JMC11 RNA was dramatically reduced to only 2% of that obtained with JMC8 RNA. These reductions in cleavage efficiency in vitro reflect our results observed from plasmid transfection experiments. Thus, the sequence requirements for cleavage and polyadenylation in vitro parallel those required in vivo. Analysis of pA^+ and pA^- RNA, for both JMC10 and JMC11, revealed that RNA cleaved at the poly A site was polyadenylated (Fig. 2, compare poly A^+ RNA with poly A^- RNA tracks), indicating that while sequences downstream from the poly A site are involved in cleavage they do not reduce the efficiency of polyadenylation. Reactions with JMC11 RNA also generated small amounts of two additional cleavage products with sizes of 113 and 108 bases (arrowed in Fig. 2); the shorter of these products appeared to be polyadenylated while the longer transcript was polyadenylated to a level of 50% (Fig. 2).

The nuclear extract characterised in Fig. 2 also produced RNA polyadenylated at the 3' terminus of the precursor. We have other extracts which polyadenylate only after cleavage and these were used in subsequent experiments.

Formation of RNA/Protein Complexes In Vitro

To characterise complexes which may play a role in cleavage and polyadenylation, RNA/protein complexes formed with JMC8, JMC10 and JMC11 precursor RNAs were compared with those produced by two control transcripts; the first contained Ad2 L3 poly A site sequences, known to be efficiently processed by the nuclear extract (18), while the second comprised pGEM1 plasmid sequences only and was not a substrate for cleavage/polyadenylation. Polyadenylation reactions containing the different precursors were incubated at 30°C for 20min to allow complex formation and treated with heparin prior to loading onto a non-denaturing polyacrylamide gel. Previous

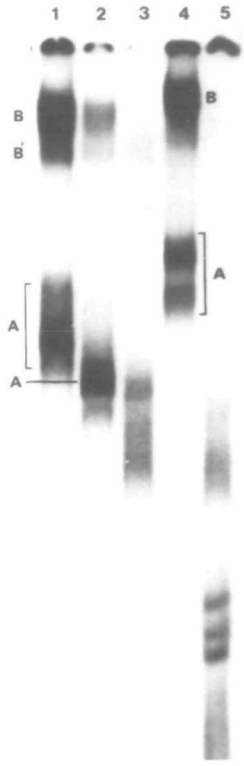

Fig. 3 RNA/protein complexes formed with different precursor RNAs. Polyadenylation reactions were incubated at 30°C for 20min and then treated with heparin prior to loading on a non-denaturing polyacrylamide gel. Precursor RNAs used were as follows: lane 1, JMC8 RNA; lane 2, JMC10 RNA; lane 3, JMC11 RNA; lane 4, Ad2 L3 RNA; lane 5, pGEM1 RNA.

analysis indicated that the Ad2 L3 precursor formed two complexes of 35S and 50S on glycerol gradients (19) which, following treatment with heparin, migrated on non-denaturing polyacrylamide gels as two bands, A and B respectively (Fig. 3, lane 4; 22). The 35S peak often migrates as a smear on polyacrylamide gels however in Fig. 3 it has separated into two bands. Complex A contains only precursor RNA while both precursor RNA and processed RNA are present in complex B (22,23). Both JMC8 and JM10 RNAs formed complexes corresponding in mobility to the Ad2 L3 band B (Fig. 3, lanes 1 and 2). The

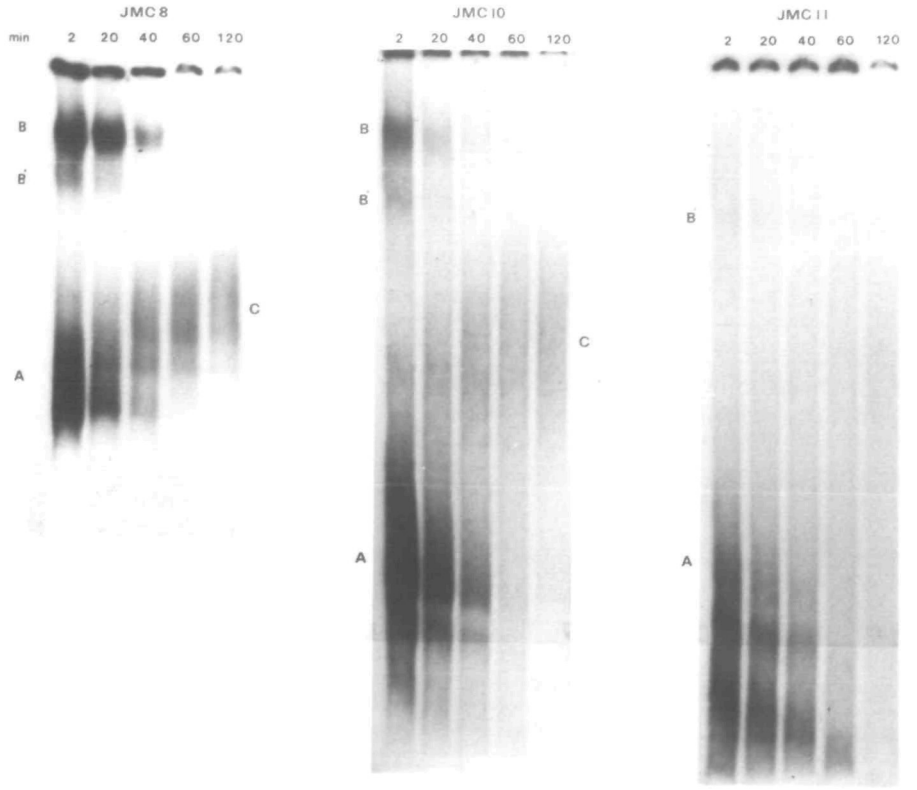

Fig. 4 Time-course of complexes formed with JMC8, JMC10 and JMC11 precursor RNAs. Polyadenylation reactions were incubated at 30°C for times ranging from 2min to 120min and then loaded on non-denaturing polyacrylamide gels following treatment with heparin. Complexes formed during the reaction are indicated.

somewhat greater mobility of complex B formed with JMC8 and JMC10 RNAs as compared to that with Ad2 L3 RNA may be a result of the 119 base longer length of the Ad2 L3 precursor. Complex B is barely detectable with JMC11 RNA which lacks the GU-rich sequence and is not efficiently processed (Fig. 3, lane 3). However, JMC11 does form a complex, B', which has a slightly greater mobility than band B. JMC8 and JMC10 RNAs also produce this B' complex as does the pGEMI precursor (Fig. 3, lane 5); this band is slightly larger with Ad2 L3 RNA and is masked by the abundance of complex B.

A second major product obtained with JMC8 RNA consists of a broad band which probably corresponds to the A complex observed with Ad2 L3 RNA. With the JMC10 precursor, the A complex has a greater mobility than the equivalent JMC8 band. This change in mobility of complex A with JMC8 and JMC10 RNAs is unlikely to be a result of the 7 base difference in length between the two precursors. Rather, the difference in mobilities may be due to the removal of downstream sequences, such as the G-rich tract, which contribute to the lower efficiency of processing of JMC10 RNA. JMC11 RNA also forms a complex which comigrates with band A formed with JMC10 but with reduced efficiency. The other complexes produced with JMC11 RNA show a similar banding pattern and mobility to those formed with pGEMI RNA; these complexes are considered indicative of non-specific interactions.

These data imply that the production of complex B depends on the presence of GU-rich sequences downstream from the poly A site and that formation of this complex is necessary for efficient cleavage and polyadenylation. This agrees with other studies which indicate that complex B is involved in the processing of RNA at poly A sites (22-25). Formation of complex A also appears to be dependent on the presence of downstream sequences, however, the role of this complex in RNA processing is less certain.

Kinetics of Complex Formation

The rapidity with which complexes A and B were formed with JMC8 RNA was analysed to determine the relationship between complex formation and efficiency of cleavage/polyadenylation at various times during the reaction. JMC8 RNA was incubated in a polyadenylation reaction at 30°C for times ranging from 2min to 120min, and RNA/protein complexes and polyadenylated RNA products were analysed at five different timepoints. Complexes A and B formed rapidly and were present after 2min incubation (Fig. 4). With increasing time, both complexes gradually disappeared and a third diffuse complex, C, was detected which migrated between complexes A and B; after 60min, complex C was the major species detected. For JMC8 RNA, complex A disappeared more rapidly than complex B, however, the opposite effect was

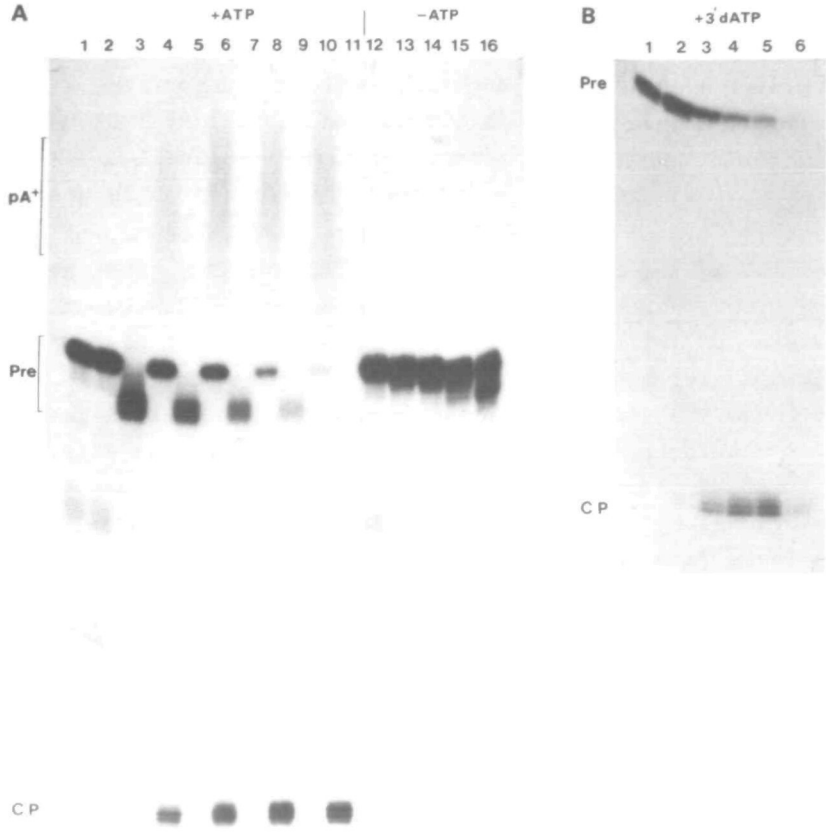

Fig. 5 Analysis of RNA products at different incubation times in the presence and absence of ATP and in the presence of 3'dATP. JMC8 RNA was incubated in polyadenylation reactions for the following times: lanes 2, 3, 12 in (A) and lane 2 in (B), 2min; lanes 4, 5, 13 in (A) and lane 3 in (B), 20min; lanes 6, 7, and 14 in (A) and lane 4 in (B), 40min; lanes 8, 9, 15 in (A) and lane 5 in (B), 60min; lanes 10, 11, 16 in (A) and lane 6 in (B), 120min. In panel (A), reactions were performed in the presence (lanes 2 to 11) and absence of ATP (lanes 12 to 16); in panel (B), reactions contained 3'dATP. RNA products were electrophoresed directly on non-denaturing polyacrylamide gels apart from reactions containing ATP, where JMC8 RNA products were examined by hybridisation/RNase T2 analysis with complementary RNA (lanes 3, 5, 7, 9 and 11) to determine the efficiency of cleavage. Lane 1 in (A) and (B) contains unreacted JMC8 precursor RNA. The positions of polyadenylated RNA (pA^+), full-length precursor RNA (Pre) and the 5' portion of RNA cleaved at the poly A site (CP) are indicated.

found with JMC10 RNA. Analysis of RNA products at the various incubation times revealed that production of polyadenylated RNA was related to the appearance of complex C (Fig. 5A, lanes 2, 4, 6, 8 and 10). Electrophoresis of the RNAs present in complexes on denaturing polyacrylamide gels revealed that complex A contains precursor RNA, both precursor and polyadenylated RNAs are present in complex B and complex C contains only polyadenylated RNA (data not shown). At longer incubation times, the length of polyadenylated RNA increased and this was reflected in a correspondingly slower mobility of complex C. Hybridisations and RNase T2 analysis showed that the polyadenylated RNA present at each timepoint was cleaved at the poly A site (Fig. 5A, lanes 3, 5, 7, 9 and 11).

The ratio of cleaved and polyadenylated RNA to precursor RNA increased for the first 60min of the reaction, however, beyond this point there was no further increase in the amount of processed RNA. Thus, although complexes A and B formed rapidly, their production was not rate limiting for RNA processing. Complex formation with JMC10 and JMC11 RNAs equally was extremely rapid and at longer incubation times complex C was detected with JMC10 RNA (Fig. 4). For JMC11 RNA, complex C was not detected, consistent with the inability of this precursor to be cleaved and polyadenylated.

Effects of ATP and 3'dATP on Complex Formation

As ATP is required for polyadenylation in vitro, complexes formed in the absence of ATP were compared to those in reactions containing ATP. For ATP^- reactions, ATP was depleted in the nuclear extract by incubation at 30°C for 20min prior to the addition of precursor RNA (30). In the absence of ATP, complexes A and B formed as rapidly as in reactions containing ATP but complex C was not present at longer incubation times (Fig. 6A). This is consistent with the failure to detect polyadenylated RNA in reactions lacking ATP (Fig. 5A, lanes 12-16). Thus, ATP does not appear to be required for complex formation under our reaction conditions.

Analysis of RNA products in ATP^- reactions revealed the accumulation of RNA cleaved 5 bases upstream from the 3' end of the precursor (Fig. 5A, lane 16). Cleavage at this site does not

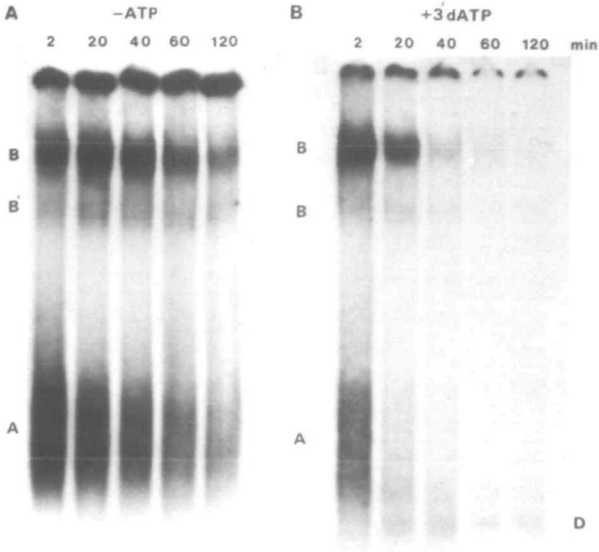

Fig. 6 Analysis of complexes formed in the absence of ATP and in the presence of 3'dATP. JMC8 RNA was incubated at 30°C for times ranging from 2min to 120min and the RNA/protein complexes loaded on non-denaturing polyacrylamide gels following treatment with heparin. In panel (A), ATP was absent in reactions while those in panel (B) contained 3'dATP.

appear to be sequence-specific as JMC10 and JMC11 precursors were also cleaved at a similar distance from their 3' termini in the absence of ATP (data not shown).

Substitution of ATP with 3'dATP in polyadenylation reactions allows cleavage but prevents polyadenylation (18). In the presence of 3'dATP, complexes A and B were of similar mobility to those formed with JMC8 RNA at early times in reactions containing ATP (Fig. 6B). As the incubation time increased, complex C was not detected, however, a third complex, D, which migrated below complex A, was produced. The amount of RNA present in complex D increased at longer incubation times as compared to that present in complexes A and B (Fig. 6B), consistent with the accumulation of cleaved RNA in the reaction

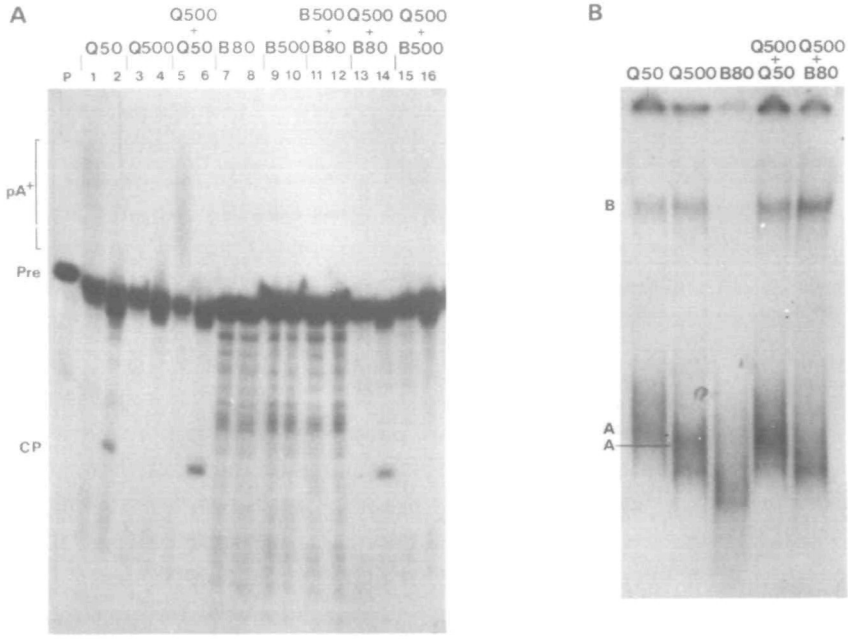

Fig. 7 (A) Separation and reconstitution of components required for in vitro cleavage and polyadenylation. Column fractions were incubated with JMC8 RNA for 2h in the presence of either ATP or 3'dATP. Odd-numbered lanes show reaction products in the presence of ATP and even-numbered lanes show reaction products with 3'dATP. The positions of polyadenylated RNA (pA^+), full-length precursor RNA (Pre) and the 5' portion of RNA cleaved at the poly A site (CP) are indicated. Unreacted precursor RNA was run in lane P.
(B) Complex formation with column fractions. JMC8 RNA was incubated at 30°C for 20min with column fractions and the RNA/protein complexes loaded on non-denaturing polyacrylamide gels following treatment with heparin. Complexes formed during the reaction are indicated.

(Fig. 5B, lanes 2-6). Analysis of the RNAs present in complex D confirmed that this complex contained only cleaved RNA (data not shown). As in reactions containing ATP, complex A disappeared more rapidly than complex B.

Fractionation and Reconstitution of Cleavage/Polyadenylation Activities

To further analyse the components involved in complex formation and cleavage/polyadenylation, crude nuclear extract

was fractionated on a Q-Sepharose column and fractions were eluted at 50mM KCl (Q50) and 500mM KCl (Q500). Fraction Q50 retained the ability to cleave and polyadenylate JMC8 RNA, however, fraction Q500 was unable to correctly process RNA (Fig. 7A). Addition of Q500 to Q50 slightly increased the efficiency of cleavage at the poly A site (Fig. 7A). Fraction Q50 was then further fractionated on a Biorex 70 column with 80mM KCl and 500mM KCl yielding two fractions, B80 and B500, neither of which was able to cleave or polyadenylate RNA even when combined; both fractions contained nuclease activity which cleaved JMC8 RNA at sites other than at the poly A site (Fig. 7A). Addition of B80, but not B500, to Q500 restored the ability to cleave JMC8 RNA at the poly A site (Fig. 7A). Thus, two inactive fractions containing components necessary for the in vitro reaction will correctly process RNA when combined.

We analysed complexes formed using JMC8 RNA together with the Q-Sepharose and Biorex 70 fractions which contained components required for cleavage and polyadenylation (Fig. 7B). Fraction Q50, which cleaved and polyadenylated RNA, formed two complexes with similar mobilities to those of complexes A and B produced with crude nuclear extract. Fraction Q500, which did not process RNA, also formed complex B but the mobility of complex A was increased relative to that obtained with crude nuclear extract or fraction Q50 and was similar to that obtained using JMC10 RNA with crude nuclear extract. Addition of Q500 to Q50 produces complex B and a complex which resembles the A complexes formed with these fractions individually. Fraction B80, which also contributes factors required for the in vitro reaction, does not form complexes A or B but does produce a complex which migrates faster than complex A. Combining the B80 and Q500 fractions, which together can process RNA, produces complexes with similar mobilities to those formed with Q500 only. The results therefore demonstrate that fraction Q500 can form complex B which is required for in vitro processing yet all the components required for cleavage and polyadenylation are not present in this fraction. Moreover, components present in fraction B80, which together with factors from Q500 allow RNA processing to occur, do not alter the mobility of complex B.

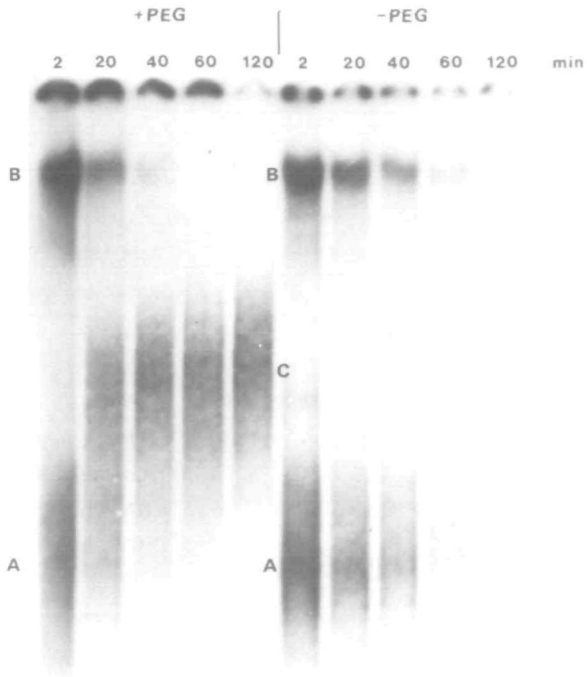

Fig. 8 Effect of PEG on complex formation. JMC8 RNA was incubated in polyadenylation reactions for times ranging from 2min to 120min in the presence and absence of PEG and the RNA/protein complexes loaded on a non-denaturing gel after treatment with heparin.

Effect of PEG on Complex Formation

Previous data has indicated that the addition of polyvinyl alcohol (PVA) to *in vitro* reactions significantly increases the level of cleavage (20,31). As an alternative to PVA we have used PEG in reactions and our comparisons of the effects of PEG and PVA reveal that both chemicals increase cleavage efficiency to the same extent (data not shown). This increase in efficiency may be due to PEG affecting the rate of complex formation. To examine this possibility, complexes formed in the absence of PEG were compared to those produced in the presence of PEG using a

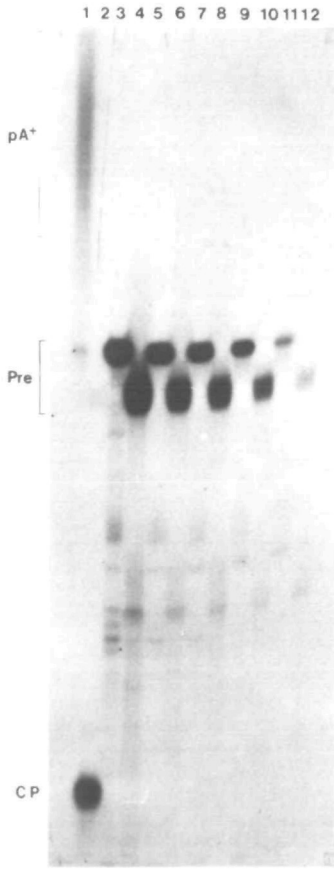

Fig. 9 Analysis of RNA products in reactions lacking PEG. JMC8 RNA was incubated in polyadenylation reactions lacking PEG for the following times: lanes 3 and 4, 2min; lanes 5 and 6, 20min; lanes 7 and 8, 40min; lanes 9 and 10, 60min; lanes 11 and 12, 120min. RNA products were either eletrophoresed on a denaturing polyacrylamide gel (lanes 3, 5, 7, 9 and 11) or examined by hybridisation/RNase T2 analysis with complementary RNA (lanes 4, 6, 8, 10 and 12). Lanes 1 and 2 contain JMC8 RNA incubated for 120min in the presence of PEG and show the positions of polyadenylated RNA (pA^+; lane 1) and the 5' portion of RNA cleaved at the poly A site (CP; lane 2); the position of full-length precursor RNA (PRE) also is indicated.

nuclear extract which requires PEG for cleavage and polyadenylation (Fig. 8). Interestingly, complexes A and B formed rapidly in the absence of PEG but complex C did not

appear at longer incubation times. This failure to form complex C reflects the inability of the nuclear extract to polyadenylate precursor RNA (Fig. 9, lanes 3-12). These data further demonstrate that although the formation of complex B is required for cleavage and polyadenylation, this complex may not contain all the components necessary to produce polyadenylated RNA.

DISCUSSION

We show here that sequences downstream from a HSV-2 poly A site are required for efficient formation of mRNA 3' termini *in vitro*. Further, these sequence requirements are similar both *in vivo* and *in vitro*, confirming results obtained with other poly A sites (14,25,32). Moreover, the use of pre-synthesised precursor RNA as a substrate demonstrates that the downstream sequences function in RNA. The data presented here confirm our previous proposal that a GU-rich sequence, which is concordant with the consensus YGUGUUYY and is located approximately 30 bases beyond the polyadenylation signal, is required for efficient mRNA 3' end formation (12). Sequences located further downstream also modulate the level of 3' end processing and, in HSV, G-rich tracts are often present beyond the GU-rich region. Thus, sequences downstream from the poly A site, in particular the GU-rich sequence, are involved in accurate cleavage of precursor RNA at the poly A site.

We identify two complexes, A and B, which form rapidly at the onset of the reaction with RNA comprised of HSV-2 poly A site sequences and these complexes have similar mobilities to those formed with Ad2 L3 poly A site sequences (22). From other reports, complex A is considered a non-specific complex which forms with RNA lacking poly A site sequences. However, our deletion analysis shows that removal of G-rich sequences increases the mobility of complex A and this change in migration is unlikely to be a result of the different lengths of precursors. A similar increase in the mobility of complex A is observed in our fractionation studies using precursor RNA which contains G-rich sequences; possibly a component which recognises G-rich sequences has been removed in such fractions. The altered migration of complex A does not appear to reflect a dramatic

reduction in the efficiency of RNA processing, thus, the role of this complex in in vitro polyadenylation remains unclear.

By contrast, formation of complex B does appear to be a prerequisite for cleavage at poly A sites and this complex contains both processed and unprocessed RNA. Mutations in the AAUAAA signal which prevent processing of RNA also abolish complex B formation (22-24). Moreover, sequences downstream from the poly A site are an additional requirement for complex B formation (22,25) and we have shown that the GU-rich sequence is an essential component in the production of this complex. The GU-rich sequence appears to function in concert with the AAUAAA sequence since precursors containing a mutated AAUAAA signal and an intact GU-rich sequence fail to compete with RNA in complex B (23,24). Moreover, RNase T1 digestion of RNA in complex B shows that a 67 base region encompassing the AAUAAA signal and GU-rich region in the precursor RNA is protected from digestion (23). However, the nature of any interaction at the GU-rich region is not known. Results by complementary oligonucleotide/RNase H analysis reveal that an extract component recognises the AAUAAA signal (33). Using the same assay, we have obtained similar results but no interaction could be detected at either the GU-rich sequence or poly A site (J. McLauchlan, S. Simpson and J.B. Clements, manuscript in preparation). Possibly, interaction with sequences other than the AAUAAA signal may be transient or unstable and therefore not detectable by this method.

Complexes A and B form extremely rapidly at the onset of the reaction and, at longer incubation times in the presence of ATP, a third complex, C, appears which migrates between A and B and contains polyadenylated RNA. Replacing ATP with 3'dATP does not influence either the production of complexes A and B or the rate of the cleavage reaction. Since RNA is not polyadenylated in reactions containing 3'dATP, complex C is not detected, however, another complex, D, containing cleaved but non-polyadenylated RNA is produced which migrates faster than complex A. The length of polyadenylated RNA as compared to the length of cleaved but non-polyadenylated RNA may contribute to the different mobilities of complexes C and D. In addition, the RNA/protein interactions in complexes C and D may not be

identical since poly A binding proteins may be present in the nuclear extract.

Our fractionation studies reveal that complex B can be produced in the absence of cleavage and polyadenylation. Similarly, the absence of PEG in reactions does not prevent the formation of complex B although RNA processing does not occur; these data suggest that this complex does not contain all the components required for 3' end formation. This is consistent with other data which indicates that complexes purified on glycerol gradients are unable to cleave and polyadenylate RNA (21). While formation of complex B is a prerequisite for efficient processing, we show that its production need not reflect the rate of cleavage and polyadenylation. From our identification of factors involved in cleavage which do not contribute to complex formation, we suggest that certain essential components may interact transiently with complex B. Based on this information, we propose the following model for polyadenylation in vitro. At the onset of the reaction, RNA is sequestered between complexes A and B and these complexes may be in equilibrium. Precursor RNA in complex B is available for cleavage, however, the interaction of certain factors with complex B which are essential for processing is a rate limiting step in the reaction; these factors may not contribute to the initial formation of complex B. Following interaction of these factors with complex B, RNA cleavage and polyadenylation can occur. These events lead to the breakdown of complex B and processed RNA is released from complex B to form a third complex. Dissociation of complex B alters the equilibrium between complexes A and B and as a result, precursor RNA is released from complex A to form complex B.

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