Variation in ATP requirement during influenza virus transcription

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The ATP requirement of influenza A virus RNA-dependent RNA polymerase was studied during in vitro transcription reactions. In complete transcription reactions, the $K_m$ for ATP was 10-fold higher than the $K_m$ values for the other NTPs. However, during transcription elongation the $K_m$ for ATP was as low as the $K_m$ values for the other NTPs, suggesting a special requirement for ATP during transcription initiation. Gel analysis of RNA products of transcription initiation reactions showed that the incorporation of AMP into nascent RNA was more efficient at positions 4, 6 and 7 relative to the template RNA than at position 5. The polymerase produced short, abortive transcripts with lengths corresponding to positions 3 and 4 relative to the template but never to position 5 or longer. These results suggest that incorporation of AMP at position 5 induces the influenza A virus polymerase to go through a transition from a transcription initiation to an elongation complex. This functional change of the polymerase complex rather than a requirement for ATP $\beta-\gamma$ bond hydrolysis is the most likely reason for the particularly high $K_m$ for ATP during the early phase of transcription. This conclusion is supported by the fact that the ATP analogue ATP$_{\gamma}$S [adenosine 5'-$\gamma$-(3-thiotriphosphate)] can efficiently replace ATP in in vitro transcription reactions and shows a comparable drop of $K_m$ between transcription initiation and elongation.

Introduction

RNA-dependent RNA polymerases (RdRps) are the key enzymes for the multiplication of many RNA viruses. They typically are relatively large, multifunctional proteins or protein complexes responsible for virus specific, regulated RNA synthesis during the transcription and replication of viral genomic RNA. These enzymes catalyse reactions that are essential for virus multiplication but they show considerable differences from cellular RNA synthesizing proteins. They therefore constitute potential targets for antiviral chemotherapy (Meanwell & Krystal, 1996; Tisdale et al., 1995). The actual polymerase active site of RdRps may have evolved from a common, ancestral polymerase module (Poch et al., 1990), but biochemically RdRps have not yet been as well characterized as DNA-dependent polymerases or reverse transcriptases. A better understanding of similarities and differences between individual cellular and viral polymerases will support the development of new antiviral strategies.

The influenza A virus genomic ribonucleoprotein (RNP) constitutes the template for transcription by its specific RdRp. The genome consists of eight different single-stranded RNA segments (vRNAs) that all contain highly conserved and partially complementary sequences of 13 nucleotides at the 5' end and 12 nucleotides at the 3' end (Skehel & Hay, 1978; Robertson, 1979; Desselberger et al., 1980; Stoeckle et al., 1987). The genomic RNA is covered with nucleoprotein (NP), which is responsible for the formation of the specifically coiled ribonucleoprotein structure (RNP) with the RNA-bound NP polymer folded back on itself in a looped structure (Compans et al., 1972; Jennings et al., 1983; Ruigrok & Baudin, 1995). The role of structural characteristics like the coiled RNP fold-back structure (Compans et al., 1972; Jennings et al., 1983) and the fact that NP binding to the genomic RNA exposes all Watson–Crick positions of the bases to the solvent (Baudin et al., 1994; Klumpp et al., 1997) for transcription activity is at
present still unclear. The influenza A virus specific RdRp is a heterotrimeric protein complex composed of the subunits PA, PB1 and PB2 (Lamb, 1989; Lamb & Krug, 1996). Transcription activity can be followed in vitro with detergent disrupted virus particles or purified RNPs in the presence of RNA primers (Krug et al., 1989). In virions, the polymerase complex is bound to the conserved 3' and 5' ends of the single-stranded genomic RNA (Klumpp et al., 1997). Transcription activity is dependent on the presence of these ends for transcription initiation (Krug et al., 1989; Hagen et al., 1994; Fodor et al., 1994, 1995) and NP binding to the template RNA is required for efficient elongation (Honda et al., 1988).

In vitro, influenza virus transcription and replication occur in the nucleus of infected cells (Herz et al., 1981; Jackson et al., 1982). The RNA polymerase initiates transcription at the 3' termini of the genomic RNA segments using as primer a 9–15 nucleotide capped RNA fragment derived from the 5' ends of cellular mRNAs (Bouloy et al., 1978; Krug et al., 1979; Beaton & Krug, 1981). It appears that the polymerase preferentially uses RNA primers that are able to undergo base-pairing interactions at least with the 3'-terminal uridine of the vRNA template, although base-pairing between primer and template is not essential for transcription initiation (Beaton & Krug, 1981; Krug et al., 1980; Lamb et al., 1981; Hagen et al., 1995). The polymerase can also initiate transcription by using small RNA oligonucleotides like ApG, GpG or ApGpC as primers, which are complementary to the vRNA 3' end. There is no obligatory site of transcription initiation. Depending on the primer sequence, transcription initiation can occur with GTP or CTP between positions 2–4 of the vRNA 3' end (Hagen et al., 1994, 1995; Plotch et al., 1981; Honda et al., 1986).

We have analysed the nucleotide triphosphate requirements of the influenza virus RdRp during transcription initiation and elongation. The $K_m$ for ATP in in vitro transcription reactions is known to be at least 10-fold higher than the $K_m$ values for the other three NTPs (Stridh & Datema, 1984), but the reason for this difference is unknown. Relatively high $K_m$ values for specific purine nucleotides have been documented before in studies with other RNA polymerases for initiating NTPs (Testa & Banerjee, 1979; Anthony et al., 1969). However, during primed transcription, the influenza virus polymerase neither initiates with ATP nor is it dependent on ATP for the formation of the first phosphodiester bond. We have studied separate phases of influenza virus transcription in vitro in order to better understand this specific requirement for ATP.

Methods

Materials. Influenza virus A/PR/8/34 RNPs were prepared by standard procedures as described (Klumpp et al., 1997). RNases for RNA sequencing, SP6 RNA polymerase and unlabelled ribonucleoside 5'-triphosphates were purchased from Pharmacia, S-adenosyl-L-methionine (SAM), the dinucleotide ApG, kinase inhibitors and ATP analogues from Sigma, radiolabelled ribonucleotide 5'-triphosphates (3000 Ci/mmol) from Amersham and vaccinia virus guanylyltransferase from Gibco BRL. Recombinant ribonuclease inhibitor RNasin was from Promega.

Assay of influenza virus specific transcription. Except when indicated so in the figure legends, complete ApG transcription reactions were performed at 30°C in 50 µl reaction mixtures containing 0.1–0.4 nM RNP, 50 mM Tris–HCl, pH 7.8, 100 mM KCl, 10 mM NaCl, 10 mM DTT, 0.4 FS25 U/µl RNasin, 0.2 µg/µl BSA, 0.4 mM ApG, 0.5 mM GTP, CTP, UTP and 1 mM ATP, keeping the $\alpha^{-32}$P-labelled NTP at 50 µM for the times indicated in the figure legends. Reactions were stopped by the addition of 50 µl 25% trichloroacetic acid (TCA), 2% Casamino acids and incubated for 30 min on ice. Samples were filtered through a Millipore AP15 glass-fibre filter using a Bio-Rad BioDot microfiltration device and washed with 10% TCA. Retained radioactivity was measured by scintillation counting.

Preinitiated polymerase transcription complexes were prepared by incubating 0.4–0.8 nM RNP under the same conditions as described above either in the absence of UTP or in the presence of 0.1 µM UTP for 1–10 min at 30°C. The reactions were then passed through 1 ml Sephadex G-50 columns (Pharmacia) equilibrated in transcription buffer, leading to a 10³–10⁴-fold separation of NTPs and ApG from RNP. Transcription elongation rates were measured after the addition of fresh NTPs (0.5 mM) and [$\alpha^{-32}$P]NTP at 50 µM. Reactions were stopped and analysed as described above. For gel analysis of transcription products, reactions were stopped by the addition of 40 mM EDTA. RNA was purified by phenol extraction and ethanol precipitation following standard protocols (Sambrook et al., 1989), resolubilized in denaturing gel loading buffer (7 M urea, 20% sucrose) and loaded onto 20% acrylamide gels containing 7 M urea. The polymerase elongation complexes were strictly dependent on UTP for the resumption of transcription activity and full elongation was dependent on the addition of all four NTPs, indicative of an efficient separation of NTPs and primer during G-50 column chromatography. RNA primed transcription reactions were performed under the same incubation conditions as described above using 0.1 nM RNP, 60 nM capped GEM-RNA (see below) and NTP concentrations as described in the figure legends.

In vitro RNA synthesis and RNA capping reaction. In vitro transcription with SP6 RNA polymerase was done according to standard procedures (Promega protocols and applications guide) using as template DNA Smal-digested pGEM-7Zf+ plasmid (Promega). The use of the resulting 68 nucleotide GEM-RNA for influenza virus specific endonuclease reactions has been described previously (Hagen et al., 1994; Chung et al., 1994). In vitro transcribed RNA was purified by electrophoresis on 12% acrylamide–7 M urea gels; bands were visualized by UV shadowing, excised and eluted overnight in 0.6 M ammonium acetate, 1 mM EDTA, 0.1% SDS. Unlabelled, capped RNA was produced by performing the transcription reaction in the presence of 1.5 mM capping analogue (m$^p$GpppG, Boehringer), 0.3 mM GTP, which results in the capping of > 50% of the transcripts. Vaccinia virus guanylyltransferase (Gibco BRL) was used to specifically label GEM-RNA in the cap structure. RNA (10 µg) was incubated with 6 µg guanylyltransferase in 50 mM Tris–HCl, pH 8, 1.25 mM MgCl$_2$, 6 mM KCl, 5 mM DTT, 0.6 µl RNasin, 1 mM SAM and 1 µl [$\alpha^{-32}$]GTP in a 30 µl volume for 1 h at 37°C. The reaction was stopped with denaturing RNA gel loading buffer and cap-labelled GEM-RNA was purified by denaturing gel electrophoresis as described above. Direct sequencing reactions of labelled RNA with RNases T1 and U2 were done as described (Klumpp et al., 1997).

ATPase assays. For thin-layer chromatography, 1 nM purified RNP was incubated with 2 nM [$\alpha^{-32}$]ATP (specific activity 3000 Ci/mmol) and 0.5 µM cold ATP in transcription buffer (see above) for 30–60 min at 30°C in 20 µl total volume. Control reactions were
performed with ATP in the absence of RNP (C-), with ATP in the presence of 2 mM T4 polynucleotide kinase and 100 ng DNA oligonucleotide (C+; production of ADP and labelled phosphate, control for the absence of phosphatase activity in RNP preparations). A 1 µl sample of the reactions was spotted onto polyethyleneimine-coated TLC plates (Schleicher and Schuell) and separation was in 1 M LiCl, 0.5 M formic acid. For HPLC analysis of ADP production, complete transcription reactions, transcription initiation or elongation reactions were performed as described above, but with all NTPs at 0.5 mM concentration, RNP at 1 nM and using 100 µl reaction volumes. Transcription reactions were stopped by the addition of EDTA to 40 mM and loaded onto a 2 ml Waters Symmetry C18 column, equilibrated with 0.1 M potassium phosphate, pH 6.5, 5 mM tetrabutylammonium hydroxide (TBA). Bound nucleotides were eluted with a 0–50% methanol gradient in the same buffer.

Results

ATP requirements of the influenza virus specific transcription reaction

We studied the kinetic parameters of influenza virus specific RNA synthesis in a system using virion derived RNPs, which represent preformed polymerase–template complexes, in the presence of the dinucleotide primer ApG and various amounts of nucleotide triphosphates (NTPs). $K_m$ values for the different NTPs were determined under initial velocity conditions and reproduced with independent virus preparations, RNP preparations and separate batches of NTP stocks. Fig. 1(a) shows example plots of data fitted to a hyperbolic equation using the least squares method or plotted in a linear fashion according to the Hanes–Woolf equation (insets) (Cleland, 1979; Rudolph & Fromm, 1979). $K_m$ values were determined from both hyperbolic and linear fits. The results are summarized in Table 1. Lineweaver–Burk, Hanes–Woolf, Eadie–Hofstee and direct plots gave comparable results in all experiments (the deviations in calculated $K_m$ values were smaller than the standard deviation between independent experiments). In agreement with previous results using a different virus strain (Stridh & Datema, 1984), we found that the $K_m$ for ATP in such a complete transcription reaction was about 10-fold higher than the $K_m$ values for the other three NTPs. Transcription initiation occurs with CTP in this system (see template sequence scheme in Fig. 4(c)), but the $K_m$ for CTP was comparable to the $K_m$ of GTP and UTP in contrast to studies with other RNA polymerases that showed an increased $K_m$ for the NTP used for the first phosphodiester bond formation, either ATP or GTP (Testa & Banerjee, 1979; Anthony et al., 1969; Osumi-Davis et al., 1992; Bonner et al., 1992).

The $K_m$ for ATP is reduced in elongation reactions

To determine if there was a specific requirement for ATP during transcription initiation, we established a system to perform initial velocity measurements of RNA synthesis selectively during elongation by using preinitiated transcription complexes separated from primer dinucleotides and NTPs by size exclusion chromatography on G-50 columns (see Methods). In vitro transcription reactions were performed with purified elongation complexes in the presence of increasing NTP concentrations and $K_m$ values were calculated by fitting hyperbolic and linear equations to the data points as described above (Table 1 and example curves in Fig. 1b). We found that in elongation reactions the $K_m$ for ATP was low and comparable to the $K_m$ values for the other NTPs. When the $K_m$ values for the NTPs in elongation reactions were compared to complete transcription reactions only the $K_m$ for ATP had significantly changed (16-9-fold decrease), whereas the $K_m$ values for the other NTPs had decreased by a factor of only 3. These results suggested that there was a specific requirement for ATP during transcription initiation, which was different from the requirement during elongation. There was a difference in the maximal velocity of nucleotide incorporation between complete transcription reactions and elongation reactions, which was presumably caused by the loss of actively transcribing polymerase complexes due to nonspecific adsorption to the column material (about 60% of loaded protein was recovered from the column) and to the dissociation of stalled transcription complexes. The concentration of active protein in the assay was therefore lower in elongation reactions as compared to complete reactions.

A second ATP-dependent active site apart from the polymerase active site could theoretically be involved in transcription initiation, but not elongation. If this hypothetical enzymatic function had a high $K_m$ for ATP, this could be a reason for measuring a high $K_m$ (ATP) only in complete transcription reactions, but not in elongation reactions. Although influenza virus transcription initiation is independent of ATP (see below and Figs 3 and 4), it would still be possible that ATP was specifically required for promoter clearance. Therefore, we performed a series of experiments to test if ATP $\beta\gamma$ bond hydrolysis was required for influenza virus specific transcription in vitro.

Influenza virus specific transcription in the presence of $\beta\gamma$ blocked ATP analogues

ATP could be efficiently replaced by ATP-S [adenosine 5'-O-(3-thiotriphosphate)] in influenza virus transcription reactions, but no significant activity was detectable in complete transcription reactions with either AMPNP [5'-adenylylimido(diphosphate)] or AMPPCP (5'-methyleneadenosine 5'-triphosphate) (Fig. 1c). In elongation reactions, the apparent $K_m$ values were low for both ATP-S and ATP, indicating that they were used with equal efficiency by the polymerase (Table 1 and Fig. 1c). Transcription activity, albeit about 5-fold lower than with ATP, became detectable with AMPNNP, but not with AMPPCP, in elongation reactions (Fig. 1c). Similar differences in the enzymatic activity have also been observed with other polymerases in the presence of ATP analogues.
Fig. 1. NTP requirements of the influenza A virus RdRp for incorporation of $\alpha$-$^{32}$P-labelled CTP or GTP into TCA insoluble material. (a) complete transcription, (b) elongation, (c) ATP-analogue replacing ATP in complete transcription (left) and elongation (right). Transcription assays were performed either with purified RNPs or polymerase elongation complexes obtained by preincubation of RNPs with NTPs and primer and purified by G-50 size exclusion chromatography (see Methods and legend to Fig. 2). RNP (0-4 nM) was incubated with two unlabelled NTPs at a concentration of 500 µM, 400 µM ApG, 50 µM of one $\alpha$-$^{32}$P-labelled NTP and various concentrations of the fourth NTP for 15 min at 30°C. $K_m$ values were estimated from fitting hyperbolic curves to the data and linear regression analysis respectively, and results are summarized in Table 1. The insets show the same data plotted according to the Hanes–Woolf equation ($s/v - 1/V_{max} = s + K_m/V_{max}$). The experiments were reproduced with different virus and RNP preparations using reaction times between 5 min and 20 min. Incorporation of labelled NTP was linear for at least 1 h under these conditions.

(Testa & Banerjee, 1979; Fujioka et al., 1991; Lofquist et al., 1993; Timmers, 1994). They are most likely due to a lower affinity of the polymerase for imido and methylene analogues of ATP than for ATP itself. These results could not formally exclude the requirement for an ATP hydrolysing activity during transcription initiation, although the efficient use of ATP$\gamma$S argued against this possibility. It was therefore interesting to investigate if ADP was produced during transcription initiation. We analysed the release of labelled ADP from $[\alpha$-$^{32}$P]ATP in transcription reactions by separating the nucleotides on thin-layer chromatography sheets. We found that, depending on the virus preparation, the cor-
responding RNP preparations could possess apparent ATPase activity (Fig. 2c). The ADP production, which varied considerably between virus batches, could also be analysed by reverse-phase chromatography (Fig. 2a, b). At the moment it is not clear if this ATPase activity simply represents a contamination of RNP preparations with a cellular protein or if it has any significance for the virus. We could not correlate the amount of copurifying ATPase activity with differences in the transcription activity of RNP preparations. Nevertheless, to exclude any interference with the present kinetic analysis, the data presented in this paper were obtained from independent RNP preparations, all without apparent ATPase activity (Fig. 2a, c). These RNPs showed no apparent ADP production during either transcription initiation or elongation suggesting that ATP β-γ bond hydrolysis was not required for influenza virus specific in vitro transcription (Fig. 2a). Highly selective, low level phosphorylation of a polymerase subunit may result in ADP levels that could possibly escape detection in the above control experiments. A kinase with characteristics of casein kinase II has been found in variable concentrations in virus particles (Tucker et al., 1990), but a possible function for viral transcription was not investigated. We did not see any stimulatory effect of adding casein kinase II to in vitro transcription reactions, nor did DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole), an inhibitor of casein kinase II, influence RNA synthesis by influenza virus RdRp at DRB concentrations up to 200 μM (Table 2; Zandomeni et al., 1982).

**AMP incorporation into RNA products occurs more efficiently at position 4 than at position 5 of the vRNA template**

ATP usage during transcription initiation was studied in more detail by analysing RNA products on denaturing acrylamide gels. Transcription reactions were primed with capped GEM-RNA (see experimental procedures). GEM-RNA is cleaved by the influenza virus endonuclease to a so-called G11 primer containing ApG at the 3’ end (Hagen et al., 1994) (Fig. 3a, right panel, lanes ‘RNP’). The G11 primer is able to base-pair to the first two bases of the vRNA template, and the polymerase preferentially initiates transcription with CTP to produce a 12 nucleotide RNA, G11 + 1 nt (Fig. 3a, right panel, lane ‘RNP + CTP’). This initiation reaction was then performed with cap-labelled GEM-RNA and increasing concentrations of CTP; the reaction products were separated on acrylamide gels, cut out and quantified by scintillation counting. The incorporation of the first nucleotide appeared to be very efficient under these conditions and an apparent Kₘ of 10 nM was estimated from fitting a hyperbolic curve to the data points (Fig. 3a, left panel, and 3b). Similar results were obtained using unlabelled GEM-RNA and labelled CTP to study transcription initiation. The initiated G11 + 1 nt RNA is shown again in Fig. 4 (left panel, lane 5). Corresponding reactions are shown on the right panel of Fig. 4 after a longer exposure of the acrylamide gel. The 12 nucleotide RNA could be further elongated with either GTP (lane 4) or ATP (lane 2) to a 13 nucleotide RNA (G11 + 2 nt). The RNA products migrated slightly differently according to the nucleotides incorporated, which confirmed that elongation was due to either AMP or GMP incorporation at position 4 on the template RNA according to the sequence heterogeneity of the 3’ ends of the influenza A virus vRNA segments at this position (see Fig. 3c). Intermediate size transcripts were produced in the presence of ATP, GTP and CTP (Fig. 4, lane 1) and long transcripts in the presence of all four NTPs (lane 3). Interestingly, AMP incorporation was much more efficient at position 4 of the template (giving rise to G11 + 2 nt) than at the subsequent template positions. In the presence of CTP and ATP mainly 13 nucleotide RNA (G11 + 2

### Table 1. Nucleotide requirements for influenza virus specific transcription in vitro

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>ATP</th>
<th>GTP</th>
<th>CTP</th>
<th>UTP</th>
<th>ATP₂S</th>
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<tr>
<td>Kₘ values for complete reaction* (initiation + elongation)</td>
<td>54 ± 18 (9)†</td>
<td>4’7 ± 1’8 (3)</td>
<td>4’9 ± 4’5 (6)</td>
<td>5’2 ± 2’3 (4)</td>
<td>28 ± 13 (5)</td>
</tr>
<tr>
<td>Kₘ values for elongation‡</td>
<td>3’2 ± 1’6 (8)</td>
<td>1’7 ± 1’3 (3)</td>
<td>1’4 ± 0’6 (3)</td>
<td>1’8 ± 0’4 (2)</td>
<td>2’8 ± 2’0 (3)</td>
</tr>
<tr>
<td>Reduction (-fold) in Kₘ after transcription initiation</td>
<td>16’9</td>
<td>2’8</td>
<td>3’5</td>
<td>2’9</td>
<td>10</td>
</tr>
</tbody>
</table>

* ApG primed transcription reaction.
† The Kₘ values in μM for the different NTPs as well as for the ATP analogue ATP₂S, determined as described in the legends of Figs 1 and 3 for ApG primed influenza A virus specific in vitro transcription reactions.
‡ No. of independent experiments from which the standard deviation was deduced.
§ Preinitiation of transcription with 400 μM ApG, 500 μM ATP, CTP, GTP, followed by size exclusion chromatography and incubation with NTPs in the absence of primer.
Fig. 2. Analysis of ATPase activity in RNP preparations. (a, b) Separation of nucleotides and small oligonucleotides produced in RNP transcription reactions by reverse-phase HPLC. Peaks were identified by running nucleotide standards in parallel on the same column. (a) Time-course of the synthesis of transcription initiation product ApGpC (AGC) in the absence (traces 4–7) or presence (traces 8–11) of ATP. There was no detectable production of ADP, indicating the absence of ATPase or kinase activity in the RNP preparation. (b) Reverse-phase HPLC separation of nucleotides and small oligonucleotides. Some RNP preparations contained low level ATPase activity. For these preparations ADP was produced in the presence of RNP (traces 1, 3 and 5), but not in the absence of RNP (traces 2 and 4). (c) Thin-layer chromatography separation of nucleotides. Six different RNP preparations originating from two different influenza A/PR/8/34 virus preparations were tested in in vitro ATPase assays for the production of labelled ADP from [α-32P]ATP. RNP preparations 1–3 showed no detectable ADP production, whereas RNP preparations 4–6 from a different virus source produced low amounts of labelled ADP. ‘C–’ is a negative control of [α-32P]ATP incubated in ATPase reaction buffer, ‘C+’ is a positive control showing [α-32P]ADP production from [α-32P]ATP by 2 mU T4 polynucleotide kinase in the presence of 100 ng DNA oligonucleotide substrate. RNP preparations without detectable ADP production [similar to the examples shown in (a) and (c), lanes 1–3] were used for kinetic analysis of in vitro transcription.

nt) was produced, i.e. one CMP and one AMP incorporated, although up to four AMPs could in theory be incorporated into the nascent RNA according to the conserved vRNA template sequence (Fig. 3c). Also, much larger amounts of short transcripts than long RNAs were produced in the complete transcription reactions (lane 3). A large proportion of the short RNAs represented abortive transcripts, because they could not be elongated by the addition of excess NTPs. The short transcripts were of 12 and 13 nucleotides length (G11 + 1 nt, +2 nt), but, as indicated by the gap between these short transcripts and intermediate size products (black line in Fig. 4), transcription was much more processive as soon as the third nucleotide had been incorporated, i.e. as soon as position 5 on the template had been passed.

Incorporation of more than one AMP could be observed at higher concentrations of ATP. We performed transcription
initiation reactions under initial velocity conditions with increasing amounts of ATP in the presence of GEM-RNA and labelled CTP. As described before, products of 13 nucleotides length (G11 + 2 nt) were detectable earlier than longer RNAs. At higher ATP concentrations the immediate incorporation of four AMP residues was observed (giving rise to G11 + 5 nt), but no intermediate transcripts containing two or three AMP residues incorporated were detectable (Fig. 5). At the highest ATP concentration transcripts corresponding to G11 + 6 nt were produced, presumably due to a misincorporation at position 8 of the vRNA template (Fig. 5 and sequence scheme in Fig. 3c). These results suggest that the incorporation of the third nucleotide into nascent RNA (position 5) is much less efficient than incorporation of the second nucleotide (position 4), although ATP is used in both cases. The incorporation of nucleotides beyond this critical position 5 of the template RNA is again of higher efficiency as suggested by the absence of intermediate products between position 4 and 7 of the template (i.e. between products G11 + 2 nt and G11 + 5 nt). Similar results were obtained with transcription reactions performed with labelled GEM-RNA primer and unlabelled CTP (not shown). Bands corresponding to G11 + 2 nt and G11 + 5 nt were cut out of the gel and quantified by scintillation counting. The relative band intensities plotted against the corresponding ATP concentration could be fitted to a hyperbolic equation and gave apparent $K_m$ values of 1 µM and 50 µM for the formation of the G11 + 2 nt and the G11 + 5 nt products respectively (Fig. 5). Interestingly, the apparent $K_m$ value for ATP measured for the production of the G11 + 5 nt RNA was similar to the ‘high’ $K_m$ measured for ATP in complete ApG primed transcription reactions (Table 1). These results indicate a significant mechanistic difference between AMP incorporation at position 4 as compared to AMP incorporation at position 5. This is consistent with the observation that larger amounts of short transcripts were produced as compared to longer RNA products, and these shorter transcripts were stopped at position 4. Apparently, only a fraction of initiation events escaped into the elongation phase by passing position 5 under these conditions. These results suggest that a conformational change of the polymerase is induced by the incorporation of the AMP at position 5 relative to the template RNA. This incorporation, which commits the polymerase to processive elongation, requires higher concentrations of ATP than is required for AMP incorporation at other template positions and explains the measurement of a high apparent $K_m$ for ATP in complete transcription reactions.

**Table 2. Transcription activity in the presence of DRB**

<table>
<thead>
<tr>
<th>DRB (µM)</th>
<th>Activity (%)</th>
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<tr>
<td>200</td>
<td>96</td>
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<tr>
<td>100</td>
<td>104</td>
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<tr>
<td>50</td>
<td>93</td>
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<td>20</td>
<td>96</td>
</tr>
<tr>
<td>10</td>
<td>106</td>
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<td>5</td>
<td>113</td>
</tr>
<tr>
<td>1</td>
<td>98</td>
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<tr>
<td>0</td>
<td>100</td>
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In the present study we established *in vitro* systems to separately analyse and compare influenza virus specific transcription initiation and elongation reactions. The determination of $K_m$ values for the NTP substrates of ApG primed RNA synthesis revealed a specific requirement for ATP during transcription initiation. In transcription elongation reactions the $K_m$ for ATP was low and comparable to the $K_m$ values for the other NTPs. The $K_m$ values for the NTPs other than ATP were similar for both complete transcription and elongation reactions. The significant change of the $K_m$ for only ATP in the reactions suggested a specific requirement for ATP during transcription initiation. The synthesis pattern of transcription products as observed on denaturing acrylamide gels showed that RNA synthesis by the influenza virus polymerase could be connected to the production of significant amounts of short abortive transcripts indicative of the transition between an initiation and an elongation phase of transcription similar to reports for other RNA polymerases (Carpousis & Gralla, 1980; Hansen & McClure, 1980; Yamakawa et al., 1981; Luse & Jacob, 1987; Levin et al., 1987; Martin et al., 1988; Sun et al., 1996). The ability of the influenza virus polymerase to reiteratively produce trinucleotide abortive transcripts *in vitro* has been described before (Horisberger, 1982). The maximal size of the influenza virus specific abortive oligonucleotides corresponded to a stop of transcription at position 4 relative to the template, i.e. the primer RNA plus two nucleotides in the capped GEM-RNA system. The synthesis of much larger amounts of transcripts, which are stopped at position 4, compared to the amounts of longer RNAs was even more intriguing when considering the fact that up to four AMP residues could be incorporated at positions 4 to 7 relative to the template (see Fig. 4), but one was incorporated more efficiently than the others. An estimation of AMP incorporation at other template positions and explains the measurement of a high apparent $K_m$ for ATP in complete transcription reactions.

**Discussion**

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Fig. 3. Endonuclease and transcription initiation reactions with cap-labelled GEM-RNA. (a) Left panel: transcription initiation reaction performed with 0-1 nM RNP in the presence of increasing concentrations of CTP (0·0001, 0·0005, 0·001, 0·005, 0·01, 0·05, 0·1, 0·5, 1, 5, 10, 50, 100, 500 µM, from left to right) for 2 min at 30 °C. Right panel: transcription initiation reaction performed with 0·1 nM RNP and 1 µM CTP (lane 'RNP,CTP'), endonuclease reaction in the absence of CTP (lanes 'RNP') and RNA sequencing reactions of GEM-RNA using RNase T1 (G residues) and RNase U2 (A residues). Transcription products were separated on a 20% acrylamide gel. The G11 endonuclease product migrates slightly slower than the corresponding RNase product in the T1 lane, because the former contains a hydroxyl group at the 3' end whereas the latter has a phosphate group. The positions of the full-length GEM-RNA, the endonuclease product (G11) and the initiation product (G11 +1nt) are indicated on the left. Additional bands are due to unspecific degradation of the labelled substrate RNA under the incubation conditions. (b) Band intensities of GEM-RNA, G11 and G11 +1 nt were measured by cutting the corresponding bands from the gel and scintillation counting. Relative band intensities of G11 +1nt were plotted against CTP concentration and...
ATP requirement of influenza virus transcription

GTP and UTP from the reaction leading to a forced stop at position 7 (G11+5 nt). The absence of RNA products corresponding to positions 5 and 6 of the template therefore suggests that passing position 5 is followed by a concomitant increase of AMP incorporation efficiency. The apparent $K_m$ (ATP) observed for passing position 5 is very similar to the 'high' $K_m$ (ATP) obtained in complete ApG primed transcription reactions, supporting the notion that the low

fitted to a hyperbolic curve. The graph shows the quantification of the first seven lanes (up to 0-1 mM CTP) to emphasize the quality of the hyperbolic fit from low to saturating CTP concentrations. The apparent $K_m$ value for CTP for the first phosphodiester bond formation was estimated to be 10 nM. The increase in band intensity for the formation of the G11+1 band at different CTP concentrations was linear for about 30 min under these conditions. (c) Nucleotide sequences of the G11 primer and the conserved influenza A virus vRNA 3’ end, which is the immediate template for transcription. The G11 primer can form two base-pairs with the template RNA, and transcription preferentially initiates with CTP. There is sequence heterogeneity at position 4 of the template with segments 1–3, 5 and 7 containing C and segments 4, 6 and 8 containing U at this position in the case of influenza virus A/PR/8/34. Transcription reactions were done in a mixture of all vRNA segments.
efficiency step of AMP incorporation at position 5 accounted for the ‘high’ $K_m$ for ATP measured in complete transcription reactions. Interestingly, the influenza virus specific endonuclease activity, which provides primer RNA for transcription initiation, was apparently not rate limiting in this RNA synthesis system. The cleavage of precursor RNA and the incorporation of the first nucleotide, CMP, was fast compared to the production of a transcript having two nucleotides incorporated.

We were able to detect this significant change in $K_m$ (ATP) early in transcription, indicative of the transition of influenza virus RdRp from transcription initiation to elongation, because the specific sequence of the influenza virus vRNA 3’ end requires AMP incorporation at four subsequent sites. The present results pose the question whether this polymerase transition also occurs at template position 5 with primers that are initiated at position 2 instead of position 3, as is the case with GEM-RNA. Such studies are in progress, and they will determine if it is either the length of the nascent RNA passing a threshold value or the incorporation of a crucial nucleotide at a fixed position relative to the template that induces the structural change of the polymerase.

Recent studies on eukaryotic RNA polymerase II suggest that a $\beta-\gamma$ hydrolysable ATP cofactor may be specifically required for promoter escape during early transcription (Goodrich & Tjian, 1994; Dvir et al., 1996). Evidence for a
specific ATP requirement during transcription initiation was also found in one study with *E. coli* RNA polymerase (Fujikawa *et al.*, 1991), and was also described in association with the requirement for specific initiation factors (Popham *et al.*, 1989; Kustu *et al.*, 1991). Other RNA polymerases are independent of ATP hydrolysis and even RNA polymerase II can function in the absence of hydrolysable ATP analogues on supercoiled DNA templates (Timmers, 1994; Goodrich & Tjian, 1994). The requirement for ATP hydrolysis may therefore be determined by the promoter type and template topology and be due to the recruitment of accessory factors like nucleic acid helicases or transcription enhancers rather than to a basic function of nucleic acid polymerase active sites.

Is the transition from initiation to elongation, which we observe with the influenza virus RdRp, coupled to ATP hydrolysis? Our present results show that transcription was similarly efficient in the presence of either ATP or ATPγS, arguing against the need for β-γ bond hydrolysis. ATPγS can be used as a substrate by certain kinases, but the RNP preparations showed no detectable kinase activity in control experiments (see below). On the other hand, AMPPNP and AMPPCP could not replace ATP in the same assay and the use of AMPPNP, but not AMPPCP, for RNA synthesis became measurable only in elongation reactions. The most likely explanation for this result is an apparently low affinity of the influenza virus polymerase for these ATP analogues, as has been observed before in other polymerase systems (Testa & Banerjee, 1979; Lofquist *et al.*, 1993; Timmers, 1994; Gershowitz *et al.*, 1978). Transcription activity levels of roughly 10% with AMPPNP as compared with ATP would remain undetected in our assays. The apparent increase in affinity for AMPPNP during the elongation phase is consistent with a conformational change of the polymerase, connected with the change from a promoter-bound to a forward-moving conformer.

Together, the findings describe an *in vitro* system to study biochemical parameters of the influenza virus RdRp activity. We observe a specific requirement for ATP during transcription initiation and the production of distinct products of abortive transcription indicative of an ATP-induced polymerase transition from transcription initiation to elongation at position 5 relative to the template RNA. This transition appeared to be independent of ATP β-γ bond hydrolysis. It may be mechanistically similar to the passage of pause sites by RNA polymerases, which has also been shown to result in an apparent increase in the *Km* value for the substrate needed to induce polymerase translocation (Kingston *et al.*, 1981; Guajardo & Sousa, 1997).

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