Influenza virus is a negative-sense, segmented RNA virus encoding its own RNA-dependent RNA polymerase. It contains three subunits, PB1, PB2 and PA, that together with the viral nucleoprotein are required for transcription and replication of its genome (reviewed by Fodor & Brownlee, 2002; Lamb & Krug, 2001; Neumann et al., 2004). Polymerase activity requires a specific virion RNA (vRNA) or cRNA promoter. The minimal vRNA and cRNA promoters are conserved sequences of short, non-coding sequences (14–15 nt in length) at the 5′ and 3′ ends of the gene segments that base pair partially with each other (Fodor et al., 1993; Pritlove et al., 1994), yet retain short, terminal 5′ and 3′ hairpin loops in a corkscrew configuration (Azzeh et al., 2001; Brownlee & Sharps, 2002; Crow et al., 2004; Flick & Hobom, 1999; Flick et al., 1996).

Recently, we described an in vitro assay for the initiation of replication by a partially purified recombinant RNA polymerase (3P) isolated by transient transfection of 293T cells (Deng et al., 2006). In this assay, the dinucleotide pppApG was synthesized in the presence of ATP and [α-32P]GTP and either a model cRNA or vRNA promoter, but in the absence of any added primer.

The aim of the study here was, firstly, to extend these findings to study the initiation of replication by a recombinant influenza ribonucleoprotein (RNP) complex in vitro. Secondly, in order to characterize further the mechanism of initiation, we assessed whether adenosine, AMP and ADP could substitute for ATP in our assay. Thirdly, we compared the efficiency of initiation by PB1–PA and, for comparison, PB1–PB2 dimeric polymerase with that of heterotrimeric RNA polymerase, as PB1–PA dimers were reported to be active in replication (Honda et al., 2002).

Influenza RNP complex was prepared, as before, by transient transfection of 293T cells with pcDNA expression plasmids for PB1, PB2, PA and NP of influenza A/WSN/33 virus and a negative-sense CAT reporter (pPOLI-cCAT) flanked with influenza cRNA promoter ends (Crow et al., 2004). To aid affinity purification of the RNP complex, the PB2 subunit was C-terminally tagged with a tandem affinity purification (TAP) tag (Puig et al., 2001). After partial purification and cleavage with tobacco etch virus (TEV) protease, the tagged PB2 has a 46 aa C-terminal extension. Such C-terminal tags are known to be compatible with both transcription and replication and do not apparently interfere with polymerase assembly (Deng et al., 2005).

We first confirmed [Fig. 1(a)] (lane 2) that the PB1–PB2tap, PA and NP components of the RNP could be visualized readily on SDS-PAGE and were present at levels similar to those of the 3P (lane 1) complex, partially purified in parallel. A minor component, Hsp90, was detected, as before, fractionating close to the PB1 band (Deng et al., 2005). NP was present, as expected, in the RNP complex (lane 2), but was absent in the 3P complex (lane 1). The RNP and 3P complexes were estimated to be <25% pure because of contamination with TEV protease, IgG, Hsp70 and other, mainly lower-molecular-mass components.

Fig. 1(b) (lane 1) shows the dinucleotide pppA32P synthesized by the RNP complex in the presence of ATP and [α-32P]GTP under conditions of incubation similar to before (in 3 μl for 1·5 h at 30 °C) (Deng et al., 2006), except...
that the final ATP concentration was 1.6 mM. The yield of pppA\(^{32}\)pG (lane 1) was significant and was estimated quantitatively, by phosphorimage analysis, as 1.8% of the input \(\alpha\)-\(^{32}\)P-labelled GTP. When ATP was replaced with ADP, AMP or adenosine, slower-moving products were observed (Fig. 1b, lanes 5, 9 and 13), consistent with their identification as ppA\(^{32}\)pG, pA\(^{32}\)pG and A\(^{32}\)pG, respectively. The yields of products relative to the input \(\alpha\)-\(^{32}\)P-GTP at the highest concentrations of the various substrates tested were as follows: pppA\(^{32}\)pG, 1.8%; ppA\(^{32}\)pG, 1.1%; pA\(^{32}\)pG, 9.2%; A\(^{32}\)pG, 4.3%. Thus, the order of preference of substrates was AMP > adenosine > ATP > ADP. The high yields of dinucleotides suggest that this assay measures a very active catalytic property of the enzyme.

The yields of pppA\(^{32}\)pG, ppA\(^{32}\)pG and pA\(^{32}\)pG were not reduced markedly when the ATP, ADP or AMP concentrations were reduced from 1.6 to 0.2 mM (Fig. 1b, lanes 1–4, 5–8, 9–12). However, in the case of adenosine (lanes 13–16), which, because of its lower solubility, was tested over a lower concentration range (0.1–0.8 mM), the yield of A\(^{32}\)pG was reduced from 4.3% of the input \(\alpha\)-\(^{32}\)P-GTP at 0.8 mM adenosine to 1.3% at 0.1 mM adenosine.

The identity of A\(^{32}\)pG was confirmed by gel elution and the finding that it co-migrated with an unlabelled ApG marker by polyethyleneimine (PEI)–cellulose thin-layer chromatography (TLC) in 0.7 M ammonium sulphate (Randerath & Randerath, 1967) (results not shown). The identity of pA\(^{32}\)pG was confirmed by gel elution followed by T\(_2\) RNase digestion and the fact that it co-migrated on PEI–cellulose TLC in 0.7 M ammonium sulphate (Randerath & Randerath, 1967) (results not shown). The identity of ppA\(^{32}\)pG was deduced from the isolation of a T\(_2\) RNase-digestion product, presumed to be ppA\(^{32}\)p, migrating, as expected, just slower than marker ATP on PEI–cellulose TLC, and the fact that it had only a marginally slower mobility than pppA\(^{32}\)pG on 25% PAGE in 6 M urea (Fig. 1b).

Surprisingly, ADP consistently gave rise to both ppA\(^{32}\)pG and pA\(^{32}\)pG (lanes 5–8), suggesting that a contaminant in our enzyme preparation – possibly a nucleotide pyrophosphatase, as suggested before (Deng et al., 2006) – converted ppA\(^{32}\)pG to pA\(^{32}\)pG during or after dinucleotide synthesis. Given this conversion, we might also have expected a partial conversion of pppA\(^{32}\)pG to ppA\(^{32}\)pG by the same pyrophosphatase contaminant when synthesis of dinucleotides was performed with ATP. Indeed, evidence that this occurred was the presence of a minor product in a position consistent with ppA\(^{32}\)p after T\(_2\) RNase digestion on PEI–cellulose TLC (results not shown). This minor conversion of pppA\(^{32}\)pG to
ppA\textsuperscript{32}pG was not visible in Fig. 1(b) (lanes 1–4) because of lack of resolution of the di- and triphosphorylated forms.

As different strategies are used by influenza RNA polymerase to initiate replication on its vRNA and cRNA promoters (Deng et al., 2006), it was of interest to compare initiation by ATP, ADP, AMP and adenosine with the vRNA and cRNA promoters, separately. To do this, partially purified recombinant RNA polymerase (3P) was prepared as before (Deng et al., 2006) (Fig. 1a, lane 1). The polymerase preparations were incubated with [\(\alpha\)-\textsuperscript{32}P]GTP and initially ATP (1 mM) under the same conditions as for Fig. 1(b), except for the addition of either a model vRNA or cRNA promoter (Fig. 3b, c). Fig. 2(a) (lanes 3 and 15) confirms our previous results (Deng et al., 2006) that the 3P complex can initiate synthesis, giving rise to ppA\textsuperscript{32}pG with either added model vRNA or cRNA promoter. However, the efficiency of synthesis of ppA\textsuperscript{32}pG was on average twofold lower than with the RNP complex (Fig. 1), presumably because of inefficient assembly of the model promoters with the polymerase. As expected, dinucleotide synthesis required both the 5’ and 3’ strands of the promoters. There was no activity if either the 5’ or 3’ strand alone of either of the promoters was used [compare lanes 1–3 and 13–15 in Fig. 2(a)].

Interestingly, the efficiency of initiation by ATP, ADP, AMP and adenosine in the synthesis of their respective dinucleotides differed for the vRNA and cRNA promoters. Thus A\textsuperscript{32}pG (Fig. 2a, lane 24) was present in higher yield than ppA\textsuperscript{32}pG, ppA\textsuperscript{32}pG or pA\textsuperscript{32}pG for the cRNA promoter, whereas A\textsuperscript{32}pG (lane 12) was in lower yield than ppA\textsuperscript{32}pG, ppA\textsuperscript{32}pG or pA\textsuperscript{32}pG for the vRNA promoter. Moreover, the yields of pA\textsuperscript{32}pG (Fig. 2a, lane 21) were higher than those of ppA\textsuperscript{32}pG (lane 15) for the cRNA promoter, but similar for...
the vRNA promoter (compare lanes 3 and 9). These differences suggest that the accessibility of ATP, AMP and adenosine for the polymerase differs between the two promoters. This is consistent with our previous results that viral RNA polymerase initiates replication internally from positions 4 and 5 of the cRNA promoter and then realigns to the 3′-terminal residues 1 and 2 for subsequent elongation. However, for the vRNA promoter, initiation occurs at positions 1 and 2, giving rise to the dinucleotide pppApG, followed by elongation (Deng et al., 2006).

In order to test which initiating nucleotide is favoured in the initiation of replication by the polymerase, an equimolar mixture of ATP, AMP and adenosine at concentrations varying between 0·0·8 and 0·0·9 mM was tested with the 3P complex and the model vRNA promoter. All three possible dinucleotide products were synthesized, but A32pG was present in the lowest yield with both model vRNA (Fig. 2b) and cRNA (results not shown) promoters. Thus, the differences, i.e. the yield of ApG, observed with the two different promoters (Fig. 2a) was at least partially masked when mixtures of ATP, AMP and adenosine were used. Despite substituting efficiently for ATP, ADP and AMP when used alone (Figs 1b, 2a), adenosine did not compete efficiently when present at the same concentration as the other nucleotides.

Fig. 2(c) confirms that there is competition between ATP and adenosine in the synthesis of dinucleotides by the RNP complex, as increasing concentrations of ATP from 0·0·6 to 1 mM (lanes 2–6) in the presence of a constant amount (0·0·8 mM) of adenosine caused a progressive increase in the yield of pppA32pG, while the yield of A32pG decreased progressively. At approximately similar concentrations of adenosine (0·0·8 mM) and ATP (1 mM), ATP was preferred over adenosine (lane 6), in agreement with the results for the 3P complex (Fig. 2b).

By showing that ADP and adenosine were substrates for the initiation of replication, we significantly extend previous knowledge that a virus-derived (non-recombinant) RNP complex can initiate with ATP and AMP (Kolpashchikov et al., 2004). The 5′ triphosphate group is critical not required for initiation. Moreover, we were able here to study initiation with recombinant polymerase in addition to the RNP complex, demonstrating differences in the properties of the vRNA and cRNA promoters with respect to initiation by ATP, ADP, AMP and adenosine. In addition, the dinucleotides ApG and pApG migrated slower than pppApG (Fig. 1b), allowing more sensitive detection than pppApG, because pppApG migrated close to [α-32P]GTP. Therefore, the use of either adenosine or AMP, rather than ATP, provides a convenient, sensitive and easy assay of replication initiation. Overall, our results raise the possibility that not all replication products of the influenza viral genome necessarily contain a 5′ triphosphate end group—perhaps 5′ diphosphate, 5′ monophosphate or 5′ hydroxyl end groups could occur. A 5′ monophosphate end group is consistent with a report indicating that influenza vRNA segments can be circularized by RNA ligase without prior pyrophosphatase treatment (Szymkowiak et al., 2003). However, given the low concentrations of free ADP, AMP and adenosine (about 50 μM, <50 μM and 1 μM, respectively) compared with ATP (about 5 mM) in eukaryotic cells (Brosnan et al., 1990; Mills et al., 1976), it is likely that the majority of the replication products of the influenza viral genome will initiate with ATP and contain a 5′ triphosphate end group.

Finally, we tested the ability of PB1–PA and PB1–PB2 dimeric RNA polymerase to synthesize pppA32pG, using ATP and [α-32P]GTP as substrates. PB1–PAtap and PB1–PB2tap dimers were expressed in 293T cells and purified by TAP affinity purification (Fig. 1a, lanes 3, 4) (Deng et al., 2005). Fig. 3(a) shows that no pppA32pG products were detected with either dimer, either with the model vRNA (lanes 4, 5) or cRNA (lanes 8, 9) promoters. By contrast, the 3P complex synthesized a product in good yield (lanes 3 and 7). However, we cannot exclude the possibility that dimers have residual activity below our detection limit (<5% of the 3P complex) in this assay. Furthermore, addition of recombinant NP did not influence the yield of pppA32pG product synthesized by the 3P complex (results not shown), in agreement with there being no direct involvement of NP in the initiation of replication (Lee et al., 2002; Mullin et al., 2004; Vreede et al., 2004).

The results presented here are apparently inconsistent with a report (Honda et al., 2002) that PB1–PA, expressed in insect cells, was active in the de novo synthesis of a 53 nt long transcript directed by a model vRNA promoter, but was much less active with model cRNA promoters. The reasons for these contradictory results are unknown. However, limitations in one or other of the assays, the fact that different host cells (human 293T cells or insect cells) were used to express the recombinant polymerase, potentially different post-translational modifications of the polymerase and differences in the purity of the expressed polymerase could all have contributed.

Nevertheless, the lack of enzymic activity of the dimers in initiating replication found here is consistent with our recent report that such dimers show no detectable enzymic activity either in transcription with capped primers or in ApG-primed activity, even though the PB1–PA dimer could bind the promoter (Deng et al., 2005).

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References


