

Purification, thioredoxin renaturation, and reconstituted activity of the three subunits of the influenza A virus RNA polymerase

(*in vitro* transcription)

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ABSTRACT The virion-associated RNA polymerase and the structural nucleoprotein of influenza A virus were separated by sodium dodecyl sulfate/PAGE, electroblotted to a polyvinylidene membrane, and eluted with good recovery from the membrane. After renaturation by incubating with *Escherichia coli* thioredoxin, these proteins were active in a reconstituted *in vitro* transcription reaction with purified genomic RNAs. All four proteins (i.e., the three subunits of the RNA polymerase as well as the structural nucleoprotein) were required for activity. The RNA products were plus-strand, mRNA-sized species.

Influenza virus is an enveloped single-stranded RNA-containing virus whose genome is of negative sense and is in eight segments. The three largest genomic segments encode an RNA-dependent RNA polymerase composed of two basic subunits (PB1 and PB2) and an acidic subunit (PA) (1), which associate to form the active enzyme. Transcription of the genomic segments by the influenza RNA polymerase results in the synthesis of virus-specific mRNAs that contain a capped 5' end consisting of 10-13 nucleotides derived from host cell heterogeneous nuclear RNA and a 3' poly(A) tail. The virus mRNAs are incomplete copies of the genomic RNAs in that transcription begins at the penultimate base at the 3' end of the template and terminates 17-22 nucleotides from the 5' end (2). Studies of *in vitro* transcription by influenza ribonucleoprotein complexes (RNPs) containing the enzyme plus genomic RNA encapsidated with nucleoprotein (NP) have indicated that PB2 is involved in the binding of the polymerase to capped host cell mRNAs (3-5) and the subsequent endonucleolytic cleavage of the capped 10- to 13-nucleotide-long oligonucleotide derived from host mRNA (6), which serves as primer in the synthesis of transcripts. PB1 has been shown to catalyze the addition of nucleotides to the nascent mRNA chains (3, 7); the role of PA in transcription has yet to be defined. The replicative mode of influenza-specific RNA synthesis results in the synthesis of full-length plus and minus sense RNAs (8); no data are available concerning the nature of the enzyme that catalyzes this reaction although it is presumed that its composition is somewhat similar to that of the transcriptase.

Several labs have constructed plasmids that express the three polymerase subunits in eukaryotic (and prokaryotic) cells (9-13). These studies have shown that the polymerase subunits are all transported to the host cell nucleus where they, in large part, exist in a sizable pool of tightly associated polymerase complexes that are unassociated with RNPs (12).

To extend the biochemical studies of the mechanism of action of the influenza transcriptase and the role(s) of each subunit, we began work to purify the individual subunits of the virion-associated transcriptase in active form.

In this report, we describe a reconstituted *in vitro* influenza transcription reaction that relies on the three individual, purified, renatured transcriptase subunits, purified NP protein, and genomic RNA for activity. Purification of active enzyme subunits was accomplished by using a procedure that utilizes sodium dodecyl sulfate (SDS)/PAGE resolution of the proteins followed by blotting of the proteins to a polyvinylidene difluoride membrane from which proteins can be quickly eluted with good recovery and renatured following incubation with *Escherichia coli* thioredoxin (14).

MATERIALS AND METHODS

Virus and Virion RNP Complexes. Assorted strains of influenza A virus, all NWS derivatives, were grown in 10-day-old embryonated eggs and purified as described previously (15). Virus was Pronase treated to remove hemagglutinin and neuraminidase proteins (15).

Purified virus cores were treated for 10 min at 0°C with 20 mM Hepes (pH 7.4) containing 0.25% Triton N-101, 1 M urea, and 1.2 mM dithiothreitol and were layered onto a 25% (vol/vol) glycerol cushion containing 20 mM Hepes (pH 7.4), 10 mM KCl, 1.5 mM magnesium acetate, 0.5 mM dithiothreitol, 0.25% Triton N-101, and 1 M urea. The samples were then centrifuged for 2 hr at 45,000 rpm in a Beckman SW 50.1 rotor. This method to produce RNP complexes did not completely remove all of the matrix protein but did produce RNP complexes that were very active in *in vitro* transcription reactions, and they retained activity for several days when stored at 4°C.

In Vitro Transcription Reactions. These assays were carried out in a reaction mixture (200 μ l) containing 30 mM Hepes (pH 7.7), 60 mM KCl, 3 mM magnesium acetate, 1 mM dithiothreitol, 1 mM GTP, 1 mM ATP, 1 mM CTP, 5 μ Ci of [α -³²P]UTP (New England Nuclear, 3000 Ci/mmol; 1 Ci = 37 GBq), 0.05% Triton N-101, 5 units of human placental RNase inhibitor (Pharmacia), 0.5 mM ApG or alfalfa mosaic virus (AIMV) RNA 4, and either purified virion RNPs or renatured proteins isolated from SDS/PAGE gels of RNPs as described below. Reactions were incubated at 31°C; duplicate 20- μ l samples were removed at various times and processed for trichloroacetic acid-precipitable radioactivity (16).

To analyze the RNA products of the *in vitro* reactions, the reaction was stopped by the addition of SDS to 0.5%, and the reaction mixtures were extracted twice with an equal volume of phenol/chloroform, 1:1, (vol/vol). Sodium acetate was added to the aqueous phase to 0.2 M, and RNA was precipitated by the addition of 3 vol of ethanol and storage at -20°C for 12 hr. The precipitates were collected by centrifugation and were washed twice with 70% ethanol/30% 0.2 M

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Abbreviations: RNP, ribonucleoprotein complex; NP, nucleoprotein; AIMV, alfalfa mosaic virus.

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sodium acetate. RNA samples were then analyzed on a 0.9% agarose gel containing 10 mM methylmercury (II) hydroxide (17).

Purification of the Polymerase Subunits and NP Protein. Virion RNP complexes were disrupted by boiling for 5 min in 50 mM Tris-HCl (pH 6.8) containing 1% (wt/vol) SDS, 2% (vol/vol) 2-mercaptoethanol, and 5% (wt/vol) sucrose. The RNP proteins were then resolved on 10% SDS/PAGE gels (16). After electrophoresis, the proteins were electroblotted onto polyvinylidene difluoride membranes (Immobilon, Millipore) at 4°C overnight at 20 V. After electrotransfer, the protein bands were stained with 0.2% amido black 10B in water for 20 min and were destained in distilled water. This method of staining does not lead to denaturation of the proteins on the membrane and has a sensitivity comparable to that of amido black in alcohol/acetic acid. Individual protein bands were eluted from the Immobilon membranes essentially as previously described (18).

The bands corresponding to PB1, PB2, PA, and NP proteins were excised with a small sharp dissecting scissors and were placed in an Eppendorf tube containing 0.2–0.5 ml of eluant (1% Triton X-100 in 50 mM Tris at pH 9.0) per cm² of Immobilon. The samples were then centrifuged for 10 min at room temperature to release the bound proteins, the Immobilon membrane was removed, and the supernatant was centrifuged again for 5 min to remove any insoluble material. If the samples were to be concentrated, the protein-containing eluant was added to 4 vol of acetone in a dry ice/ethanol bath and was allowed to precipitate for 60 min, and the protein was then collected by centrifugation for 15 min in an Eppendorf centrifuge.

Purification of Influenza Virion RNA. The virus was mixed with an equal volume of 20 mM Tris-HCl, pH 7.9/1 mM EDTA/1% SDS and was extracted twice with phenol/chloroform, 1:1. The RNA was then ethanol precipitated, washed twice with 70% ethanol, and dissolved in 10 mM Tris acetate (pH 7.5) containing 0.5 M NaCl and 1 mM EDTA. The solution was passed through an Elutip-r column (Schleicher & Schuell). The adsorbed RNA was eluted with 8 M guanidine hydrochloride/0.1 M sodium acetate, reprecipitated with cold ethanol, and redissolved in 10 mM Tris, pH 7.4/1 mM EDTA.

Reconstitution of the *in Vitro* Transcriptase Reaction. The three individual polymerase subunits and NP protein were excised from the Immobilon membrane, and strips containing ≈1 μg of total polymerase and 10 μg of NP were eluted as described above. The pH of the samples was lowered to 7.0 by adding 5 μl of 1 M HEPES (pH 7.0). The enzyme subunits plus NP protein were mixed and were then incubated with reduced thioredoxin (Chemical Dynamics, South Plainfield, NJ) (50 μM) and virion RNA (≈1 μg) overnight at 4°C in the presence of 50 units of RNasin. The mixture was then incubated for 1 hr at 31°C after the addition of fresh virion RNA (≈1 μg); an equal volume of 2× transcription mixture was added, and transcription was carried out as described above.

Preparation of Radiolabeled Probes. Purified virion RNA was 5' end-labeled by using T4 polynucleotide kinase (Bethesda Research Laboratories) as described (19).

Slot Blot and Northern Blot Analyses. RNA products of the *in vitro* transcription reactions were denatured with 1.6 M glyoxal for 1 hr at 50°C and blotted to nitrocellulose membranes in a Bethesda Research Laboratories slot blot apparatus. The membranes were baked for 2 hr at 80°C and were prehybridized for 12 hr at 42°C in 5× SSC (1× = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/5× Denhardt's solution (1× = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/50 mM sodium phosphate, pH 6.8/0.1% SDS/5 mM EDTA/yeast RNA at 50 μg/ml/sheared salmon sperm DNA at 50 μg/ml/50% (vol/vol)

formamide. Hybridization was at 42°C for 48 hr in the same solution but with 45% formamide.

For Northern blotting, the RNA samples were resolved on a 1.2% agarose gel in methylmercury (II) hydroxide, RNA was transferred to nitrocellulose overnight, and hybridization was performed as above.

RESULTS

Purification of the Influenza Polymerase Subunits and NP Protein. All three polymerase subunits are packaged within the virus particles in tight association with the RNP complex (6) as a multisubunit enzyme (12). The dissociation of the polymerase subunits from the RNP templates and from each other has been achieved only by the use of cesium trifluoroacetate gradients (6) or by the use of SDS (1). However, strong detergents such as SDS result in denaturation of the proteins and loss of biologic activity. We initially attempted to use conventional methods of protein purification to obtain individual active subunits including the following: (i) selective dissociation of the subunits from virion RNPs with high salt concentrations and nonionic detergents, (ii) molecular-sieve chromatography of the RNP proteins in the presence of high salt and nonionic detergents, and (iii) use of antibody affinity columns. None of these methods produced separation of the subunits from each other or from NP protein. Furthermore, we observed that the polymerase proteins, once solubilized, demonstrated a marked tendency to tightly adsorb to glass and plastic, which resulted in large losses during each step of purification.

We have recently observed (18) that proteins of various molecular weights could be quickly and efficiently eluted from Immobilon membranes in a pH-dependent manner, and we therefore used this technique in our attempts to purify the influenza polymerase subunits. Fig. 1 shows that all three

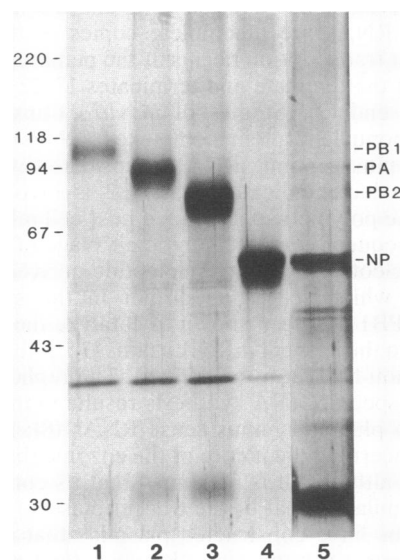


FIG. 1. Purification of influenza A proteins by elution from Immobilon membranes. The proteins of the purified influenza RNP complexes were separated on a 10% SDS/PAGE gel, transferred to an Immobilon membrane, and stained with amido black. The individual bands were excised and eluted, and the eluted proteins were precipitated with acetone and reanalyzed on a 10% SDS/PAGE gel with silver staining. The band at a molecular weight of ≈40,000 was an artifact of the elution procedure and was present in controls without protein. Lanes: 1, PB1; 2, PA; 3, PB2; 4, NP; 5, influenza virion RNP complexes. The identity of the enzyme subunits in the gels was confirmed by immunoblotting with rabbit polyclonal antisera. The positions of molecular weight standards ($\times 10^{-3}$) are indicated at left.

subunits as well as NP protein, when reanalyzed by SDS/PAGE after elution from the Immobilon membranes, were clearly separated with very little degradation.

Renaturation of the Polymerase Subunits. It is perhaps likely that transfer of proteins from SDS/PAGE to membrane matrices such as nitrocellulose or Immobilon leads to a partial renaturation as evidenced by the ability of specific antibodies directed against the native proteins to react with the blotted proteins in Western analyses. However, in many cases the transferred proteins are not active enzymatically or biologically. Recently, the efficacy of thioredoxin in the renaturation of proteins was reported (14), and it was suggested that this protein act efficiently to catalyze the formation of correct disulfides during protein refolding. Furthermore, recent work in our lab showed that *E. coli* RNA polymerase subunits purified on Immobilon membranes as described above and renatured with thioredoxin resumed 80% of the original enzymatic activity when reconstituted (18). We therefore eluted the polymerase subunits and NP protein from the Immobilon membranes and incubated these proteins plus influenza RNA with thioredoxin and then tested this reconstituted mixture *in vitro* for incorporation of radio-labeled UTP into acid-insoluble material in a transcription reaction.

Fig. 2 shows that, when the reaction contained all three subunits, NP protein, and purified virion RNA plus either ApG or AIMV RNA 4 as primer, there was incorporation into acid-insoluble material, which was usually in the range of 10–20% of that incorporated by purified virion RNPs. Incorporation was often linear for up to 3 hr. If the eluted proteins were not incubated with thioredoxin, there was no incorporation in the *in vitro* reaction (data not shown).

The Renatured RNA Polymerase Synthesizes Plus-Stranded RNA Products. To establish the polarity of the RNA products

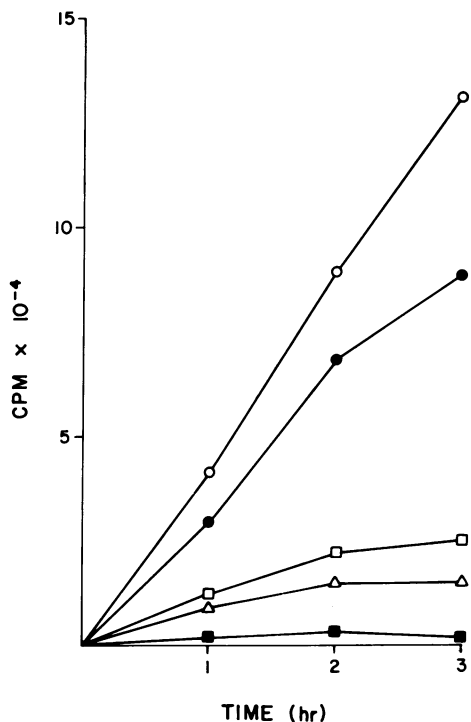


FIG. 2. Synthesis of RNA in a reconstituted *in vitro* transcription reaction. The reaction mixture (200 μ l) was as described in *Materials and Methods*. ●, Purified virion RNP complexes (10 μ g of total protein) plus 0.5 mM ApG as primer; ○, the same as symbol ● plus 4 μ g of AIMV RNA 4 as primer; □, renatured polymerase subunits and NP protein (10 μ g of total protein) plus purified virion RNA and 0.5 mM ApG; △, the same as symbol □ plus 4 μ g of AIMV RNA 4; ■, reaction mixture plus virion RNA and no proteins.

synthesized *in vitro* by the renatured polymerase subunits, we blotted unlabeled RNA products from the *in vitro* reaction to nitrocellulose in a slot blot apparatus and then probed them with ³²P-end-labeled virion RNAs. As can be seen in Fig. 3, both virion RNPs (slots 1 and 2) and reaction mixtures containing renatured polymerase subunits plus NP protein and virion RNA (slots 3 and 4) all synthesized significant amounts of positive-strand RNAs.

Slots 5 and 6 contained RNA products from *in vitro* reactions containing polymerase subunits and NP protein that had been eluted from Immobilon membranes by using an elution buffer at pH 8.0. The reduced amount of RNA detected in slots 5 and 6 (when compared with slots 3 and 4) probably reflects the pH dependence of elution of proteins from Immobilon (18), and therefore these reactions probably contained less enzyme (18). This was confirmed by the reduced amount of RNA detected in reactions reconstituted with enzyme eluted from Immobilon with transcription buffer at pH 7.7. This might be expected to result in a further decrease in enzyme recovery from the membranes. It can also be seen when comparing slot 1 with slot 2 as well as slot 3 with slot 4 that AIMV RNA 4 at 4 μ g per reaction, which is about a 60 nM concentration, served as a much better primer in this reaction than did 0.5 mM ApG, an observation previously reported by Plotch and coworkers (20).

Size of Products Synthesized in the *in Vitro* reaction. The size distribution of the RNA products was analyzed on denaturing agarose gels, and, as shown in Fig. 4, they were heterogeneous in size and were in the same molecular weight range as the five small influenza genomic segments (\approx 900–1800 nu-

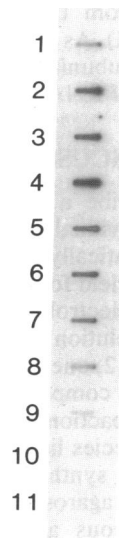


FIG. 3. Synthesis of plus-strand RNA in the *in vitro* reconstituted reaction: effects of pH and primer on synthesis. *In vitro* transcription was as described in *Materials and Methods* with purified native RNPs and renatured polymerase subunits plus NP protein and virion RNA. Unlabeled RNA products were analyzed by slot blot with a 5' (³²P) end-labeled virion RNA probe, and then the blot was subjected to autoradiography for 48 hr. Slot 1, virion RNPs (\approx 1 μ g) plus 0.5 mM ApG. Slot 2, virion RNPs (\approx 1 μ g) plus 4 μ g of AIMV RNA 4. Slot 3, reaction mixture containing renatured polymerase subunits and NP (\approx 10 μ g of total protein) eluted from Immobilon with 1% Triton X-100 in 50 mM Tris-HCl (pH 9.0). Primer was 0.5 mM ApG. Slot 4, same as slot 3, but primer was 4 μ g of AIMV RNA 4. Slot 5, renatured polymerase reaction was the same as slot 3 except elution was at pH 8.0. Primer was 0.5 mM ApG. Slot 6, same as slot 5 but with AIMV RNA 4 as primer. Slot 7, renatured polymerase reaction, but elution with transcription buffer at pH 7.7 and 0.5 mM ApG as primer. Slot 8, same as slot 7, but AIMV RNA 4 as primer. Slot 9, same as slot 5, but with 3% bovine serum albumin added to the elution buffer. Slot 10, same as slot 3, but without added virion RNA template. Slot 11, virion RNA alone (no added proteins).

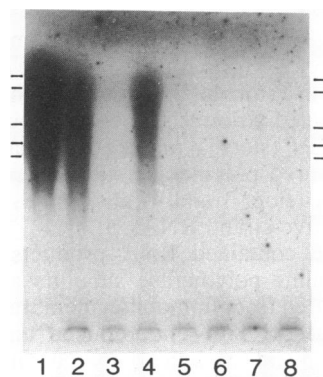


FIG. 4. Size analysis of the RNA products of the reconstituted *in vitro* reaction on methylmercury (II) agarose gels. *In vitro* reactions each contained 0.5 mM ApG as primer. The reactions were carried out for 3 hr, and the RNA was analyzed on denaturing gels, which were subjected to autoradiograph. Lane 1, purified virion RNP complexes. Total protein was $\approx 10 \mu\text{g}$. Lane 2, the same as lane 1 except that total protein was $\approx 0.3 \mu\text{g}$. Lane 3, a reaction containing purified virion RNA alone. Lane 4, a reaction containing the three polymerase subunits plus NP protein eluted from Immobilon and renatured. Total protein was about $10 \mu\text{g}$. Lane 5, same as lane 4, but NP protein was not added. Lane 6, same as lane 4, but PB1 was not added. Lane 7, same as lane 4, but PB2 was not added. Lane 8, same as lane 4, but PA was not added. The positions of influenza virion RNA markers for segments 4–8 stained with ethidium bromide are indicated at the right and left.

cleotides). Furthermore, the products synthesized by the reaction containing all four renatured proteins (Fig. 4, lane 4) were indistinguishable from those synthesized by intact virion RNPs (Fig. 4, lane 1). As can be seen, omission of any one of the polymerase subunits or NP from the reaction resulted in no synthesis of RNA (Fig. 4, lanes 5–8).

DISCUSSION

In this report, we describe a method for the successful isolation of the three individual subunits of the influenza A transcriptase in an enzymatically active form. Activity can be recovered in reasonable yield following electrophoretic separation by SDS/PAGE, electroblotting to Immobilon membranes, and subsequent elution and renaturation with thioredoxin. As shown in Fig. 2, one can usually recover 10–30% of the enzyme activity as compared to native virion RNPs, and this reconstituted reaction incorporated radiolabeled nucleotides into RNA species linearly for 2–3 hr. When the size of the RNA species synthesized in this reaction was examined in denaturing agarose gels (Fig. 4), the RNA species were heterogeneous and had a size range that overlapped that of authentic influenza genomic RNA markers and products synthesized by intact virion RNPs. As was expected, these RNA species were plus sense as shown by dot blot analysis (Fig. 3). The fact that omission of any one of the polymerase subunits from the *in vitro* reaction resulted in lack of synthesis of product RNAs was not surprising based on previous studies of the mechanism of action of holoenzyme (7). However, the observation that addition of renatured NP protein was necessary for the synthesis of these RNA species implied that there probably was at least a partial assembly of RNP complexes from NP and virion RNA.

A previous report by Kato *et al.* (6) showed that a complex containing purified influenza A RNA polymerase plus genomic RNAs would cleave capped poly(A) endonucleolytically at 10–12 nucleotides from the 5' end *in vitro* and would also add several nucleotides to an ApG primer if genomic RNA was included in the reaction. The authors suggested that the RNA polymerase complex was able to carry out at

least limited elongation in the absence of NP protein. In our studies, we did not specifically look for smaller oligonucleotides in the absence of NP protein (Fig. 4, lane 5), but none were immediately apparent in gels of products of the *in vitro* reaction. The results reported here will now enable us to study whether any one purified polymerase subunit has an endonucleolytic activity when incubated with capped eukaryotic mRNA, which subunit will bind ApG and which constituents are necessary for elongation.

The reconstitution conditions utilized in these experiments were selected to utilize RNP proteins in molar ratios close to that of native RNP particles. If the reconstituted transcription reaction was dependent on the assembly of RNP particles, the amount of NP protein added might have been limiting. This may explain the recovery of only partial activity levels. Recently, Kingsbury *et al.* (21) reported that influenza A NP protein expressed in *E. coli*, when purified, readily forms RNP complexes with single-stranded RNA from influenza virus or with RNA of nonviral origin (e.g., ribosomal RNA) but not with double-stranded RNA or DNA. Electron micrographs of purified NP from *E. coli* and eukaryotic ribosomal RNA showed that the RNP complexes formed displayed a helical morphology similar to influenza RNPs. We are currently examining the morphology of the reconstituted transcription complexes under the electron microscope. Kingsbury *et al.* (21) showed that binding of NP to single-stranded RNA was rapid (30 min at 21°C) and that 100% binding occurred at a ratio of four bases per NP protein molecule.

The successful use of SDS/PAGE and Immobilon membranes to purify and thioredoxin to renature the RNA polymerase subunits of this tightly associated (6, 9–13) large enzyme provides a practical means to obtain reasonable amounts of active purified proteins that have proved too difficult to purify by classical biochemical methods. As suggested by Pigiet and Schuster (14), this technique may also be quite useful in the refolding of recombinant proteins produced in bacteria, which are often insoluble, denatured, and inactive.

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