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Complex Formation between Influenza Virus Polymerase Proteins Expressed in Xenopus Oocytes

PAUL DIGARD, VIVIAN C. BLOK, AND STEPHEN C. INGLIS

Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QH

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All three influenza virus polymerase (P) proteins were expressed in Xenopus oocytes from microinjected in vitro transcribed mRNA analogs, with yields of up to 100 ng per oocyte. To examine the functional state of the Xenopus-expressed P proteins, the polypeptides were tested for their ability to form stable complexes with each other. As seen in virus-infected cells, all three P proteins associated into an immunoprecipitable complex, suggesting that the system has considerable promise for the reconstruction of an active influenza RNA polymerase. Examination of the ability of paired combinations of the P proteins to associate indicated that PB1 contained independent binding sites for PB2 and PA, and so probably formed the backbone of the complex. Sedimentation analysis of free and complexed P proteins indicated that PB1 and PB2 did not exist as free monomers, and that similarly, complexes of all three P proteins did not simply consist of one copy of each protein. The heterodisperse sedimentation rate seen for complexes of all three P proteins did not appear to result from their binding to RNA, suggesting the incorporation of additional polypeptides in the polymerase complex.

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INTRODUCTION

Influenza virus gene expression depends on the coordinated transcription and replication of eight segments of negative-sense single-stranded RNA. This is mediated by the three viral polymerase proteins PB1, PB2, and PA in association with the nucleoprotein (Inglis et al., 1976), and possibly with other viral proteins or unidentified host cell factors. On infection of a permissive cell the ribonucleoprotein–polymerase complexes migrate to the nucleus where viral mRNAs are transcribed (Herz et al., 1981). These mRNAs are initiated by, and contain, 5’-capped RNA fragments generated by a viral cap-dependent endonuclease from host cell mRNAs (Plotch et al., 1979). They are also polyadenylated, but lack sequences complementary to the extreme 5’-end of their corresponding genome RNA template (Hay et al., 1977a). At later times, dependent on the production of virus specific proteins, full-length, nonpolyadenylated, noncapped copies of the genomic RNAs are made, which then serve as templates for production of more vRNA (Hay et al., 1977b). The polymerase (P) proteins have been shown to exist and probably function as a complex both in virions (Braam et al., 1983) and in the infected cell (Detjen et al., 1987; Akkina et al., 1987), but little is known about the role of the individual proteins, or the precise composition of the (presumably differing) complexes which catalyze the synthesis of the three types of virus RNA.

To address the structure–function relationship of the polymerase proteins a system is needed whereby individual virus proteins can be expressed in an appropriate environment in sufficient quantity to facilitate reconstitution of activity. It should then be possible to dissect the system to reveal the individual functions of particular proteins. Our approach has been to seek expression of the polymerase-associated polypeptides through transcription of cloned DNA into artificial mRNA analogs using the bacteriophage SP6 RNA polymerase, and translation of these mRNAs in Xenopus oocytes. Such a system offers the advantage that proteins may be produced individually, or in any desired combination, simply by translating the appropriate mRNA mixture. In addition, since the polypeptides can be produced simultaneously in an environment similar to that in the infected cell, the likelihood is that they will display appropriate physiological interactions. Thus, the system offers considerable promise for the reconstitution of enzyme activity.

In this report we describe the establishment of such a system and its preliminary characterization. In addition, as a first step toward reconstitution of enzyme ac
tivity, we show here that in the absence of any other virus specific polypeptides all three P proteins can interac
t to form complexes, and report preliminary char-
acterization of these complexes.

MATERIALS AND METHODS

Materials

All enzymes were obtained from Boehringer-Mann-
heim, and all radiochemicals from Amersham (En-
gland). Nuclease-treated rabbit reticulocyte lysate was
obtained from Dr. T. Hunt (Cambridge). Influenza strain
A/PR8/34 was propagated in, and purified from, embry-
onated eggs as previously described (Inglis et al.,
1976). Infected cell lysates were prepared at 6 hr post-
fection from confluent monolayers of chick embryo
fibroblasts infected at a multiplicity of infection of
around 10.

Plasmids

Transcription vectors for the three polymerase
genes were constructed by the insertion of cDNA cop-
ies of the relevant influenza A/PR8/34 segments (the
generous gift of Dr. P. Palese) into a plasmid containing
an SP6 RNA polymerase promoter. The SP6 vector
used, pSP64-T (Krieg and Melton, 1984; obtained from
Dr. A. Colman), also provides flanking 5' - and 3'-non-
coding sequences from the Xenopus globin gene,
which enhance the translation of foreign genes in oo-
cytes (Drummond et al., 1985). Copies of segments 1,
2, and 3 were excised from the plasmids pAPR101,
pAPR206, and pAPR303 (Young et al., 1983) by diges-
tion with the restriction enzymes BamHI, HindIII, and
EcoRI, separately. Segment 1 was inserted directly
into the BglII site separating the 5' - and 3'-globin non-
coding sequences in pSP64-T, while segments 2 and
3 were end-filled by Klenow fragment DNA polymerase
(Boehringer) and blunt-end-ligated into a similarly end-
filled pSP64-T. The resulting plasmids containing the
PB1, PB2, and PA genes, in positive orientation relative
to the SP6 promoter, were designated pST1+, pST2+,
and pST3+, respectively. All manipulations were car-
ried out according to standard procedures (Maniatis et
al., 1982).

In vitro transcription and translation

In vitro transcription and translation was carried out
as previously described (Brierley et al., 1987). Briefly,
the three transcription plasmids were linearized down-
stream to the globin 3'-noncoding sequence by diges-
tion with Smal, and SP6 RNA polymerase run-off tran-
scripts were synthesized under conditions which re-
sulted in the incorporation of a synthetic 5'-mGpppG
suitable in the incorporation of a synthetic 5'-mGpppG
cap structure (New England Bio-Labs). The product
RNA was then phenol-extracted and checked for struc-
tural integrity by agarose gel electrophoresis (Maniatis
et al., 1982). For in vitro translation, messenger-depen-
dent reticulocyte lysate (MDL; Pelham and Jackson,
1976) was programmed with mRNA to a final concen-
tration of around 0.1 μg/μl and incubated at 30° for 1 hr.

Microinjection of Xenopus oocytes

Oocytes were taken from the frog, maintained, and
injected essentially according to standard procedures
(Colman, 1984). Each oocyte received a maximum of
50 ng of RNA in a constant injection volume of 50 nl.
At 2 hr postinjection, groups of eight oocytes per RNA
were transferred to Modified Barth’s Saline (MBS) con-
taining [35S]methionine (sp act 1150 Ci/mmol; Amers-
ham, England) at 1.0 mCi/ml (10 μCi/oocyte) and sub-
sequently harvested by mechanical disruption into
TKM buffer (20 mM Tris-HCl, pH 7.6, 100 mM KCl, 5
mM MgCl2, 1 mM phenylmethylsulfonyl fluoride
(PMSF), 50% glycerol, 10 μl/oocyte) at 5 hr postinjec-
tion. The resulting lysates were then clarified by micro-
centrifugation and stored at −20° prior to analysis.

Preparation of monospecific antisera
to the P proteins

The production of antisera directed against the PB2
protein has already been described (Brierley et al.,
1987), and a similar strategy was used to prepare anti-
sera against PB1 and PA. Briefly, portions of P protein
coding sequence were expressed in bacteria as C-ter-
ninal fusions with β-galactosidase, using the pEX se-
ries of plasmids (Stanley and Luzio, 1984). These fu-
sion proteins were then purified by gel elution and used
to immunize rabbits. In this study, antibodies raised to
amino acids 50–370 of PB1, 342–463 of PA, and 585–
759 of PB2 (F5) were used.

Immunoprecipitation from oocyte lysates

For immunoprecipitation, 10 μl of [35S]methionine-la-
beled oocyte lysate (containing the equivalent of one
oocyte) was diluted to 100 μl with oocyte immunopre-
cipitation buffer, (OIPB; 50 mM Tris–HCl, pH 7.6, 100
mM KCl, 5 mM MgCl2, 1% Triton X-100, 1% sodium
deoxycholate, 0.1% SDS, 1 mM PMSF) and left on ice
for 30 min before the addition of 4 μl of rabbit antise-
rum. After a further 30 min on ice, 50 μl of a 50% sus-
pension of protein A–Sepharose (Sigma) in OIPB was
added, and the tubes were rotated at 4° for 30 min.
Finally, the Sepharose-bound material was collected
by centrifugation, washed once with 1 ml of OIPB, and
PBID
PB2D IPA
abcdefghij

Fig. 1. Expression of the influenza virus P proteins in Xenopus oocytes, and their reactivity with the monospecific antipolymerase sera. Lanes a, d, g, j—unprecipitated lysates: oocytes microinjected with pST1+, pST2+, pST3+, H2O, respectively. Lanes c, f, i—precipitated with α-PB1, α-PB2, α-PA, respectively. Lanes b, e, h—precipitated with the corresponding preimmune bleeds.

eluted in Laemmli sample buffer (Laemmli, 1970). Proteins contained in the supernatant were separated on a 10% polyacrylamide gel and detected by autoradiography.

Immunological detection of nitrocellulose-bound proteins

Samples were subjected to polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) and transferred to nitrocellulose according to standard procedures (Towbin et al., 1979). The nitrocellulose was blocked by incubation with a solution of 4% BSA in PBS for 1 hr at 37°C, followed by a 1 hr incubation at room temperature with the antiserum diluted 1 in 200 in 4% BSA, 2% newborn calf serum in PBS. The blot was then rinsed with 1% NP-40 in PBS, before incubation with 1 μCi of [125I]-labeled protein A for 1 hr. A final wash with 1% NP-40 in PBS was carried out before the blot was air-dried and exposed to film.

Velocity gradient centrifugation

Oocyte lysates (from 10 oocytes) were layered on top of linear 5–20% (w/v) sucrose gradients in 20 mM Tris–Cl, pH 7.6, 100 mM KCl, 5 mM MgCl2, 0.1% NP-40, 1 mM PMSF and centrifuged at 100,000 g, for 12 hr. Fractions were then collected, adjusted to 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and analyzed for their P protein content by immunoprecipitation and PAGE. All gradients had bovine serum albumin (BSA; M, 68,000) and apoferritin (M, 440,000) included as internal standards.

RESULTS

Expression of the three P proteins in Xenopus oocytes

To test the capacity of Xenopus oocytes for expression of the influenza P proteins, artificial mRNAs corresponding to each gene were transcribed in vitro using the SP6 RNA polymerase and microinjected into the oocytes. After a 2 hr recovery period, the cells were labeled by incubation with [35S]methionine for 3 hr, harvested, and analyzed by gel electrophoresis before and after immunoprecipitation with specific anti-P protein sera (Fig. 1). In the unprecipitated tracks, strong bands of the expected mobility could be seen [lane a, pST1+ transcript (PB1); lane d, pST2+ transcript (PB2); lane g, pST3+ transcript (PA)] and were not seen in control water-injected oocytes (lane j). In each case these polypeptides were specifically precipitated by the homologous antiserum (lanes c, f, i), but not by the corresponding preimmune sera (lanes b, e, h).

The extent of radiolabeling of the P proteins in the above experiment suggested that their production was quite efficient. To obtain a quantitative estimate, the accumulation of the P proteins was examined over a period of time after microinjection of mRNA, by West-
ern blotting with the directed antisera. The result of such an experiment for PB1 is shown in Fig. 2A. The antiserum specifically detected a single band in lysates from pST1+-injected oocytes which comigrated both with \(^{[35}S]\)methionine-labeled PB1 (synthesized in MDL using pST1+ transcript) and with PB1 from purified virions. The results indicated that the total amount of PB1 increased up to 40 hr postinjection and then declined slightly. Known amounts of purified virus were also included in the Western blot as standards to quantify the amount of PB1 produced. Since the equivalent of half an oocyte was loaded in each track, it was estimated from densitometry scans that after 40 hr each oocyte had accumulated on average 100 ng of PB1 [assuming that PB1 comprises 1% w/w purified influenza virions (Inglis et al., 1976)].

Similar analyses were carried out for the other P proteins (data not shown). The accumulation of PA was similar to that of PB1, but PB2 was produced less efficiently (approximately 10 ng per oocyte). This appears to be the result of protein turnover, since microinjected \(^{[35}S]\)methionine-labeled PB2 (synthesized prior to injection in MDL programmed with pST2+ transcript) was found to have a half-life of 3 hr, and since the accumulation of PB2 was similar to that of the other P proteins up to about 3 hr postinjection (data not shown).

A further experiment was carried out to assess P protein production in oocytes relative to virus-infected cells; Fig. 2B shows a Western blot comparing the amount of PB1 accumulated per half oocyte 36 hr after microinjection, with that present in an equivalent amount (in terms of total protein) of chick embryo fibroblast (CEF) cells at 6 hr postinfection (approximately \(10^6\) cells). A more intense signal was observed from the pST1+-injected oocyte track than that from the infected cell track. However, the rate of production in the infected cell must be higher given the difference in incubation times. Nevertheless, the overall quantities seem comparable.

Formation of P protein complexes in oocytes

An important initial step in the biogenesis of the influenza polymerase is likely to be the formation of a complex of the three P proteins; accordingly, the oocyte-expressed P proteins were examined for their ability to associate with each other. Groups of a dozen oocytes were injected with solutions containing one, two, or all three of the mRNA analogs. For these experiments, the RNAs were mixed in equal quantities and single RNAs were diluted in H2O to keep the concentration of any one transcript constant throughout. The oocytes were then metabolically labeled and harvested as before. A 3-hr labeling period was employed to avoid the problem of the instability of PB2, and allow the production of approximately equal amount of all three proteins. Following harvest, the oocyte lysates were analyzed by immunoprecipitation and PAGE (Fig. 3). The antisera can be seen to be truly monospecific in that none of the P proteins were significantly precipitated by the heterologous antisera when expressed in isolation (lanes a–i). However, each antiserum precipitated all three P proteins from oocytes injected with a mixture of all three transcripts (lanes j–l). This specific coprecipitation provides evidence that when cotranslated in an oocyte, the P proteins are present as a complex.

Given that all three P proteins would associate into a complex, it was of interest to determine which of the polypeptides were interacting with which. Therefore, paired combinations of the mRNA analogs were microinjected, and their products assayed for association as before by immunoprecipitation with specific antisera (Fig. 3, lanes m–r). PB1 and PB2 (lanes m and n), and PB1 and PA (lanes q and r) were found to coprecipitate, but not PB2 and PA (lanes o and p), thus indicating that PB1 may act as the backbone of the complex, and also implying discrete binding sites on PB1 and PB2 and PA.

Sedimentation analysis of individual and complexed P proteins

In order to characterize further the association of the three P proteins in oocytes, individual and complexed
P proteins were analyzed by velocity gradient sedimentation. The aims of this were twofold; first to confirm the physical existence of complexes, by showing an increased sedimentation rate for the coexpressed P proteins, and second, to examine the size of complex formed.

Lysates from oocytes microinjected with single or mixed P protein mRNAs were fractionated on sucrose gradients, and then each fraction was assayed for its P protein content by immunoprecipitation. Figure 4 shows the result of this experiment for individually expressed P proteins. PA migrated slightly faster than the BSA marker (as expected for an M, 82,000 protein), but surprisingly PB1 and PB2, which are similar in size, sedimented as much larger and more heterogeneous bodies. The majority of the proteins sedimented in fractions corresponding to a size of around M, 250,000, but significant amounts of material were also present in fractions corresponding to much larger sizes. For PD1 and PD2 then, it seemed likely that demonstration of a convincing mobility difference between the individual and complexed form would be difficult. However, such an experiment remained possible for PA, given its lower and more discrete sedimentation rate.

Accordingly, oocyte lysates containing either PA alone or PA in combination with PB1 and PB2 were fractionated on a gradient, and the fractions immunoprecipitated with anti-PA serum. The results of this experiment are shown in Fig. 5. Again, PA alone migrated as a reasonably defined band near the top of the gradient (top panel). However, in the presence of the other two P proteins, it sedimented throughout the whole of the gradient (middle panel), suggesting its inclusion in complexes. In addition, the coprecipitation of PB1 and PB2 with PA could also be seen, further confirming the existence of an interaction between the polypeptides.

It is interesting to note, however, that the ratio of the three polypeptides present in the complexes was not constant throughout the gradient. In particular, the ratio of PB1 and PB2 to PA increased in the faster sedimenting species, suggesting either that PA has more than one binding site for each of the basic P proteins, or perhaps more likely, that the former proteins can form complex structures linked through self-association.

From the migration pattern of PA seen in complexes, the marked heterogeneity in sedimentation rate of PB1 and PB2 appears to be extended to a complex of all three P proteins. In view of the fact that the P proteins must interact with RNA, it seemed possible that the high sedimentation values obtained for individually expressed PB1 and PB2 (Fig. 4) and for complexes containing PB1 and PB2 could have arisen from the poly-
peptides binding to RNA present in the lysate. If this was the case, RNase treatment of the lysate prior to gradient fractionation should significantly decrease the sedimentation rate of the complexes. The bottom panel in Fig. 5 shows the result of such RNase treatment; no difference in mobility between treated and untreated complexes could be seen. Furthermore, RNase treatment of lysates containing individually expressed PB2 also failed to affect its sedimentation pattern (not shown), suggesting that the heterogeneous size distribution of the P protein complexes is not the result of association with RNA.

**DISCUSSION**

A major goal in the study of the influenza polymerase is the reconstitution of an active enzyme from cloned components. This would then allow detailed analysis of the biochemical reactions catalysed by the enzyme, while manipulation of the DNA templates would facilitate structural and functional analysis of individual components. Here, we have demonstrated the feasibility of producing all three influenza virus polymerase proteins for functional studies by the translation of *in vitro* transcribed mRNA analogs in *Xenopus* oocytes. High levels of expression were achieved for PB1 and PA after prolonged incubation, reaching 100 ng per oocyte. However, PB2 appeared to be unstable, with a half-life of about 3 hr, which meant maximal expression was around 10 ng per oocyte. Other groups have noted the instability of PB2 when expressed in isolation; PB2 expressed in NIH 3T3 cells using an inducible bovine papilloma virus vector has a similar half-life of around 3 hr (Braam-Markson *et al.*, 1985). Nevertheless, 10 ng of each individual polymerase protein is equivalent to the amount present in about 1 μg of purified virus, and virion transcriptase activity can easily be detected in reactions containing only 5–10 μg of purified virus (Bishop *et al.*, 19/1). Therefore, we believe that the system offers considerable promise for the reconstitution of enzymatic activity.

Our results indicate that all three P proteins expressed in oocytes formed a complex. This parallels the situation seen in the infected cell, where similarly, all three proteins associate, and also confirms the observations of other workers that a complex of the P proteins can exist in virus-infected cells independently of virus RNPs (Detjen *et al.*, 1987; Akkina *et al.*, 1987). It is also the first direct demonstration that all three artificially expressed P proteins can reassociate into a complex, probably a vital preliminary step toward reconstitution of the influenza polymerase. In a previous report, where the influenza P proteins were expressed using baculovirus vectors (St. Angelo *et al.*, 1987), only PB1 and PR2 formed an immunoprecipitable complex. The authors suggested from this that the presence of another influenza gene product was needed for the incorporation of PA into a stable complex. In the light of the results presented here this seems unlikely; an alternative explanation is that amphibian cells provide a more suitable environment for the complex formation than do insect cells. For example some kind of post-translational modification of the P proteins might be necessary for formation of a full complex and this could be more faithfully carried out in *Xenopus* oocytes. The factors involved are likely to be quite subtle, because when the P proteins are cotranslated in MDL, no complex formation can be detected (not shown).

Akkina *et al.* (1987) reported that most of the P proteins found in the cytoplasm of virus-infected cells were not in the form of complexes. However, our work would suggest that a functional nucleus is unlikely to be necessary for complex formation, as the P proteins do not localize to the nucleus in *Xenopus* oocytes, and furthermore, all three proteins associate normally in enucleated oocytes (not shown).

Expression of pairs of P proteins indicated that PB2 and PA can associate independently with PB1, yet cannot form a complex directly with each other. Thus PB1 can be considered the “backbone” of the complex. This idea is consistent with its suggested central role as the protein responsible for elongation (Braam *et al.*, 1983), with the other two polypeptides as adjuncts fulfilling more peripheral functions such as substrate selection or cap-binding.

Gradient analysis of individually expressed P proteins indicated that PB1 and PB2 did not exist as free monomers, but rather as heterogeneous populations of high-molecular-weight aggregates. It is not clear at present whether these structures represent self-aggregation, or the proteins binding to cellular components. This property of a large and heterogeneous sedimentation rate was also a feature of complexes of all three P proteins, as seen in the markedly different migration of PA when expressed alone or in the presence of the other two P proteins. Most of the complexed material sedimented with an apparent molecular weight much greater than 250,000, the expected size for a simple trimolecular complex. Again, this could reflect some kind of association with a cellular component, but it is unlikely to be the result of the complexes binding to RNA present in the lysate, as RNase treatment prior to gradient analysis does not reduce their rate of sedimentation (Fig. 5), or that of PB2 expressed alone (not shown). A second possibility is that the larger complexes contain extra copies of the P proteins. Support
for this idea comes from the observation that the faster sedimenting forms of the complex appear to contain an increased ratio of PB1 and PB2 to PA, suggesting the existence of different stoichiometric forms of complex, varying in their relative content of PB1 and PB2. These might arise from PA binding directly to more than one copy of each protein, but given the heterogeneous size distribution of individually expressed PB1 and PB2 (and the lack of any observable direct interaction between PA and PB2), the existence of PA-associated multimers of the basic P proteins seems a more likely explanation. It has also been suggested previously that the influenza polymerase is a multimeric structure (Krystal et al., 1986). It is possible that PB1 and PB2 behave in a manner analogous to that of SV-40 large T antigen, a large multifunctional protein which exists in different oligomeric forms with discrete biochemical activities (reviewed in Rigby and Lane, 1983).

In respect of the varying ratios of the P proteins found in the Xenopus-expressed complexes, it is interesting to note that complexes detected in infected cells do not necessarily seem to consist of equimolar quantities of all three proteins. Akkina et al. (1987) reported that complexes deficient in PB2 were present in the cytoplasm, and showed data to suggest the existence of RNP-associated complexes with a less than equimolar ratio of PA (our own unpublished observations support the latter observation). It is therefore possible that different functions of the influenza RNA polymerase may be attributable to different forms of the complex.

The successful reconstruction of the influenza polymerase complex provides an indication of the potential of the Xenopus system for reconstruction of an active polymerase. As yet we have been unable to observe reassociation of the Xenopus-expressed polymerase complex with vRNA, the next step toward reassembly of the transcription complex, because vRNA is rapidly degraded in oocytes (data not shown). However, it may be possible to circumvent this problem by partially purifying the complexes from an oocyte lysate before incubating them with the RNA substrate, or alternatively, by providing natural or artificially assembled RNP structures.

Various other expression systems have been used to study the influenza polymerase. Krystal et al. (1986) constructed cell lines expressing all three P proteins and showed that these were able to functionally complement viruses bearing ts lesions in the P protein gene. However, no biochemical data were reported on this system, possibly because of the low levels of expression obtained. St. Angelo et al. (1987) expressed the P proteins in recombinant baculoviruses, but although the system offered considerable promise in terms of the levels of protein expressed, as discussed above, only an incomplete polymerase complex was formed.

Recently, it was demonstrated that an active influenza polymerase could be reassociated from the purified polypeptide components of disrupted virion RNPs by renaturation with Escherichia coli thioredoxin (Szewczyk et al., 1988). Although this is a significant result and provides an exciting system for the study of the polymerase, it is ultimately limited to the examination of wild-type proteins. Our approach has the significant advantage that mutant polypeptides can be generated easily by manipulation of the DNA protein coding sequence. Characterization of such altered polypeptides will allow a more detailed analysis of the structure and function of the polymerase proteins.

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