Rescue of Sendai virus cDNA templates with cDNA clones expressing parainfluenza virus type 3 N, P and L proteins

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Several years ago, we reported that a Sendai virus (SeV) defective genome (DIH4) could be rescued in vivo with human parainfluenza virus type 1 (hPIV1) and bovine PIV3 but not by measles virus or vesicular stomatitis virus. It was concluded that the cis-acting RNA sequences were conserved within the SeV/PIV1/PIV3 group but that interactions between the polymerase complex (P–L) and the template protein N were unique for each virus. We have re-examined these conclusions using proteins expressed from cloned N, P and L genes for SeV and PIV3. The results demonstrate the specificity of the protein–protein interactions between polymerase and template, and confirm the prediction of the earlier work that PIV3 N, P and L proteins are capable of assembling and replicating SeV minigenomes also expressed from a cDNA clone.

Paramyxoviruses are enveloped viruses containing a ca. 15 kb non-segmented RNA genome of negative polarity. Together with the rhabdoviruses and the filoviruses they form the Mononegavirales superfamily. All these viruses replicate their genomes via a positive-stranded antigenomic intermediate which, like the genome, is found in the cytoplasm of infected cells as a helical nucleocapsid containing 96% nucleocapsid (N) protein by mass (N:RNA; Egelman et al., 1989). It is this structure, rather than RNA, that serves as the template for viral RNA synthesis. This explains why, in contrast to the situation with positive-stranded RNA viruses, neither naked RNA nor plasmid-based DNA genomes are capable of initiating a viral infection when introduced into cells.

The viral polymerase is composed of two proteins, namely the L (large protein) and the P (phosphoprotein). Virion N:RNA is associated with ca. 50 L and 300 P proteins (Lamb et al., 1976) and these proteins can be stripped from the nucleocapsid with high salt or non-ionic detergent, conditions which keep the N:RNA complex intact and active as a template (Curran et al., 1992). The L protein is believed to contain most, if not all, the catalytic activities associated with the viral polymerase (e.g. RNA-dependent RNA polymerase, capping, methylation and polyadenylation). The P protein, which is found as a homo-trimer (P3) (Curran et al., 1995b), serves as a ‘cofactor’ for the polymerase since only the P–L complex is active in viral RNA synthesis (Hamaguchi et al., 1983). It is thought that the viral polymerase engages the template via interactions between the P3 protein (of the P–L complex) and the exposed C-terminal tail of the assembled N of the N:RNA (Ryan et al., 1991; Buchholz et al., 1994). The L protein alone is unable to interact with the N:RNA (Mellon & Emerson, 1978; Horikami & Moyer, 1995). The C-terminal 20% of the N protein is hypervariable amongst the paramyxoviruses and is required for template function but not for RNA encapsidation (Buchholz et al., 1993; Curran et al., 1993).

During transcription, the polymerase enters at the 3′ end of the template and by stopping/restarting at the intercistronic junctions makes a short leader RNA followed by a series of 5′ capped and 3′ polyadenylated mRNAs. During antigenome and genome synthesis (replication) these cis-acting template sequences are ignored and the polymerase makes a complete copy of its template. It is currently accepted that the switch between transcription and replication is controlled by the level of unassembled N (N°) protein (Lamb & Kolakofsky, 1996), which is found in the cell as a P–N° complex in which the P protein acts as a chaperone for N by preventing its illegitimate assembly or aggregation (Curran et al., 1995a). Replication can be reconstituted in vitro by combining extracts in which P–N and P–L have been separately co-expressed indicating that these complexes are essential for genome amplification (Horikami et al., 1992). In addition, regions of the P protein have been identified by deletion mutagenesis which are required for assembly (with N) but not RNA synthesis (with L) and vice versa, indicating that different modular domains are required for these dual functions of the P protein (Smallwood et al., 1994; Curran et al., 1994; Curran, 1996; J. Curran and others, unpublished).

Several years ago, we reported that parainfluenza virus type 1 and type 3 (PIV1 and PIV3) could replicate a small Sendai virus (SeV) copy-back defective genome (DIH4)
inactivated stock (referred to as DIH4 UV) and then super-
and then normalized to the levels in the TE control which was set at 1.
then incubated on ice for 60 min. As a negative control, the extract was
(St) by UV-inactivation. Cells were infected with this UV-
and were analysed on a 1.5% agarose-formaldehyde gel. (b) Template-
also incubated with an equivalent volume of TE buffer. Nucleocapsids
panel). The binding of
extract prepared from A549 cells transfected with hPIV3 pGEN-P HA, and
bands corresponding to
reaction products were isolated by pelleting through a 5.7 M-CsCl cushion
infected A549 cells transfected with either SeV or PIV3 pGEN-P HA, and
binding studies with the hPIV3 P HA protein. One μg of SeV and bPIV3
nucleocapsids (RNPSev and RNPv3) were mixed with a cytoplasmic
extract prepared from A549 cells transfected with hPIV3 pGEM-PHA, and
then incubated on ice for 60 min. As a negative control, the extract was
also incubated with an equivalent volume of TE buffer. Nucleocapsids
were recovered by pelleting through 50% glycerol–TNE and their
presence in the pellet was verified by immunoblotting using a combination
of monoclonal antibodies against both SeV and PIV3 N protein (upper
panel). The binding of PHA protein to the nucleocapsid pellets was also
analysed by immunoblotting using an anti-HA monoclonal antibody (lower
panel). The bands corresponding to PHA were quantified on a densitometer
and then normalized to the levels in the TE control which was set at 1.

**Fig. 1.** (a) *In vitro* transcription studies. Cytoplasmic extracts from vTF7-3-
infected A549 cells transfected with either SeV or PIV3 pGEM-PHA plus
pGEM-L (lanes [P+L]) were incubated with N:RNA templates isolated
from SeV- and bPIV3-infected cells, in the presence of [32P]GTP. As a
control, the templates were also incubated with mock-transfected cell
extracts (lanes vacT7). All reactions were performed in duplicate. The
reaction products were isolated by pelleting through a 5.7 m-CsCl cushion
and were analysed on a 1.5% agarose–formaldehyde gel. (b) Template-
binding studies with the hPIV3 P HA protein. One μg of SeV and bPIV3
nucleocapsids (RNPSev and RNPv3) were mixed with a cytoplasmic
extract prepared from A549 cells transfected with hPIV3 pGEM-PHA, and
then incubated on ice for 60 min. As a negative control, the extract was
also incubated with an equivalent volume of TE buffer. Nucleocapsids
were recovered by pelleting through 50% glycerol–TNE and their
presence in the pellet was verified by immunoblotting using a combination
of monoclonal antibodies against both SeV and PIV3 N protein (upper
panel). The binding of PHA protein to the nucleocapsid pellets was also
analysed by immunoblotting using an anti-HA monoclonal antibody (lower
panel). The bands corresponding to PHA were quantified on a densitometer
and then normalized to the levels in the TE control which was set at 1.

Generating chimeric nucleocapsids (Curran & Kolakofsky, 1991). The more distant morbilliviruses and rhabdoviruses
could not substitute. In these experiments, the SeV defective stock was freed of the homologous non-defective helper virus
(St) by UV-inactivation. Cells were infected with this UV-
inactivated stock (referred to as DIH4 UV) and then super-
infected with the heterologous helper. However, when
DIH4 UV-infected cells were incubated for 5 days in the absence
of helper virus, PIV3 rescue became dependent upon the
presence of fresh SeV polymerase which was introduced into
the cells as a UV-inactivated SeV St stock. From this
experiment it was concluded that although the *cis*-acting RNA
sequences necessary for replication (promoter and
encapsidation site) were conserved within the PIV1/PIV3
virus group, important interactions between the polymerase
and the template N protein were unique for each virus, i.e. a
PIV3 polymerase complex could only read-out a SeV RNA
template if that template had been assembled with the PIV3 N
protein.

It is now possible to rescue SeV mini-genomes and
infectious virus back from DNA using viral proteins expressed
from cDNA clones (Garcin et al., 1995). To test directly the
interpretation of our previous results we decided to prepare
functional cDNA clones of PIV3 with the aim of studying
polymerase–template interactions and to re-examine the
heterologous rescues using cloned genes. In the first
experiment, we examined whether the SeV and PIV3 polymerases
(P-L) were capable of transcribing RNA from a heterologous
template. This served also as a functional test for the cloned
polymerase genes. Core nucleocapsids were prepared from
bovine PIV3 (bPIV3)- and SeV-infected BHK cells by banding
twice on 20–40% CsCl gradients (Curran et al., 1994).
Nucleocapsids were diluted threefold in TE (10 mM-Tris
pH 7.4, 1 mM EDTA) before pelleting through a cushion of
50% glycerol–TE at 40000g, 4 °C for 90 min in an SW 60
rotor. The pellets were resuspended in TE–1% DTT–10%
glycerol and stored at −70 °C until use. Human A549 cells
(seeded on 5 cm Petri dishes) were infected with 2 p.f.u. per cell of
a vaccinia virus recombinant expressing T7 RNA poly-
merase (vTF7-3; Fuerst et al., 1986). Transcription of all the
clones was under the control of a T7 promoter. At 30 min
post-infection (p.i.) the cells were transfected with either (a)
SeV – 2.5 μg pGEM-PHA [the influenza virus HA1 epitope tag
was fused to the N terminus of the P protein (Field et al., 1988;
Curran et al., 1994)] and 0.5 μg pGEM-L – or (b) PIV3 – 5.0 μg
pGEM-PHA [an N-terminally tagged form of the human (h)
PIV3 P protein (Galinski et al., 1986b). An Nol site created at
the P start codon was used to introduce an *Nol* cassette
carrying the influenza virus HA1 epitope tag] and 1.0 μg
pGEM-L [a cDNA clone from bPIV3 (unpublished)]. At 34 h
p.i. crude cytoplasmic extracts were prepared in transcription
buffer (150 mM-HEPES pH 8.5, 150 mM-NH4Cl, 4.5 mM-magnesium
acetate, 1 mM-DTT, 20 μg/ml actinomycin D, 0.5 mM-
ATP/CTP/UTP, 40 U/ml creatine phosphokinase, 1 mM-
creatine phosphate) and mixed with 1 μg of either SeV or
bPIV3 core NCs in the presence of [α-32P]GTP (the precise
conditions used for the *in vitro* transcription reactions are
described in Curran et al., 1994). As a control for the presence
of endogenous polymerase on the purified N:RNA, the
templates were added to extracts prepared from mock-
Fig. 2. (a) Rescue of the SeV cDNA template clone pSV-DIH4 with clones expressing the PIV3 N, P<sup>HA</sup> and L proteins. A549 cells infected with vTF7-3 were transfected in duplicate with pSV-DIH4 and pGEM plasmids expressing either the SeV or PIV3 N, P<sup>HA</sup> and L proteins (see text for details). As a negative control the pGEM-P<sup>HA</sup> and -L were removed (lanes [N]). Cells were harvested at 36 h p.i. and nucleocapsids were recovered by banding on linear CsCl gradients. Rescue of the SeV template was analysed by Northern blot using a riboprobe of (+) polarity (upper panel). Expression of the PIV3 N and P<sup>HA</sup> proteins in the various cell transfected cells. RNA was recovered by pelleting through a 5.7 M-CsCl cushion and analysed on a 1.5% agarose–formaldehyde gel. As shown in Fig. 1(a), the viral polymerases were only capable of transcribing RNA from their homologous nucleocapsids. The major band in both the SeV [P + L] and PIV3 [P + L] duplicate lanes corresponds to the N mRNA, and the band migrating slightly slower is the P mRNA. Since the polymerase is thought to engage its template through the P protein, we examined the specificity of this interaction using the P<sup>HA</sup> protein of hPIV3. A cytoplasmic extract, prepared from cells transfected with hPIV3 pGEM-P<sup>HA</sup>, was mixed with 1 µg of either bPIV3 or SeV core N:RNAs. After incubation on ice for 60 min, nucleocapsids were recovered by pelleting through 50% glycerol–TNE (10 mM-Tris pH 7.4, 30 mM-NaCl, 1 mM-EDTA) at 16000 g for 1 h at 4 °C. The presence of nucleocapsids in the pellet was confirmed by Western-ELISA using a mixture of PIV3 anti-N and SeV anti-N monoclonal antibodies (Fig. 1b, upper panel). Bound P<sup>HA</sup> protein was also detected by immunoblotting with an anti-HA monoclonal antibody (Berkeley Antibody Co. USA). The lower panel in Fig. 1(b) shows that some hPIV3 P<sup>HA</sup> protein traversed the glycerol cushion in the absence of nucleocapsid (TE control), possibly due to a fraction of the protein aggregating or interacting with other large macromolecular structures present in the extract (although the presence of EDTA in the cushion would preclude ribosomal structures), and this amount remained unchanged after incubation with N:RNA from SeV. However, in the presence of bPIV3 nucleocapsids the level of bound P<sup>HA</sup> protein increased ca. fivefold. This correlation between transcription and P binding suggests that within the SeV/PIV3 group, protein–protein (i.e. the P and N of the nucleocapsid) rather than protein–RNA interactions, dictate the specificity of the polymerase-template interaction.

Earlier work suggested that the cis-acting RNA template sequences were conserved within the SeV/PIV3 group (Curran & Kolakofsky, 1991). If this is correct, PIV3 N, P and L proteins, expressed from cloned genes, should be able to assemble and amplify an SeV ‘mini-genome’ also expressed from DNA. Two such SeV template clones have been described. The first is a cDNA clone of the copy-back DIH4 described earlier (pSV-DIH4; Calain et al., 1992), and the second is a cDNA clone of another natural SeV defective, an internal deletion DI called E307 (pSV-E307; Engelhorn et al., 1993). Both clones can be expressed as positive-sense (anti-genomic) transcripts by T7 polymerase. The E307 RNA contains three extra G’s at its 5’ end, whereas DIH4 starts at the + 1 A of the viral template. The precise viral 3’ end of each transcript is defined by the hepatitis delta ribozyme which is positioned just downstream of the cDNA clone. These extracts was monitored by immunoblotting using a combination of anti-N and anti-HA monoclonal antibodies (lower panel). (b) Rescue of the SeV cDNA template clone pSV-E307 with clones expressing the PIV3 N, P<sup>HA</sup> and L proteins. This was performed as outlined in (a).
plasmids were co-transfected into vTF7-3-infected A549 cells with either SeV or PIV3 clones expressing N alone, or N, P\textsuperscript{HA} and L in the PIV3 series the N and P\textsuperscript{HA} genes came from the human strain (Galinski et al., 1986a, b) and the L gene from the bovine strain. Cells were harvested at 36 h.p.i. and nucleo-
capsids were isolated by banding on high salt gradients. N and P\textsuperscript{HA} protein expression in the extracts was detected by immunoblotting (the lower panel in Fig. 2a shows the result for the PIV3 rescue), and amplification of the cloned template was followed by Northern blot using a riboprobe of +ve polarity (i.e. the same sense as the T7 transcript derived from the template cDNA) complementary to the 5' end of the genome (pEX5'; Mottet et al., 1990). As shown in Fig.2, the PIV3 proteins were able to assemble and amplify both SeV cloned templates, although usually somewhat less efficiently than the SeV proteins. This lower efficiency of amplification may, in part, be explained by the level of viral proteins expressed from the cloned genes. Immunoblots using the anti-
HA monoclonal antibody show that the level of PIV3 P\textsuperscript{HA} protein expression is considerably lower than that expressed from SeV P\textsuperscript{HA}. This may also explain the lower level of transcription observed with the PIV3 extracts (Fig. 1a). In addition, it has been noticed that co-transfection of equal amounts of the PIV3 N and P\textsuperscript{HA} plasmids resulted in a severe interference in P\textsuperscript{HA} but not N protein expression (data not shown). To compensate for this effect the quantity of PIV3 pGEM-N DNA co-transfected with pGEM-P\textsuperscript{HA} was titrated down to give maximal expression of both proteins. These conditions also gave maximal DI genome amplification (data not shown). Note that the amount of PIV3 pGEM-N DNA transfected in these experiments was fivefold lower than that used for SeV. Since we have no antibodies against the PIV3 L protein it was not possible to examine directly any interference effects on L protein expression. The quantity of pGEM-L used was therefore optimized empirically using amplification of the mini-genome as the read-out.

In this paper we have demonstrated that functional cDNA clones of PIV3 N, P and L are capable of rescuing a heterologous (SeV) 'mini-genome' also expressed from DNA, thereby confirming the predictions of our earlier work (Curran & Kolakofsky, 1991). This 'set' of PIV3 proteins is active despite the fact that they are derived from both the human (N and P proteins) and bovine (L protein) strains of the virus, indicating that important protein–protein interactions have been conserved between the two strains. A similar conservation of protein functional domains was observed between SeV (or murine PIV1) and the closely related hPIV1 (Curran et al., 1993; unpublished observations). However, no mixed combination of PIV3/SeV N, P and L proteins can support the rescue of natural or cloned SeV templates (Curran et al., 1995a), and we have been unable to demonstrate the formation of heterologous P–N and P–L complexes between SeV and PIV3 (data not shown). Likewise, the P proteins of SeV and PIV3 interact only with their homologous templates and this correlates with transcriptional activity. The C-terminal tail of the nucleocapsid protein is required for template function, presumably because this is the region that interacts with both the P of the P–L complex and the supplemental P (Buchholz et al., 1994; Curran, 1996). The poor conservation of this region between SeV and PIV3 (Curran et al., 1993) may explain the specificity of the P–N:RNA interaction. However, it is unclear whether all the determinants for template function and specificity reside in this C-terminal region. It will therefore be interesting to examine P binding to templates containing chimeric N proteins generated by fusing the more conserved N-terminal nucleocapsid assembly region (Buchholz et al., 1993; Curran et al., 1993) to the hypervariable C-terminal tail.

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References


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