VIROLOGY 235, 261–270 (1997)
ARTICLE NO. VY978702

The Sendai Virus C Protein Binds the L Polymerase Protein to Inhibit Viral RNA Synthesis

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Received April 11, 1997; returned to author for revision May 7, 1997; accepted June 23, 1997

The Sendai virus nested set of C proteins which are expressed in an alternative open reading frame from the P mRNA has been shown to downregulate viral RNA synthesis. Utilizing a glutathione *S*-transferase (gst) C fusion protein (gstC), we have shown that C protein forms a complex with the L, but not the P, subunit of the viral RNA polymerase. When P, L, and gstC are coexpressed, an oligomer of P, through its interaction with L, is also bound to beads. Since binding of C to L in the P-L complex does not disrupt P binding, the C and P binding sites appear to be different. GstC binding to L occurs only when the proteins are coexpressed in the same cell. The gstC, but not gst, protein inhibits viral transcription *in vitro*, showing that the fusion protein retains biological function. Pulse–chase experiments of the various complexes show that L protein synthesized alone has a half-life of 1.2 hr, which is increased 12.5-fold by binding P, but is not significantly increased by binding gstC. Analyses of complex formation with truncations of L protein show that the C-terminal 1333 amino acids of L are not required for binding C. The dose–response curves show that replication of the genomic DI-H RNA is more sensitive to inhibition by C protein than is the synthesis of DI leader RNA, suggesting that the downregulation of RNA synthesis may be more complex than just the inhibition of the initiation of RNA synthesis.

INTRODUCTION

Paramyxoviruses are enveloped viruses with a single-stranded, negative-sense, nonsegmented RNA genome of 15–16 kb (for reviews, Kingsbury, 1991; Lamb and Kolakofsky, 1996). The genome is completely encapsidated by the nucleocapsid protein, NP, which renders the RNA nuclease resistant. Associated with the nucleocapsid is an RNA-dependent RNA polymerase consisting of a complex of two virus-encoded subunits, the large (L) protein and the phosphoprotein (P) (Horikami *et al.,* 1992), where the L protein is thought to possess the catalytic activities. The nucleocapsid, not naked RNA, is the template for all RNA synthesis. Transcription occurs by the sequential synthesis of leader RNA and then the NP, P/C/V, M, F, HN, and L mRNAs. Genome RNA replication occurs via a positive sense, antigenomic RNA intermediate and the synthesis of both the genome and the antigenome is coupled to the encapsidation of the RNAs by the NP protein. It has been shown that the NP and P proteins form a complex (Homann *et al.,* 1991) which is used for the encapsidation of RNA during replication (Horikami *et al.,* 1992). P protein also binds to nucleocapsids (Ryan and Kingsbury, 1988; Ryan and Portner, 1990; Ryan *et al.,* 1991). Studies of the replication of Sendai virus RNA have been facilitated by the use of a defective interfering particle (DI-H) as the template (Carlsen *et al.,* 1985). The DI-H RNA (1410 nucleotides) contains the 5′ terminal sequences of the wild-type (wt) genome RNA and has copyback termini with the 3′ terminus complementary to the 5′ terminus (Calain and Roux, 1993).

The P gene is multifunctional in paramyxoviruses and in Sendai virus encodes the P protein, a nested set of C proteins, and the V protein. The longest open reading frame encodes the Sendai P protein [568 amino acids (aa)] from nucleotide (nt) 104, while in the +1 reading frame four translational start sites (nt 81, 114, 183, and 201) are used for the simultaneous translation of the C proteins (C′, C, Y1, and Y2) from the P mRNA (Curran and Kolakofsky, 1988; Gupta and Patwardhan, 1988). The C open reading frame is present in the paramyxovirus genus of viruses, including all the morbilliviruses and parainfluenza viruses. For Sendai and PIV1 the C proteins are expressed as a C-coterminal nested set of proteins, while for measles and PIV3 there is only a single C protein (Boeck *et al.,* 1992). The C open reading frame, in contrast, is absent in the rubulavirus genus of viruses, including Newcastle disease virus, mumps virus, and simian virus 5 (Lamb and Kolakofsky, 1996). P gene transcription of most, but not all, paramyxoviruses also shows a unique feature designated P mRNA editing (Lamb and Kolakofsky, 1996). For Sendai virus there is the addition of a single nontemplated G residue at nt 1053, yielding an mRNA encoding the V protein which shares the N-terminus with P, but has a unique C-terminus (Curran *et al.,* 1991).

The Sendai virus C proteins are small, abundant basic proteins, yet they are present in only small amounts in the virion, apparently in association with the nucleocapsid

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0042-6822/97 $25.00

(Yamada *et al.,* 1990). Immunofluorescence of infected cells with antibodies to C proteins showed that they are distributed throughout the cytoplasm and are found in all cytoplasmic fractions by cell fractionation (Portner *et al.,* 1986). C protein made *in vitro* also binds to nucleocapsids from infected cells (Ryan and Kingsbury, 1988), but not to polymerase-free nucleocapsids (Curran *et al.,* 1992). The Sendai C proteins act as regulators of RNA synthesis. Initially they were postulated to specifically inhibit viral transcription, but not genome replication (Curran *et al.,* 1992); however, more recently they were also shown to inhibit wt genome and internal deletion DI replication (Cadd *et al.,* 1996). The effect on a copy-back DI (DI-H), however, was more complicated since C did not inhibit DI-H replication *in vivo,* but did *in vitro,* so inhibition appeared to be promoter-specific under certain conditions. Since the C proteins interfere with viral RNA synthesis, we tested if they might interact with one or more of the viral proteins (L, P, and NP) required for these processes. We show here that C protein forms a complex with the L, but not the P, subunit of the viral RNA polymerase where the C-terminal half of L is dispensable for binding. In addition, the data suggest that the downregulation of DI-H genome replication *in vitro* appears to be more complex than the simple inhibition of the initiation of RNA synthesis.

MATERIALS AND METHODS

Cells, viruses, and plasmids

The growth and purification of wt Sendai virus and the Sendai virus-defective interfering particle, DI-H (Harris strain), were described previously (Horikami *et al.,* 1992). Recombinant vaccinia virus containing the gene for phage T7 RNA polymerase (VVT7) (Fuerst *et al.,* 1986) was grown in Vero cells. Protein and RNA syntheses were performed in human A549 cells (American Type Culture Collection). The plasmids pGEM-NP, pGEM-Pstop (not expressing any of the C proteins, designated pGEM-P), and pGEM-L were described previously (Curran *et al.,* 1991). The plasmids with L gene truncations were described in Chandrika *et al.* (1995). The pGEM-C plasmid was constructed by subcloning the *Hin*dIII and *Sac*I fragment containing the entire C open reading frame gene from pgfp14 (Gupta and Patwardan, 1988) into those sites in pGEM. The plasmid pgfp14 had the P AUG start codon changed to CUG and so could not express the P protein. The pGEM-C plasmid thus synthesizes all the C-related proteins, C′, C, Y1, and Y2, but not P (Gupta and Patwardan, 1988). All of the viral genes were cloned downstream of the phage T7 RNA polymerase promoter. The plasmid pTM1-gstC contained the glutathione *S*-transferase (gst) gene fused in frame to the 5′ end at nucleotide (nt) 81 of the Sendai C gene (gstC) and thus included the entire C open reading from C′. The plasmid was constructed by PCR amplification of the C gene from pGEM-C with primers containing *Bam*HI and *Xho*I restriction sites to allow cloning into pTM1-gst (Chandrika *et al.,* 1995) at those sites and the construct was confirmed by sequencing. The translation product of pTM1-gstC thus consists of the C′ protein encompassing the entire C open reading frame with 2 and 4 extra aa at the N- and C-termini of C′, respectively, due to cloning and does not synthesize fusions with the individual C, Y1, or Y2 proteins.

Protein analysis

A549 cells in 35-mm dishes were infected with VVT7, at an m.o.i. of 2.5 PFU/cell and transfected with 1.7 μg of pGEM-L, pGEM-P, and pGEM-NP and 0.2 μg of pTM1-gstC at 37° with lipofectin (Gibco-BRL) as indicated in the figures. The cells were incubated with Tran^{35}S-label (66 μCi/ml) at 4.5 hr posttransfection (pt) for 1 hr in methionine- and cysteine-free medium. Cell extracts were prepared immediately (pulse, P) or in some experiments following a chase for various times in medium containing a 10-fold excess of methionine and cysteine, as indicated in the figure legends. Cell extracts were prepared at 4° in 300 μl reaction mix salts (RM salts: 0.1 *M* HEPES, pH 8.5, 0.05 *M* NH_4Cl, 7 m*M* KCl, and 4.5 m*M* magnesium acetate), containing 0.25% NP-40 and 1 μg/ml aprotinin. The lysate was clarified at 13,000 rpm for 30 min at 4°. For immunoprecipitation, samples of the ^{35}S-labeled supernatants were incubated with 1 μl each of α-SV, α-L, and α-gst antibodies (Chandrika *et al.,* 1995) and the antigen/antibody complex was collected with inactivated *Staphylococcus aureus,* Cowan strain, as described previously (Carlsen *et al.,* 1985; Horikami *et al.,* 1992). For analysis of complexes with gstC, VVT7-infected cells were transfected with various combinations of plasmids, and the cells were radiolabeled as above. Cytoplasmic cell extracts were prepared and samples (75 μl) immunoprecipitated as above. For bead-binding glutathione–Sepharose 4B beads (15 μl per reaction, Pharmacia Biotech) were prepared by washing twice in RM salts followed by blocking for 15 min at 4° in 1 ml of RM salts containing 0.1% NP-40, 0.5% nonfat dry milk, and 10 mg/ml BSA. The blocked beads were washed twice in RM salts. The ^{35}S-labeled extracts (75 μl) were incubated with the blocked beads for 15 min at 4°, washed with RM salts containing 0.25% NP-40 and 1 μg/ml aprotinin, and then immunoprecipitated, and bound proteins were analyzed by 7.5% SDS–PAGE and autoradiography. Quantitation of the bands was performed on the Phosphorimager (Molecular Dynamics).

In vitro RNA synthesis

Subconfluent A549 cells in 60-mm dishes were infected with VVT7 and transfected for transcription assays with pGEM-P (1.5 μg) and pGEM-L (0.5 μg) and for replication assays with pGEM-P (5 μg), pGEM-L (0.5

μg), plus pGEM-NP (2 μg), amounts which were previously shown to be optimal for each process (Horikami *et al.,* 1992; Chandrika *et al.,* 1995). Increasing amounts of pTM1-gstC or pGEM-C were added as indicated in the figures with compensating amounts of pGEM vector, so the total DNA transfected remained constant. At 18 hr pt cytoplasmic cell extracts were prepared by lysolecithin permeabilization at 4° in reaction mix, as described previously (Chandrika *et al.,* 1995). Transcription or replication was carried out *in vitro* with the addition of [α-^{32}P]CTP and polymerase-free wt Sendai (2 μg) or DI-H (1 μg) template, respectively, prepared as described previously (Horikami *et al.,* 1992) for 2 hr at 30°. The mRNA products were purified by pelleting through 5.7 *M* CsCl. The RNA pellet was analyzed by RNase protection with a riboprobe complementary to the 3′ end of the NP mRNA and analyzed by electrophoresis on a 5% polyacrylamide–8 *M* urea gel and autoradiography as described previously (Horikami *et al.,* 1992). For replication, the products were treated with micrococcal nuclease and banded on 20–40% CsCl gradients, and the nucleocapsid-associated RNA was extracted and analyzed by electrophoresis on 1.5% agarose–acid–urea gels and autoradiography as described previously (Horikami *et al.,* 1992). For DI leader RNA synthesis, total unlabeled product RNA was extracted, separated by electrophoresis on an 8% polyacrylamide–8 *M* urea gel, and electroblotted onto Hybond-*N*-nitrocellulose. The DI leader RNA was detected by Northern analysis with a 55-nt complementary ^{32}P-end-labeled oligonucleotide probe (Chandrika *et al.,* 1995) and the products were quantitated on the Phosphorimager.

RESULTS

The Sendai C protein binds the L polymerase protein

To determine if the C protein might interact with any of the viral proteins required for transcription and replication, since C inhibits these reactions, we employed a glutathione *S*-transferase-tagged C protein, gstC, constructed as described under Materials and Methods. Complex formation can be measured by the cobinding of a candidate protein with gstC to glutathione–Sepharose beads. VVT7-infected cells were transfected with combinations of the plasmids encoding the P, L, NP, and gstC proteins and incubated with Tran^{35}S-label. Cytoplasmic cell extracts were either immunoprecipitated to measure viral protein synthesis or incubated with beads to test for interactions with the gstC protein. Gel analysis showed that gstC and the other viral proteins were all synthesized (Fig. 1A). The other bands represent vaccinia proteins that were nonspecifically immunoprecipitated as shown in infected, but not transfected cells (Fig. 1A, lane 1). Compared to the synthesis of amount of L alone, there was increased synthesis of the L protein when it was coexpressed with P (Fig. 1A, lanes 7 and 11) as reported earlier (Horikami *et al.,* 1992), as well as a further significant stimulation in each case by coexpression with gstC (lanes 8 and 12).

When the extracts were incubated with beads, the gstC protein bound in every case and there was little or no binding of any proteins in the absence of gstC (Fig. 1B). Neither the NP nor P proteins expressed alone or together bound to gstC (Fig. 1B, lanes 4, 6, and 10). In some lanes there was a small amount of nonspecific binding of a vaccinia protein with the same mobility as NP. L expressed with gstC, however, in both the absence and the presence of P cobound to the beads (lanes 8 and 12), suggesting that C interacts with the L protein. It was shown previously that the P and L proteins form the polymerase complex (Horikami *et al.,* 1992), and, therefore, P cobound to beads through its interaction with L, since P alone did not bind gstC (Fig. 1B, lanes 12 and 6). Thus the data suggest that the C and P binding sites on L are independent of one another.

As a control for the specificity of the C–L interaction, infected cells were transfected with the gst plasmid in the absence or presence of both the P and L plasmids. Immunoprecipitation showed that the proteins were expressed (Fig. 2A, lanes 1 and 2) and while gst bound to the beads, the P and L proteins did not cobind (lanes 3 and 4). These data suggest that the interaction with L is mediated through the C portion of the fusion protein. We also asked if the gstC-L interaction required expression of the proteins in the same cell by a mixing experiment. GstC expressed alone bound beads, while P and L synthesized in the absence of gstC bound in trace amounts (Fig. 2B, lanes 2 and 3, respectively). The coexpression of gstC, P, and L gave significant binding of both P and L with gstC to beads (Fig. 2B, lane 4) as observed in Fig. 1B. In contrast, when separate extracts containing gstC and P + L were incubated together, there was just a small amount of L and P bound to the beads over the background binding (Fig. 2B, lane 5), suggesting that the majority of the interaction requires that the proteins be synthesized together.

The gstC protein inhibits Sendai transcription *in vitro*

Since the gstC protein was used to demonstrate the binding of L to C, we wanted to determine if the fusion protein retained its function in the inhibition of transcription. VVT7-infected cells were transfected with the P and L plasmids in the presence of increasing amounts of the gstC plasmid. Transcriptional activity of the samples was assayed by the addition of polymerase-free wt Sendai template and radiolabeled substrate to cell extracts. Following incubation, activity was determined by RNase protection assay of the NP mRNA product. The data showed decreasing mRNA synthesis with increasing amounts of the gstC plasmid (Fig. 3) and with a dose–response curve similar to that with coexpression with increasing

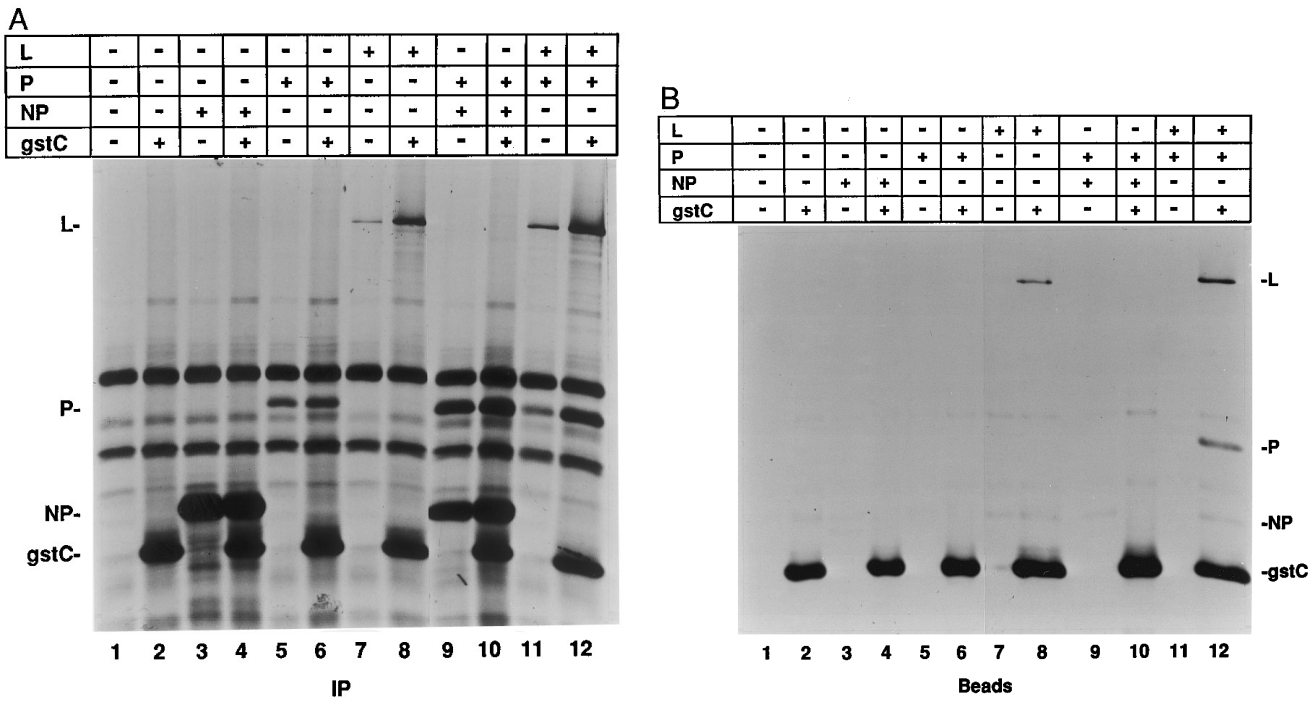

FIG. 1. Complex formation between the Sendai gstC and L proteins. VVT7-infected A549 cells were transfected with no plasmids (A and B, lanes 1) or the combinations of plasmids indicated at the top and incubated with Tran^{35}S-label as described under Materials and Methods. Samples of cytoplasmic extracts were either immunoprecipitated with α-SV, α-L, and α-gst sera (A, IP) or incubated with glutathione–Sepharose beads (B, beads) and the proteins were analyzed by SDS–PAGE. The positions of the viral proteins are indicated on the left.

C protein (data not shown). Similar experiments where increasing amounts of the gst plasmid instead of the gstC plasmid were transfected showed no inhibition of viral transcription (data not shown). These data suggest that the gst moiety of the fusion protein is not responsible for the inhibition and that gst has not interfered with the functional activity of the C protein.

Stability of the polymerase proteins

Since there appeared to be altered expression of the polymerase proteins under different conditions (Fig. 1), the expression and stability of various combinations of P and L expressed with gstC were determined using pulse–chase analysis as described under Materials and Methods. VVT7-infected cells were transfected with the indicated plasmids and pulse-labeled, and extracts were prepared immediately (0) or following a chase for various periods of time. Analysis of the proteins in the mock-transfected cells showed some nonspecific immunoprecipitation of vaccinia proteins (Fig. 4A, lanes 1–6). The gstC protein was synthesized in the pulse and was degraded with time (Fig. 4A, lanes 7–12). There was only a low level of L synthesis when it was expressed alone, which virtually disappeared by 18 hr of chase (Fig. 4A, lanes 13–18), consistent with its apparent instability reported earlier (Horikami *et al.,* 1992). Quantitation and analysis of the amount of L protein alone showed it had a half-life of 1.2 hr (Table 1). As noted in Fig. 1, coexpression of gstC with L gave a 5.4-fold stimulation of L synthesis; however, both gstC and L declined during the chase (Fig. 4B, lanes 1–6), with L having a half-life 1.9 hr only slightly more than that of L alone (Table 1). The coexpression of the P and L proteins increased the synthesis of L 2-fold and significantly stabilized the protein so it had a half-life of 15 hr (Fig. 4B, lanes 7–12; Table 1). Finally the expression of P, L, and gstC together stimulated L synthesis by 12.4-fold and the half-life of L decreased to 8 hr (Fig. 4B, lanes 13–18; Table 1).

Quantitation of the P band and the nonspecifically immunoprecipitated vaccinia proteins in this and other experiments showed that these proteins were also stimulated by gstC as well, but only by two- to fourfold. The reason for the enhanced stimulation of L by gstC is unclear. To test if L stimulation was due to the C or gst portion of the fusion protein, infected cells were transfected with the gst plasmid in various combinations with the viral plasmids. Immunoprecipitation and quantitation of the proteins showed that gst did not stimulate L protein synthesis (Table 1), nor did it stimulate the synthesis of other Sendai or vaccinia proteins (data not shown). Thus protein synthesis enhancement appears to be due to the C portion of the fusion protein.

Binding of L protein truncations to gstC

In order to begin to map the C binding site on the L protein we used the C-terminal truncations of L protein

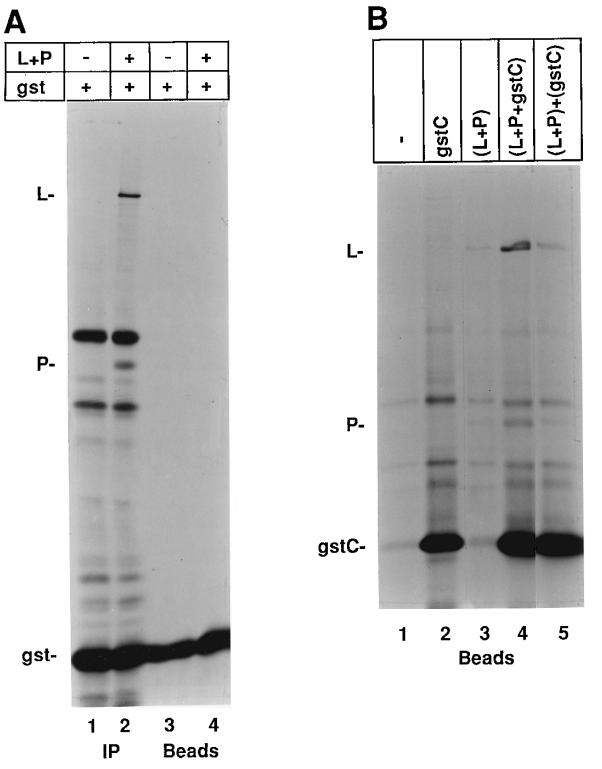

FIG. 2. The binding of gstC with L protein is specific for the C moiety and requires coexpression of the proteins. (A) VVT7-infected cells were transfected with the gst plasmid in the absence or presence of the L and P plasmids as indicated at the top and incubated with Tran^{35}S-label. Cytoplasmic cell extracts were either immunoprecipitated with α-SV, α-L, and α-gst sera (IP) or incubated with beads and the proteins were analyzed by SDS-PAGE. (B) VVT7-infected cells were transfected in duplicate with no plasmid (−, lane 1), gstC alone (lane 2), or the combinations of plasmids indicated within parentheses (lanes 3 and 4) and incubated with Tran^{35}S-label for 1 hr. Samples of extracts containing gstC and (L + P) (lanes 2 and 3) were mixed and incubated for 30 min at 30° prior to analysis (lane 5). The samples were incubated with beads and the proteins analyzed by SDS−PAGE. The positions of the proteins are indicated.

shown in Fig. 5 (Chandrika *et al.,* 1995) and tested for their binding to gstC. VVT7-infected cells were transfected with the plasmids for the wt and deleted L proteins in the absence or presence of the gstC and P plasmids and incubated with Tran^{35}S-label. Immunoprecipitation of samples of cytoplasmic extracts showed that the proteins were all synthesized (data not shown). As demonstrated above, wt L expressed with gstC in both the absence and the presence of P protein bound to beads, while L alone did not (Fig. 5, lanes 2, 3, and 1, respectively). Similarly, Lfs truncated at aa 1571 also bound the beads only in the presence of gstC (Fig. 5, lanes 4 and 5), and the Lfs−P interaction was not disrupted (lane 6). In the latter case the reduced amounts of both Lfs and P were a reflection of their reduced expression in the total sample (data not shown). In contrast, both the LΔBsp (containing a deletion of aa 895−1543) and LΔBB (truncated at aa 895) proteins bound gstC; however, P protein was not cobound (Fig. 5, lanes 8 and 9 and 11 and 12, respectively). This is consistent with our previous observations that the P−L complex did not form with these truncations (Chandrika *et al.,* 1995). These data suggest that the N-terminal 895 aa of L protein contain the C binding site which is independent of the P binding site.

The C protein differentially inhibits DI-H genome and leader RNA synthesis

Originally Curran *et al.* (1992) reported that the C proteins inhibit Sendai virus transcription, but not replication. Subsequently, Cadd *et al.* (1996) found that the situation was more complicated in that wt genome and internally deleted DI replication were also inhibited *in vivo* by C proteins; however, the copy-back DI-H replicated well *in vivo.* Surprisingly DI-H replication was inhibited by the C proteins *in vitro.* To further examine the effect of C proteins on DI-H replication *in vitro,* VVT7-infected cells were transfected with the L, P, and NP plasmids in the presence of increasing amounts of the C plasmid and extracts of these cells were incubated with polymerase-free DI-H template and [α-^{32}P]CTP. The nuclease-resistant nucleocapsid products were purified by banding on CsCl gradients and the RNA was extracted and analyzed by gel electrophoresis. The DI-H template replicated well in the absence of C proteins, and no product was synthesized in the mock-transfected sample as expected (Fig. 6A, lanes 2 and 1, respectively). The addition of increasing amounts of C plasmid, corresponding to increasing expression of the C proteins (data not shown), gave increasing inhibition of replication (Fig. 6A), confirming the results of Cadd *et al.* (1996).

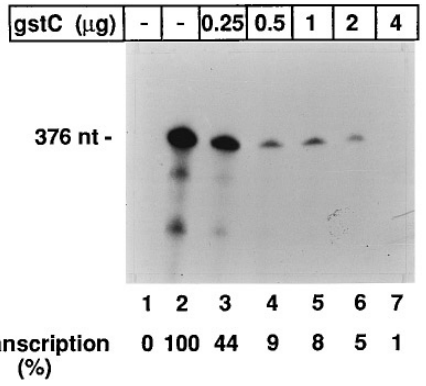

FIG. 3. Effect of gstC on Sendai virus transcription *in vitro.* VVT7-infected cells were transfected with no plasmids (lane 1) or the P and L plasmids in the absence (lane 2) or presence of increasing amounts of the gstC plasmid as indicated at the top and incubated overnight. Cytoplasmic cell extracts were incubated with polymerase-free wt Sendai virus template and [α-^{32}P]CTP and N mRNA synthesis was determined by RNase protection assay as described under Materials and Methods. The bands (376 nt) were quantitated and the percentage of transcription (bottom) was calculated relative to transcription with P and L in the absence of gstC as 100%.

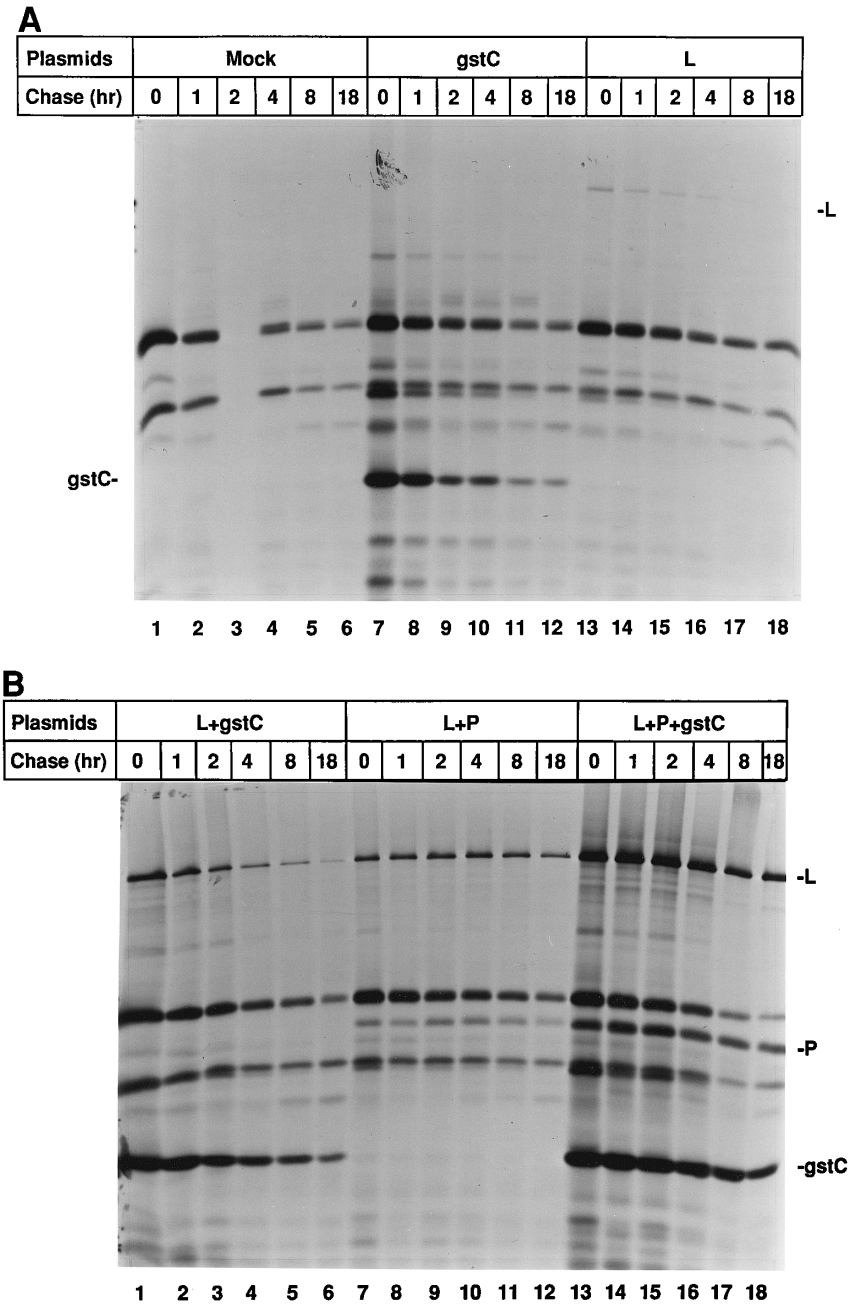

FIG. 4. Pulse–chase analysis of Sendai virus proteins expressed with gstC. Multiple dishes of VVT7-infected cells were transfected with no plasmids (mock) or the combinations of plasmids indicated at the top and incubated with Tran^{35}S-label for a 1-hr pulse (0-hr chase). Cytoplasmic cell extracts were prepared immediately or after a chase of 1, 2, 4, 8, or 18 hr as described under Materials and Methods. The samples were immunoprecipitated with α-SV, α-L, and α-gst antibodies and analyzed by SDS–PAGE. The sample in A, lane 3, was lost. The positions of the proteins are indicated.

We also tested if there was inhibition by the C proteins of the first product synthesized from the DI template, the 55-nt DI leader RNA. Extracts of VVT7-infected cells transfected as above were incubated with the DI-H template and the RNA products were detected by Northern blot analysis with an oligonucleotide probe specific for DI leader RNA. Full-length DI leader RNA synthesized in the absence of the C proteins was a discrete size (55 nt) with a distinct pattern of apparently early termination products (Fig. 6B, lane 2) as reported earlier (Chandrika *et al.,* 1995). The coexpression of the viral proteins with increasing amounts of the C proteins did not show the same dose–response of leader RNA inhibition (Fig. 6B, lanes 3–6) that was observed for genome replication (Fig. 6A). DI leader RNA remained high up to 4 μg of C plasmid and decreased significantly only at higher levels of plasmid. Quantitation of the product bands plotted relative to the control in each case showed that genome

replication was more sensitive to inhibition than leader RNA synthesis (Fig. 6C). These data suggest that C proteins inhibit a step in replication *in vitro* subsequent to the initiation of RNA synthesis, although ultimately at high levels of C initiation of RNA synthesis is also inhibited.

DISCUSSION

Since the Sendai C proteins downregulate viral RNA synthesis, we tested if C might bind to any of the three proteins, NP, P, and L, which are required for synthesis. Using a gst-tagged C protein which retains inhibitory function and cobinding to glutathione beads, C was shown to specifically bind L protein, and not the P or NP proteins (Fig. 1B). Thus C interferes with RNA synthesis by direct action on L protein, the presumed catalytic subunit of the RNA polymerase. A C–L interaction is consistent with the observed low level of C in virions (Yamada *et al.,* 1990) where L is in low abundance and with the ability of C to bind nucleocapsids containing associated polymerase, but not polymerase-free nucleocapsids (Ryan and Kingsbury, 1988; Curran *et al.,* 1992). GstC–L complex formation requires the coexpression of the proteins in the same cell (Fig. 2B), which is consistent with the previous observation that C inhibition of transcription occurs only when C is coexpressed with P and L (Curran *et al.,* 1992).

When P, L, and gstC are coexpressed, P through its interaction with L is also bound to beads. Since the binding of C to L in the P-L complex does not disrupt P binding, the C and P binding sites appear to be different. Indeed, C protein can still bind to an L truncation containing only the N-terminal 895 aa (Fig. 5), a protein which does not bind P (Chandrika *et al.,* 1995). The L proteins of (−) strand RNA viruses share six domains (I to VI) of conserved sequences that are thought to be important

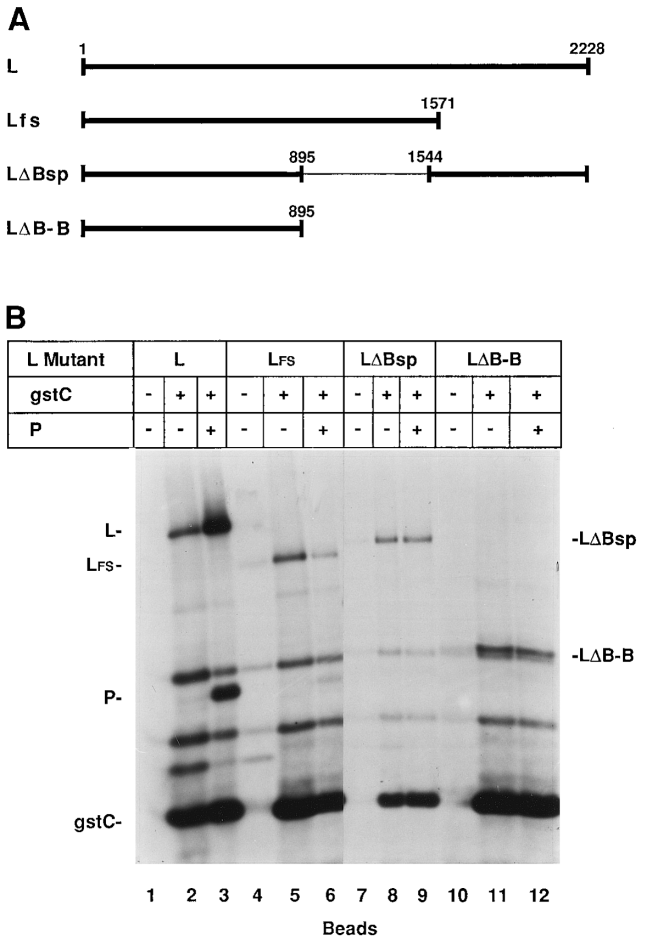

FIG. 5. Binding of gstC protein to deletions of the L protein. (A) A schematic of the L protein deletions is outlined, with the solid bar indicating the protein and the thin line indicating the deleted sequences. (B) VVT7-infected cells were transfected with the wt L plasmid or the indicated L deletion plasmids alone or in the presence of the gstC and P plasmids as indicated at the top and incubated with Tran^{35}S-label. Cytoplasmic cell extracts were incubated with beads and analyzed by SDS-PAGE. The positions of the proteins are indicated.

TABLE 1

Stimulation and Stabilization of the Sendai L Protein by the P and GstC Proteins

Proteins coexpressed[a]	L stimulation[b]	L half-life (hr)[c]
L	1.0	1.2
L + gstC	5.4	1.9
L + gst	1.0	ND
L + P	1.9	15.0
L + P + gstC	12.4	8.4
L + P + gst	2.0	ND

[a] VVT7-infected cells were transfected with the plasmids for the indicated proteins which were analyzed as described in the legend to Fig. 4.

[b] The amount of pulse-labeled L expressed under the indicated conditions was determined on the PhosphorImager and normalized to the amount of L expressed alone as 1. A second identical experiment gave similar data with the values differing by less than 10%.

[c] The half-life was determined from the decay of L protein under different conditions in the chase.

for the function of the protein (Poch *et al.,* 1990; Sidhu *et al.,* 1993). The L protein, Lfs containing 1571 aa, binds both P and C and includes all of domains I through V. The N-terminal 895 aa of L which binds only C encompasses domains I through III. Further studies are needed to precisely map the binding sites for both P and C to determine if they correlate with particular domains on the L protein.

Earlier experiments suggested that the Sendai L protein is unstable in the absence of P protein (Smallwood *et al.,* 1994). In these studies pulse–chase analysis of the various complexes, in fact, shows that L protein synthesized alone has a half-life of 1.2 hr, which is significantly increased (to 15 hr) by binding P, but is increased only a small amount (to 1.9 hr) by binding gstC (Fig. 4, Table 1). We suggest that P acts to facilitate the proper folding of L into the stable polymerase complex. With the coexpression of gstC and both polymerase subunits the half-life of the P–L complex is decreased to 8.4 hr. In a

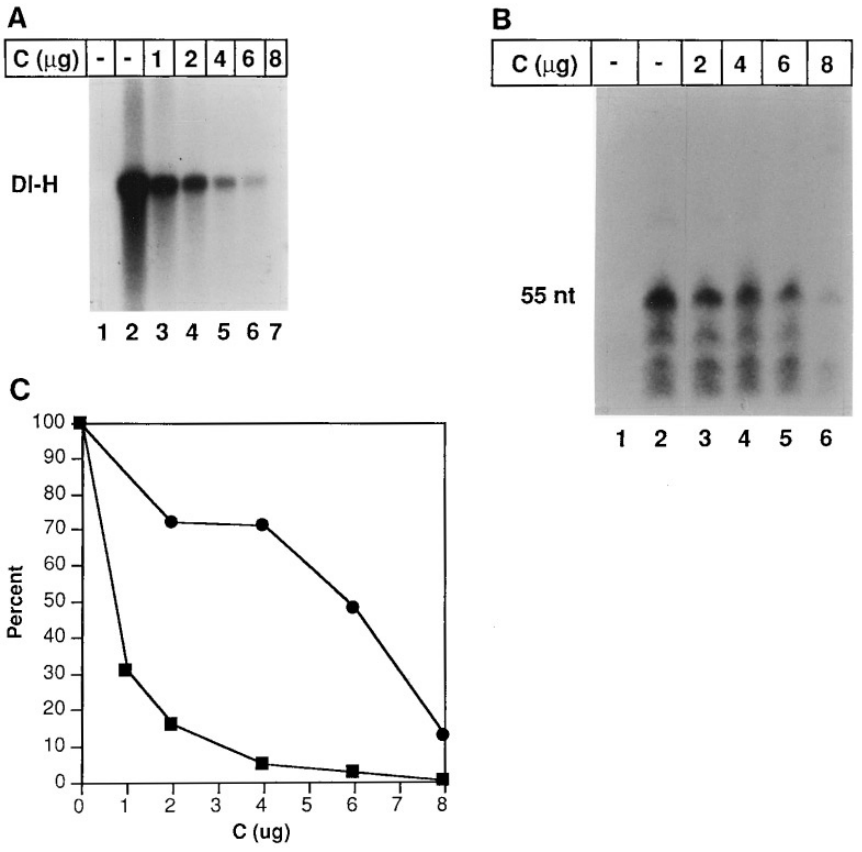

FIG. 6. The Sendai C proteins differentially inhibit DI-H genome replication and DI leader RNA synthesis *in vitro.* VVT7-infected cells were transfected with no plasmids (A and B, lanes 1) or with the P, L, and NP plasmids in the absence or presence of increasing amounts of the C plasmid as shown at the top and incubated overnight. (A) Cytoplasmic cell extracts were incubated with polymerase-free DI-H template and [α-^{32}P]CTP, the nuclease-resistant nucleocapsid products were purified, and the RNA was extracted and analyzed by agarose acid–urea gel electrophoresis. The position of DI-H RNA is indicated on the left. (B) Cytoplasmic cell extracts were incubated with polymerase-free DI-H template and the leader products detected by Northern blotting with a radiolabeled probe specific for DI leader RNA as described under Materials and Methods. The position of the 55-nt leader RNA is indicated. (C) The DI-H genomic (■) and leader (●) products were quantitated in two separate experiments and the average of each (which differed by less than 10%) was plotted relative to its control reaction in the absence of the C proteins as 100%.

normal virus infection the P, L, and C proteins are also all expressed together and based on the data for coexpression of just the three proteins above, we predict that the half-life of the polymerase during infection would also be about 8 hr. In VSV, a negative-strand RNA virus of the rhabdovirus family, the L protein expressed alone is more stable with a half-life of 3 to 6 hr, which increases to 16 hr when coexpressed with VSV P (Canter and Perrault, 1996), so in this case as well P stabilized the L protein. In the Sendai pulse–chase experiments it was also quite striking that gstC, but not gst, greatly stimulated the expression particularly of the L protein (Fig. 4, Table 1). The reason for this stimulation in the absence of any effect on the stability of L is unknown, but could be due either to gstC enhancing transcription of the L plasmid by T7 RNA polymerase or to a stimulation of translation of the L mRNA without contributing to the proper folding of the L protein.

The Sendai and mumps virus P proteins expressed in the absence of other viral proteins have been previously shown to form homotrimers (Curran *et al.,* 1995), where the oligomerization domain is also required for P binding to nucleocapsids (Ryan *et al.,* 1991). Since the P–L polymerase complex binds the template through the P subunit (Horikami and Moyer, 1995), it was suggested that a trimer of P binds each L monomer (Curran *et al.,* 1995). In the bead binding experiments the P–L polymerase complex is selected via the gstC interaction, so by quantitating the radioactivity in the P and L bands one should be able to determine the molar ratio of P to L in the complex. In multiple experiments (6) where equal amounts of the P and L plasmids were transfected, P bound to beads was always an oligomer, but the P:L ratio ranged from 2:1 to 4:1 with an average of 3:1. In experiments where the amount of P protein was both lowered and raised relative to L by changing the amount of P plasmid transfected, the ratio of P to L in the complex was also 3 ($\pm$1):1 (data not shown). Thus, the exact P oligomerization state in the polymerase complex could not be definitively determined by this methodology.

Recent studies on the formation of the P–L complex in VSV have defined an essential role for P oligomerization (Gao and Lenard, 1995a,b; Gao *et al.,* 1996). Phosphorylation of P is absolutely required for homotrimer formation, which in turn is necessary for binding to the L protein and for transcriptional activity. L binding stabilizes the P trimer (P_3) and prevents subunit exchange, which can readily occur in the absence of L. In addition, the P_3–L complex binds to the template more strongly than P_3 alone. One difference between the Sendai and VSV P oligomers is that for Sendai the P protein does not appear to exchange even in the absence of L protein (Curran *et al.,* 1995; Horikami *et al.,* 1996, unpublished data).

An interesting and unexpected feature of the effect of C protein on DI-H replication *in vitro* is that genome synthesis is more sensitive to inhibition than is DI leader RNA synthesis, which is eventually also turned off (Fig. 6). In the current model for replication (Lamb and Kolakofsky, 1996) leader RNA is synthesized first, and with a sufficient concentration of the NP_0–P complex the initation of encapsidation on leader RNA occurs with the subsequent simultaneous synthesis and encapsidation of the full-length genome. In the absence of encapsidation for a DI template only DI leader RNA is synthesized. It is possible that C binding to L alters the ability of the polymerase to recognize and/or utilize the NP_0–P complex *in vitro,* leading to a cessation of encapsidation and thus of replication. Since DI-H replication is not affected by C *in vivo* (Cadd *et al.,* 1996), the intact cellular environment, by an unknown mechanism, seems to overcome this effect and permit replication. The mechanism of C inhibition of transcription will also be of interest since in this case RNA synthesis is clearly directly affected (Curran *et al.,* 1992). Future experiments will examine the effect of C protein on the synthesis of (+) strand leader RNA, the first transcription product from the wt genome.

The function of the C gene has been investigated in two other negative-strand RNA viruses. The measles C gene was shown to be nonessential since virus lacking this gene gave titers identical to those of wt virus in tissue culture cells (Radecke and Billeter, 1996). The intracellular levels of viral RNA synthesized during infection with the wt and C-deficient viruses were not compared, so it is not known if measles C also modulates RNA synthesis. For measles virus a gstC fusion protein synthesized in *Escherichia coli* was used in an *in vitro* binding assay with viral and host proteins expressed in cells (Liston *et al.,* 1995). The C protein failed to bind other viral proteins, although there was an interaction with one cellular protein. If the measles L and C proteins do form a complex and coexpression is required for binding as it is in Sendai, it is probable that this *in vitro* binding assay would not have detected this interaction. In addition, C expressed in the yeast two-hybrid system did not interact with the measles P, V, or NP proteins, or with itself (Liston *et al.,* 1995); however, the L protein was not tested in this system.

VSV also encodes two small, basic C proteins in an overlapping reading frame of the P mRNA (Spiropoulou and Nichol, 1993). As in measles virus the VSV C gene can be deleted without apparent effect on the production of virus in tissue culture cells (Kretzschmar *et al.,* 1996). In the latter studies deletion of the C gene did not affect the intracellular levels of viral RNA and protein, suggesting that the VSV C proteins do not regulate viral RNA synthesis, unlike the inhibition of RNA synthesis seen with the Sendai virus C proteins.

ACKNOWLEDGMENT

This work was supported by NIH Grant AI 14594 (S.A.M.)

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