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Biochemistry

Expression of a cDNA encoding a functional 241-kilodalton vesicular stomatitis virus RNA polymerase

(complementation/simian virus 40 late promoter/COS cells/antibodies against synthetic peptides)

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ABSTRACT **The large gene, *L*, of vesicular stomatitis virus (VSV), which codes for the multifunctional RNA-dependent RNA polymerase, was assembled from five overlapping cDNA clones. The sequence of the 6.4-kilobase gene of the final construct was identical to the consensus sequence reported earlier. The gene was inserted into the simian virus 40 transient expression vector pJC119. Antibodies directed against synthetic peptides corresponding to the amino and carboxyl termini of the L protein were raised in rabbits. Both antibodies specifically immunostained the cytoplasm of COS cells that had been transfected with the vector DNA. The expressed L protein was immunoprecipitated from cell extracts and it was identical in size to the L protein of the virion (241 kilodaltons). Most importantly, COS cells that expressed the recombinant L protein transcribed, replicated, and consequently complemented and rescued temperature-sensitive RNA polymerase mutants of VSV at the nonpermissive temperature. The kinetics of virus release were similar to those of a wild-type VSV infection. We conclude that the recombinant RNA polymerase protein L is indistinguishable in its size and its functions from the VSV polymerase.**

All nonsegmented negative-strand RNA viruses such as paramyxo- and rhabdoviruses contain a large gene (*L*) that codes for an RNA-dependent RNA polymerase required for transcription and replication of the virus. In the case of the rhabdovirus vesicular stomatitis virus (VSV), the polymerase gene makes up about 60% of the entire genome. The 6.4-kilobase (kb) gene codes for a single protein, L, with a molecular mass of 241 kilodaltons (kDa) (1). The L protein, together with the small 30-kDa nonstructural (NS) protein (2, 3), sequentially transcribe the viral ribonucleoprotein template into five monocistronic capped, methylated, and polyadenylylated messenger RNAs (4–6). There is evidence that besides polymerization, functions such as capping (7), methylation (8–10), and polyadenylylation (11–14) of the messages may also be carried out by the polymerase complex, rendering this cytoplasmic virus independent of the host cell in respect to these functions. Because the polymerase protein L is so large, we suspect that it carries out most, if not all, of these functions.

cDNA cloning and extensive sequence analysis of the polymerase gene of VSV was our first step to dissect the functional domains of the protein. This communication describes our second step: the functional expression of a recombinant DNA coding for the RNA polymerase L of VSV. We demonstrate not only that the assembled and expressed polymerase gene is transcribed and translated into the 241-kDa L protein but also that the protein is functional. It transcribes and replicates temperature-sensitive polymerase mutants of VSV and thereby complements and rescues these mutants at the nonpermissive temperature.

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MATERIAL AND METHODS

Cells and Virus. COS cells (15), were used for DNA transfection experiments. Baby hamster kidney cells (BHK 21) were used for virus titrations and to obtain virus stocks. The two temperature-sensitive mutants of VSV Indiana serotype, ts G114 and T1026, have been described (16, 17).

cDNA Clones and Plasmid Vectors. The cDNA clones used for the assembly of the *L* gene have been described (1). pJC119 and pRL1 were a generous gift from R. A. Lazzarini (18). The individual steps for the assembly of the *L* gene were done according to standard procedures with only minor modifications (19). The strategy is briefly outlined in *Results*.

Peptide Synthesis and Antibodies. The peptides corresponding to the amino terminus, positions 5–19, and the carboxyl terminus, positions 2084–2098, of the L protein (1) were synthesized by using a Beckman 990 automated peptide synthesizer and purified by HPLC, and antibodies against these peptides were raised in rabbits as previously described (20). Neutralizing rabbit anti-VSV antibodies directed against whole virus were purchased from Microbiological Associates (Bethesda, MD). Goat anti-rabbit IgG antibodies conjugated with rhodamine were obtained from Cappel Laboratories (Cochranville, PA).

DNA Transfections. COS cells were grown to about 60–80% confluency in a 3.5-cm dish. They were transfected with 2–4 μg of doubly banded plasmid DNA (pSV-VSL1 or pJC119), together with 10 μg of sonicated salmon sperm DNA, by using the calcium phosphate coprecipitation procedure (21).

Immunofluorescent Staining. COS cells were grown on glass slides. They were either infected with VSV or transfected with plasmid DNA. At appropriate times, cells were fixed for 15 min at −20°C in methanol and then acetone. Antibodies directed against the amino or carboxyl terminus of the L protein were added, and these were subsequently stained with goat anti-rabbit IgG antibody conjugated with rhodamine.

Immunoprecipitations. Transfected COS cells were grown in 15-cm culture dishes and were labeled with 1 mCi (1 Ci = 37 GBq) of [^{35}S]methionine. After 24 hr, cell extracts were prepared and antibodies directed against the L protein were added. Immunocomplexes were isolated by binding to *Staphylococcus aureus* protein A coupled to Sepharose beads. The immunocomplexes were denatured and applied to a sodium dodecyl sulfate/polyacrylamide gel (22).

RESULTS

Assembly of the *L* Gene. While sequencing the polymerase gene of VSV, we detected 16 nucleotide differences between

Abbreviations: VSV, vesicular stomatitis virus; SV40, simian virus 40; kb, kilobase; pfu, plaque-forming unit.

various cDNA clones derived from genomic RNA of the same plaque-purified virus preparation (1). There was evidence that some of these mutations may surprisingly be present in genomic RNA itself and were not introduced during reverse transcription. Most of the differences consisted of single-base substitutions, but we also frequently detected single-base deletions and single-base insertions. Since our intention was to assemble a polymerase gene that could be expressed into a functional protein, we could not afford to have any of these mutations in the final construct. Single-base insertions or deletions would obviously interrupt the open reading frame for translation, and base substitutions would often lead to amino acid changes in the protein. Their effect on the multiple functions of the polymerase could not be evaluated. Therefore, we chose a strategy of assembly that excluded all of these mutations. The complete gene was assembled by using five overlapping cDNA clones (1) as shown in Fig. 1 *Upper*.

The construction involved the following steps, which are outlined briefly (see Fig. 1). Clone 649, which contains the 5′ end of the message, was trimmed back with nuclease BAL-31 close to the start codon for L protein translation and an *Xho* I restriction site was inserted. Portions of clones 649, 455, and 679 were then sequentially ligated together at the indicated unique restriction sites (Fig. 1 *Upper*), which completed the 3′ half of the gene.

Clones 321 and 661, which overlapped for only about 30 base pairs, were linked by first digesting the overlapping ends with exonuclease III, which specifically digests the 3′ ends of double strands and leaves the 5′ ends single stranded and intact. Because clones 321 and 661 were positioned in opposite directions within pBR322 relative to the *L* gene orientation, it was possible to hydrogen bond the two plasmid inserts specifically at the 30-base overlap and ligate one of the inserts into the other plasmid at the unique *Eco*RI site of pBR322. Larger single-stranded 5′ overhangs of about 100 bases on both sides of the 30-base-pair duplex that originated from pBR322 were trimmed back after transformation inside the *Escherichia coli* cell. The region containing the overlap was sequenced for about 300 bases in both directions and was found to be identical to the consensus sequence, demonstrating accurate repair by the cell. A *Xho* I restriction site was introduced at the 5′ end of the gene and the two halves were ligated at the unique *Bam*HI restriction site. The assembled gene was inserted at the unique *Xho* I site of the expression vector pJC119. The construction required eight subcloning steps, all carried out with pBR322 and pRL1, which is a pML2 plasmid vector containing a polycloning site. Every DNA piece that was chosen for the final construct of pSV-VSL1 (Fig. 1 *Lower*) had been sequenced previously and contained the consensus sequence, derived from, on the average, three cDNA copies as published earlier (1).

Expression of the *L* Gene. In the expression vector pSV-VSL1 (Fig. 1 *Lower*), the *L* gene is under control of the late promoter of SV40, downstream of the splice donor and acceptor sites of SV40 late messages and upstream of the polyadenylylation site (18). We expected that if all transcription and splice signals of SV40 were correctly used and no

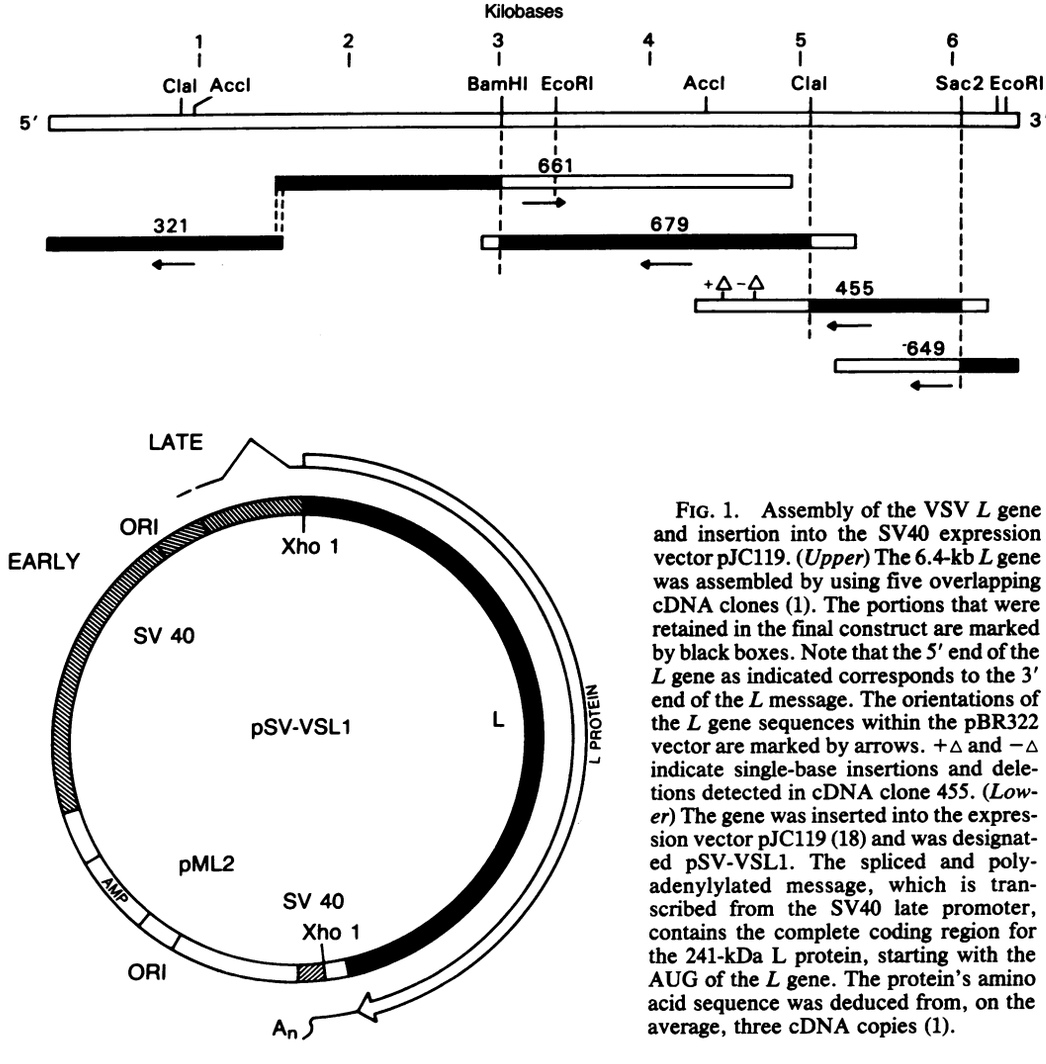

FIG. 1. Assembly of the VSV *L* gene and insertion into the SV40 expression vector pJC119. (*Upper*) The 6.4-kb *L* gene was assembled by using five overlapping cDNA clones (1). The portions that were retained in the final construct are marked by black boxes. Note that the 5′ end of the *L* gene as indicated corresponds to the 3′ end of the *L* message. The orientations of the *L* gene sequences within the pBR322 vector are marked by arrows. +Δ and −Δ indicate single-base insertions and deletions detected in cDNA clone 455. (*Lower*) The gene was inserted into the expression vector pJC119 (18) and was designated pSV-VSL1. The spliced and polyadenylylated message, which is transcribed from the SV40 late promoter, contains the complete coding region for the 241-kDa L protein, starting with the AUG of the *L* gene. The protein's amino acid sequence was deduced from, on the average, three cDNA copies (1).

potential cryptic splice signals or termination sites existed within the large *L* gene itself, the resulting message should be translated, starting with the first methionine of the L protein and yielding a complete 2109 amino acid RNA polymerase protein with a molecular mass of 241 kDa.

To detect the expressed protein in COS cells, two pentadecapeptides, Asp-Phe-Glu-Thr-Asp-Glu-Phe-Asn-Asp-Phe-Asn-Glu-Asp-Asp-Tyr and Asn-Arg-Arg-Ile-Ser-Lys-Glu-Asp-Arg-Ser-Ile-Leu-Met-Leu-Lys, corresponding to the amino terminus, positions 5–19, and to the carboxyl terminus, positions 2084–2098 of the L protein (1), respectively, were synthesized. Antibodies against these peptides were raised in rabbits. Both antibodies specifically immunoprecipitated the L protein found in the virion as well as in VSV-infected cells (data not shown), demonstrating specific binding. Immunofluorescent staining using each antibody is shown in Fig. 2. COS cells were infected with VSV, fixed 5 hr after infection, and allowed to react with serum containing antibodies with one or the other specificity. More than 90% of the VSV-infected cells showed strong immunofluorescent staining of the cytoplasm when either antibody was used.

In another set of experiments, COS cells were transfected with the expression vector with the *L* gene insert (pSV-VSL1) or a control without it (pJC119) (Fig. 2). One day after transfection, the cells were fixed and stained, using the same set of antibodies as with infected cells. Cells transfected with the vector without the *L* gene insert showed no staining or only weak background staining. On the other hand, COS cells transfected with the vector containing the *L* gene showed strong immunofluorescent staining of some cells with either antibody, indicating that the amino and carboxyl termini of the L protein were translated in these cells (Fig. 2). As with VSV-infected cells, immunofluorescent staining was detected exclusively in the cytoplasm. The transfection efficiency with the calcium phosphate coprecipitation procedure (21) was 1–5%, as judged after 24 hr; however, many more cells, up to 10%, showed bright immunofluorescent staining 2 days after transfection (data not shown). This suggests that the transfection efficiency was relatively high and that the protein accumulated in the cytoplasm of individual transfected COS cells at different rates within the 2-day period.

Recombinant L Protein Is Identical in Size to Virion L Protein. The immunofluorescent staining experiments do not rule out rearrangements of the vector *L* gene insert or posttranslational processing of the protein. Since the L protein is very large, it was important to determine the size of the expressed protein. COS cells were transfected with pSV-VSL1 or pJC119, and the proteins were labeled with [^{35}S]methionine for 24 hr, starting 20 hr after transfection. Cytoplasmic extracts were prepared and both antibodies of each specificity were added. Immunocomplexes were isolated by binding to protein A-Sepharose beads and were subsequently separated by electrophoresis on a discontinuous sodium dodecyl sulfate/polyacrylamide gel.

As shown in Fig. 3, lanes 2 and 3, both antibodies precipitated the L protein expressed in cells transfected with pSV-VSL1 DNA. In contrast, immunoprecipitations of cell extracts from cells transfected with pJC119, without the *L* gene insert, did not result in the precipitation of a protein of equivalent size (lanes 4 and 5). The consistently observed coprecipitation or nonspecific binding of cellular protein species to protein A-Sepharose beads was independent of the antibody or cell extract used. This pattern was identical in lanes 2–5. The only protein species that was detected specifically after transfection with pSV-VSL1 was the protein marked with arrowheads in lanes 2 and 3. This protein migrated with the L protein found in the virion (lane 1), and we conclude that this recombinant L protein is identical in size to the virion polymerase L. The resolution in the molecular mass range of 241 kDa is low; however, the use of antibodies directed against synthetic peptides corresponding to the amino and carboxyl termini of the L protein (as deduced from the nucleotide sequence) rules out any posttranslational processing of the L protein that could have led to a major reduction in its size. Small modifications at either terminus in the order of 5 to 10 amino acids, however, would not affect the epitopes at the opposite terminus and would not have been detected.

The Recombinant Polymerase L Is Functional. To determine whether the expressed polymerase protein was functional, an assay system had to be established that would be sensitive enough and would take into account that only a small percentage of the cells express the L protein transiently. The genetics of VSV have clearly shown that group I mutants are polymerase L mutants. One of the best-characterized VSV L gene mutants is ts G114 (16). *In vitro* transcriptions by reconstitution of the polymerase complex (L protein plus NS protein plus template) revealed that the temperature-sensitive phenotype is caused by a mutant L protein (23). The mutant is unable to synthesize RNA at 40°C *in vitro* as well as *in vivo*, unlike T1026, which is capable of primary but not

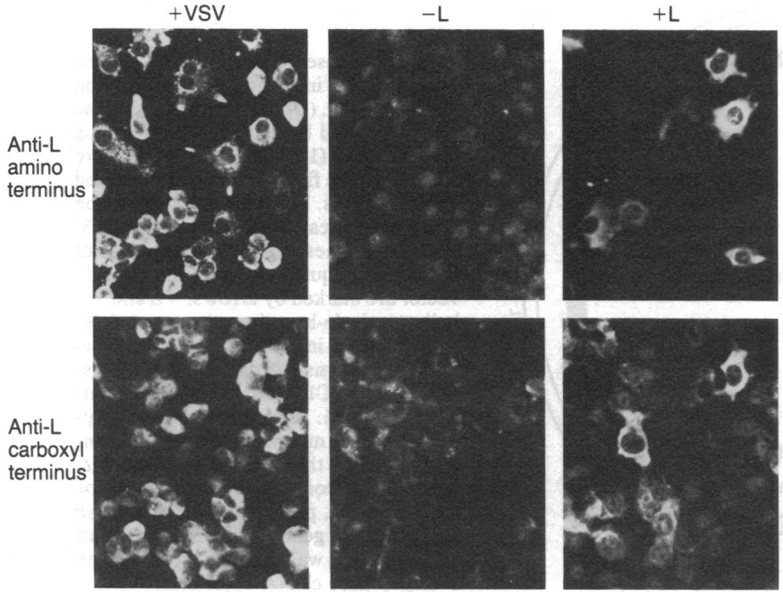

FIG. 2. Immunofluorescent staining of recombinant L protein expressed in COS cells. COS cells were infected with VSV at a multiplicity of infection of 3 (+VSV), transfected with pJC119 DNA (−L), or transfected with pSV-VSL1 DNA (+L). Five hours after infection or 24 hr after transfection, respectively, the cells were fixed and incubated with rabbit serum containing antibodies directed against the amino and carboxyl termini of the L protein, respectively. Bound antibodies were stained by using goat anti-rabbit IgG antibodies conjugated with rhodamine.

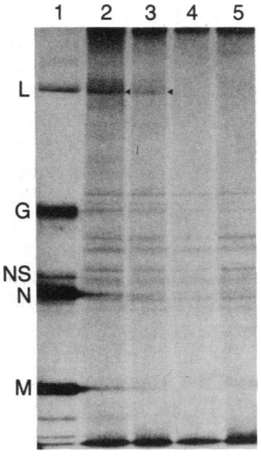

FIG. 3. Immunoprecipitation of recombinant L protein expressed in COS cells. COS cells were transfected with pSV-VSL1 (lanes 2 and 3) or pJC119, which lacks the *L* gene insert (lanes 4 and 5). Twenty-four hours after transfection the cells were labeled with [^{35}S]methionine for 1 day and cell extracts were prepared. Antibodies directed against the amino (lanes 2 and 4) and carboxyl termini (lanes 3 and 5) of the L protein were added and the immunocomplexes were isolated by binding to protein A-Sepharose beads. The proteins were denatured and separated next to ^{35}S-labeled virion proteins (lane 1) on a denaturing discontinuous polyacrylamide gel.

secondary transcription (17, 24). The main advantage of using ts G114 is that it does not seem to revert. ts G114, therefore, appeared to be a perfect candidate to test whether the recombinant L protein is functional. If the L protein is functional, we anticipated that released virus would be detected in the supernatant of cells that were transfected with pSV-VSL1 and superinfected with ts G114 at 40°C.

The protocol of our complementation assay is shown in Fig. 4. The results of a typical complementation assay are shown in Table 1. As can be seen with both polymerase mutants, ts G114 and T1026, virus was released from COS cells at the nonpermissive temperature only when the cells were previously transfected with pSV-VSL1 and not when the cells were transfected with pJC119, which does not contain the *L* gene insert. Over a period of 24 hr after infection, the pfu/ml of medium increased from $<10^2$ pfu/ml to 2.6×10^5 pfu/ml in the case of ts G114, or to 5×10^4 pfu/ml in the case of T1026, suggesting that both polymerase mutants were rescued by cells that expressed the recombinant L protein. In addition, released virus, when titered in BHK 21 cells at 40°C instead of 32°C, did not form any plaques, demonstrating that released virus still exhibited the same temperature-sensitive phenotype as the input mutant virus.

The kinetics of virus release at 40°C is shown for ts G114 in Fig. 5. Instead of harvesting released virus 24 hr after infection, we replaced the media in 2-hr intervals with fresh media over a 24-hr period and determined the titer of released virus with BHK 21 cells at 32°C. COS cells that were transfected with pJC119 DNA did not release any virus particles throughout the 24-hr period (the limit of detectability during titration was 10 pfu/ml). Cells transfected with pSV-VSL1, on the other hand, released temperature-sensitive virus as early as at 2 hr after infection and reached a level of about 10^5 pfu/ml after 24 hr. The kinetics of virus release was similar, compared to a wild-type VSV infection. Virus was released with similar kinetics whether the *L* gene was expressed in transfected cells for 1 or 2 days prior to superinfection with the temperature-sensitive mutant. However, the titer of released virus was always lower from infected cells that had expressed the *L* gene for 2 days. This result suggests that the accumulation of L protein does not lead to a more efficient complementation.

These complementation data, taken together with the immunoprecipitation experiments, strongly suggest that the recombinant L protein is indistinguishable from the virion polymerase not only in its size but also in its functions. Thus, expression of the recombinant L protein from pSV-VSL1 appears to support transcription of functional messenger RNAs as well as replication, which is a prerequisite for the complementation of the temperature-sensitive mutants.

Table 1. Complementation of VSV *L* gene mutants in COS cells

Mutant	Vector		Released virus (pfu/ml) at restrictive temperature (40°C)		
			0 hr after infection, titer at 32°C	24 hr after infection	
				Titer at 32°C	Titer at 40°C
ts G114	pJC119	(−L)	$<10^2$	$<10^2$	ND
ts G114	pSV-VSL1	(+L)	$<10^2$	2.6×10^5	$<10^2$
T1026	pJC119	(−L)	$<10^2$	$<10^2$	ND
T1026	pSV-VSL1	(+L)	$<10^2$	5×10^4	$<10^2$

Transfections and infections were carried out as described in the legend to Fig. 4. ND, not determined; pfu, plaque-forming units.

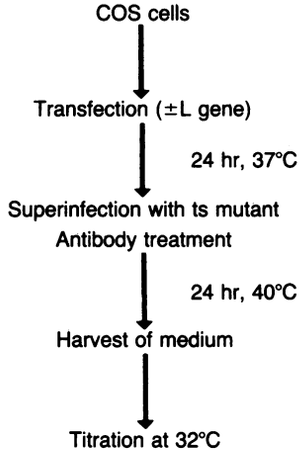

FIG. 4. Protocol of the *L* gene complementation assay. COS cells were transfected with either pJC119 or pSV-VSL1 and incubated at 37°C for 24 hr. Temperature-sensitive polymerase mutants were adsorbed to the cells at a multiplicity of infection of 3 for 30 min at 37°C. Unadsorbed virus was neutralized with rabbit anti-VSV antibodies. Neutralized virus and antibodies were removed by five serial washings. The cells were kept for 24 hr at 40°C, the restrictive temperature for the mutant. Released virus was titered on BHK 21 cells at 32°C.

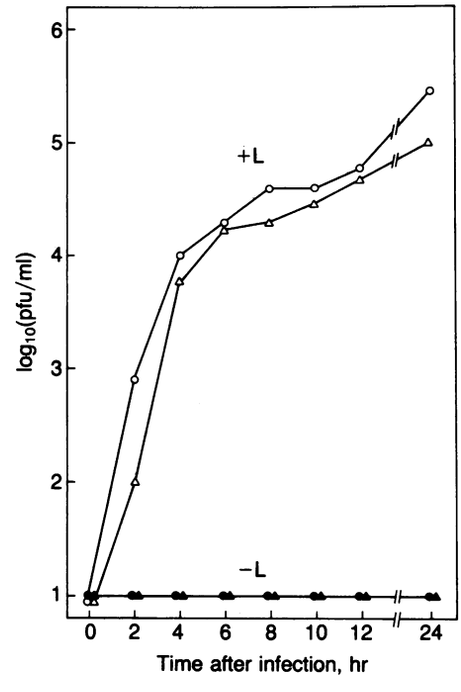

FIG. 5. Kinetics of mutant virus release at the restrictive temperature. COS cells were transfected with pJC119 (−L) (●, ▲) or pSV-VSL1 (+L) (○, △) and superinfected 24 hr (●, ○) and 48 hr (▲, △) after transfection with the polymerase mutant ts G114 as outlined in the legend to Fig. 4. The cells were incubated at 40°C, the nonpermissive temperature of the mutant, and media were replaced in 2-hr intervals. Released virus was titered on BHK 21 cells at 32°C.

DISCUSSION

In this communication, we reported the assembly and insertion of the VSV RNA polymerase gene, *L*, into the eukaryotic expression vector pJC119 (18). The expression of a functional polymerase depended on excluding any of the mutational changes previously described from the final construct. The assembled gene, described here, contains the consensus sequence derived from three cDNA copies (1). It was uncertain whether the expected approximately 8-kb messenger RNA would be spliced and polyadenylylated without interrupting the reading frame for this large protein. Immunoprecipitation of the expressed L protein, however, demonstrated that the recombinant L protein is identical in size to the virion polymerase. The use of monospecific antibodies directed against the amino or carboxyl terminus of the protein ruled out any modifications of the L protein that could have caused a substantial reduction of its size. The complementation of well-defined temperature-sensitive polymerase mutants by expression of the recombinant L protein resulted in the rescue and release of virus particles. Released virus showed the same temperature-sensitive phenotype as the mutant, which ruled out reversion or recombinational events between RNA strands, which have never been observed with negative-strand RNA viruses.

Sequential transcription of the VSV genome favors the genes positioned close to the 3′ terminus, such as *N* and *NS* (25). L message is the least abundant among VSV messages, suggesting that the L protein is needed in catalytic amounts. As yet, the optimal L protein concentration required for transcription and replication is unknown. The wide range in the level of L expression between individual COS cells, as detected by immunofluorescent staining, suggests that transfected COS cells may not rescue the mutant with the same efficiency. A wild-type VSV infection yields approximately 10^3 pfu per cell. In comparison, approximately 10^5 pfu were released from maximal 10^6 COS cells. Based on the immunofluorescent staining for the L protein, a maximum of about 1/10 of the cells had the potential to rescue the mutants. Recent double immunofluorescent staining of cells, using antibodies directed against the amino terminus of L (the L-expressing cells) and the viral nucleocapsid protein N (the complementing cells), surprisingly revealed that cells with high levels of L expression never show immunofluorescent staining for the N protein, while cells with low levels of L expression do. It appears that high levels of L protein *or* L message may arrest transcription or replication of the mutant ts G114. The mechanism of this interesting phenomenon is unknown. Because of the overestimation for the number of complementing COS cells, the virus yield per cell needs adjustment and is probably closer to 10–100 pfu per cell. This may only be a factor of 10 less than during a wild-type VSV infection (10^3 pfu per COS cell).

We can assume that, during complementation in our system, temperature-sensitive L protein is simultaneously synthesized in addition to the recombinant L protein. This mutant L protein may interfere with the "wild-type" L protein encoded by the vector and thereby reduce the virus yield. Intragroup or possibly intragenic complementation between L gene mutants has been reported (26, 27), which raises the possibility that the polymerase complex may contain more than one L protein subunit. A mosaic polymerase complex, consisting of a temperature-sensitive L protein and a recombinant L protein, may not be as efficient in performing all the essential functions during transcription and replication. This, of course, would most likely reduce the virus yield, a situation that somewhat resembles a homotypically autointerfered infection by ts *L* gene mutants at the restrictive temperature (28).

The complementation assay, which yields at least a 10^4 to 10^5 increase in released virus, will allow the study of the organization of the multiple functional domains of the polymerase complex. It will be interesting to see whether the entire L protein is needed for complementation or whether portions can be deleted without affecting, e.g., the polymerization function while, e.g., the methylase or capping functions are removed or inactivated. The accuracy of the polymerase can be studied and the mutation rate may be affected by introducing mutational changes in the recombinant polymerase gene itself. A cell line with every cell constitutively expressing the recombinant L protein should allow plaque purification and propagation of defective interfering particles, such as DI-LT (29–33), in the absence of parental helper virus. The cell-killing DI-LT particle transcribes the *N*, *NS*, *M*, and *G* genes of VSV, while the *L* gene is almost completely deleted. Interference by coexpression of a mutant L protein, as described above, could not occur. Polymerase mutants of VSV usually account for almost 90% of all spontaneous or induced VSV mutants analyzed (27). Since the L protein would be provided in *trans* by the cell, it would be advantageous to study DI-LT mutants of the other four genes on a homogeneous *L* gene background.

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