An L (Polymerase)-Deficient Rabies Virus Defective Interfering Particle RNA Is Replicated and Transcribed by Heterologous Helper Virus L Proteins

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A rabies virus-derived defective interfering particle (DI) was isolated and characterized. The DI genome contained an internal deletion of 6.4 kb spanning the 3′ moiety of the pseudogene region (Ψ) and most of the L gene. DI-specific monocistronic N, NS, and M mRNAs as well as a G/L fusion mRNA were transcribed in cells coinfected with DI and helper virus. In addition, polycistronic DI RNAs and standard virus RNAs with internal A stretches and intergenic regions were found. Superinfection experiments showed that heterologous rabies-related viruses (Lyssavirus serotypes 2, 3, and 4) can complement the L deficiency of the DI genome. The heterologous polymerase proteins recognize correctly the replicational and transcriptional signal sequences of the Lyssavirus serotype 1-derived DI.

INTRODUCTION

Rhabdoviruses possess a single-stranded negative sense RNA genome encoding five major proteins. The viral RNA is tightly associated with the nucleoprotein (N), the phosphoprotein (NS), and the polymerase (L) to form a nucleocapsid which constitutes the active template both for transcription and replication of the viral genome. Transcription successively gives rise to a short leader RNA and five monocistronic, capped, and polyadenylated mRNAs which encode the viral proteins (for review see Banerjee, 1987). The transcriptase is thought to enter the template near the 3′ end and to sequentially synthesize the leader and the N, NS, M (matrixprotein), G (glycoprotein), and L mRNA by terminating and restarting at defined signals flanking each cistron. In the replication process, however, these signals are not recognized by the polymerase (replicase). The resulting full-length positive- and negative-stranded genomic RNAs are only present as nucleocapsids. The switch from transcription to replication is proposed to be regulated mainly by the amount of free N protein available within the cell. Concurrent encapsidation of the nascent RNA chain would be responsible for the polymerase to ignore the transcriptional start- and stop-signals (Blumberg *et al.*, 1983; Banerjee *et al.*, 1984; Arnheiter *et al.*, 1985; Banerjee, 1987).

The genome of rabies virus, the prototype of the Lyssavirus genus, is organized similar to that of vesicular stomatitis virus (VSV), the prototype rhabdovirus (genus Vesiculovirus) and the basic transcription and replication mechanisms seem to be identical (Tordo *et al.*, 1986a, 1986b, 1988; Conzelmann *et al.*, 1990). Putative transcriptional start- and stop/polyadenylation signals flanking the genes show high homology in both viruses. In contrast to VSV, however, the intergenic regions of rabies virus are variable in length, namely, two nucleotides (nts) in VSV and two to 24 nts in rabies virus. The most striking difference in genome organization is the presence of a pseudogene region (Ψ) between the G and L gene in rabies virus (Tordo *et al.*, 1986a). The Ψ is always terminated by an intact transcriptional stop signal and is transcribed as the 3′ portion of a G/Ψ mRNA of 2.3 kb in all rabies virus strains examined so far (Tordo and Poch, 1988; Morimoto *et al.*, 1989; Conzelmann *et al.*, 1990).

In VSV considerable insight into replication and transcription mechanisms has been obtained by the study of defective interfering (DI) particles which lack part(s) of the parental genome and whose propagation depends on helper virus proteins. DI particle and standard virus genomes share the minimum essential characteristics for replication, e.g., terminal promoter sequences, and in some cases also for transcription (for reviews see Lazzarini *et al.*, 1981; Perrault, 1981; Schlesinger, 1988). As in most negative-stranded RNA viruses DI particles are also frequently generated in lyssaviruses (Crick and Brown, 1974; Kawai and Matsumoto, 1977; Wiktor *et al.*, 1977; Clark *et al.*, 1981). Recent cloning and sequencing of cDNA that spans the whole genome of rabies virus allow identification and detailed characterization of transcription and replication products from both standard and defective rabies virus particles by the use of specific hybridization probes and primers. In the present communication we

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describe the isolation and the first detailed characterization of a rabies virus-derived defective interfering particle (SAD DI-1) which is defective in the L (polymerase) gene. Its replication and transcription by the L proteins of homologous rabies viruses (Lyssavirus serotype 1) and heterologous "rabies related" helper viruses (Lyssavirus serotypes 2, 3, 4; for review see King and Crick, 1988) was studied *in vivo* by superinfection experiments.

MATERIALS AND METHODS

Cells and viruses

All viruses were grown in BHK-21 cells (clone BSR) as described (Conzelmann *et al.*, 1990). Virions and DI particles were purified from supernatants (Wiktor *et al.*, 1977) and genomic RNA was isolated according to Chirgwin *et al.* (1979). The virus strains used in this study are: SAD B19 (Street Alabama Dufferin B19), CVS (Challenge virus standard), ERA (Evelyn–Rokitnicky–Abelseth), W187 (a rabies street virus isolated from a rabid fox and passaged twice in BHK-21 cells), PM (Pitman–Moore), HEP (Flury High Egg Passage), LEP (Flury Low Egg Passage), PV (Pasteur virus) [all serotype 1]; LBV (Lagos Bat virus) [serotype 2]; MOK (Mokola virus) [serotype 3]; and DUV (Duvenhage) strains 1 to 5 (Human South Africa, Bat South Africa, Bat Hamburg, Bat Stade, Bat Bremerhaven, respectively) [serotype 4]. SAD B19/MB is an SAD B19 stock.

For superinfection experiments 5×10^6 cells were infected with helper viruses at an m.o.i. of 0.1 to 1. Six hours p.i. 2 μl of a solution containing purified SAD DI-1 particles was added in cell culture medium containing 1% DEAE-Dextran. After 30 min adsorption at room temperature cells were washed twice and incubated at 37°C in Eagle's medium supplemented with 10% calf serum in a 5% CO_2 atmosphere. Cells and supernatants were harvested 30 h p.i.

Oligonucleotides and cDNA probes

Deoxyoligonucleotides were synthesized on a Biosearch 8700 DNA Synthesizer and purified by denaturing polyacrylamide gel electrophoresis. The oligonucleotides used in this study were: G1P (5′-dATGGGTCTCGATGCAA-3′; nt 4090 to 4105), G5P (5′-dTAGCGGACTCAGGATGAG-3′; nt 5214 to 5231), L1P (5′-dTGCCTGTGAACCGGAT-3′; nt 11789 to 11804), S1P (5′-dCCGTGAAGGCCAGAGAAG-3′; nt 5257 to 5265 and 11700 to 11708), Leader1P (5′-dACGCTTAACAA-3′; nt 1 to 11), and the respective complementary oligonucleotides G1M, G5M, L1M, and S1M. SAD B19 cDNA fragments used for gene specific hybridizations are: *Eco*RI/*Eco*RI 0.9 kbp, nt 225 to 1112 (N); *Bgl*II/*Xba*I 0.9 kbp, nt 1521 to 2430 (NS); *Xba*I/*Xba*I 0.7 kbp, nt 2430 to 3095 (M); *Hin*dIII/*Apa*I 1.3 kbp, nt 3401 to 4714 (G); and *Hin*dIII/*Sal*I 2.6 kbp, nt 5337 to 7894 (L). All nucleotide positions mentioned refer to the SAD B19 rabies virus genome sequence (Conzelmann *et al.*, 1990).

Labeling and hybridization

Fifty nanograms of oligonucleotides were labeled in 30 μl of 70 m*M* Tris, pH 7.6, 10 m*M* $MgCl_2$, 5 m*M* DTT, 4 U T4 PNK, and 50 μCi [γ-^{32}P] ATP (3000 Ci/mMol; Amersham). Hybridization to Northern filters (Thomas, 1980) was performed at 45°C (G1P/M and L1P/M) or 50°C (S1P/M) overnight in 1 *M* NaCl, 0.1 *M* Tris, 20 m*M* sodium phosphate, 1 μ*M* EDTA, 0.5% SDS, 10× Denhardt's solution, and 50 μg of tRNA per ml. The filters were washed three times at hybridization temperature in 5× SSC, 0.1% SDS for 20 min each and exposed to Kodak X-Omat AR films at −70°C with an intensifying screen (Agfa Curix MR 800). cDNA fragments were labeled with [α-^{32}P] dCTP (3000 Ci/mMol) by nick-translation (Nick-Translation Kit, Amersham) as recommended by the supplier. Northern blots to nylon membranes (Duralon-UV, Stratagene) were hybridized with cDNA probes in a solution containing 7% SDS, 1 m*M* EDTA, 0.5 *M* NaH_2PO_4, pH 7.2, and washed three times for 15 min each in 1% SDS, 1 m*M* EDTA, 40 m*M* NaH_2PO_4, pH 7.2.

RNA sequencing

One microgram of genomic DI RNA or 10 μg of total cellular RNA were annealed with 5 ng of labeled primer in a 10 or 15 μl vol, respectively, and sequenced with AMV reverse transcriptase (Life Sciences) in 50 m*M* KCl, 50 m*M* Tris–HCl (pH 8.3), 6 m*M* $MgCl_2$, 10 m*M* DTT, 200 μ*M* of dNTPs, 100 μ*M* of dideoxynucleotides, except for ddTTP which was 160 μ*M*. After incubation for 30 min at 43°C, the reaction was chased with 500 μ*M* dNTP for 10 min.

Synthesis, cloning, and sequencing of cDNA

After isolation of total RNA of infected cells (Chirgwin *et al.*, 1979) poly(A)$^+$ RNA was enriched by oligo-dT-cellulose chromatography (Aviv and Leder, 1972). cDNA was synthesized according to Gubler and Hofmann (1983), ligated with synthetic *Eco*RI adaptors (Pharmacia) and cloned in λZAPII phages (Stratagene). G specific clones were identified by plaque hybridization with a 1.3-kb *Hin*dIII/*Apa*I fragment from the SAD B19 genomic cDNA clone pSAD13 (Conzelmann *et al.*, 1990) and excised *in vivo* as recommended by the supplier. The terminal regions of the inserts were se-

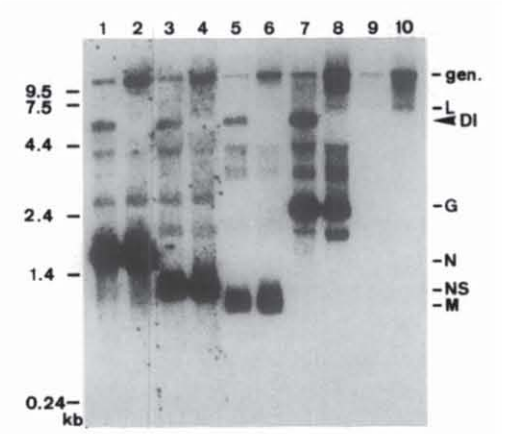

FIG. 1. Identification of rabies virus RNAs. Northern blots of total RNA from cells infected with SAD B19/MB (lanes 1, 3, 5, 7, 9) and PV (lanes 2, 4, 6, 8, 10) were hybridized with gene-specific SAD B19 cDNA fragments (lanes 1, 2, N; 3, 4, NS; 5, 6, M; 7, 8, G; 9, 10, L). Exposure time was 4 to 16 h for the different probes. Genomic RNAs (gen) and monocistronic mRNAs (M, NS, N, G, L) are indicated. A 5.5-kb RNA (arrowhead) specific for SAD B19/MB infected cells contains sequences homologous to N, NS, M, and G genes.

quenced on double-stranded plasmids according to Zhang *et al.* (1988).

RESULTS

Identification of SAD DI-1-specific RNAs

In tissue culture cells infected with rabies virus large amounts of viral mRNAs and genome length negative- as well as positive-stranded RNAs can be demonstrated. The pattern of bands generated by Northern hybridizations is highly similar when different fixed rabies virus strains are used for infection (Tordo and Poch, 1988; Conzelmann *et al.*, 1990). In cells infected with SAD B19/MB stock at an m.o.i. of 0.5, however, an additional prominent 5.5-kb RNA was detected in preparations of total cellular RNA (Fig. 1). Northern hybridizations with gene-specific SAD B19 cDNA fragments showed that the 5.5-kb RNA contained sequences homologous to rabies virus N, NS, M, and G genes (Fig. 1) and also to the Ψ region (data not shown). No hybridization could be demonstrated with a probe spanning 2.6 kb of the L gene (Fig. 1, lane 9). Bicistronic, and to a smaller degree also polycistronic viral mRNAs, can readily be detected in cells infected with rabies virus. According to its size and hybridization features, the 5.5-kb RNA could represent such a polycistronic mRNA including the N, NS, M, and G cistrons. After separation of poly(A)$^+$ and poly(A)$^-$ RNA by oligo(dT)cellulose chromatography, however, the 5.5-kb RNA together with full-length viral RNA was found in the poly(A)$^-$ fraction (Fig. 2), whereas both mono- and polycistronic mRNAs had been removed entirely from this fraction.

In order to determine the polarity of the 5.5-kb RNA hybridizations with strand-specific oligonucleotides were performed. First, two oligonucleotides derived from the G coding region of SAD B19 were used (G1P and G1M). The 5.5-kb RNA and full-length viral genomic RNA showed hybridization with G1P, recognizing negative-stranded RNA (Fig. 3). The complementary oligonucleotide G1M also gave a positive signal with the 5.5-kb RNA. Thus, as is found for standard virus genomes, both positive- and negative-stranded RNA are present in the 5.5-kb band and we concluded that the 5.5-kb RNA represents a defective rabies virus genome (SAD DI-1). With G1M the 2.3-kb G/Ψ mRNA and an RNA population which most likely represents M + G/Ψ bicistronic mRNA (3.3 kb) can be detected. In the RNA from ERA-infected cells, which was included as a control, in addition to the common 2.3-kb G/Ψ mRNA a 1.8-kb G mRNA which lacks the Ψ (Conzelmann *et al.*, 1990) is recognized by G1M (Fig. 3, lane 4).

Taking into account the hybridization results obtained with the gene-specific cDNA probes it was concluded that the SAD DI-1 genomic RNA is colinear to the SAD B19 genome RNA up to the Ψ including an intact 3′ end. The replication model proposed for rhabdoviruses depends on identical sequences at the 3′ termini of both positive- and negative-strand genomic RNA (Banerjee, 1987). Accordingly, the SAD DI-1 RNA should also possess an intact 5′ end region or the complement of its 3′ end sequence. To determine whether

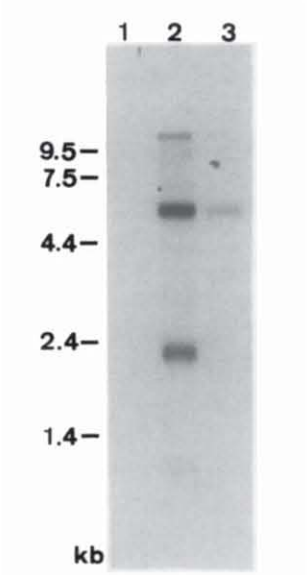

FIG. 2. Standard viral 12-kb RNA and 5.5-kb RNA are present in the poly(A)$^-$ RNA fraction (lane 3). Lanes 1 and 2 represent total RNA from noninfected and SAD B19/MB-infected cells, respectively. The Northern blot was hybridized with a G-specific SAD B19 probe.

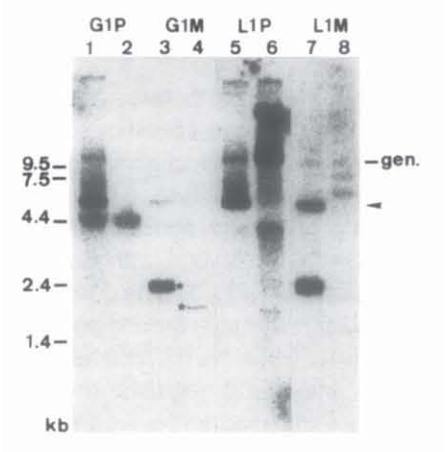

FIG. 3. Strand-specific oligonucleotide hybridizations with total RNA of cells infected with SAD B19/MB (lanes 1, 3, 5, 7) and ERA (2, 4, 6, 8). The 5.5-kb RNA (arrowhead) is recognized both by oligonucleotides deduced from the positive and negative strand G (G1P, G1M) and L (L1P, L1M) SAD B19 sequence. With G1M the G/Ψ mRNAs (2.3 kb) of SAD B19 and ERA and the G mRNA of ERA (1.8 kb) are recognized (asterisks). A 2.3-kb positive-stranded RNA containing L homologous sequences is present only in SAD/B19 MB-infected cells (L1M, lane 7). Exposure time was 12 h (lanes 1 to 6) and 36 h (lanes 7, 8).

parental 5′ terminal sequences are present in the DI genome, hybridizations were performed with oligonucleotides located close to the SAD B19 genome 5′ end (L1P/L1M, Fig. 3). Positive hybridization signals were obtained with the 5.5-kb RNA and thus we assumed that the SAD DI-1 genome possesses both intact 3′ and 5′ ends and (an) internal deletion(s) of more than half of the original genome length comprising most of the L gene.

In addition to the DI genomic RNA, L1M recognizes RNA molecules which represent the L mRNA (6.5 kb) and a G/Ψ + L bicistronic mRNA (approximately 9 kb) as well as positive-stranded standard virus genomic RNA both in ERA and SAD B19-infected cells (Fig. 3, lanes 7 and 8). Only in SAD B19/MB-infected cells, however, an intensive signal was found with an RNA which comigrated with the 2.3-kb G/Ψ-mRNA of SAD B19 (Fig. 3, lane 7). Further experiments showed that this positive-stranded RNA was polyadenylated (data not shown) and thus most likely represented an mRNA. This was the first indication that not only replication of the DI genomic RNA but also transcription of DI specific mRNAs occurs in infected cells.

RNA sequencing

In order to determine the exact location of the deletion in the DI genome we sequenced the negative strand of the 5.5-kb RNA directly in total RNA of infected cells. This was possible because only a relatively small amount of standard virus genomic RNA compared to the DI RNA was present in the RNA preparations (see Fig. 1). An oligonucleotide (G5P) which was derived from the sequence of the SAD B19 Ψ and which proved to hybridize with the DI genome after northern blotting (data not shown) was chosen as a primer. As a control total RNA from cells infected with plaque purified SAD B19 virus was included. The sequence obtained from the SAD B19 standard virus genome was identical to the published SAD B19 sequence. The DI sequence was colinear up to nt 5265 and continued with a sequence homologous to the SAD B19 genome corresponding to nt 11700 to 11928 (Fig. 4A). A strong terminal band indicated that the DI genomic RNA possesses a 5′ end homologous to the SAD B19 5′ end. Thus the SAD DI-1 genome has been generated from that of its progenitor virus by the deletion of nt 5266 to 11699. The calculated length of the DI genome with 5494 nts correlates well with the observed length of 5.5 kb. Therefore, putative additional deletions in other regions of the genome should be very small.

The sequence near the 3′ end of the DI genome was determined using a 10 mer nucleotide which is comple-

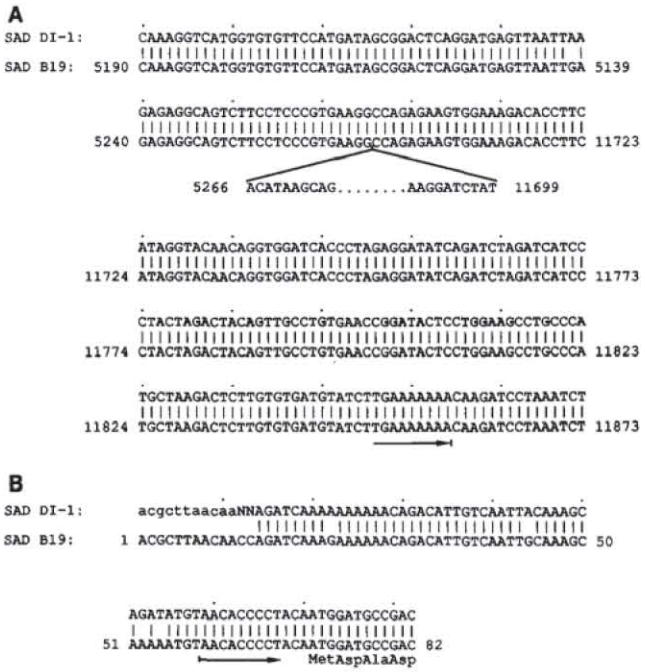

FIG. 4. Comparison of SAD B19 standard virus and SAD DI-1 particle nucleotide sequences (positive strand). (A) Location of the SAD DI-1 internal deletion; the transcriptional stop/polyadenylation signal of the SAD B19 L mRNA and SAD DI-1 G/L fusion mRNA is marked by an arrow. (B) 3′ terminal sequences of SAD B19 and SAD DI-1 with the N transcriptional start signal (arrow) and the first encoded amino acids. Lower-case letters show the primer sequence used for sequencing the RNA and "N" represent not determined nucleotides.

mentary to the 3′ end of the SAD B19 genome (nt 2 to 11). This oligonucleotide was able to prime reverse transcription of the DI genomic RNA and thus the hypothesis that SAD DI-1 possesses also a 3′ terminal sequence which is highly similar to the rabies virus 3′ consensus sequence was verified. An RNA sequence up to the coding region of the N gene could be determined with the 3′ primer. Compared to the corresponding SAD B19 sequence four differences in the leader region were detected but none in the first 50 nts of the N cistron including the rabies virus transcriptional start signal consensus sequence (Fig. 4B). Accordingly a DI-specific N mRNA might be initiated correctly and, presupposing the colinearity of the DI genome with that of SAD B19, the following cistrons would also give rise to mRNAs.

cDNA cloning of SAD DI-1-specific mRNAs

The presence of a 2.3-kb polyadenylated RNA containing L-specific sequences (Fig. 3) indicated transcription of specific mRNAs from the DI genome. According to the assumption that the DI genome possesses only the large 6.4 kb deletion detected and otherwise is colinear to the genome of SAD B19, the 2.3-kb mRNA should represent a fusion mRNA starting at the G transcriptional start signal and ending at the L stop/polyadenylation signal. This RNA would be only 64 nucleotides longer than the G/Ψ mRNA of SAD B19 and comigrate with this mRNA in our standard Northern gels (see Fig. 3). For further analysis oligo(dT) primed cDNA from poly(A)$^+$ RNA of cells infected with SAD B19/MB was cloned. The resulting recombinant phages were screened with gene-specific probes (see Materials and Methods). Eight individual clones which gave a positive hybridization signal with the G-specific probe were subjected to *in vivo* excision and analyzed further by restriction mapping and nucleotide sequencing of their termini (pG1 to pG8).

By their restriction patterns the clones could be divided into two groups (pG1 to 4 and pG5 to 8). pG1, 3, and 4 must originate from DI mRNAs since their sequence exhibited exactly the same deletion as the DI genome (Fig. 4A) and correct polyadenylation at the L stop/polyadenylation signal. Clones pG5 to pG8 terminated at the pseudogene stop/polyadenylation signal which is deleted in the DI genome and thus represent cDNA derived from helper virus mRNA. Clone pG2 also showed the DI-specific deletion but no poly(A) tract. It started within the 3′ trailer of the M gene, passed the M and L stop/polyadenylation signals and may thus be derived from a DI particle genomic RNA molecule accidentally present in the poly(A)$^+$ RNA preparation. The transcriptional start signal of the G gene is located at nt 3290 of the SAD B19 genome. cDNA clones pG1, 3, 6, and 7 start at nt 3312, 3417, 3320, and 3320, respectively. Thus we assume that those four cDNA clones originate from correctly initiated monocistronic mRNAs. The other four clones, however, contain additional sequences not templated by a monocistronic G mRNA. At the 5′ ends of clones pG4, 5, and 8, poly(A) stretches of 42, about 100, and 39 residues, respectively, are found, followed by the M/G intergenic region and then the G cistron sequence (Fig. 5). We conclude that these clones originate from bi- or polycistronic mRNAs which possess internal poly(A) stretches. According to our results obtained so far cDNA clones originating from bicistronic mRNAs do always contain an internal poly(A) stretch. This suggests that most bicistronic M + G mRNAs do neither result from simple transcriptional overreading of the SAD B19 or SAD DI-1 M polyadenylation signal nor from putative switching from replication to transcription. Instead the polymerase—during or after the polyadenylation process—apparently fails to release the M mRNA and/or to recognize the nearby G transcriptional start signal.

Isolation of SAD DI-1 genomic RNA and DI particles

To prove the existence of DI particles which are exported from the infected cell, RNA was isolated from cell culture supernatants. After infections at an m.o.i. of 5 more than 90% of RNA found in supernatants consisted of 5.5-kb RNA while 12-kb standard virus RNA prevailed after infection at an m.o.i. of 0.01 (Fig. 6). By hybridization with an SAD DI-1-specific oligonucleotide (S1P) which was deduced from the sequence of the DI "splice"-site it could be demonstrated that the 5.5-kb RNA isolated from cell culture supernatants is identical to the 5.5-kb RNA in infected cells (not shown). By velocity centrifugation through sucrose gradients particles which contain the SAD DI-1 genome could be separated from standard virions (data not shown).

Superinfections

To investigate whether replication of the SAD DI-1 genome is restricted to cells infected with homologous helper viruses, a series of superinfections with sucrose gradient purified DI particles was performed. Cells were first infected with different rabies virus strains as well as rabies-related viruses. Six hours p.i. the cells were submitted to the second infection with purified SAD DI-1 particles. By Northern hybridizations of total cellular RNA, efficient DI replication was demonstrated with all serotype 1 viruses examined (ERA, CVS, W187, PM, HEP, LEP, PV; Fig. 7, lanes 2 to 8). As can be seen by the "disappearance" of the genomic helper virus RNAs, SAD DI-1 strongly interfered with the growth of

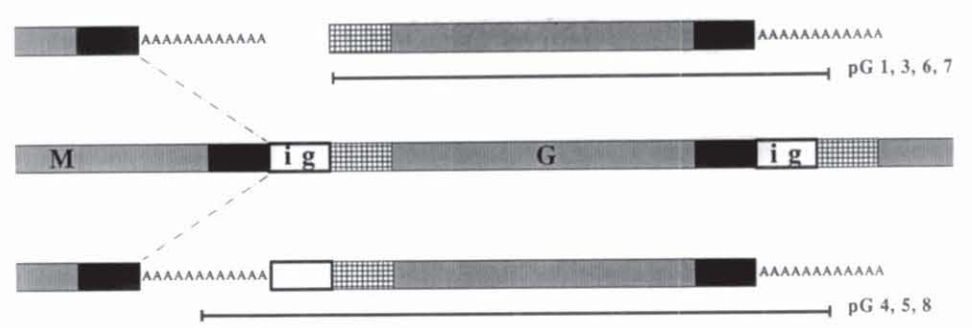

FIG. 5. Schematic presentation of the poly $(A)^+$ cDNA clones pG 1 to pG 8. The virus genome is shown in the center with the transcriptional stop/polyadenylation signals (black boxes), start signals (grid) and the intergenic regions (ig). Monocistronic and polycistronic mRNA templates are shown above and below the genome. In clones pG 1, 3, and 4 the DI deletion is found and the G stop/polyadenylation signal is replaced by the L signal (not shown).

all rabies viruses. The efficiency of DI genome replication seems to be similar for all serotype 1 viruses and to depend mainly on the amount of helper virus present during coinfection (compare lanes + and − DI).

DI genomic RNA could be demonstrated in significantly larger amounts than in the control experiments (Fig. 7, lanes 1+/−) not only in cells infected with rabies viruses but interestingly also after infection with the various rabies-related viruses (Lagos Bat Virus, serotype 2; Mokola, serotype 3; Duvenhage 1, 2, 3, 4, 5, serotype 4). The hybridization assays were performed at highly stringent conditions, so that only serotype 1 RNA is recognized by the SAD B19 G-specific probe. This allowed identification not only of the DI genomic RNA but also of the DI-specific G mRNA in cells coinfected with the rabies-related viruses (Fig. 7, lanes 9 to 15). Thus the SAD DI-1 genome is not only replicated but also at least the G/L fusion mRNA is transcribed from the DI genome by heterologous helper virus proteins.

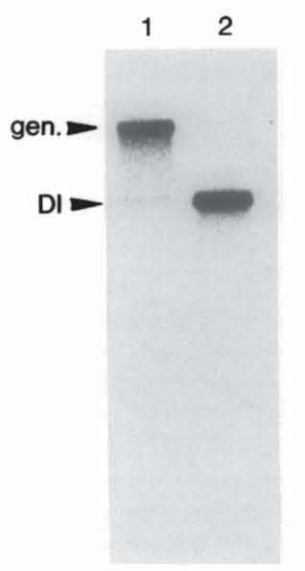

FIG. 6. Standard virus and DI genomic RNAs isolated from cell culture supernatants of cells infected with SAD B19/MB at an m.o.i. of 0.01 (1) and 5 (2). RNA was isolated from pelleted virions 30 hp.i. and hybridized with the G probe. gen, SAD B19 genomic RNA; DI, SAD DI-1 genomic RNA.

Except for the DI-specific G/L fusion mRNA the hypothetical DI N, NS, and M mRNAs should correspond to standard virus mRNAs and could thus not be identified by the above described hybridization experiments in cells coinfected with DI and the homologous SAD B19 virus. The possibility, however, to distinguish serotype 1-specific RNAs from those of the other serotypes by high stringency hybridization could be utilized to prove the existence of additional DI specific transcripts. Total RNA of cells infected with DUV 2 (South Africa Bat virus) or DUV 3 (Bat Hamburg virus) and superinfected with SAD DI-1 was screened with the gene-specific probes. As an example the hybridization with the NS probe is shown (Fig. 8). Besides the monocistronic NS mRNA (1.2 kb) polycistronic RNAs are also present. With the N and M probes the respective DI mRNAs could be demonstrated, too (not shown). All monocistronic and polycistronic DI mRNAs correspond in size exactly to the respective SAD B19 transcripts. These data suggest that SAD DI-1 encodes its own functional N, NS, M, and G proteins and is dependent only on the helper virus L protein.

DISCUSSION

The analysis of an unusual 5.5-kb RNA in cells which were infected with a particular stock (MB) of the SAD B19 rabies virus strain showed that this RNA represents the genome of a rabies virus defective interfering particle (SAD DI-1). The SAD DI-1 genome is characterized by a large internal deletion spanning the 3′ portion of the Ψ region and almost the entire L gene. DI-specific N, NS, and M mRNAs, which are by size indistinguishable from standard virus mRNAs, are tran-

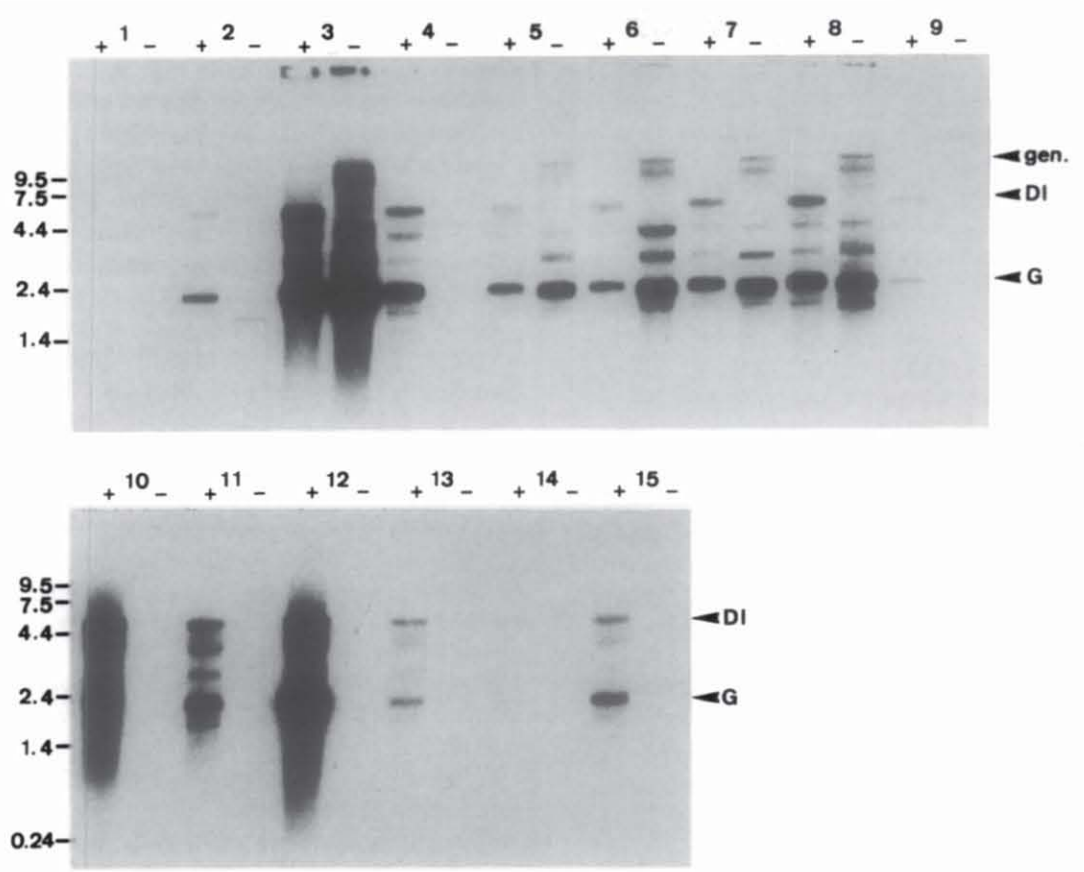

FIG. 7. Superinfections of rabies virus (2–8) or rabies-related virus (9–15)-infected cells with purified SAD DI-1 particles. Total RNA from cells infected only with helper virus (−) or superinfected 6 h p.i. with SAD DI-1 (+) was isolated 30 h p.i. Northern blots were hybridized with a G Probe. 1, no helper virus; 2–8, helper viruses CVS, ERA, W187, PM, HEP, LEP, PV4, respectively (all serotype 1); 9, LBV (serotype 2); 10, Mokola (serotype 3); 11–15, Duvenhage 1, 2, 3, 4, 5, respectively (serotype 4); gen, helper virus genomic RNA; DI, SAD DI-1 genomic RNA; G, G mRNA.

scribed from the DI genome. Corresponding to the genomic deletion, a SAD DI-1-specific G mRNA with the 3′ part of Ψ substituted by L sequences is present in infected cells. Additional deletions in the DI genome cannot yet be ruled out, but taking into account the observed lengths of the DI genomic and messenger RNAs such putative deletions should be very small. The partial sequences obtained from the DI genome at the 5′ region of the N gene and the 5′ and 3′ sequences of the G mRNA strongly suggest that at least the DI-specific G and N mRNAs are translated in the cell to yield DI-specific proteins. Whether any of the SAD DI-1 encoded proteins are functionally active will be addressed in further experiments.

The vast majority of VSV DI particle genomes represent the 5′ half of the standard virus genome and have a nonparental sequence at their 3′ end which is complementary to the normal 5′ terminus (Perrault and Semler, 1979; Lazzarini *et al.*, 1981; Kolakofsky, 1982). Those "snapback" or "panhandle" type DI genomes are thought to arise by a switch of the replication complex from the original template to the nascent RNA and subsequent back-transcription (Huang, 1977; Leppert *et al.*, 1977). While such defective genomes are replicated in the presence of helper virus, they are transcriptionally inactive and thus depend on the whole set of helper virus proteins. Only few VSV DI particles belong to the "internal deletion" type whose genomes possess both the original 3′ and 5′ end. And only one of these, "DI-LT," has been reported to be transcriptionally active to synthesize its own N, NS, M, and G mRNAs and proteins (Prevec and Kang, 1970; Johnson and Lazzarini, 1977; Perrault and Semler, 1979; Herman and Lazzarini, 1981; Meier *et al.*, 1981). The genome organization of SAD DI-1 is highly similar to that of the unique VSV DI-LT which also possesses only one large deletion in the L gene. In addition to SAD DI-1 we have recently isolated a rabies virus DI particle derived from the PV strain (PV DI-1). Most surprisingly PV DI-1 apparently also represents an internal deletion type DI. Similar to SAD DI-1 and DI-LT a major deletion of approximately 4.5 kb has occurred in the L gene. We have evidence that PV DI-1 is also transcriptionally active (unpublished results). In contrast to transcribing

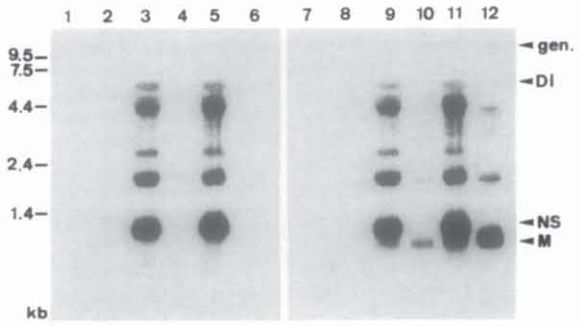

FIG. 8. Demonstration of SAD DI-1-specific NS mRNA. Total RNA from cells infected with DUV 2 (lane 4) and DUV 3 (lane 6) or coinfected with DUV 2/SAD DI-1 (lane 3) and DUV 3/SAD DI-1 (lane 5) was hybridized under stringent conditions with the SAD B19 NS probe. Both monocistronic NS mRNA (1.2 kb; NS) and polycistronic RNAs are present. As a control RNA of cells infected only with SAD DI-1 (1) and of noninfected cells (2) is included. After reprobing the same blot (without stripping the SAD B19 probes) with an M-specific DUV 3 cDNA fragment (lanes 7–12), in addition, helper virus genomic (gen) and M mRNA (M) was identified.

VSV DI particles, SAD DI-1 and PV DI-1 show a high degree of genetic stability. With SAD DI-1 more than 20 passages have been performed up to now and no changes with regard to size, hybridization with various probes, and degree of interference with SAD B19 have been observed.

The finding that the first two analyzed rabies virus DI particles belong to the internal deletion type and that both are transcriptionally active raises the question whether there are differences in the DI generation mechanisms between VSV and rabies virus. By analysis of additional DI particles it remains to be determined whether internal deletion type DIs are created preferentially in rabies virus in contrast to VSV. Hints on the exact mechanism which most likely involves a translocation of the replicase complex along the parental template (Lazzarini *et al.*, 1981) might be found by future sequence analyses of internal deletion DI genomes.

Interference of DI particles with their helper viruses usually is a result of competition for viral proteins (Giachetti and Holland, 1988; Pattnaik and Wertz, 1990). In the case of SAD DI-1 at least a functional L protein has to be provided in the infected cell. From our superinfection experiments it is obvious that the vast amount of DI transcription and replication is due to the L protein of the homologous or heterologous helper virus used for the first infection and neither to L protein packaged in the DI particle nucleocapsids nor to residual contaminating SAD B19 virus nucleocapsids (Fig. 8, lane 1). The ability of the L proteins from members of all lyssavirus serotypes to replicate the serotype 1 DI genome and to transcribe DI-specific RNAs correctly show the functional homology of these multifunctional enzymes. We conclude from the superinfection and hybridization experiments that the replication signals and also the transcriptional start and stop/polyadenylation signals of SAD DI-1 (serotype 1) are strictly recognized and followed by the heterologous proteins. Moreover it is likely that these signals are highly similar or identical in all lyssavirus serotypes. A genus-specific consensus genomic end sequence 3′UGCGAAUUGUU has been proposed for lyssavirus by sequence comparison of Mokola virus (serotype 2) and rabies virus (serotype 1) (Bourhy *et al.*, 1989). This consensus sequence, supposed to be involved in replication initiation as well as the rabies virus consensus transcription start signal AACAYYNCT, (mRNA sense) and stop/polyadenylation signal $^{A}/_{T}$GAAAAAAAC (Conzelmann *et al.*, 1990) have also been found in the Duvenhage 3 (serotype 4) virus genome (unpublished data).

It is unclear how the functional transcriptase or replicase complexes are made up in cells coinfected with SAD DI-1 and heterologous viruses. In VSV it has been shown that the L protein of VSV_{NJ} can interact with the heterologous VSV_{IND} NS to form active polymerase complexes *in vitro* whereas the VSV_{IND} L may only interact with the homologous VSV_{IND} NS protein (De and Banerjee, 1984; Moyer, 1989). Lyssavirus serotypes appear to be related more closely than VSV serotypes as can be estimated by heterologous hybridization experiments and sequence analyses. The NS and N proteins of rabies virus and Duvenhage 3 are 63 and 89% homologous, respectively (unpublished data) whereas only 32 and 80% homology is found between VSV_{NJ} and VSV_{IND} NS and N proteins, respectively (Banerjee *et al.*, 1984; Gill and Banerjee, 1985). Thus combinations of rabies helper virus L with the putative DI N and NS proteins might be functional. An efficient *in vitro* transcription system for rabies virus that would allow to directly assay the synthetic activity of heterologous transcriptase complexes is not yet available. The analysis of SAD DI-1 particles isolated from cells coinfected with heterologous helper viruses and DI particles might shed some light on the question whether the L proteins of the different rabies virus serotypes may use only homologous (helper virus) NS and N proteins or whether combinations between helper virus L and the putative heterologous DI NS and N proteins are possible.

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