JOURNAL OF VIROLOGY, May 1989, p. 1938–1944
0022-538X/89/051938-07$02.00/0

Vol. 63, No. 5

Detection of Virus-Specific RNA-Dependent RNA Polymerase Activity in Extracts from Cells Infected with Lymphocytic Choriomeningitis Virus: In Vitro Synthesis of Full-Length Viral RNA Species†

FRANCES V. FULLER-PACE* AND PETER J. SOUTHERN

Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037

Received 17 November 1988/Accepted 19 January 1989

We have developed an in vitro assay for the lymphocytic choriomeningitis virus (LCMV) RNA-dependent RNA polymerase with ribonucleoprotein complexes extracted from acutely infected tissue culture cells. The RNA products synthesized in vitro corresponded in size to the full-length genomic L and S RNAs and subgenomic NP and GP mRNAs normally produced in vivo during acute LCMV infection. In a temporal analysis spanning the first 72 h of acute infection, the in vitro polymerase activity of ribonucleoprotein complexes was maximal at 16 h and declined significantly at later times. In contrast, the intracellular levels of the viral L protein (the putative polymerase protein) appeared to be maximal at 48 to 72 h postinfection. Our results suggest that the accumulation of L protein correlates with reduced viral replication and transcription at later times in acute infection and may be involved in the transition from acute to persistent LCMV infection.

Lymphocytic choriomeningitis virus (LCMV), the prototype arenavirus, has been studied extensively as a model system for virus-host interactions (8, 9, 18). Recent interest in the molecular details of infection by LCMV and the other arenaviruses has prompted studies on the structure and organization of the viral genome and on regulatory mechanisms that influence viral gene expression (30–32, 34). The LCMV genome consists of two single-stranded RNA segments, designated L and S, with approximate lengths of 7.2 and 3.4 kilobases, respectively (26, 36). The S RNA segment has an ambisense coding arrangement (2) that directs synthesis of the three major structural proteins: an internal nucleoprotein (NP; molecular weight 63,000) that is associated with the genomic RNA, and two surface glycoproteins, GP-1 (molecular weight 43,000) and GP-2 (molecular weight 36,000) (5), that are derived by posttranslational cleavage of a precursor polypeptide, GP-C (6). The L RNA segment encodes a high-molecular-weight protein (molecular weight ca. 200,000), thought to be part or all of the viral RNA-dependent RNA polymerase (14, 32), and the possible presence of a second, L-encoded protein is currently under investigation (M. Salvato, personal communication).

Genetic mapping studies have clearly implicated the viral L RNA segment in lethal LCMV infection of adult guinea pigs (29) and in the altered biological properties of variant viruses recovered from the spleens of persistently infected mice (1). In the guinea pig infection, it is not clear whether an L-encoded protein is directly pathogenic or whether the LCMV (WE strain) polymerase supports more rapid initial virus replication to precipitate a lethal infection via unidentified secondary mechanisms. Similarly, precise molecular explanations are not currently available for altered immune responses in mice infected with the spleen-variant viruses, but the altered phenotype maps to the L RNA segment and may be associated with replication of the variant viruses in lymphoid cells (1, 25).

To date, characterization of the RNA-dependent RNA polymerase of LCMV and other arenaviruses has been confined to virion-associated enzymes. Leung et al. (19), using purified Pichinde virions, described an in vitro polymerase activity that synthesized a heterogeneous population of RNAs complementary to virion RNA. With Tacaribe virus, Boersma and Compans (3) coupled a viral in vitro transcription reaction with a rabbit reticulocyte lysate translation system to demonstrate the synthesis of virus-specific polypeptides. For LCMV, Bruns et al. (4) have reported an RNA polymerase activity that is associated with nucleocapsids derived from purified virions. In the present study, we have begun to examine the properties of the LCMV RNA-dependent RNA polymerase and have developed an in vitro assay for polymerase activity that uses extracts from acutely infected cells. Using this system, we have demonstrated synthesis in vitro of full-length genomic L and S RNAs and subgenomic NP and GP mRNAs and have monitored apparent changes in the polymerase activity during the course of an acute LCMV infection. In previous studies, we have analyzed the steady-state levels of intracellular viral RNA species that accumulate over the time course of acute infection (13, 34) and can now make comparisons between these intracellular species and the in vitro reaction products synthesized from cell extracts harvested at various times postinfection. Our eventual goal is to determine whether changes in polymerase activity are involved in the regulation of viral replication and transcription during various stages of infection.

MATERIALS AND METHODS

Cells and virus. BHK-21 cells were grown in Falcon T175 flasks in Dulbecco-Vogt modified Eagle medium supplemented with 5% fetal calf serum. The 50% confluent monolayers were infected with LCMV (Armstrong strain CA-1371) at a multiplicity of infection of 5, and extracts were

* Corresponding author.

† Publication No. 5492-IMM from the Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, CA 92037.

prepared for Western blotting (immunoblotting) and polymerase assays at various times after infection.

Preparation of cell extracts. Extracts enriched for ribonucleoprotein (RNP) complexes were prepared as described by Hill and Summers (16). Monolayers (in T175 flasks) were washed with phosphate-buffered saline, scraped off into phosphate-buffered saline, and collected by centrifugation at 1,000 × *g* for 5 min. The cell pellet was suspended in lysis buffer (10 mM KCl, 1.5 mM magnesium acetate, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4], 0.5 mM dithiothreitol) (1 ml per T175 flask), allowed to swell for 5 min on ice, and then disrupted with 30 strokes of a Wheaton B Dounce homogenizer. Nuclei, cell membranes, and other cell debris were removed by centrifugation at 10,000 × *g* for 20 min at 4°C. NaCl and Triton X-100 were added to 0.5 M and 1%, respectively, and RNP complexes were pelleted by centrifugation through 2.5 ml of 50% glycerol, in the above lysis buffer, for 2.5 h at 45,000 rpm in a Beckman SW50.1 rotor, at 4°C. The pellet was suspended in cold 2× reaction buffer (200 mM KCl, 100 mM Tris hydrochloride [pH 7.5], 10 mM magnesium acetate, 4 mM dithiothreitol) (50 μl for each T175 flask) and used in polymerase assays immediately.

Western blotting. Proteins in the cell extracts were separated by electrophoresis in 7% polyacrylamide–sodium dodecyl sulfate (SDS) gels (17) and transferred electrophoretically to nitrocellulose filters (0.2 μm pore size) overnight at 250 mA, with a recirculation cooling system. The filters were incubated with antipeptide antibodies directed against regions of NP and L (kindly provided by M. J. Buchmeier). Bound immunoglobulin was detected with ^{125}I-labeled *Staphylococcus aureus* protein A as described previously (32).

Assays for RNA polymerase activity. Unless specified otherwise, assays were carried out for 1 h at 30°C in 100-μl volumes containing 50 μl (200 μg of protein) of cell extract in 2× reaction buffer, 1 mM each unlabeled ATP, CTP, and UTP, 10 μM unlabeled GTP, and 50 μCi of [α-^{32}P]GTP (Du Pont NEN Products; 600 Ci/mmol). Where necessary, multiple 100-μl reaction mixes were used. These gave higher incorporation than reactions performed in larger volumes. Reactions were stopped by the addition of unlabeled GTP and EDTA (final concentrations, 1 and 10 mM, respectively), followed by the addition of 100 μl of 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–100 mM NaCl–0.1% SDS. Unincorporated nucleotides were removed with a Sephadex G-50 spin column (20), and after phenol extraction, RNAs in the reaction mixture were precipitated in ethanol and suspended in sterile distilled water for subsequent analysis.

RNA gel electrophoresis and Northern blots. RNAs in the polymerase reaction mixes were denatured with glyoxal and separated by agarose gel electrophoresis in 10 mM $NaPO_4$, pH 6.5 (21). Unless specified otherwise, the gels were dried onto DE81 paper at 80°C under vacuum and exposed for autoradiography with Kodak XRP-1 film. In some cases, after electrophoresis, the RNAs were transferred to nitrocellulose filters by capillary diffusion of 20× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature (35) and baked for 2 h at 80°C under vacuum. After autoradiography, the ^{32}P was allowed to decay, and the filters were then hybridized with LCMV probes to confirm the specificity of the labeled polymerase reaction products.

Southern blots. Plasmids containing cDNA fragments from LCMV L and S segments were digested with appropriate restriction enzymes to give discrete LCMV-derived DNA fragments and transferred to nitrocellulose (33). cDNAs derived from 28S rRNA were used as controls. After being baked at 80°C under vacuum, the filters were hybridized with the polymerase reaction products as described below.

Alkaline hydrolysis. Partial hydrolysis of RNAs in the polymerase reaction mixes was achieved by incubation for 1 h at 40°C in 50 mM Na_2CO_3, followed by neutralization with 150 mM sodium acetate (pH 5). This procedure gave RNA fragments ranging in size from 100 to 300 nucleotides.

Synthesis of hybridization probes. For hybridization with LCMV-derived probes, restriction fragments from the LCMV L and S segment cDNAs were purified from preparative agarose gels (37) and then labeled in vitro with Klenow DNA polymerase I in a modified version of the original nick translation reaction (28).

Hybridization. For hybridization with cDNA probes, the filters were prehybridized at 37°C for 3 h in 50% deionized formamide–5× SSC–2.5× Denhardt solution (1× Denhardt solution is 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone) with 100 μg of denatured sonicated salmon sperm carrier DNA per ml; for hybridization with polymerase assay reaction products, the filters were prehybridized for 3 h at 55°C in 50% formamide–4× SSC–2× Denhardt solution, with 100 μg of denatured salmon sperm DNA per ml and 100 μg of yeast tRNA per ml as carriers. In each case, hybridization was carried out for 20 to 24 h under the conditions used for prehybridization. Filters were washed twice in 2× SSC–0.1% SDS at 37°C, then at 60°C in the same solution, and finally at 60°C in 0.1× SSC–0.1% SDS–0.1% Tween-20. All washes were for 30 min. Autoradiography was carried out at −70°C with Kodak XAR-5 film and Du Pont Cronex Lightning Fast intensifying screens. Before subsequent hybridizations, probes were stripped from filters by washing in 0.1× SSC–0.1% SDS–0.1% Tween-20 for 2 h at 85°C. Filters were then prehybridized as before.

RESULTS

Detection of viral proteins in intracellular RNP complexes. Intracellular RNP complexes were prepared from cultures of acutely infected BHK cells (see Materials and Methods) at various times postinfection. Viral proteins were detected by Western blotting with antipeptide antibodies specific for NP, GP-2, or L (the putative polymerase) protein (7, 32). The amounts of both NP and L protein increased for 24 to 72 h (Fig. 1), whereas GP-2 could not be detected within the intracellular RNP complexes (data not shown). Analysis of different cellular fractions by this Western blotting approach showed that L protein was only detectable in the RNP fraction (data not shown). Based on these results, cell extracts enriched for viral RNP complexes were initially prepared at 72 h postinfection in order to ensure a high concentration of L protein for the in vitro polymerase reactions.

Synthesis of virus-specific RNAs in vitro. Concentrated preparations of intracellular RNP complexes extracted from acutely infected BHK cells 72 h postinfection and parallel cultures of mock-infected BHK cells were suspended in reaction buffer (see Materials and Methods) and incubated in the presence of [α-^{32}P]GTP at 30°C. We observed a linear, time-dependent increase in the incorporation of radioactivity into trichloroacetic acid (TCA)-precipitable counts in a 1-h incubation but could find no reproducible difference between the infected and uninfected RNP preparations in this system.

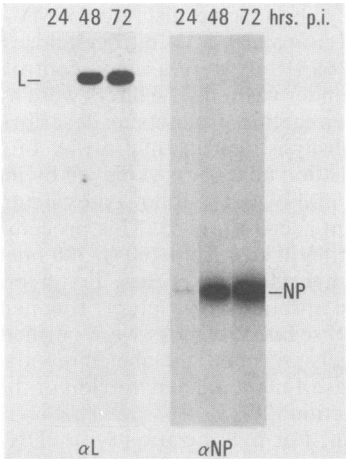

FIG. 1. Western blot showing accumulation of viral L protein and nucleoprotein (NP) in RNP complexes extracted from cells at various times postinfection (p.i.). In each case, 25 μg of protein was loaded on an SDS-polyacrylamide gel and separated by electrophoresis. Viral proteins were detected by using monospecific rabbit anti-L (αL) or anti-NP (αNP) antipeptide antibodies, followed by treatment with ^{125}I-labeled *S. aureus* protein A. Relative exposures of autoradiographs: L, 72 h; NP, 3 h.

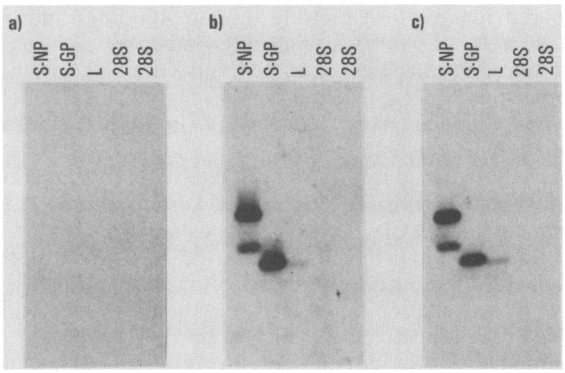

FIG. 2. Hybridization reactions with ^{32}P-labeled RNAs synthesized in vitro. Plasmid DNAs were digested with restriction enzymes to release different cDNA target sequences derived from the genomic S or L RNAs or host 28S RNA. The DNA fragments were separated by agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized with the following probes: (a) ^{32}P-labeled RNAs synthesized with an extract from uninfected cells; (b) ^{32}P-labeled RNAs synthesized with an extract from infected cells; (c) as in panel b, but the labeled RNAs were partially hydrolyzed by treatment with alkali before the hybridization reaction was initiated. Target cDNA sequences are identified as follows. S-NP, cDNA from bases 1665 to 3342 in LCMV S sequence (31). This gives two fragments, 1,043 and 634 base pairs, on digestion with *Pst*I. S-GP, cDNA from bases 11 to 435 in LCMV S sequence (31). L, L122 cDNA clone from LCMV L segment (32); bases 511 to 1208 in L sequence (M. Salvato, E. Shimomaye and M. B. A. Oldstone, submitted for publication). This gives two fragments of 426 and 271 base pairs on digestion with *Pst*I. 28S, cDNAs from mouse 28S rRNA as non-LCMV-specific control. First 28S lane, bases 3855 to 4147; second 28S lane, bases 1499 to 1917 (P. J. Southern, unpublished observations).

Furthermore, extraction of nucleic acid from the reaction mixtures prior to TCA precipitation and several variations in the conditions for the actual TCA precipitation still did not provide any numerical discrimination between the infected and uninfected reactions (data not shown).

As an alternative approach to demonstrate virus-specific RNA synthesis, we used the in vitro reaction products as hybridization probes against target cDNAs derived from the viral genome or, as a control, cDNAs from host 28S rRNA sequences. With the extract harvested from infected cells 72 h postinfection, there was strong hybridization to viral target sequences from the genomic S RNA segment (NP and GP coding regions) and a low level of hybridization to an L-derived target sequence, but no hybridization to the 28S rRNA target sequences (Fig. 2b). None of the target sequences was detected when the reaction products from the uninfected cell extract were used as hybridization probes (Fig. 2a). As an additional control, similar hybridization reactions were repeated, and identical results were obtained with probes that had been partially hydrolyzed by treatment with alkali. This control was included because in preliminary experiments with the cell extracts and [α-^{32}P]UTP, we found that preexisting rRNAs in the reaction could become labeled at the 3′ terminus (data not shown). Cellular poly(U) polymerases are known (19), but there are no reports of endogenous cellular poly(G) polymerase activity; thus, we chose labeled GTP to follow the reaction. Nevertheless, for the results from the hybridization assays to be valid, it was critical to distinguish between terminal labeling of preexisting full-length viral RNAs and de novo synthesis of RNAs in vitro. If the full-length RNAs present in the enriched RNP preparations were being end-labeled in the in vitro reactions, alkaline hydrolysis of the probe would only allow hybridization with cDNA targets corresponding to the 3′ ends of the RNAs. In these experiments, the cDNAs were derived from internal regions of the viral genomic or rRNAs, and the observed hybridization after alkaline hydrolysis was therefore consistent with de novo synthesis of uniformly labeled RNAs. In addition, we subsequently observed that neither viral nor 28S rRNAs were labeled if [α-^{32}P]GTP was used in the absence of any other ribonucleotides (data not shown).

Temporal analysis of polymerase activity during LCMV infection. The L protein was not detected in infected cell extracts at 24 h after infection but had accumulated significantly by 48 to 72 h postinfection (Fig. 1). In contrast, the accumulation of LCMV mRNAs in vivo and the release of infectious virions were maximal at 16 to 24 h postinfection under our standard conditions for virus infection (multiplicity of infection of 5) (8; our unpublished observations). This suggested that there may be a disparity between the levels of L protein and the intracellular activity of the viral polymerase. Therefore, in vitro reactions were performed with infected-cell extracts harvested at 24, 48, and 72 h postinfection, and the reaction products were analyzed by denaturing agarose gel electrophoresis (Fig. 3). Separation of the in vitro reaction products by gel electrophoresis provided an immediate method for monitoring polymerase activity. Although the hybridization method had the advantage of showing virus-specific RNA synthesis, the electrophoresis approach was considerably less cumbersome and allowed direct analysis of the reaction products.

Individual in vitro reactions were performed immediately after extraction and concentration of the RNP complexes, and then the labeled RNA products were recovered and stored for gel analysis. These results indicated that infected-cell extracts, harvested at 24 h postinfection, were capable of synthesizing both genome-sized RNA and subgenomic mRNAs in vitro and that, at later times, the polymerase activity was markedly diminished. The labeled RNAs synthesized in vitro corresponded to the genomic and mRNA

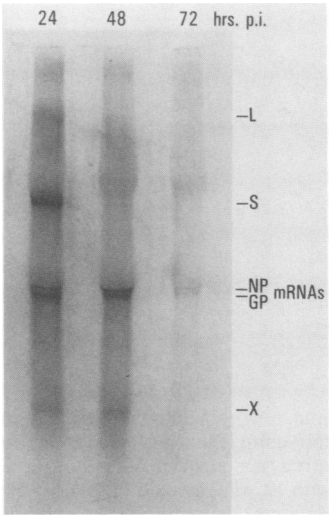

FIG. 3. Autoradiograph of a denaturing agarose gel showing the ^{32}P-labeled RNA products that were synthesized in vitro with extracts harvested at various times postinfection (p.i.). The total products from three 100-μl polymerase reaction mixes were loaded on the gel for each time point. RNAs corresponding to LCMV RNAs are indicated. L, genomic L RNA; S, genomic S RNA; NP, nucleoprotein mRNA; GP, glycoprotein mRNA; X, novel subgenomic RNA.

species normally produced during an acute infection of tissue culture cells (see below and Fig. 6). There was one novel product formed during the in vitro reaction (Fig. 3, band marked X) that may represent a premature termination product. The derivation of this discrete RNA species (length, approximately 300 to 500 bases) is currently under investigation.

Determination of optimal time for extraction of RNP from infected cells. The relatively high polymerase activity observed at 24 h after infection prompted us to study changes in the in vitro activity of infected cell extracts at earlier times following LCMV infection. The accumulation of reaction products was clearly maximal in the RNP fraction extracted

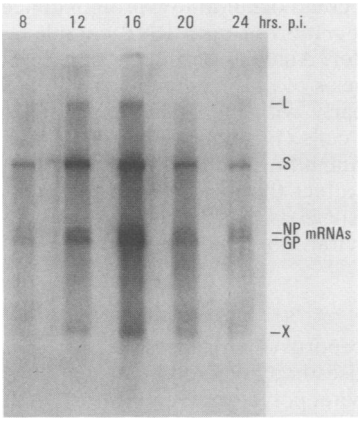

FIG. 4. Autoradiograph of a denaturing agarose gel showing the ^{32}P-labeled RNA products that were synthesized in vitro with extracts harvested between 8 and 24 h postinfection (p.i.). Products from three 100-μl polymerase reaction mixes were loaded on the gel for each time point. RNAs corresponding to LCMV RNAs are indicated. See Fig. 3 legend for details.

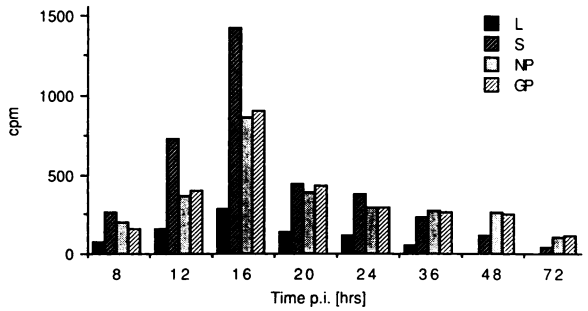

FIG. 5. Relative levels of incorporation of [^{32}P]GMP into individual viral RNA species. Reactions were performed in vitro with extracts harvested at various times during the first 72 h of acute LCMV infection. Incorporation of radioactivity is expressed as counts per minute in each RNA species and was obtained by direct scanning of dried agarose gels with an AMBIS β scanner. Scanning was carried out overnight, and the counts obtained were individually corrected for background within each lane of the gel. The standard deviation as calculated by the scanner was 1 to 3%. Average counts from two or three assays (three 100-μl reaction mixes in each case) are represented for each time point. L, genomic L RNA; S, genomic S RNA; NP, nucleoprotein mRNA; GP, glycoprotein mRNA.

at 16 h postinfection (Fig. 4). The unidentified RNA, X, was also most highly labeled in the 16-h sample. None of the labeled RNAs synthesized in vitro, including X, was produced in reactions with RNP complexes from uninfected cells.

In a subsequent experiment, the polymerase activity over the first 72 h after infection was monitored to compare the synthesis of the individual viral RNAs. RNA products from reactions carried out at various times postinfection were separated by gel electrophoresis, and the incorporation of [α-^{32}P]GMP into the individual RNAs was quantitated directly by scanning the dried agarose gels with an AMBIS β scanner (Fig. 5). The patterns of synthesis of L and S genomic RNA were parallel over the 72-h period of infection, although L RNA was synthesized in three- to fivefold-lower amounts. Likewise, NP and GP mRNAs were synthesized in parallel throughout this time course and at each time point were found in approximately equal amounts. The synthesis of both genome-sized RNAs (L and S) and subgenomic mRNAs (NP and GP mRNAs) increased coordinately and peaked at 16 h postinfection. From 16 to 20 h postinfection, both decreased sharply in parallel. After 20 h, the decrease was considerably more gradual but the synthesis of genome-sized RNAs declined more rapidly; this is particularly evident in Fig. 3.

Comparison between viral RNAs synthesized in vitro and viral RNAs synthesized in vivo during acute infection of BHK cells. The labeled RNAs from in vitro reactions, performed with either infected or uninfected extracts, were separated by denaturing agarose gel electrophoresis and transferred to a nitrocellulose membrane. A sample of RNA, extracted from the intracellular RNP fraction prior to initiating the in vitro reactions, was denatured, electrophoresed, and transferred to the same nitrocellulose membrane. Direct autoradiography of the filter indicated that genome-sized L and S RNAs, subgenomic NP and GP mRNAs, and the novel subgenomic RNA (X) were all synthesized in vitro (Fig. 6a). After the ^{32}P was allowed to decay (approximately 2 months), the filter was hybridized sequentially with nick-translated probes specific to the GP and NP coding regions

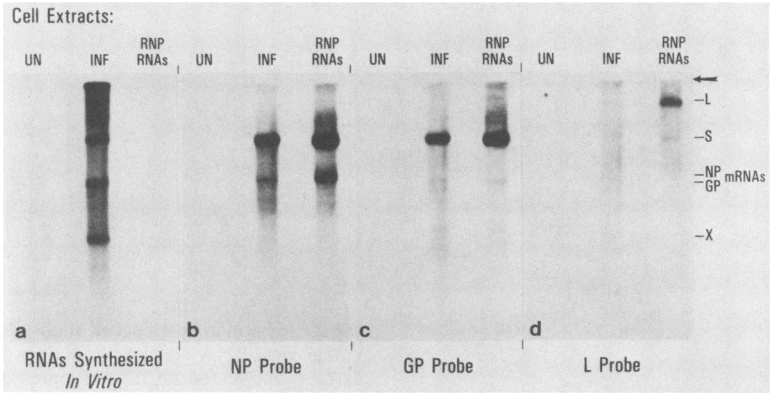

FIG. 6. Comparison between viral RNAs synthesized in vitro and viral RNAs that accumulate within acutely infected tissue culture cells. (a) ^{32}P-labeled RNA products from in vitro LCMV polymerase reactions carried out with RNP complexes harvested 16 h after infection. The RNA species were separated by electrophoresis in a denaturing agarose gel and then transferred to a nitrocellulose filter for autoradiography. After the ^{32}P was allowed to decay, the nitrocellulose filter was hybridized sequentially with LCMV-specific cDNA probes. (b) NP probe derived from the 3′ end of the S segment; bases 2299 to 3342 in S RNA sequence (31). This detects genomic S RNA and NP mRNA. (c) GP probe derived from the 5′ end of the S segment; bases 11 to 435 in S RNA sequence (31). This detects genomic S RNA and GP mRNA. The low level of hybridization to GP mRNA reflects the low amount of this RNA present in infected cells at this time post infection (13). (d) L probe derived from the L segment; L122 cDNA clone (32), bases 511 to 1208 in L RNA sequence (Salvato et al., submitted). This detects genomic L RNA. This autoradiograph was exposed for 72 h, compared with 24 h for the NP and GP probes). UN, Extract from uninfected cells; INF, extract from infected cells; RNP RNAs, infected-cell extract prior to polymerase reaction. Arrow, Gel origin.

of the S RNA segment and an L probe (Fig. 6b, c, and d, respectively). This analysis clearly demonstrated that the in vitro reaction products were indistinguishable in size from the intracellular viral RNAs that accumulate during acute LCMV infection.

Time course of polymerase-dependent incorporation of label into RNA products. Reactions performed with cell extracts harvested at 16 h postinfection were allowed to proceed for various lengths of time ranging from 0 to 4 h. In this experiment, the synthesis of L and S genomic RNA and NP and GP mRNA products increased linearly for the first hour of the polymerase reaction, and then essentially no further incorporation of radioactivity occurred (Fig. 7). Therefore, to obtain some estimate of the specific activity for the polymerase in cell extracts, reactions were stopped after 40 min—a time point well within the linear portion of the graph (Fig. 7). Under these assay conditions, the specific activity of the LCMV polymerase was calculated by estimating the total [^{32}P]GMP incorporation into full-length LCMV genomic and mRNAs. An average of 10 assays gave a specific activity of approximately 50 fmol of nucleotide incorporated per mg of cell extract protein per h at 30°C.

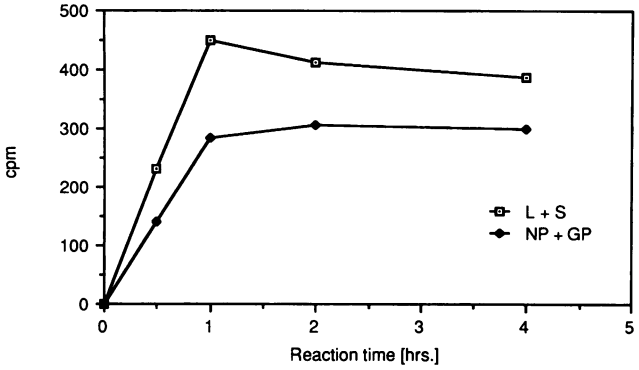

FIG. 7. Time course for incorporation of label into in vitro reaction products. The incorporation of [^{32}P]GMP into L and S genomic RNAs and NP and GP mRNAs was measured in in vitro reactions. The products from individual 100-μl reaction mixes were separated by denaturing agarose gel electrophoresis, and after drying, the gel was scanned. For these comparisons, counts from L RNA were considered to represent genomic L RNA synthesis. To date, we have been unable to identify a discrete L mRNA species, and it may be electrophoretically indistinguishable from genomic L RNA. Therefore, some of the counts in L RNA may be due to L mRNA rather than genomic L RNA synthesis. However, as the overall incorporation of ^{32}P into L RNA was relatively low, any incorporation resulting from L mRNA synthesis would not have affected the overall genomic/mRNA synthesis ratio significantly.

Titration of protein concentration for the in vitro polymerase reaction. Reaction mixes with different amounts of infected cell extract (0 to 240 μg of total protein), harvested 16 h after infection, included a compensatory amount of uninfected-cell extract to bring the total protein concentration in all the reaction mixes to 240 μg. The constant protein concentration was maintained in order to minimize possible problems that could occur at low protein concentrations due to either dissociation of viral RNP complexes or loss of a cellular cofactor. Analysis of the reaction products indicated that the synthesis of both genome-sized RNAs and mRNAs increased linearly with increasing concentration of protein from infected cells (Fig. 8). Moreover, addition of dactinomycin and α-amanitin, which inhibit cellular RNA polymerases, did not affect the synthesis of these RNAs (data not shown). This suggests that the observed polymerase activity was due to a viral, not a cellular, polymerase.

DISCUSSION

The initial reports of viral polymerase activity associated with arenaviruses had involved the use of purified virions as the source of viral polymerase and RNA templates (3, 4, 19). In contrast, we chose to use RNP complexes extracted from acutely infected cells for two reasons: (i) the study of LCMV polymerase activity in cellular extracts will facilitate direct comparisons of the polymerase activities in acutely and persistently infected cells and may provide valuable information on the role of the viral polymerase in the molecular

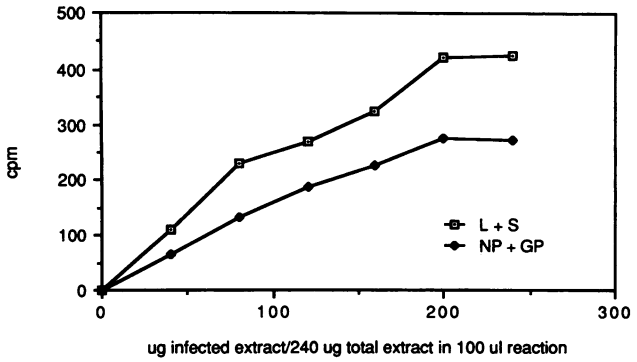

FIG. 8. In vitro RNA synthesis as a function of the protein concentration derived from infected-cell extracts. The total incorporation of [^{32}P]GMP into L and S genomic RNAs (see legend to Fig. 7) and NP and GP mRNAs was determined over a range of infected-cell extract concentrations. All in vitro reactions were carried out at 30°C for 40 min. In each case, the total protein concentration was 240 μg. Data were obtained by scanning a dried agarose gel (see Fig. 7). Counts are for individual 100-μl reaction mixes.

mechanism of viral persistence, and (ii) optimal replication and transcription may depend on interaction of the viral polymerase with cellular factors that may not be present in purified virions. It is not known whether cellular proteins are involved in arenavirus replication and transcription, but there have been several reports of cellular proteins enhancing or being required for in vitro RNA synthesis in other RNA viruses, including vesicular stomatitis virus (VSV), Sendai virus, and poliovirus (15, 23, 24). In the case of measles virus, polymerase activity was found to be 10-fold higher in RNP complexes from infected cells than in purified virions, although no direct role for a cellular cofactor has yet been identified (27).

In our in vitro assay for the LCMV polymerase, we observed synthesis of full-length, LCMV-specific RNAs corresponding in size to the genomic L and S RNAs and the NP and GP mRNAs (Fig. 4 and 6). A similar assay system, with cytoplasmic RNP complexes, demonstrated the synthesis of full-length genome-sized RNA and mRNA in vitro for influenza virus (10). The RNAs synthesized in vitro hybridized specifically with LCMV-derived cDNAs (Fig. 2). Other hybridization experiments with strand-specific M13 subclones derived from various regions of the LCMV L and S segments have shown that full-length genomic sense and genomic complementary-sense RNAs were synthesized in this in vitro assay system (our unpublished observations). The absence of RNA synthesis in extracts from uninfected cells (Fig. 2 and 6), the correlation of RNA synthesis with the concentration of infected-cell RNP extract (Fig. 8), and the insensitivity of the polymerase to dactinomycin and α-amanitin strongly suggest that the polymerase is viral.

The level of in vitro polymerase activity in extracts harvested at various times during the first 72 h of acute infection approximately mirrors the accumulation of intracellular viral RNAs that are present at equivalent times postinfection (13, 34). The rates of both in vitro synthesis and in vivo accumulation of viral RNA were reduced after 16 to 24 h; this correlates with a previously observed decrease in the production of infectious virus (8). The reduction in in vitro polymerase activity contrasts with the obvious accumulation of L (the putative polymerase) protein in these extracts (Fig. 1) and suggests a loss or inhibition of activity at high concentrations of L protein. A similar observation was made in VSV complementation experiments, where replication of a temperature-sensitive L mutant virus could be supported in host cells expressing low levels of L protein but replication of the mutant was inhibited in the presence of high levels of L protein (21). Likewise, high levels of L protein also inhibited replication of wild-type VSV. The reduced replication may be due to formation of aberrant complexes involving other viral proteins that are required for replication. It is not known which viral proteins play a direct role in LCMV replication or transcription, but the higher amount of L protein at later times in infection might affect the association of L with the RNA-NP complex and therefore the replication and transcription efficiency. This type of regulatory mechanism may reflect a common feature in the control of negative-strand and ambisense RNA virus polymerases.

The in vitro synthesis of L and S genomic RNAs and NP and GP mRNAs closely paralleled each other between 8 and 20 h after infection, with a pronounced peak at about 16 h (Fig. 4 and 5). After 16 h there was a biphasic decrease in polymerase activity, with a rapid fall between 16 and 20 h followed by a more gradual decline. By 48 h, however, the synthesis of genome-sized RNAs fell to virtually undetectable levels, while that of subgenomic mRNAs decreased much more slowly (Fig. 3 and 5). This suggests an uncoupling of the two processes and may reflect changes in the polymerase, perhaps caused by accumulating levels of L protein or interaction with another viral protein or a cellular factor.

LCMV readily establishes persistent infections in cultured cells and in mice infected within the first 24 h of life. Persistent infections are characterized by reduced viral replication, decreased accumulation of viral glycoproteins at the surface of infected cells, the generation of interfering particles, and the appearance of novel viral RNAs. However, it is not known what is responsible for the switch from acute to persistent infection. The reduction in the generation of infectious virus (8), appearance of novel subgenomic viral RNAs (11, 12), and generation of interfering particles (18) may be due to an altered RNA polymerase or replicase activity. We are currently investigating whether there are any differences in the viral polymerase activity or in the RNAs synthesized in vitro with RNP extracts from persistently infected cells.

ACKNOWLEDGMENTS

We are grateful to John Fazakerley, David Meek, Michael Oldstone, and Jacques Perrault for constructive comments on the manuscript and to Michael Oldstone for continuing interest and support. We thank Charles Frankel and David Meek for help with the AMBIS β scanner and Gay Schilling for excellent secretarial assistance.

This manuscript was supported in part by Public Health Service grants NS-12428, AG-04342, and AI-25224 from the National Institutes of Health. F. V. Fuller-Pace is a recipient of a postdoctoral fellowship from the Juvenile Diabetes Foundation.

LITERATURE CITED

1. **Ahmed, R., R. S. Simon, M. Matloubian, S. R. Kolhekar, P. J. Southern, and D. M. Freedman.** 1988. Genetic analysis of in vivo-selected viral variants causing chronic infection: importance of mutation in the L RNA segment of lymphocytic choriomeningitis virus. J. Virol. **62**:3301–3308.
2. **Auperin, D. P., V. Romanowski, M. Galinski, and D. H. L. Bishop.** 1984. Sequencing studies of Pichinde virus S RNA indicate a novel coding strategy, an ambisense viral S RNA. J.

Virol. **52**:897–904.
3. **Boersma, D. P., and R. W. Compans.** 1985. Synthesis of Tacaribe virus polypeptides in an *in vitro* coupled transcription and translation system. Virus Res. **2**:261–271.
4. **Bruns, M., W. Zeller, H. Rhodewohld, and F. Lehmann-Grube.** 1986. Lymphocytic choriomeningitis virus. IX. Properties of the nucleocapsid. Virology **151**:77–85.
5. **Buchmeier, M. J., J. H. Elder, and M. B. A. Oldstone.** 1978. Protein structure of lymphocytic choriomeningitis virus: identification of the virus structural and cell associated polypeptides. Virology **89**:133–145.
6. **Buchmeier, M. J., and M. B. A. Oldstone.** 1979. Protein structure of lymphocytic choriomeningitis virus: evidence for a cell-associated precursor of the virion glycopeptides. Virology **99**:111–120.
7. **Buchmeier, M. J., P. J. Southern, B. S. Parekh, M. K. Wooddell, and M. B. A. Oldstone.** 1987. Site-specific antibodies define a cleavage site conserved among arenavirus GP-C glycoproteins. J. Virol. **61**:982–985.
8. **Buchmeier, M. J., R. M. Welsh, F. J. Dutko, and M. B. A. Oldstone.** 1980. The virology and immunology of lymphocytic choriomeningitis virus infection. Adv. Immunol. **30**:275–331.
9. **Compans, R. W., and D. H. L. Bishop.** 1985. Biochemistry of arenaviruses. Curr. Top. Microbiol. Immunol. **114**:153–175.
10. **del Rio, L., C. Martinez, E. Domingo, and J. Ortin.** 1985. *In vitro* synthesis of full-length influenza virus complementary RNA. EMBO J. **4**:243–247.
11. **Francis, S. J., and P. J. Southern.** 1988. Deleted viral RNAs and lymphocytic choriomeningitis virus persistence *in vitro*. J. Gen. Virol. **69**:1893–1902.
12. **Francis, S. J., and P. J. Southern.** 1988. Molecular analysis of viral RNAs in mice persistently infected with lymphocytic choriomeningitis virus. J. Virol. **62**:1251–1257.
13. **Fuller-Pace, F. V., and P. J. Southern.** 1988. Temporal analysis of transcription and replication during acute infection with lymphocytic choriomeningitis virus. Virology **162**:260–263.
14. **Harnish, D. G., K. Dimock, D. H. L. Bishop, and W. E. Rawls.** 1983. Gene mapping in Pichinde virus: assignment of viral polypeptides to the genomic L and S RNAs. J. Virol. **46**: 638–641.
15. **Hill, V. M., S. A. Harmon, and D. F. Summers.** 1986. Stimulation of vesicular stomatitis virus *in vitro* RNA synthesis by microtubule-associated proteins. Proc. Natl. Acad. Sci. USA **83**:5410–5413.
16. **Hill, V. M., and D. F. Summers.** 1982. Synthesis of VSV RNPs *in vitro* by cellular VSV RNPs added to uninfected HeLa cell extracts: VSV protein requirements for replication *in vitro*. Virology **123**:407–419.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Lehmann-Grube, F.** 1984. Portraits of viruses: arenaviruses. Intervirology **22**:121–145.
19. **Leung, W.-C., M. F. K. L. Leung, and W. E. Rawls.** 1979. Distinctive RNA transcriptase, polyadenylic acid polymerase, and polyuridylic acid polymerase activities associated with Pichinde virus. J. Virol. **30**:98–107.
20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **McMaster, G. K., and G. G. Carmichael.** 1977. Analysis of single-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA **74**:4835–4838.
22. **Meier, E., G. G. Hermison, and M. Schubert.** 1987. Homotypic and heterotypic exclusion of vesicular stomatitis virus replication by high levels of recombinant polymerase protein L. J. Virol. **61**:3133–3142.
23. **Morrow, C. D., G. F. Gibbons, and A. Dasgupta.** 1985. The host protein required for *in vitro* replication of polio virus is a protein kinase that phosphorylates eukaryotic initiation factor-2. Cell **40**:913–921.
24. **Moyer, S. A., S. C. Baker, and J. L. Lessard.** 1986. Tubulin: a factor necessary for the synthesis of both Sendai virus and vesicular stomatitis virus RNAs. Proc. Natl. Acad. Sci. USA **83**:5404–5409.
25. **Oldstone, M. B. A., M. Salvato, A. Tishon, and H. Lewicki.** 1988. Virus-lymphocyte interactions. III. Biologic parameters of a virus variant that fails to generate CTL and establishes persistent infection in immunocompetent hosts. Virology **164**:507–516.
26. **Pedersen, I. R.** 1971. Lymphocytic choriomeningitis virus RNAs. Nature (London) New Biol. **234**:112–114.
27. **Ray, J., and R. S. Fujinami.** 1987. Characterization of in vitro transcription and transcriptional products of measles virus. J. Virol. **61**:3381–3387.
28. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
29. **Riviere, Y., R. Ahmed, P. J. Southern, M. J. Buchmeier, and M. B. A. Oldstone.** 1985. Genetic mapping of lymphocytic choriomeningitis virus pathogenicity: virulence in guinea pigs is associated with the L RNA segment. J. Virol. **55**:704–709.
30. **Romanowski, V., Y. Matsuura, and D. H. L. Bishop.** 1985. Complete sequence of the S RNA of lymphocytic choriomeningitis virus (WE strain) compared to that of Pichinde arenavirus. Virus Res. **3**:101–114.
31. **Salvato, M., E. Shimomaye, P. Southern, and M. B. A. Oldstone.** 1988. Virus-lymphocyte interactions. IV. Molecular characterization of LCMV Armstrong (CTL^+) small genomic segment and that of its variant, clone 13 (CTL^-). Virology **164**: 517–522.
32. **Singh, M. K., F. V. Fuller-Pace, M. J. Buchmeier, and P. J. Southern.** 1987. Analysis of the genomic L segment from lymphocytic choriomeningitis virus. Virology **161**:448–456.
33. **Smith, G. E., and M. D. Summers.** 1980. The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl-paper. Anal. Biochem. **109**:123–129.
34. **Southern, P. J., M. K. Singh, Y. Riviere, D. R. Jacoby, M. J. Buchmeier, and M. B. A. Oldstone.** 1987. Molecular characterization of the genomic S RNA segment from lymphocytic choriomeningitis virus. Virology **157**:145–155.
35. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**:5201–5205.
36. **Vezza, A. C., J. P. Clewley, G. P. Gard, N. Z. Abraham, R. W. Compans, and D. H. L. Bishop.** 1978. Virion RNA species of arenaviruses Pichinde, Tacaribe, and Tamiami. J. Virol. **26**: 485–497.
37. **Vogelstein, B., and D. Gillespie.** 1979. Preparation and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA **76**:615–619.