JOURNAL OF VIROLOGY, Oct. 1977, p. 74-81
Vol. 24, No. 1

Printed in U.S.A.

Visna Virus RNA Synthesis

M. BRAHIC,[1]* P. FILIPPI,[2] R. VIGNE,[2] AND A. T. HAASE[1]

University of California, San Francisco, Veterans Administration Hospital, San Francisco, California 94121[1]; and Laboratoire de Virologie, Faculté de Médecine, Secteur Nord, 13326 Marseille Cedex 3, France[2]

Received for publication 20 May 1977

Visna is a classical slow infection in which virus characteristically persists in the face of the host immune response. The agent of this disease belongs to the retravirus group. The persistence of infection and the slow spread of virus are at least in part a consequence of restriction of the expression of virus genetic information in tissues of an infected animal (A. T. Haase et al., Science 195:175-177, 1977), but the point at which the virus life cycle is interrupted in vivo and the mechanism of restriction are unknown. We have embarked on a molecular analysis of restriction, focusing first on transcription. In this paper we have established the levels of viral RNA synthesis under permissive conditions, as a base line for subsequent studies in vivo. We show that (i) uninfected cells do not contain RNA sequences related to the visna virus genome, (ii) parental RNA is rapidly transported to the nucleus of the infected cell, (iii) virus RNA is synthesized in the nucleus and then transported to the cytoplasm, (iv) synthesis of RNA proceeds mostly exponentially to reach levels of about 4,000 copies per cell at the end of the growth cycle, (v) nuclear and cytoplasmic RNA sediment in two size classes, 35S and 10-20S, (vi) viral mRNA has the same polarity as genome RNA and also sediments in two size classes of 35S and 10-20S.

Visna is a classical model of slow virus infection of the central nervous system of sheep. The causative agent is closely related to RNA tumor viruses in structure and in the characteristic transfer of genetic information from RNA to DNA during replication (9).

There are two particularly interesting aspects of the infection caused by visna virus. In the infected animal, virus persists despite the immune response that it evokes; and the disease process evolves slowly, usually over a period of months or years. Both of these features of in vivo infection may be a consequence of a low level of expression of viral genetic information: we have recently shown that proviral DNA is synthesized in cells in tissues of experimentally infected animals, whereas viral proteins are almost totally absent in the same cells. This conversion to latency in most infected cells in vivo allows the virus to persist; the occasional breakdown of repression in some cells leads to continued production of small amounts of virus and continued but slow spread of infection. The level at which this host restriction is effected has not yet been identified. It could be that of proviral DNA transcription or a later step in the process of protein synthesis.

This restriction in vivo is in marked contrast to the situation in tissue culture cells (in vitro). In lytic infection of permissive cells in vitro, the viral life cycle is completed by 72 h, in a one-step growth cycle, and about 100 to 500 infectious progeny virus are released per cell (9).

In the present series of investigations, we have embarked on a molecular analysis of host restriction in vivo. To obtain base line information for our study of proviral DNA transcription in vivo, we decided to investigate the RNA metabolism of visna virus in in vitro acutely infected permissive cells in which the virus production is maximal. In this study we have followed the fate of parental RNA, determined the time course of progeny RNA synthesis, and examined the sizes of the different intracellular RNAs.

MATERIALS AND METHODS

Cells and virus. Visna virus strain 1514 was used throughout this work.

Sheep choroid plexus cells were grown in vitro and used at passage 5 or 6. The growth medium was Leibowitz medium 15 (L-15) supplemented with 15% fetal calf serum.

Confluent cell monolayers were synchronously infected as follows. The growth medium was removed, and the cell monolayer was washed with ice-cold phosphate-buffer saline (PBS) buffer. The cells were then infected with 3 PFU of visna virus per cell. Adsorption was carried out for 2 h at 4°C. At the end of this period, the viral inoculum was removed and replaced by warm (37°C) L-15 medium containing

2% lamb serum. The infected cells were then incubated at 37°C. The time at which the cells were shifted to 37°C was taken as time zero of the infection.

In preliminary experiments we showed, by infectious-center assay, that under these conditions 90% or more of the cells are infected and that the time course of cytopathic effect and virus production was not affected by the adsorption at 4°C. This temperature did block replication, however. We found that, at 4°C, virus particles were adsorbed but that the early events in the life cycle, in particular synthesis of viral DNA, did not begin until the cells were shifted to 37°C (B. Traynor, P. Ventura, A. Haase, and M. Brahic, in preparation).

RNA extraction. For all the experiments described in this article, the glassware was heated at 180°C for at least 2 h, and the buffers were treated with 0.2% diethylpyrocarbonate (DEP) for 1 h and then autoclaved.

Total cytoplasmic RNA was extracted as follows. The cells were trypsinized and washed once in PBS buffer containing 3 mM $MgCl_2$. The cell pellet was suspended ($\simeq 2 \times 10^7$ cells/ml) in ice-cold reticulocyte standard buffer (10 mM NaCl-10 mM Tris-hydrochloride [pH 7.4]-1.5 mM $MgCl_2$) containing 1% Nonidet P-40, transferred to a Dounce homogenizer, and subjected to 10 strokes of a tight-fitting pestle. Nuclei were sedimented at 600 × *g* for 4 min at 4°C. The salt concentration of the supernatant was adjusted to 200 mM NaCl, 10 mM EDTA, and 1% sodium dodecyl sulfate (SDS). RNA was extracted by the phenol-chloroform technique described by Penman (15) with the following modification: chloroform was not used alone but was mixed in a 1:1 ratio with redistilled phenol.

Nuclear RNA was extracted according to the procedure of Penman (15), including the double detergent washing of the nuclei pellet. As for the purification of cytoplasmic RNA, chloroform was used mixed in a 1:1 ratio with redistilled phenol.

For the experiments shown in Fig. 1 to 3, cytoplasmic and nuclear RNAs prepared according to the Penman procedure were subjected to a DNase treatment before being hybridized: the RNA pellets were dissolved in 1 ml of a mixture of 10 mM NaCl, 5 mM Tris-hydrochloride, pH 7.5, and 5 mM $MgCl_2$ and incubated for 30 min at 37°C with 20 μg of RNase-free pancreatic DNase per ml. EDTA and SDS were then added to a final concentration of 10 mM and 0.5%, respectively, and the incubation mixture was extracted twice with phenol-chloroform.

After two ethanol precipitations, the RNA pellets were dried under vacuum and dissolved in a small volume of 20 mM Tris-hydrochloride, pH 7.5-0.1% DEP. The UV absorbance (*A*) was measured at 260 and 280 nm. The A_{260}/A_{280} ratio was always greater than 1.9.

Contamination of nuclear RNA by cytoplasmic RNA was monitored in the following way. Sheep choroid plexus cells were labeled with [^{3}H]uridine for 12 h, and nuclear RNA was extracted. Analysis of this RNA (2×10^5 cpm) on a sucrose gradient did not show a peak of 18S ribosomal RNA taken as a marker of cytoplasmic RNA (15). We concluded that the nuclear RNA preparations were essentially free of cytoplasmic contaminants.

The possibility that viral RNA might leak during the purification of the nuclei was also investigated. Nuclei were prepared from 48 h-infected cells, and the amount of viral RNA was measured, as described in the legend of Fig. 2, after one, two, or four washes with double detergent mixture. Since no differences in the amount of viral RNA were observed, we concluded that washing nuclei did not result in the leakage of nuclear viral RNA into the cytoplasmic fraction.

Polysome preparation. The medium of the cells was changed to growth medium 1 h before trypsinization, and cycloheximide was added at a final concentration of 1 μg/ml 30 min before trypsinization to increase the size of the polysomes and to prevent "runoff" of ribosomes. After trypsinization, the cells were washed once in PBS containing 3 mM $MgCl_2$. The cell pellet was suspended ($\simeq 10^8$ cells/ml) in buffer A (300 mM NaCl-50 mM Tris-hydrochloride [pH 7.5]-8 mM $MgCl_2$-1 mM dithiothreitol-1 mg of heparin per ml) containing 2% Nonidet P-40, transferred to a Dounce homogenizer, and subjected to 10 strokes of a tight-fitting pestle. The nuclei were sedimented at 600 × *g* for 4 min. The supernatant was diluted to 6 ml with buffer A and layered on top of a discontinuous gradient consisting of 3 ml of 2 M sucrose and 3 ml of 0.5 M sucrose in buffer A. The gradient was spun for 1.5 h at 40,000 rpm at 4°C in an SW41 rotor.

In some experiments, the polysome pellet was resuspended in 0.5 ml of buffer A and layered on top of a linear 0.5 to 1.5 M sucrose gradient in buffer A. The gradient was centrifuged at 4°C for 90 min at 30,000 rpm in an SW41 rotor. The sedimentation profile of the polysomes was determined by measuring the A_{260} of each fraction collected from the gradient.

In other experiments, RNA was extracted from the polysome pellet. The pellet was suspended in 1 ml of 100 mM NaCl-10 mM Tris-hydrochloride (pH 7.5)-1 mM EDTA-0.5% SDS and extracted with phenol-chloroform as described above.

From the hybridization kinetics ($C_rt_{1/2}$) of total cytoplasmic RNA and polysomal RNA prepared from the same infected cells, it was calculated that approximately 5% of the total cytoplasmic virus-specific RNA was recovered in the polysomal RNA.

RNA-DNA hybridization. Viral RNA sequences were detected by hybridization in solution with ^{3}H-labeled complementary DNA ([^{3}H]cDNA). The [^{3}H]-cDNA was prepared by an endogenous reaction in which purified visna virus was used as a source of both RNA-dependent DNA polymerase and viral 60-70S RNA. The reaction was carried out in the presence of 100 μg of actinomycin D per ml. The exact conditions for the reaction have been described (8). The specific activity of [^{3}H]cDNA was 4.3×10^4 cpm/ng. Each [^{3}H]cDNA preparation was characterized for both the extent of hybridization with purified 60-70S viral RNA and the amount of [^{3}H]cDNA necessary to protect 100% of ^{32}P-labeled 60-70S viral RNA against digestion by pancreatic RNase. In all cases, more than 90% of the [^{3}H]cDNA hybridized to viral 60-70S RNA with a $C_rt_{1/2}$ of 1×10^{-2} to 2×10^{-2} mol·s/liter, and the [^{3}H]cDNA was able to protect

100% of the ^{32}P-labeled 60-70S viral RNA at a DNA/RNA ratio of less than 10.

To measure the amount of viral sequences present in a given RNA preparation, the rate of hybridization of [^{3}H]cDNA to this RNA preparation was compared with the rate of hybridization of [^{3}H]cDNA to purified 60-70S viral RNA (12). Hybridization reactions were performed in solution in a final volume of 20 μl per time point. The hybridization mixture contained 600 mM NaCl, 10 mM Tris-hydrochloride (pH 7.5), 3 mM EDTA, 0.1% SDS, 1 mg of carrier RNA per ml, 600 to 800 cpm per 20 μl of [^{3}H]cDNA, and varying amounts of RNA. Hybridization was performed at 68°C. The extent of hybridization was assayed by the resistance of [^{3}H]cDNA to digestion with S_1 nuclease (12). Hybridization kinetics were performed by varying either the time of hybridization or the RNA concentration and were plotted as a function of the C_rt parameter (RNA concentration · time of hybridization expressed in mole·second per liter). The C_rt values were normalized to the standard salt concentration (2). Since the value of C_rt at which 50% of [^{3}H]cDNA hybridizes to an RNA sample ($C_rt_{1/2}$) is inversely proportional to the concentration of viral RNA in this sample, it was possible to express the amount of viral RNA as the number of 60-70S RNA equivalents present per cell using the following equation: number of 60-70S RNA equivalent = ($C_rt_{1/2}$ of 60-70S RNA)/($C_rt_{1/2}$ of RNA sample) × (amount of RNA per cell)/(amount of RNA per virion). The amount of RNA per cell was taken as 8×10^{-6} μg, and the amount of 60-70S RNA per virion was assumed to be 1.7×10^{-11} μg.

Sucrose gradient centrifugation. All gradients used for sizing viral RNA were 5 to 20% linear sucrose gradients in a mixture of 100 mM NaCl, 10 mM Tris-hydrochloride (pH 7.5), 1 mM EDTA, and 0.5% SDS. Centrifugations were performed in an SW41 rotor at 18°C and 40,000 rpm. One hundred micrograms of RNA was used per gradient. The RNA sample was disaggregated by heating to 100°C for 30 s in a solution of 100 mM NaCl, 10 mM Tris-hydrochloride (pH 7.4), and 1 mM EDTA before layering on top of the gradient.

The sedimentation profiles of viral RNA were determined by hybridization with [^{3}H]cDNA. Two hundred micrograms of carrier RNA was added to each fraction of the gradient, and the RNA of each fraction was precipitated by 2 volumes of ethanol. After centrifugation, the RNA pellets were dried under vacuum and dissolved in 30 μl of a solution of 20 mM Tris-hydrochloride (pH 7.5) and 0.1% DEP. Ten microliters of each fraction was hybridized with [^{3}H]cDNA in a final volume of 50 μl using the salt and temperature conditions described above. The extent of hybridization was determined after digestion with S_1 nuclease. Hybridization was performed in a large RNA excess and for a time period sufficiently short such that the percentage of [^{3}H]cDNA hybridized was proportional to the concentration of viral RNA present.

RESULTS

Absence of RNA sequences related to visna virus genome in uninfected sheep cells.

Before studying visna virus RNA metabolism, we decided to investigate the possibility that uninfected sheep cells might contain RNA sequences that can hybridize to viral [^{3}H]cDNA. Such sequences have been described in chicken cells and mouse cells in the cases of, respectively, avian and murine RNA tumor viruses (18, 5).

Cytoplasmic RNA was extracted from uninfected SCP cells and hybridized with [^{3}H]cDNA as described in Material and Methods, up to a C_rt of 10^5 mol·s/liter. Figure 1 shows that no hybridization was observed even for the highest C_rt value attained. Since a $C_rt_{1/2}$ of 5×10^4 mol·s/liter corresponds to the presence of 0.1 viral genome equivalent per cell, we concluded that RNA sequences related to visna virus are virtually absent from uninfected sheep cells. The study of visna virus RNA metabolism was therefore possible without the problems created by "background" hybridization.

Time course of viral RNA synthesis. The major purpose of this study was to determine the time course and the extent of transcription of visna proviral DNA during the lytic replication cycle of the virus. Therefore, permissive cells derived from SCP were synchronously infected with 3 PFU of visna virus per cell and harvested at different times after infection. Cytoplasmic and nuclear RNAs were extracted, and the amount of viral RNA was determined in both fractions 0.5, 1.5, 3, 6, 9, 12, 24, 48 and 72 h after infection. The results of the hybridization reactions are presented in Fig. 2.

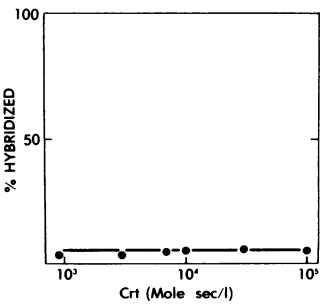

FIG. 1. *Quantitation of viral RNA in the cytoplasm of uninfected sheep choroid plexus cells. Cytoplasmic RNA was prepared from 100 uninfected 75-cm² tissue culture flasks. The RNA was hybridized with virus-specific [³H]cDNA prepared by an endogenous reverse transcriptase reaction. The final RNA concentration in the hybridization mixture was 6 mg/ml. The various C_rt values were attained by varying the time of incubation. C_rt values are in mole·seconds per liter. The extent of hybridization was assayed by resistance to the S_1 nuclease.*

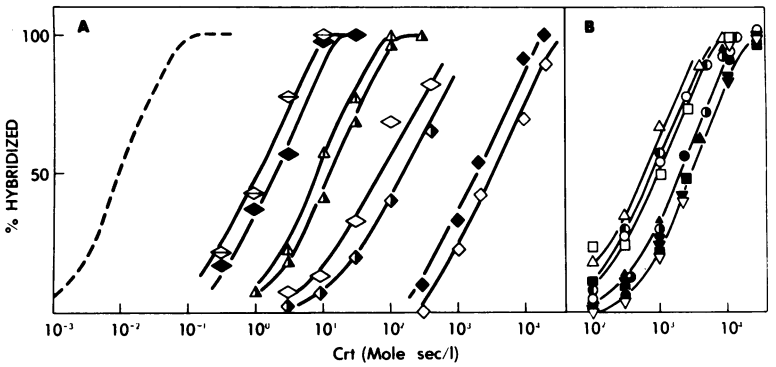

FIG. 2. *Quantitation of virus-specific RNA in the cytoplasmic and nuclear fractions of sheep choroid plexus cells at different times of a synchronous infection. For each time point, 45 tissue culture flasks (75 cm²) were synchronously infected with 3 PFU of visna virus per cell. At each time point, the cells were harvested and fractionated into cytoplasmic and nuclear fractions, and RNA was extracted from both fractions. All RNA samples were dissolved in 50 μl of 20 mM Tris (pH 7.5)-0.1% DEP. The different RNAs were hybridized with virus-specific [³H]cDNA in a final volume of 20 μl per C_rt value examined. Quantitation of viral RNA in nuclei can be unreliable because nuclear RNA cannot be prepared entirely free of DNA, which introduces uncertainty into RNA concentration determined solely from the A_{260}. This problem was obviated by preparing nuclear and cytoplasmic RNA from the same cells, dissolving both in the same volume, and using the RNA concentrations from cytoplasmic RNA to construct the C_rt curves of both cytoplasmic and nuclear RNA. The number of viral genome equivalents in the nuclei was computed from the $C_rt_{1/2}$ of nuclear and cytoplasmic RNA, since the $C_rt_{1/2}$ is inversely proportional to the concentration of viral RNA sequences, and both nuclear and cytoplasmic RNA were prepared from the same number of cells and dissolved in the same volume. The RNA concentrations for cytoplasmic RNAs were: 0.5 h, 3,480 μg/ml; 1.5 h, 4,000 μg/ml; 3 h, 2,560 μg/ml; 6 h, 3,920 μg/ml; 9 h, 3,920 μg/ml; 12 h, 3,760 μg/ml; 24 h, 180 μg/ml; 48 h, 196 μg/ml; 72 h, 59 μg/ml. For purified 60-70S viral RNA, the RNA concentration was 0.2 μg/ml. The extent of hybridization was assayed by resistance to the S_1 nuclease. Symbols (- - -) 60-70S RNA, (◐) 0.5-h cytoplasmic, (◑) 0.5-h nuclear, (○) 1.5-h cytoplasmic, (●) 1.5-h nuclear, (△) 3-h cytoplasmic, (▲) 3-h nuclear, (□) 6-h cytoplasmic, (■) 6-h nuclear, (▽) 9-h cytoplasmic, (▼) 9-h nuclear, (◇) 12-h cytoplasmic, (◆) 12-h nuclear, (◈) 24-h cytoplasmic, (◇) 24-h nuclear, (⍙) 48-h cytoplasmic, (▲) 48-h nuclear, (⊖) 72-h cytoplasmic, (◆) 72-h nuclear. For the sake of clarity, C_rt curves have been represented in two parts of the same figure. (A) C_rt curves for 60-70S RNA and 12-, 24-, 48-, and 72-h RNAs. (B) Curves for 0.5-, 1.5-, 3-, 6-, and 9-h RNAs.*

The data obtained from these hybridization studies are summarized in Table 1 and Fig. 3, which also presents the time course of production of infectious virions.

During the first 3 h of the infection, we detected a total of eight to nine viral genome equivalents per cell. This value is in reasonably good agreement with the multiplicity of infection used (3 PFU/cell), taking into account a particle-to-PFU ratio of 5 in the case of visna virus (9). Two copies of viral genome were present in the nucleus even at the earliest time studied (30 min). Therefore, a fraction of the infecting RNA molecules must be rapidly transported to the nucleus (see Discussion). Between 6 and 12 h postinfection, there was a marked decrease in viral RNA content in the cytoplasm, whereas the amount of viral RNA remained constant in the nucleus. These phenomena were observed in three independent experiments. The accumulation of viral RNA was first detected in the nucleus 10 to 12 h postinfection and in the cytoplasm approximately 2 h later. By 24 h after infection, the amount of viral RNA was still four times greater in the nucleus than in the cytoplasm; at the same time, the first progeny viral particles were detected in the culture medium. Between 24 h postinfection and the end of the lytic cycle (72 h), the amount of viral RNA increased almost exponentially in both the nucleus and the cytoplasm and was always higher in the cytoplasm. At 72 h postinfection, the cells contained a total of about 4,000 viral genome equivalents.

Size of cytoplasmic and nuclear viral RNA. The visna virus genome is a 60-70S RNA molecule made of two or three 35S RNA subunits (21). Rapidly harvested virions contain free 35S RNA subunits that assemble to form the 60-70S RNA complex during aging of the particle (1). One would, therefore, expect at least part of the viral intracellular RNA to consist of 35S RNA molecules. We investigated this point by examining the size of viral cytoplasmic and nuclear RNA 48 h after infection, a time that corresponds to the active period of RNA synthesis

TABLE 1. *Amount of viral RNA present in the cytoplasm and the nucleus of sheep choroid plexus cells at different times after synchronous infection with 3 PFU of visna virus per cell*

Time after infection (h)	Cytoplasmic RNA		Nuclear RNA	
	$C_rt_{1/2}$[a]	No. of viral genome equivalents per cell[b]	$C_rt_{1/2}$[a]	No. of viral genome equivalents per cell[b]
0.5	7.5×10^2	6	2.3×10^3	2
1.5	7.5×10^2	6	2.3×10^3	2
3	6.4×10^2	7	2.3×10^3	2
6	1.0×10^3	5	3.0×10^3	2
9	3.0×10^3	2	3.0×10^3	2
12	3.0×10^3	2	2.0×10^3	3
24	2.0×10^2	23	6.0×10^1	77
48	1.0×10^1	471	1.4×10^1	336
72	1.2×10^0	2,350	1.8×10^0	1,570

[a] C_rt values are in moles·second per liter. $C_rt_{1/2}$ values were graphically determined from the curves presented in Fig. 2.

[b] Numbers of viral genome equivalents per cell were calculated as indicated under Material and Methods and in the legend of Fig. 2. The number given in the table is the nearest integer to the calculated value. The $C_rt_{1/2}$ for purified viral 60–70S was determined from Fig. 2. The value was 10^{-2} mol·s/liter.

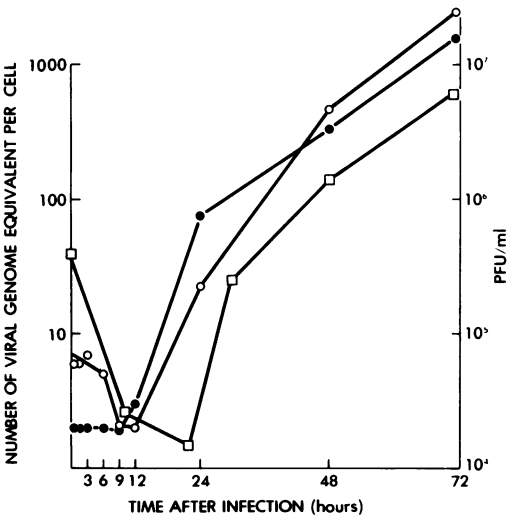

FIG. 3. *Time course of accumulation of viral RNA in the nucleus (●) and the cytoplasm (○) of sheep choroid plexus cells synchronously infected with visna virus. The amount of viral RNA is expressed in number of viral genome equivalents per cell, and the values were taken from Table 1. Symbol: (□) time course of appearance of infectious viral particles in the culture medium (PFU per milliliter).*

(Fig. 3). Cytoplasmic and nuclear RNAs were prepared as described in Materials and Methods and sedimented through a 5 to 20% sucrose gradient. To determine the sedimentation profile of viral RNA, the RNA present in each fraction was hybridized to [^{3}H]cDNA. Ribosomal and transfer RNA were used as sedimentation coefficient markers.

Two classes of viral RNA were found in both the nucleus and the cytoplasm (Fig. 4). The first is an RNA species with sedimentation coefficient of 35S identical to that of the genome subunits. The second class sedimented between 20S and 10S with a constant peak at 20S. However, the sedimentation profile in this part of the gradient was variable from one experiment to another.

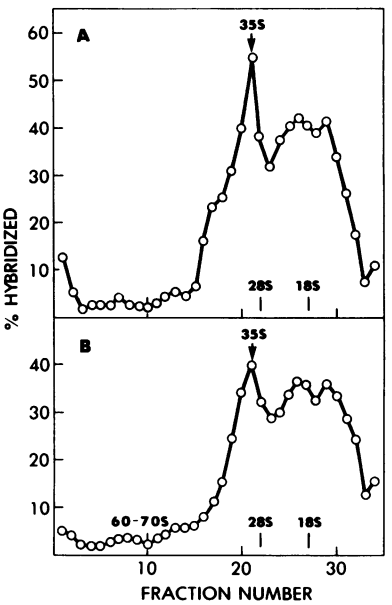

FIG. 4. *Sedimentation profile of virus-specific RNA extracted from (A) the cytoplasm and (B) the nucleus of sheep choroid plexus cells 48 h after infection with visna virus. Twenty 75-cm² tissue culture flasks were used in each case. Cytoplasmic and nuclear RNA were extracted as described in Materials and Methods and dissolved in the same volume of 100 mM NaCl–10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA –0.5% SDS –0.1% DEP. One hundred micrograms of cytoplasmic RNA and the corresponding volume of nuclear RNA were heated to 100°C for 30 s to disaggregate RNA and layered on top of a 5 to 20% sucrose gradient. Centrifugation was from right to left in an SW41 rotor at 40,000 rpm for 120 min. A mixture of ^{32}P-labeled 60-70S RNA and [^{3}H]rRNA was centrifuged in a separate tube of the same rotor to serve as a sedimentation coefficient marker. After fractionation, the RNA present in each fraction was concentrated by ethanol precipitation in the presence of 200 μg of carrier RNA per fraction and hybridized with [^{3}H]cDNA. The extent of hybridization was assayed by the resistance to S_1 nuclease digestion. The locations of 60-70S, 28S, and 18S marker RNAs are indicated.*

Identification of viral mRNA's. Visna virus is a member of the family of retraviruses (9). Genome RNA is therefore expected to have the same polarity as viral mRNA. It follows that [^{3}H]cDNA should hybridize to viral RNA sequences that cosediment with polyribosomes, and further, these sequences should be released when the polysomes are disrupted with EDTA (16). In the experiment described below, we demonstrate that these predictions were fulfilled.

Purified polyribosomes from infected cells were divided into two fractions, and EDTA, at a final concentration of 20 mM, was added to one. The two fractions were then sedimented separately in linear 0.5 to 1.5 M sucrose gradients. The sedimentation profile of total cellular polyribosomes was determined from the A_{260} of each fraction, and that of viral sequences was determined by hybridizing the RNA of each fraction with [^{3}H]cDNA. Figure 5 shows the result of the experiment. Before EDTA treatment, the hybridization profile and the A_{260} were nearly coincident. Treatment with EDTA disrupted the cellular polyribosomes, as shown by the shift of all UV-absorbing material to the top of the gradient. The same treatment also resulted in displacement of viral RNA sequences to the top of the gradient. From the hybridization profiles of Fig. 5, it can be estimated that more than 85% of the viral RNA, which cosedimented with cellular polyribosomes before treatment, was displaced to the top of the gradient. From this experiment we conclude that more than 85% of the viral RNA sequences associated with purified polyribosomes behave like mRNA. The experiment also shows that the sedimentation profile of virus-specific polyribosomes is very similar to that of the bulk of cellular polyribosomes.

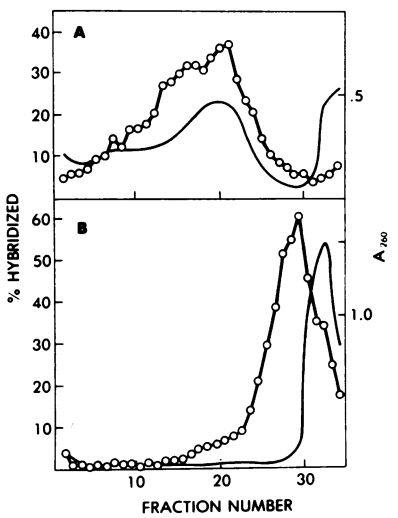

FIG. 5. *Virus-specific RNA in purified polyribosomes. Purified polyribosomes were prepared from 40 75-cm² tissue culture flasks 48 h after infection with visna virus. The final polyribosome pellet was divided in half. One-half was directly layered on top of a 0.5 to 1.5 M linear sucrose gradient; the other half was treated with 20 mM EDTA before being layered on top of a similar sucrose gradient. Centrifugation was for 90 min at 30,000 rpm and 4°C in an SW41 rotor. After fractionation of the gradients, the A_{260} of each fraction was determined. The content of each fraction was concentrated by ethanol precipitation in the presence of 200 μg of carrier RNA and hybridized with [^{3}H]cDNA. The extent of hybridization was assayed by S_1 nuclease digestion. Sedimentation was from right to left. (A) Polyribosomes not treated with EDTA; (B) polyribosomes treated with EDTA. Symbols: (——) A_{260}, (○) percentage of [^{3}H]cDNA hybridized.*

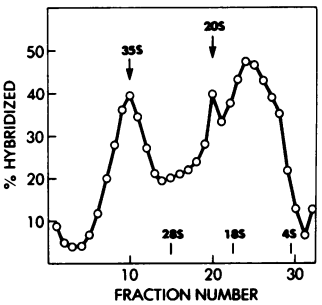

FIG. 6. *Sedimentation profile of visna virus mRNA. Polyribosomes were purified from 40 75-cm² tissue culture flasks 48 h after infection with visna virus. The RNAs present in the polyribosome pellet were extracted with the phenol–chloroform–SDS method. One hundred micrograms of polyribosomal RNA was heated to 100°C for 30 s to disaggregate the RNA and then layered on top of a 5 to 20% linear sucrose gradient. Centrifugation was, from right to left, for 3 h and 20 min at 40,000 rpm in an SW41 rotor. The amount of virus-specific RNA present in each fraction of the gradient was determined as for the experiment presented in Fig. 4. [^{3}H]rRNA's were centrifuged on a separate gradient in the same rotor and used as sedimentation coefficient markers. Their positions are indicated.*

We then examined the size of viral mRNA's. For this purpose, polyribosomes were purified from cells infected for 48 h and RNAs were extracted from the polysome pellet. The RNAs were sedimented through a 5 to 20% sucrose gradient containing SDS, and the hybridization profile with [^{3}H]cDNA was determined as described above. Figure 6 shows a typical profile. In all polyribosomal RNA preparations examined, a sharp peak sedimenting at 35S was present. This discrete RNA species was always accompanied by an almost equal amount of

smaller RNAs that constantly presented peaks or shoulders at 20S and 14S. The exact profile in this region of the gradient, however, varied from one experiment to another.

DISCUSSION

Visna virus replication is restricted in the tissues of infected animals (in vivo) (10), and this might explain the persistence of the infection and its slowness. The level at which this restriction is exerted in vivo has not yet been determined but could be that of transcription, since proviral DNA is readily detectable in brain cells of infected animals (10). We, therefore, began investigation of the modalities of proviral DNA expression in vivo and in vitro. The main purpose of the present work was to obtain base line information on proviral DNA transcription in cells infected in vitro. We have shown that, in this system, proviral DNA is extensively transcribed and that viral RNA sediments in two distinct classes: 35S RNA and 10-20S RNA.

Figure 1 demonstrates that uninfected sheep cells do not contain RNA sequences related to visna virus genome. This result extends previous observations (7) showing that visna 60-70S RNA did not hybridize to a vast excess of DNA extracted from uninfected sheep cells. Both experiments strongly suggest that visna virus is a purely exogenous virus of sheep. Since there was no transcription from endogenous sequences, backgrounds were low enough to allow us to follow the fate of the few copies of RNA introduced in the cell.

Our study of the time course of viral RNA synthesis revealed several interesting phenomena. In the first part of the lytic cycle (from 0.5 h to 10 h), we were following the fate of parental RNA. The most striking observation was that parental RNA is rapidly transported to the nucleus, a phenomenon that takes place during the first 30 min of the infection. For reasons already discussed in Materials and Methods, we believe that this was not just the result of contamination of nuclear RNA by cytoplasmic RNA. A rapid transport of parental avian sarcoma and leukosis RNA to the nucleus of chicken embryo fibroblasts has also been described (3). Interestingly, studies from this laboratory (B. Traynor et al., in preparation) have demonstrated that visna proviral DNA is first detected exclusively in the nucleus approximately 3 h after infection and that no viral DNA is found in the cytoplasm at any time of the lytic cycle. It is, therefore, likely that upon infection of sheep cells, visna virus RNA and some viral structural proteins, including the RNA-dependent DNA polymerase, are rapidly transported to the nucleus, where proviral DNA synthesis takes place. This is in contrast to the replication of avian sarcoma viruses. In this case, proviral DNA is apparently synthesized in the cytoplasm and transported to the nucleus (20). This difference in the sites of proviral DNA synthesis of visna and avian sarcoma viruses could be related to the type of tissue in which these viruses normally replicate. Visna virus multiplies essentially in nondividing cells (for example, brain cells), whereas avian sarcoma or leukemia viruses multiply in actively dividing cells. One can speculate that cellular proteins involved in the replication of cellular DNA might be required for the synthesis of type C proviral DNA. These proteins would be actively synthesized in the cytoplasm of rapidly growing cells and would therefore be available for the cytoplasmic synthesis of avian sarcoma-leukemia proviral DNA. These proteins might be present only in the nucleus of nondividing cells, which would explain why visna proviral DNA synthesis is exclusively nuclear.

A second observation concerning the fate of parental RNA is that some viral RNA remains in the cytoplasm and is degraded after 6 h (Fig. 3). It is possible that these RNA molecules correspond to viral particles that failed to reach the nucleus and whose infectious cycle had been aborted. Alternatively, they may function as mRNA for the synthesis of early essential viral protein(s). Such a phenomenon has been recently described in the case of murine sarcoma leukemia viruses (17). In agreement with this, preliminary experiments from this laboratory (J. Scott, personal communication) have shown a burst of synthesis of the major visna virus structural protein during the first hours of the lytic cycle.

The second part of the lytic cycle (between 10 and 72 h postinfection) corresponds to the phase of active RNA synthesis. The kinetics of appearance of viral RNA sequences in the nucleus and the cytoplasm and the kinetics of release of infectious virions (Fig. 3) suggest that viral RNA is transcribed in the nucleus, exported to the cytoplasm, and eventually encapsidated into viral particles. This general scheme has also been proposed in the case of the avian RNA tumor viruses (14). From several experiments analogous to that described in Table 1, we estimate that cells which have been infected by 8 to 10 viral genomes contain 4,000 to 5,000 copies of viral genome at the end of the lytic cycle.

The main size class of virus RNA in lytically infected cells cosediments with genome subunits and has the same polarity as the viral

genome. Figure 4 shows that 35S genomic RNA is found in the nucleus and is therefore a major transcription product of proviral DNA. Since we only studied steady-state nuclear RNA, our experiments do not address the issue of whether this RNA arises from the processing of a large precursor molecule.

We could not determine whether the noncovalent linkage of 35S subunits into virion 60-70S RNA complexes takes place in infected cells, because viral RNA aggregated during extraction and had to be denatured before sedimentation. A similar aggregation phenomenon has also been described in the case of intracellular RNA of murine RNA tumor viruses (see Discussion of reference 11) and avian sarcoma viruses (4).

Figure 5 demonstrates that our procedure for purifying polyribosomes yielded almost exclusively ribonucleoprotein which could be dissociated with EDTA. Therefore the viral RNA present in this fraction has the characteristic properties of mRNA. Figure 6 shows that 35S genomic RNA functions as mRNA. Smaller viral RNA molecules are also present in purified polyribosomes that may represent discrete RNA species, and not just degradation products of 35S RNA, because: (i) polyribosomes were prepared in the presence of 1 mg of heparin per ml, a good RNase inhibitor; and (ii) all preparations exhibited peaks or shoulders at 20S and 14S. Such small viral mRNA's have also been described in the case of murine leukemia viruses (6, 19). Their possible significance in the regulation of the synthesis of the various structural viral polypeptides has been discussed recently (13).

ACKNOWLEDGMENTS

We gratefully acknowledge J. Tamalet for his support during the realization of this work, P. Ventura, N. Sauze, and G. Vestris for their excellent technical assistance, and H. Lukes for her help in the preparation of the manuscript.

M. Brahic was supported by the California division of the American Cancer Society senior fellowship D-281. We acknowledge grants A6512119 and 995717 from Centre National de la Recherche Scientifique and Public Health Service grant NS11782 from the National Institute of Neurological and Communicative Disorders and Stroke. This project was VAH MRIS 3367 from the Veterans Administration.

LITERATURE CITED

1. **Brahic, M., and R. Vigne.** 1975. Properties of visna virus particles harvested at short time intervals: RNA content, infectivity, and ultrastructure. J. Virol. 15:1222-1230.
2. **Britten, R. J., and J. Smith.** 1970. A bovine genome. Carnegie Inst. Washington Yearb. 68:378-386.
3. **Dales, S., and H. Hanafusa.** 1972. Penetration and intracellular release of the genome of avian RNA tumor viruses. Virology 50:440-458.
4. **Deng, C. T., D. Stehelin, J. M. Bishop, and H. E. Varmus.** 1977. Characteristics of virus-specific RNA in avian sarcoma virus-transformed BHK-21 cells and revertants. Virology 76:313-330.
5. **Fan, H., and D. Baltimore.** 1973. RNA metabolism of murine leukemia virus: detection of virus-specific RNA sequences in infected and uninfected cells and identification of virus specific messenger RNA. J. Mol. Biol. 80:93-117.
6. **Gielkens, A. L. J., M. H. L. Salden, and H. Bloemendal.** 1974. Virus specific mRNA on free and membrane bound polyribosomes from cells infected with Rauscher leukemia virus. Proc. Natl. Acad. Sci. U.S.A. 71:1093-1097.
7. **Haase, A. T., and H. E. Varmus.** 1973. Demonstration of a DNA provirus in the lytic growth of visna virus. Nature (London) New Biol. 245:237-239.
8. **Haase, A. T., A. C. Garapin, A. J. Faras, H. E. Varmus, and J. M. Bishop.** 1974. Characterization of the nucleic acid product of the visna virus RNA dependent DNA polymerase. Virology 57:251-258.
9. **Haase, A. T.** 1975. The slow infection caused by visna virus. Curr. Top. Microbiol. Immunol. 72:101-156.
10. **Haase, A. T., L. Stowring, O. Narayan, D. Griffin, and D. Price.** 1977. Slow persistent infection caused by visna virus: role of host restriction. Science 195:175-177.
11. **Haseltine, W. A., and D. Baltimore.** 1976. Size of murine RNA tumor virus-specific nuclear RNA molecules. J. Virol. 19:331-337.
12. **Leong, J. A., A. C. Garapin, N. Jackson, L. Fanshier, W. Levinson, and J. M. Bishop.** 1972. Virus-specific ribonucleic acid in cells producing Rous sarcoma virus: detection and characterization. J. Virol. 9:891-902.
13. **Mueller-Lantzsch, N., and H. Fan.** 1976. Monospecific immunoprecipitation of murine leukemia virus polyribosomes: identification of P30 protein-specific messenger RNA. Cell 9:579-588.
14. **Parsons, J. T., J. M. Coffin, R. K. Haroz, P. A. Bromley, and C. Weissmann.** 1973. Quantitative determination and location of newly synthesized virus-specific RNA in chicken cells infected with Rous sarcoma virus. J. Virol. 11:761-774.
15. **Penman, S.** 1969. Preparation of purified nuclei and nucleoli from mammalian cells, p. 35-48. *In* K. Habel and N. P. Salzman (ed.), Fundamental techniques in virology. Academic Press Inc., New York.
16. **Perry, R. P., and D. E. Kelley.** 1968. Messenger RNA-protein complexes and newly synthesized ribosomal subunits: analysis of free particles and components of polyribosomes. J. Mol. Biol. 35:37-59.
17. **Salzberg, S., M. S. Robin, and M. Green.** 1977. A possible requirement for protein synthesis early in the infectious cycle of the murine sarcoma-leukemia virus. Virology 76:341-351.
18. **Schincariol, A., and W. Joklik.** 1973. Early synthesis of virus-specific RNA and DNA in cells rapidly transformed with Rous sarcoma virus. Virology 56:532-548.
19. **Shanmugam, G., S. Bhaduri, and M. Green.** 1974. The virus specific RNA species in free and membrane bound polyribosomes of transformed cells replicating MSV-MLV. Biochem. Biophys. Res. Commun. 56:697-702.
20. **Varmus, H. E., R. V. Guntaka, W. J. W. Fan, S. Heasley, and J. M. Bishop.** 1974. Synthesis of viral DNA in the cytoplasm of duck embryo fibroblasts and in enucleated cells after infection by avian sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 71:3874-3878.
21. **Vigne, R., M. Brahic, P. Filippi, and J. Tamalet.** 1977. Complexity and polyadenylic acid content of visna virus 60-70S RNA. J. Virol. 21:386-395.