Visna Virus Exhibits a Complex Transcriptional Pattern: One Aspect of Gene Expression Shared with the Acquired Immunodeficiency Syndrome Retrovirus

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A complex pattern of gene expression was found for visna virus in a highly permissive cell culture system in vitro. In addition to the genomic RNA (9.4 kilobases [kb]), five other mRNAs were detected. The three large RNA transcripts (5.0 kb and a doublet at 4.3 kb) arise by a single splicing event joining 5' sequences to sequences located at positions 3' to the *pol* gene. The two smallest transcripts (1.8 and 1.5 kb) are at least doubly spliced mRNAs which contain sequences derived from the 5' end of the genome, the region between the *pol* and *env* genes, and 3' terminal sequences. In addition to this complex pattern of transcription, the mRNAs appear to be regulated temporally. The 1.5-kb mRNA appears 6 h later than the other transcripts. The significance of this complex pattern of gene expression in the unique aspects of the lentivirus life cycle and pathogenesis is considered.

The lentiviruses are a family of nononcogenic retroviruses including ovine visna virus, caprine arthritis-encephalitis virus, equine infectious anemia virus, and the human immunodeficiency virus (HIV). These viruses cause chronic, progressive multiorgan diseases in their hosts (16, 23). Visna virus, the prototype of this group, causes chronic pneumonitis and a progressive inflammatory central nervous system disease months to years after the initial infection (16, 23). The slow course of the infection in vivo contrasts remarkably with the infection of cells in tissue culture. In sheep cells in culture visna virus replicates rapidly to a high titer and results in cell death (5, 17, 25). In infected animals there is little cell-free virus and no direct cell cytopathology. The target cells for visna virus in the animal are cells of monocyte-macrophage lineage (12, 27). Virus replication is associated almost exclusively with macrophages in affected tissues such as the lung, lymph nodes, and spleen. Replication is, however, restricted in these cells; the majority of cells containing viral RNA do not produce infectious virus (9, 11, 12, 26). The restriction of viral replication in the host may contribute to the slowness of the course of disease but does not explain the persistence of infectious virus in a host producing neutralizing antibody. Recently, it has been shown that there is a population of monocyte-macrophage precursor cells in the bone marrow that contain viral RNA. These cells may act as a reservoir of latently infected cells, which, when differentiated into macrophages, produce virus (12). Support for this notion comes from in vitro studies showing that monocytes removed from infected animals and allowed to mature into macrophages in culture are activated, producing high levels of viral RNA and infectious virus (12, 26). In situ hybridization has shown that the level of viral RNA in macrophages in vitro is substantially greater than that observed for the comparable macrophages in the animal (10, 12, 25). Thus, the differentiated state of the host target cells as well as host immunological factors may play roles in regulating viral gene expression. To study the mechanisms involved in restriction of viral replication at the molecular

level, viral gene expression under the highly permissive conditions for virus replication in vitro must first be understood.

The genetic structure of the lentiviruses is similar to that of other retroviruses (42); lentiviruses contain (5' to 3') the structural genes *gag*, *pol*, and *env* (33, 35, 40, 45). They contain additional open reading frames (ORFs) between *pol* and *env* and overlapping the 3' end of *env* (33, 35, 40, 45). Although the lentiviruses share limited nucleotide sequence homology in these regions (4, 13, 14, 30, 31), the presence and similar location of these ORFs in their genomes represent a common feature of lentiviruses. As such, it is quite possible that these ORFs encode proteins responsible for certain of the unique and complex aspects of the lentiviral life cycle and pathogenesis, such as restricted viral replication and latency in vivo (16, 23) and cytopathic replication (16, 25, 26) and *trans*-activation in vitro (18). In HIV at least two gene products (*tat* [1, 36, 38] and *art* [37] or *trs* [7]), derived from the ORFs between *pol* and *env* (1, 5, 36-38), have been shown to be *trans*-acting factors important for the regulation of viral gene expression (1, 7, 39). The transcription of HIV reflects the complex genetic organization of the lentiviruses with at least four spliced mRNAs in addition to the full-length viral RNA present in infected cells (32).

To determine whether this complex transcriptional pattern is a common feature of lentivirus gene expression, the virus-specific mRNAs were examined in visna virus-infected cells, and their genetic structure was determined. Infected cells contain a full-length 9.4-kilobase (kb) transcript, two singly spliced subgenomic species of 5.0 and 4.3 kb (doublet), and two multiply spliced species of 1.8 and 1.5 kb. In addition, there appears to be differential gene expression during the growth cycle. At 6 h after infection, the 9.4-, 5.0-, 4.3- (doublet), and 1.8-kb mRNAs are present, but the 1.5-kb species is not detectable. By 12 h, the 1.5-kb mRNA has increased dramatically to a level almost equivalent to that of the 1.8-kb message. Both messages continue to increase through 24 h, by which time the amounts of the 1.5- and 1.8-kb species are essentially equivalent.

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MATERIALS AND METHODS

Preparation of RNA. Cultures of primary sheep choroid plexus (SCP) cells were prepared and grown as described previously (25). Propagation of visna virus strain 1514 was as described previously (24). When confluent, SCP cells were infected with visna virus (strain 1514) at a multiplicity of 2. Total cellular RNA was isolated at the peak of the cytopathic effect (24 h after infection) by the method of Chirgwin et al. (3). In addition, the RNA was washed twice with sodium acetate, which has been reported to remove contaminating DNA and small RNAs (29). This was useful for decreasing the background when analyzing the RNA as described below. The total cellular RNA was phenol-chloroform extracted twice to remove proteins and then subjected to two cycles of poly(A)⁺ selection on oligo(dT)-cellulose (Collaborative Research, Inc.).

Northern transfers and hybridization conditions. A 800-ng sample of poly(A)⁺ RNA was electrophoresed in a 1% agarose-formaldehyde gel (19, 43) with a mini-gel apparatus (Bio-Rad Laboratories) for approximately 4 h at 45 V or until the xylene-cyanol dye marker had migrated 4.3 cm. The gel was washed for 1 h (two changes) in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then transferred to a nylon membrane (GeneScreen; New England Nuclear Corp.) overnight in 10× SSC.

Membranes were prehybridized at 42°C for 2 h in a solution containing 50% formamide (FLUKA), 6× SSC, 5× Denhardt solution (19), 20 µg of single-stranded DNA per ml, 20 µg of tRNA per ml, 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7), 1% sodium dodecyl sulfate, 100 µg of heparin per ml, and 1% glycine. Hybridization was carried out at 42°C for 20 to 24 h with 5 × 10⁶ cpm of ³²P-labeled probe. The hybridization solution had the same composition as the prehybridization solution, except that it contained 1× Denhardt solution. After hybridization filters were washed for 15 min in 2× SSC at room temperature and then at 62°C in 0.1× SSC–0.1% sodium dodecyl sulfate and exposed to X-ray film at –70°C. It was necessary to vary exposure length depending on the size of the probe and the specific activity to obtain an appropriate signal intensity. The exposure times are as follows: probe A, 65 h; probe B, 7 h; probe C, 16 h; probe D, 2.5 h; probe E, 96 h; probe F, 7 h; probe G, 72 h.

Probe construction. Probes were subcloned from the cloned visna virus genome (21) by standard recombinant DNA methods (19) into various plasmid vectors (probe A, puc18; probes B and E, pGEM2; probes C and D, puc19) or the bacteriophage M13 vector (probe G). Probe F is a *BalI*-to-*HincII* fragment that was gel purified from low-melting-point agarose by the method of Feinberg and Vogelstein (6). Plasmids were propagated in *Escherichia coli* JM109 (probes A, B, C, and D) or DH1 (probe E), whereas M13 phage were propagated in *E. coli* JM103 as described previously (19). DNA fragments were gel purified by electroelution onto DEAE membranes before insertion into the various vectors. The size and location of each probe within the proviral genome are indicated in Fig. 1. Probe A extends from an *NarI* site at position 418 just 3' of the R region to an *SstI* site at position 784. Probe B extends from an *EcoRI* site at 987 to a *SmaI* site at 1430. Probe C extends from a *KpnI* site at 2280 to a *BamHI* site at 4843. Probe D extends from a *BglII* site at 5472 to an *EcoRI* site at 5655. Probe E extends from a *PstI* site at 5876 to a second *PstI* site at 6123. Probe F extends from a *BalI* site at 6214 to a *HincII* site at 6650. Probe G extends from a *HpaII* site at 7139 to an *XbaI* site at 8025.

Labeling of probes. Subcloned probes were labeled to a specific activity of 1 × 10⁸ to 2 × 10⁸ cpm/µg (acid-precipitable counts) by nick translation (19, 34) or (in the case of probe F) the method of Feinberg and Vogelstein (6). A cDNA probe representing the entire viral genome was made as described by Taylor et al. (41). Probes were stripped from nylon membranes as described by Graham et al. (15).

Molecular weight determination of visna viral RNA. ³²P-labeled RNA transcripts of known size were synthesized as described by Melton et al. (20). The plasmid template used for this purpose was the gift of J. Corden and contains a mouse RNA polymerase II gene inserted into the pGEM1 vector (J. M. Ahearn, M. S. Bartolomei, M. L. West, and J. O. Corden, EMBO J., in press). Linearization of this plasmid with *HincII* and transcription from the SP6 promoter yields a 7.4-kb product. Linearization with *BamHI* and transcription from the SP6 promoter yields a 2.8-kb product. Transcription of the Gemini control template (Promega Biotech) with SP6 RNA polymerase yields a 1.4-kb transcript (in addition to smaller transcripts which are not present on our gels). These three labeled transcripts (7.4, 2.8, and 1.4 kb) were electrophoresed in lanes parallel to the samples and used as molecular weight standards.

Enzymes. Restriction enzymes, SP6 RNA polymerase, and T4 DNA ligase were purchased from Bethesda Research Laboratories and were used under conditions suggested by the manufacturer. Avian myeloblastosis virus RNA-dependent DNA polymerase was purchased from Life Sciences, Inc. *E. coli* DNA polymerase Klenow fragment, calf thymus primers, and T4 polynucleotide kinase were purchased from P-L Biochemicals, Inc. S1 nuclease was purchased from Boehringer Mannheim Biochemicals and P-L Biochemicals.

End labeling of the 5' ends of DNA. After digestion with *BglII*, approximately 50 µg of the clone containing the *BamHI*-*HindIII* fragment was phenol extracted and dephosphorylated with 0.75 U of calf intestinal phosphatase. After dephosphorylation, the DNA was heated at 68°C for 15 min and desalted on a G-50 Sephadex column. The small amounts of RNA present in the DNA samples were removed before end labeling by chromatography on a Sepharose 4B column equilibrated in 10 mM Tris (pH 7.4)–1 mM EDTA–0.3 M sodium acetate. DNA (1 to 5 µg) was end labeled by using 250 µCi of [³²P]ATP and 20 U of T4 polynucleotide kinase. The reaction mix was then desalted on G-50 Sephadex, digested with *BamHI*, phenol extracted, and ethanol precipitated before being used in S1 nuclease mapping experiments.

S1 nuclease mapping of viral RNA. S1 nuclease mapping was carried out by the method of Nash et al. (28) with 80,000 to 150,000 cpm of end-labeled DNA (specific activity, 1 × 10⁶ to 12 × 10⁶ cpm/µg).

RESULTS

Northern blot analysis of viral RNA. Although replication of visna virus is restricted in vivo, primary cultures of SCP cells in vitro are highly permissive for viral gene expression. During infection of these cells, visna virus replicates to high titers, cytopathic effect is evident, and viral RNA accumulates to high copy number. At 72 h post infection, the time of peak virus production, there are approximately 20,000 copies of viral RNA per cell as measured by in situ hybridization and dot-blot analysis (9, 12). Thus, cultured SCP cells provide a system for transcriptional analysis of the viral genome at the molecular level. Although more than one size class of visna viral RNA has been identified previously on

FIG. 1. Northern blot hybridization with subgenomic probes. The open bars show the visna proviral genome. The solid heavy bars in the LTRs represent the R region. The cap site is situated at nucleotide number 256 in the 5' R region, and the polyadenylation signal extends from nucleotides 9433 to 9438 in the 3' R region. Open triangles represent the location of consensus splice acceptor sites, and closed triangles represent the location of consensus splice donor sites. The stippled bars show the location and size of subgenomic probes. The probes are described in detail in Materials and Methods. The bottom portion of the figure shows the results obtained when each probe is hybridized to visna viral mRNA as described in Materials and Methods. The size of each species is indicated on the left. Lanes: a, probe A; b, probe B; c, probe C; d, probe D; e, probe E; f, probe F; g, probe G; h, poly(A)-selected RNA from uninfected cells prepared and processed exactly like RNA from infected cells. RNA in lane h was hybridized to probe G; a 3-day exposure is shown. All other subgenomic probes similarly fail to hybridize to uninfected RNA (data not shown). Blots were stripped of probe and exposed to X-ray film for 2 days to ensure complete removal of the initial probe before reuse. Lane e is the same blot as lane a (lane a is the first probing of this blot, and lane e is the second). Lane f is the same blot as lane g (lane g is the first probing of this blot and lane f is the second).

sucrose gradients (8), no transcriptional mapping studies of the visna viral genome have been conducted to date.

Viral transcripts were initially identified in RNA prepared from infected SCP cells by fractionation on denaturing gels (19, 43). After electrophoresis, RNA was transferred to a nylon membrane and hybridized with a cDNA probe representing the entire viral genome (41) (data not shown). Six species of viral RNA were identified: genome length RNA (9.4 kb) and RNAs of 5.0, 4.3 (doublet), 1.8 and 1.5 kb. By analogy to other retroviruses, the genome-length RNA should function as mRNA for the *gag* gene (coding for the core proteins) and the *pol* gene (coding for reverse transcriptase). The RNAs that are subgenomic in size are produced by splicing of genome-length RNA. In the case of other retroviruses, the *env* gene (coding for the envelope proteins) is contained on an RNA generated by a single splicing event that deletes the *gag* and *pol* regions of the genome, leaving the 5' leader sequence spliced to the *env* coding region. Therefore, a single subgenomic RNA would be expected to code for the *env* gene in visna virus-infected cells. The *env* precursor protein, which in visna virus is 150,000 kilodaltons (40, 44), would require an mRNA at least 4.0 kb in size.

To characterize these RNAs in more detail, they were mapped by using a series of probes specific for different regions of the viral genome. These probes (diagrams in Fig. 1) span the entire genome. Parallel lanes of poly(A)-selected viral RNA from infected cells (800 ng) were electrophoresed on formaldehyde gels, transferred to nylon membranes, and hybridized with ³²P-labeled DNA probes.

Transcription of retroviruses is initiated in the 5' long

terminal repeat, and a cap site situated at the 5' end of the R region is used for both spliced and unspliced mRNAs. A putative cap site has been identified in the nucleotide sequence of visna virus at this location (40). A 5'-specific DNA fragment (Fig. 1, probe A) was used as a hybridization probe on a Northern blot to determine whether transcription of the viral RNAs initiates from the 5' long terminal repeat. Six visna virus-specific mRNAs were detected with this 5'-specific fragment. These were the same six mRNAs detected with a visna virus probe representing the entire viral genome (data not shown). The RNAs include the 9.4-kb genomic RNA as well as four major subgenomic species of 5.0, 4.3 (doublet), 1.8, and 1.5 kb. The fact that all of the mRNAs both contain 5'-specific sequences and are polyadenylated makes it unlikely that any are degradation products of larger RNA species.

Both the *gag*- and *pol*-specific subgenomic probes (Fig. 1,

TABLE 1. RNA probes

RNA size (kb)	Detected with probe ^a :						
9.4	A	B	C	D	E	F	G
5.0	A			D	E	F	G
4.3 (upper)	A				E	F	G
4.3 (lower)	A					F	G
1.8	A				E	F	
1.5	A					F	

^a The locations and sizes of these probes are indicated in Fig. 1.

FIG. 2. Structures of the viral mRNA transcripts deduced from hybridization to different subgenomic probes. Open triangles above the genome indicate the location of consensus splice acceptor sites (22, 40); closed triangles represent the location of consensus splice donor sites (22, 40). Splice donor and acceptor sites which best fit the consensus are shown; other splice sites do exist and may, in fact, be used. The size and location of subgenomic probes are illustrated by the stippled boxes.

probes B and C) hybridized only to the 9.4-kb species. Thus this mRNA species must function as *gag-pol* message as well as the genomic RNA. The subgenomic probe containing *Q*-specific sequences (probe D) extends from a *Bgl*III site at position 5473 to an *Eco*RI site at 5657. The *Q*-specific probe (Fig. 1, probe D) hybridizes to the 9.4- and 5.0-kb species, but not to the 4.3- (doublet), 1.8-, or 1.5-kb bands. Thus, this 5.0-kb mRNA represents a spliced message containing 5' sequences spliced to sequences between probes C and D. Based on its hybridization to probe D it probably utilizes the consensus splice acceptor located at nucleotide 4958 (22, 40).

The sequence of the visna viral genome has been determined by Sonigo et al. (40) and in our laboratory (M. J. Braun, M. A. Gonda, and J. E. Clements, manuscript in preparation) and contains, in addition to ORFs for *gag*, *pol*, and *env*, ORFs between *pol* and *env* referred to as *Q* and *S*. To determine the genetic content of the 1.8- and 1.5-kb mRNAs two small probes were constructed. The first probe (probe E) extends from the *Pst*I site at 5876 (3' end of ORF *Q*) to a second *Pst*I site at 6123 (3' end ORF *S*) (Fig. 1). Although this probe contains 76% of the nucleotides from the *S* ORF, it does not include a consensus splice acceptor (22) located at nucleotide number 6149 (40). To detect small transcripts which may have utilized this splice acceptor, we employed another probe (probe F), which extends from a *Bal*I site at 6214 (5' end of *env*) to a *Hinc*II site at 6650 (within the *env* ORF). Probe E hybridizes to the 9.4-, 5.0-, 4.3- (upper species), and 1.8-kb messages (Fig. 1, lane e; Table 1). It does not, however, detect the lower 4.3-kb species or the 1.5-kb species. However, probe F hybridizes to both the 4.3-kb species and the 1.5-kb mRNA as well as to the other four (Fig. 1, lane f; Table 1).

These results illustrate several features of the 1.8- and 1.5-kb mRNAs. Based on the reactivity of probes E and F and the lack of reactivity of probe G (the *env* probe) (Fig. 1, lane g; Table 1), we conclude that these messages are at least doubly spliced. In addition, a probe situated between probes F and G also fails to hybridize to the small transcripts (data not shown). It is possible that the 1.8-kb message utilizes the

5' splice donor at 556 and splice acceptor at 5881. The 1.5-kb message would use, in addition to the same 5' splice donor, a potential splice acceptor at 6149 (22, 40). This is consistent with its hybridization to probe F and nonreactivity with probe E. A second consensus splice donor sequence (22) located at nucleotide number 6365 (40) near the beginning of the *env* gene and two consensus splice acceptors (22) located between 8233 and 8788 (40) could represent splice sites for the 1.8- and 1.5-kb transcripts. These splice acceptors would allow for the addition of 3' sequences containing the polyadenylation signal and site.

The *env*-specific probe (Fig. 1, probe G) hybridizes to the 9.4- and 5.0-kb species and both species at 4.3 kb but does not detect either the 1.8- or 1.5-kb mRNA. It is curious that there are two mRNAs which contain the *env* sequences. The larger species hybridizes with probe E and thus must utilize the splice acceptor at nucleotide 5881, whereas the smaller species does not hybridize to this probe and must utilize the splice acceptor at nucleotide 6149 (Fig. 1, Table 1). In Fig. 2 these results are shown as a diagram with the putative structure of each mRNA based on its hybridization with the various subgenomic probes. The location of consensus splice donor and acceptor sequences within the viral genome are indicated. Note that every virus-specific transcript can be accounted for and that there is no evidence for any message containing sequences derived solely from a 3' ORF other than the *env* ORF.

FIG. 3. S1 nuclease mapping analysis of the 3' border of the splice junction in viral RNAs. DNA probes from the region of the genome extending from the *Bam*HI site at 5.1 kb to the *Hind*III site at 6.0 kb were end labeled and hybridized to 2 μ g of total RNA from infected SCP cells. After hybridization at 56°C for 18 h, hybrids were digested with 200 U of S1 nuclease per ml and analyzed on 4% acrylamide-8 M urea gels. Lane a contains marker DNA (end-labeled fragments of lambda DNA digested with *Hpa*II and end-labeled fragments of simian virus 40 DNA digested with *Mbo*I). Lane b contains protected DNA fragments after S1 nuclease digestion of the *Bam*HI-*Bgl*III probe with total RNA from infected SCP cells. The sizes of the protected fragments are indicated in the margins. The sizes of the marker lambda fragments are 0.74, 0.44, and 0.41 kb. The sizes of the marker simian virus 40 fragments are 0.95, 0.61, 0.396, 0.384, and 0.237 kb.

S1 nuclease mapping studies on viral RNA. The location of the 5.0-kb mRNA was ascertained by S1 nuclease mapping. From the Northern blot data, this mRNA should be located in the region of the genome between nucleotides 4590 and 5480. A *Bam*HI-*Bgl*II fragment (nucleotides 4845 to 5471) 626 bases long was isolated and end labeled at the *Bgl*II site. When RNA from infected cells was hybridized to this DNA and digested with S1 nuclease, three fragments 530, 480, and 360 bases in size were detected (Fig. 3, lane b). In contrast, RNA from uninfected cells failed to protect the DNA from digestion, and genomic RNA isolated from virions protected only a full-length fragment of DNA (data not shown). The locations of the splice sites defined by S1 nuclease mapping correspond approximately to positions 4941, 4991, and 5111 on the genome map. Though we expected to find a single splice acceptor site in this region corresponding to the spliced RNA, we discovered instead that there were three closely arranged splice acceptor sites within 200 bases of one another. We sequenced the DNA from this region of the genome to see whether splice acceptor consensus sequences were located at or near the positions mapped by S1 nuclease digestion. Matches to the consensus sequence for splice acceptor sites [(T)_nN₁AG/G] (22) were found close to these positions at nucleotides 4955, 4997, and 5120 on the map. The first splice acceptor site at 4955 is an excellent match with the consensus sequence and contains a CT-rich upstream region. The second site at 4997 lacks a CT-rich upstream region; however, in addition to being part of the ORF for the *pol* gene, this region has been identified by others as part of a short, single-stranded gap involved in discontinuous plus-strand synthesis during replication (2), and there may be strong constraints on the sequence in this region. The putative splice site at 5120 is a good match to the consensus sequence, except that it contains an A rather than a G in the last position. However, several of the splice sites identified in HIV by sequencing of cDNA clones derived from spliced RNAs contain this substitution (1). Although the definitive mapping of these splice acceptor sites awaits sequencing of the protected DNA fragments or sequencing of cDNA clones derived from these mRNAs, the S1 nuclease mapping data presented here strongly suggest that at least three splice acceptor sites are utilized in this region of the viral genome to produce a family of RNAs whose function at the present time is unclear.

Temporal pattern of viral transcription. To detect any differential production of viral mRNAs during infection, a time course experiment was done, and poly(A)⁺RNA was isolated at 6, 12, and 24 h after infection. Northern blot analysis was done with a cDNA probe made from visna virus genomic RNA, which has a high specific activity (Fig. 4). As early as 6 h after infection (before cytopathic effect was observable) the full-length 9.4-kb band and the 5.0-, 4.3- (doublet), and 1.8-kb bands were detectable on the Northern blot. The 1.5-kb mRNA was not detectable even after extremely long exposures, although the 1.8-kb mRNA species could be readily detected after exposure of the Northern blot for several days. By 12 h after infection, both the 1.5- and 1.8-kb mRNAs as well as the other three messages were detectable. At 24 h after infection (when dramatic cytopathic effect was observable) all mRNA species were present and were easily detected after short exposures.

Laser densitometry of the autoradiographs was used to quantitate these results. To ensure that equal amounts of RNA were transferred onto each lane of the blot, the probe was removed, and the blot was rehybridized with a chicken actin probe (gift of D. Cleveland) (data not shown). The

FIG. 4. Northern blot hybridization and densitometer data for the time course experiment. The top portion of the figure corresponds to the bottom part of Northern blots and shows the 1.8- and 1.5-kb species hybridized to a cDNA probe (Materials and Methods). Lanes: a, 13-day exposure of 6-h RNA; b, 13-day exposure of 12-h RNA; c, 14-h exposure of 24-h RNA. The data from scanning these X-ray films with a laser densitometer are presented in the bar graph. Peak intensities were reported as absolute area by the densitometer and converted to log area for graphing purposes. The numerical data for the 24-h RNA were converted to 13-day exposure equivalents before graphing. Data are shown for one set of Northern blots; the same relationship was observed in several separate and independent experiments.

quantitative results are presented in the bar graph portion of Fig. 4; quantitation of the area corresponding to the two small transcripts is shown. No significant difference was detected in the levels of the other three transcripts at any time point. The quantitation of the 9.4-kb mRNA is included as an internal control. At 6 h after infection, no peak corresponding to the 1.5-kb message was detectable even when the densitometer was set at its most sensitive settings. This agrees with our visual observations of the film. At 12 h, two peaks were detectable corresponding to the 1.5- and 1.8-kb species. Note that the 1.5-kb message has increased dramatically, but the 1.8-kb message is still present in a greater amount. At 24 h, both messages are present in elevated and approximately equivalent amounts.

DISCUSSION

The highly productive replication of lentiviruses in appropriate cells derived from their natural hosts is one of the unique biological properties of this group of retroviruses. Visna virus, the biological prototype and the most easily cultivated virus of this group, therefore provides an excellent model for the study of regulation of gene expression of lentiviruses. For this reason, a transcriptional analysis of viral RNA produced from the visna viral genome under permissive conditions of growth in cultured SCP cells was carried out. In addition to the 9.4-kb genome-length RNA, which by analogy to other retroviruses codes for the *gag* and *pol* proteins, four other distinct viral mRNA species were

identified. All subgenomic RNAs contain a short 5' leader spliced to different regions of the genome 3' of the *pol* gene. The 5.0-kb species is singly spliced and contains the entire *Q* ORF. If this message uses the splice acceptor at nucleotide 4955 (22, 40), initiation of translation could occur at the methionine codon located at nucleotide 5223, generating a 230-amino-acid polypeptide that would be both hydrophilic and basic in nature (40). Such a base composition is consistent with the hypothesis that the *Q* gene product may interact with RNA or DNA and have a regulatory function.

The two mRNAs of 4.3 kb have different genetic structures; the larger band contains sequences from the *S* ORF, whereas the smaller species contains few or none of these sequences. Two splice acceptors are present in this region (at nucleotides 5881 and 6149) (22, 40) and probably are utilized in these mRNAs. Either or both of these transcripts could function as the *env* mRNA. However, the larger transcript contains the entire *S* ORF and thus may function to produce a protein product from this region. Such a protein would be approximately 96 amino acids in length and would be hydrophilic and basic in nature (40).

The 1.8- and 1.5-kb transcripts are (at least) doubly spliced, containing sequences from the 5' end of the genome spliced to sequences derived between *pol* and *env* which are then spliced to sequences at the 3' end of *env*. The human lentivirus HIV produces analogous transcripts (32). In HIV, one of these transcripts in the 1.8- to 2.0-kb size range yields the *trans*-acting *tat*-III protein (1, 36, 38). The coding exons for this protein are derived from the 5' half of the *S* ORF and sequences in the 3' part of the *env* ORF (36, 38). By comparison, it is possible that the visna virus 1.8-kb transcript will produce the visna virus analog of the HIV *tat*-III protein. The 1.5-kb transcript contains sequences from the 3' half of the *S* ORF spliced to sequences in the *env* ORF. A second *trans*-acting factor for HIV (*art* or *trs*) has recently been defined (7, 37). Although no specific transcript for this protein has been fully characterized, it is known to be multiply spliced and in the 1.8- to 2.0-kb size range (7, 37). In addition, the coding exons for the *art* protein are derived from the 3' half of the *S* ORF and sequences in the 3' part of the *env* ORF (37). It is therefore possible that the visna virus 1.5-kb transcript encodes the visna virus analog of the HIV *art* or *trs* protein.

In addition, our data suggest that there is a differential production of the small transcripts during the course of a visna virus lytic infection. Transcription occurs as early as 6 h after infection, yet only four of the five spliced species are detectable. The smallest 1.5-kb transcript cannot be detected at this initial time point. However, this inequality disappears by 12 h after infection, when all spliced species are present in approximately equal amounts. Thus, there appears to be a delay in the production of the 1.5-kb transcript. Further investigation is required to determine whether this delay is important in the viral life cycle. The protein encoded by this transcript may not be required until late in infection and could, in fact, be detrimental to the infectious process if present early. Thus, the production of this transcript may be regulated. The data so far do not allow us to determine whether such regulation would occur at a splicing or message stability level. Also, the requirements for the production of this transcript need to be determined. It is possible that a viral protein encoded by one of the other messages is necessary for its production. Alternatively, the action of one or more cellular proteins induced by a viral protein or some other aspect of the viral infection may be required for the formation of this spliced transcript. These considerations

stress that it will be important in the future to fully characterize the nature of the proteins encoded by the spliced transcripts and to define how they influence and interact with the numerous factors affecting lentiviral infections.

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