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Temperature-Sensitive Viral RNA Expression in Moloney Murine Sarcoma Virus ts110-Infected Cells†

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We examined the *mos*-specific intracellular RNA species in 6m2 cells, an NRK cell line nonproductively infected with the ts110 mutant of Moloney murine sarcoma virus. These cells present a normal phenotype at 39°C and a transformed phenotype at 28 or 33°C, expressing two viral proteins, termed P85$^{gag\text{-}mos}$ and P58^{gag}, at 28 to 33°C, whereas only P58^{gag} is expressed at 39°C. It has been previously shown that 6m2 cells contain two virus-specific RNA species, a 4.0-kilobase (kb) RNA coding for P58^{gag} and a 3.5-kb RNA coding for P85$^{gag\text{-}mos}$. Using both Northern blot and S1 nuclease analyses, we show here that the 3.5-kb RNA is the predominant viral RNA species in 6m2 cells grown at 28°C, whereas only the 4.0-kb RNA is detected at 39°C. During temperature shift experiments, the 3.5-kb RNA species disappears after a shift from 28 to 39°C and is detected again after a shift back from 39 to 28°C. By Southern blot analysis, we have detected only one ts110 proviral DNA in the 6m2 genome. This observation, as well as previously published heteroduplex and S1 nuclease analyses which showed that the 3.5-kb RNA species lacks about 430 bases found at the *gag* gene-*mos* gene junction in the 4.0-kb RNA, suggests that the 3.5-kb RNA is a splicing product of the 4.0-kb RNA. The absence of the 3.5-kb RNA when 6m2 cells are grown at 39°C indicates that the splicing reaction is thermosensitive. The splicing defect of the ts110 Moloney murine sarcoma virus viral RNA in 6m2 cells cannot be complemented by acute Moloney murine leukemia virus superinfection, since no 3.5-kb ts110 RNA was detected in acutely superinfected 6m2 cells maintained at 39°C. The spliced Moloney murine leukemia virus *env* mRNA, however, is found in acutely infected cells maintained at 39°C, suggesting that the lack of ts110 viral RNA splicing at 39°C is not due to an obvious host defect. In sharp contrast, however, 6m2 cells chronically superinfected with Moloney murine leukemia virus produce a 3.5-kb RNA species at 39°C as well as at 28°C and contain proviral DNAs corresponding to the two viral RNA species. In these cells, the splicing of the 4.0-kb RNA appears to remain thermosensitive; however, additional 3.5-kb RNA can be independently transcribed from its own proviral DNA at either permissive or nonpermissive temperatures, thus bypassing the usual splicing requirement.

Moloney murine sarcoma virus (MoMuSV) is a replication-defective virus containing a transforming gene (termed v-*mos*) acquired by recombination with a normal cellular sequence (c-*mos*). By UV irradiation of MoMuSV 349 virions, a subclone of the MoMuSV 124, Blair et al. obtained a thermosensitive mutant, designated MoMuSV ts110 (2). An NRK cell line nonproductively infected by this virus (6m2 cells) shows a transformed phenotype when grown at 28 or 33°C but reverts phenotypically when grown at 39°C (5a, 12). 6m2 cells contain two viral proteins, of 58,000 and 85,000 daltons (8, 23), both of which are present at the permissive temperature. However, only P58 is detected at 39°C. Immunological and structural studies have shown that P58 is a truncated *gag* polypeptide, whereas P85 is a *gag-mos* fusion protein (8, 14). A kinase activity has been detected associated with P85$^{gag\text{-}mos}$ at 28 or 33°C; however, this activity is lost at 39°C (11, 12).

6m2 cells superinfected with Moloney murine leukemia virus (MoMuLV) produce pseudotype viruses harboring two v-*mos*-containing RNA species, about 4.0 and 3.5 kilobases (kb) long (9). In vitro translation of these RNAs has shown that the 4.0-kb RNA coded for P58^{gag}, whereas the 3.5-kb RNA was translated into P85$^{gag\text{-}mos}$ (10, 14). Gallick et al. (5a) have previously shown that the 3.5-kb RNA species was absent at nonpermissive temperatures and was only detected at temperatures at which the cells were morphologically transformed. By heteroduplex studies, Junghans et al. (10) have shown that the 4.0-kb RNA species was derived from the MoMuSV 124 genome by a central deletion of about 1,500 bases. S1 mapping analyses have located the 5′ and 3′ borders of this deletion approximately at positions 2409 and 3883, respectively, by the numbering system of Van Beveren et al. (22). The 3.5-kb RNA possesses a more extended deletion on both sides. The 5′ border is approximately at position 2017 near a consensus splice donor site, and the 3′ border is located at nucleotide 3936 near a consensus splice acceptor site (15).

In this work we present further evidence that the 3.5-kb RNA is a splicing product of the 4.0-kb RNA and that the splicing reaction is thermosensitive. We also show that this defect is not host cell related but is more likely to be an inherent property of the ts110 MoMuSV viral RNA itself. Finally, we have examined the virus-specific RNA species in two cell lines containing revertants of MoMuSV ts110 and presenting a transformed phenotype at 28°C as well as 39°C.

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MATERIALS AND METHODS

Cells. The nonproducer NRK cell line infected with ts110 MoMuSV (6m2) was grown in McCoy 5a medium containing 15% (vol/vol) fetal calf serum. The 206-2IC producer cell line was generated by superinfection of 6m2 with the IC strain of MoMuLV (5). Spontaneous revertant 54-5A4 cells were obtained from 6m2 cells by recloning them in agar at 39°C. The revertant 204-3 clone was obtained by superinfection of 6m2 cells by Simian sarcoma-associated virus, after which the cell-free supernatant containing ts110 MoMuSV-simian sarcoma-associated virus pseudotypes was used to infect NRK cells and a nonproducer cell clone was selected. Both cell lines grow well in agar at 33 and 39°C and produce no detectable virus (20). Generally, all of the cells listed above were grown at 33°C and transferred to the experimental temperature 2 days before RNA extraction.

For the acute infection of 6m2 cells, fresh medium was added to MoMuLV-producing cells, harvested after 6 to 8 h, and filtered through 0.45-μm-pore-size filters. A 20-ml portion of this cell-free supernatant containing 10 μg of polybrene per ml was added per T150 flask of 20% confluent 6m2 cells equilibrated at 39°C for 6 h. After 6 h, an additional 30 ml of fresh medium was added to the flasks, and the cells were grown at 39°C overnight. Polybrene-containing medium was replaced by fresh medium, and the cells were allowed to grow at 39°C for the next 2 days. Total RNA was extracted for the experiment at 3 days postinfection. For the experiment at 15 days postinfection, cells were grown at 33°C and shifted to 39°C for the 3 days preceding RNA extraction.

Northern blot analysis. Total RNA was extracted by the hot phenol procedure (7) and selected twice on oligo-deoxythymidylate cellulose (1). Polyadenylate [poly(A)]-containing RNA was then denatured by heating for 20 min at 50°C in 50% dimethylsulfoxide–1 M glyoxal–10 mM sodium phosphate buffer (pH 7) (13) and subjected to electrophoresis in 1% agarose–10 mM phosphate buffer (pH 7) gel at 50 V for 10 h. Approximately 5 μg of poly(A)-containing RNA was loaded per lane. RNA was transferred to nitrocellulose sheets by the procedure of Thomas (21), and virus-specific RNA species were detected by hybridization to a ^{32}P-labeled DNA probe (16). The prehybridization, hybridization, and washing procedures were as described previously (6, 21). Ribosomal 28S and 18S RNA were run in a parallel lane as size markers and stained with acridine orange (13).

S1 nuclease analysis. S1 nuclease analyses were done essentially as described previously (15). Briefly, the 5′-end-labeled 359-base-pair (bp) insert from pBA.36 was hybridized to 10 μg of total cellular RNA from 6m2 cells grown at 39, 33, or 28°C in 80% formamide–40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.8)–400 mM NaCl–1 mM EDTA at 56°C for 3 h, digested with S1 nuclease (200 U/ml) at 37°C for 30 min, phenol extracted, and ethanol precipitated. The digests were dissolved in 20 μl of 10 M urea–1 mM EDTA–0.015% bromocresol green, heated to 50°C for 5 min, and analyzed on a 4% polyacrylamide gel containing 8 M urea. After electrophoresis, the gels were fixed with 50% methanol–7% acetic acid, dried, and autoradiographed.

Southern blot analysis. Cellular DNA was extracted as previously described (15). Briefly, cells were rinsed in isotonic buffer and lysed in 2% sodium dodecyl sulfate–8 M urea–0.35 M NaCl–10 mM Tris (pH 8). The cell lysate was extracted with phenol-chloroform and ethanol precipitated. Nucleic acids were then redissolved in water and digested with 50 μg of RNase per ml at 37°C for 60 min and 100 μg of proteinase K per ml for 120 min at 37°C, re-extracted with phenol-chloroform, and ethanol precipitated. DNA was digested by the appropriate restriction enzyme, subjected to electrophoresis on 0.8% agarose gel in 50 mM Tris (pH 8.3)–40 mM sodium acetate–2 mM EDTA at 40 V for 16 h, and transferred to nitrocellulose as described by Southern (18). Prehybridization and hybridization were done in the same buffers as those used for RNA blots. After hybridization, the filters were washed four times with 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate)–0.1% sodium dodecyl sulfate and twice with 0.1× SSC–0.1% sodium dodecyl sulfate at 37°C, dried, and autoradiographed.

DNA probes. Plasmid pK101 is a pKC7 plasmid into which a 1.3-kb *Bgl*II-*Hin*dIII *mos*-containing DNA fragment has been inserted (10). The insert was excised by a double digestion with *Xba*I and *Hin*dIII.

As a MoMuLV DNA probe, we used the pMLV1 plasmid kindly provided by Inder Verma (Salk Institute). The insert is a permuted 8.5-kb MuLV DNA inserted at the *Hin*dIII site of pBR322.

The inserts were separated from plasmid DNA by digestion with the appropriate restriction enzyme(s), fractionation on agarose gel, and purification by electroelution and absorption to Schleicher & Schuell Elutip-D columns. Nick translation was done by the method of Rigby et al. (16). Approximately 10^6 cpm of ^{32}P-labeled DNA was used per lane of RNA or DNA for hybridization.

RESULTS

Viral RNA species in 6m2 cells. 6m2 cells are known to contain both P85$^{gag\text{-}mos}$ and P58^{gag} at 28 or 33°C and only P58^{gag} at 39°C (5a, 9). It has been previously shown that P85$^{gag\text{-}mos}$ synthesis was discontinued within 4 h after a temperature shift from 33 to 39°C (12, 19; J. P. Horn, Ph.D. thesis, University of Texas, Houston, 1982). Moreover, pulse-chase experiments suggested that P85$^{gag\text{-}mos}$ was turned over more rapidly at 39°C than at 33°C (19). In reverse temperature shift experiments (39 to 33°C), P85$^{gag\text{-}mos}$ synthesis was found to resume after about a 3-h delay; however, the addition of actinomycin D was found to abolish the reappearance of P85$^{gag\text{-}mos}$ (9). Since the absence of detectable P85$^{gag\text{-}mos}$ at the nonpermissive temperature seemed to be the consequence of two independent phenomenona (i.e., the absence of P85$^{gag\text{-}mos}$ mRNA and instability of the protein itself), we found that it was of interest to study the virus-specific RNA species in 6m2 cells during temperature shift experiments.

As a control for the relative quantity of 4.0- and 3.5-kb RNA species in 6m2 cells equilibrated at different temperatures, Northern blot analysis of intracellular virus-specific RNA extracted from 6m2 cells grown for 48 h at 28, 33, 37, or 39°C was performed (Fig. 1). It can be clearly seen that at 39°C the only *mos*-containing RNA species detected was the 4.0-kb RNA (lane 4). At 37 and 33°C, increasing amounts of the 3.5-kb RNA could be detected, but the 4.0-kb RNA was still the predominant species (lanes 2 and 3). RNA extracted from 6m2 cells grown at 28°C (lane 1), however, contained a drastically increased amount of the 3.5-kb RNA species and an accompanying decrease in the level of the 4.0-kb RNA.

We have previously described an S1 nuclease assay capable of distinguishing between the 4.0- and 3.5-kb RNAs by using a 5′-end-labeled probe (pBA.36) which hybridizes across the *gag* gene-*mos* gene junction point (15). In this assay, the 4.0- and 3.5-kb RNAs will protect 175 and 122 bases of pBA.36, respectively, from digestion by S1 nuclease (15; see inset under Fig. 2). Nash et al. have previously

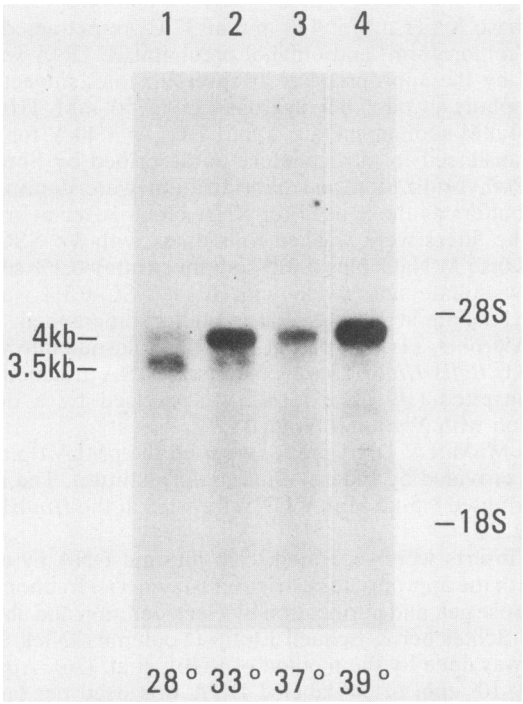

FIG. 1. Virus-specific RNA in 6m2 cells grown at different temperatures. Poly(A)-containing RNA from each cell preparation was analyzed by electrophoresis on agarose gels, transferred to nitrocellulose, and hybridized to a ^{32}P-labeled *mos*-specific probe. Lanes: 1, RNA from 6m2 cells grown at 28°C; 2, RNA from 6m2 cells grown at 33°C; 3, RNA from 6m2 cells grown at 37°C; 4, RNA from 6m2 cells grown at 39°C.

used this assay to estimate the amounts of 4.0- and 3.5-kb RNA present in 6m2 cells at 33 and 39°C and their kinetics during temperature-shift experiments (15). In the present work we wished to extend these earlier findings to the growth conditions at 28°C. Accordingly, the 5′-end-labeled 359-bp pBA.36 insert was hybridized under DNA-excess conditions to RNA from 6m2 cells grown at 33°C and shifted to 28 or 39°C. Both the 175-base (4.0-kb-RNA-specific) and 122-base (3.5-kb-RNA-specific) fragments were protected by RNA from 6m2 cells grown at 33°C, indicating the presence of both of these RNAs (Fig. 2, lane 1). RNA from 6m2 cells grown at 39°C, however, could protect only the 175-base fragment (lane 2), suggesting that only the 4.0-kb RNA is present at 39°C. Finally, RNA from 6m2 cells grown at 28°C protected more of the 122-base fragment than the 175-base fragment (lane 3), suggesting, as did the Northern blots, that the 3.5-kb RNA is the predominant ts110 viral RNA species in 6m2 cells grown at 28°C.

Evidence on the dynamics of the ts110 viral RNA species in 6m2 cells during temperature shift experiments is presented in Fig. 3. The RNA shown in lane 1 was extracted from 6m2 cells grown at 28°C for 48 h and contained a greater amount of 3.5-kb RNA than 4.0-kb RNA. The ratio of 4.0-kb RNA to 3.5-kb RNA in this preparation was the same as that observed in Fig. 1, lane 1. RNA extracted from 6m2 cells shifted up to 39°C for 6 and 24 h is shown in Fig. 3, lanes 2 and 3, respectively. As can be seen, the relative quantity of 3.5-kb RNA was dramatically reduced after a 6-h shift to 39°C, and after 24 h at 39°C, there was no detectable 3.5-kb RNA in the 6m2 cells. Reshifting cells previously shifted to 39°C for 24 h back to 28°C had, predictably, the reverse effect. RNA extracted from 6m2 cells reshifted to 28°C for 6 h after a 24-h incubation at 39°C is shown in lane 4. It can be seen that after 6 h at 28°C, the 3.5-kb RNA species was again detected. After a 24-h reshift to 28°C, the pattern of virus-specific RNA species (lane 5) was the same as that observed in 6m2 cells grown at 28°C for 48 h (lane 1), the 3.5-kb RNA being the predominant viral RNA species.

Another RNA species of about 2.3 kb in size could be frequently seen in 6m2 cells grown at 28°C (Fig. 3, lanes 1 and 5). However, this species was not detected in every experiment (Fig. 1, for example). Since we detected no viral proteins other than P58^{gag} and P85$^{gag\text{-}mos}$ in 6m2 cells, it therefore seems unlikely that the 2.3-kb RNA codes for an as yet unrecognized viral protein. Our present interpretation is that the 2.3-kb RNA is a degradation product of the 4.0-kb RNA at a specific site.

Viral RNA species in chronically and acutely superinfected 6m2 cells. In previous studies, Horn et al. have observed that 6m2 cells chronically superinfected with MoMuLV appeared to produce P85$^{gag\text{-}mos}$ at both 33 and 39°C, although the phenotype of these cells remained temperature sensitive (9). It therefore seemed possible that superinfection with MoMuLV somehow altered the temperature dependence of the transcriptional defect. To test this possibility, we performed Northern blot analysis on RNA from 6m2 cells chronically and acutely superinfected with MoMuLV. Since 28 and 39°C appeared to represent the extremes of the situation in 6m2 cells (more 3.5- than 4.0-kb RNA at 28°C;

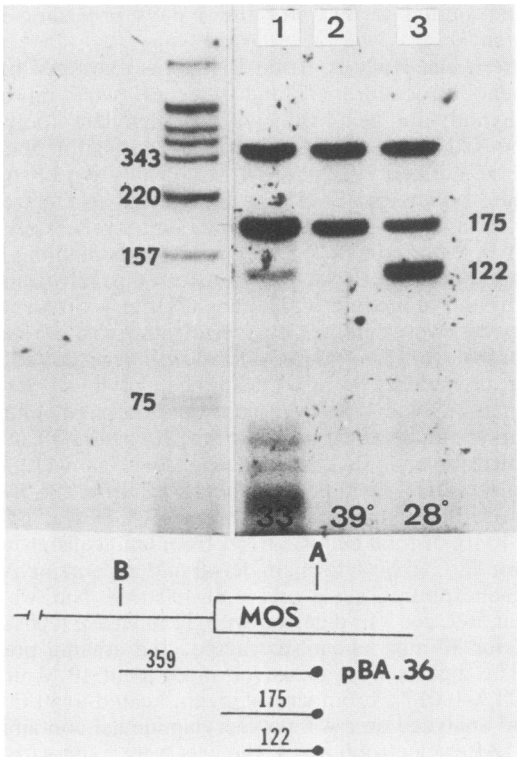

FIG. 2. S1 analysis of RNA in 6m2 cells. The same poly(A)-containing RNA preparations described in Fig. 1 were hybridized to the 5′ ^{32}P-labeled pBA36 359-bp insert and digested with S1 nuclease, and the protected fragments were analyzed on a 4% polyacrylamide gel containing 8 M urea. Protection of pBA36 DNA by RNA from 6m2 cells grown at 33°C (lane 1), 39°C (lane 2), and 28°C (lane 3). The unlabeled lane contains a 5′-end-labeled pBR322/*Hin*fI digest. B, *Bgl*II site; A, *Ava*I site.

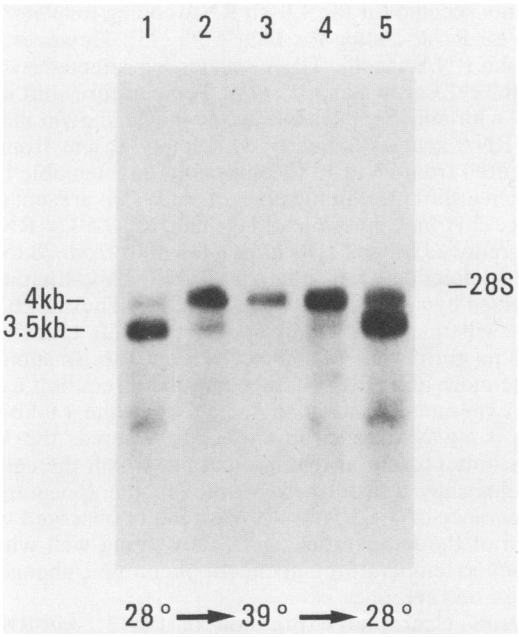

FIG. 3. Virus-specific RNA in 6m2 cells during temperature shift experiments. Poly(A)-containing RNA was analyzed as described in Fig. 1 and hybridized to a ^{32}P-labeled *mos*-specific probe. Lanes: 1, RNA extracted from 6m2 cells grown at 28°C for 48 h; 2, RNA extracted from 6m2 cells grown at 28°C and shifted up to 39°C for 6 h; 3, RNA extracted from 6m2 cells grown at 28°C and shifted up to 39°C for 24 h; 4, RNA extracted from 6m2 cells grown at 28°C, shifted up to 39°C for 24 h, and shifted back to 28°C for 6 h; 5, RNA extracted from 6m2 cells grown at 28°C, shifted up to 39°C for 24 h, and shifted back to 28°C for 24 h.

only the 4.0-kb RNA at 39°C), we performed the following experiments with cells grown at these temperatures only.

The 206-2IC cell line is a 6m2 cell line chronically infected with the IC strain of MoMuLV. Northern blot analysis of virus-specific RNA species in these cells is presented in Fig. 4. The RNA species detected with a *mos*-specific probe in 206-2IC cells grown at 28 and 39°C are shown in panel A. As was found in 6m2 cells grown at 28°C, in 206-2IC cells grown at 28°C, the 3.5-kb RNA was the predominant species (Fig. 4A, lane 1). In contrast to 6m2 cells, however, in which only the 4.0-kb RNA was observed at 39°C, 206-2IC cells grown at 39°C contained both the 4.0- and 3.5-kb RNA species (Fig. 4A, lane 2), although the 4.0-kb RNA was predominant. To detect the helper MoMuLV RNA in 206-2IC cells, we used a MoMuLV-representative probe (panel B). In addition to the 4.0- and 3.5-kb *mos*-containing RNA species, we detected the 8.3-kb MoMuLV genomic RNA, as well as the 3.0-kb spliced *env* mRNA at both 28 (Fig. 4B, lane 1) and 39°C (Fig. 4B, lane 2). We loaded less poly(A)-containing RNA (about 1 μg) in lane 2 than in lane 1. This explains the lower amount of 8.3-kb RNA detected at 39°C (Fig. 4B, lane 2).

In contrast to 206-2IC cells, 6m2 cells acutely superinfected with MoMuLV expressed a different group of viral RNAs. For this experiment, 6m2 cells were first exposed to culture fluid containing MoMuLV and then were grown at 39°C for the 3 days preceding RNA extraction. RNA was extracted 3 and 15 days after superinfection, oligodeoxythymidylate selected, and analyzed on agarose gels. After blotting, the nitrocellulose sheet was successively hybridized with a *mos*-specific probe (Fig. 5, panel A) and a MoMuLV-representative probe (Fig. 5, panel B). It can clearly be seen that the 4.0-kb RNA species was the only *mos*-containing RNA detected at 39°C either 3 (Fig. 5A, lane 1) or 15 days (Fig. 5A, lane 2) after superinfection. Both the 8.3-kb MoMuLV genomic RNA and the 3.0-kb *env* mRNA (Fig. 5B, lanes 1 and 2) are present in these cells at both the time points after superinfection.

Proviral DNA in 6m2 and 206-2IC cells. Since two ts110 MoMuSV-specific RNA species were observed in 6m2 and chronically infected 206-2IC cells, it was important to determine how many ts110 MoMuSV proviruses were present in these cell lines. Accordingly, we performed Southern blot analysis of cellular DNA digested with *Sst*I, a restriction enzyme known to cut only once in the long terminal repeat of MoMuSV (22). When hybridized with a *mos*-specific DNA probe, the blot will show the exact size of the integrated provirus, minus one long terminal repeat (Fig. 6). The hybridization of the *mos*-specific probe with high-molecular-weight DNA extracted from uninfected NRK cells is shown in lane 1. A 2.65-kb DNA fragment representing the rat c-*mos* gene can be seen. In 6m2 cellular DNA, in addition to the 2.65-kb c-*mos* DNA fragment, another DNA fragment ca. 3.85 kb in size was detected (lane 2). There was therefore only one provirus in 6m2 cells which corresponded in size to the size of the ts110 4.0-kb RNA. However, in cellular DNA extracted from 206-2IC cells, three DNA fragments 3.85, 3.35, and 2.65 kb in size could be detected (Fig. 5, lane 3). The 3.85- and 2.65-kb DNAs represented the ts110 provirus and the c-*mos* gene, respectively. The 206-2IC-unique 3.35-kb *Sst*I fragment, clearly not present in 6m2 cells, corresponded in size to the 3.5-kb *mos*-specific RNA species. It therefore seems that chronically infected 206-2IC cells contain an integrated copy of the ts110 3.5-kb RNA, which can be transcribed at both 28 and 39°C.

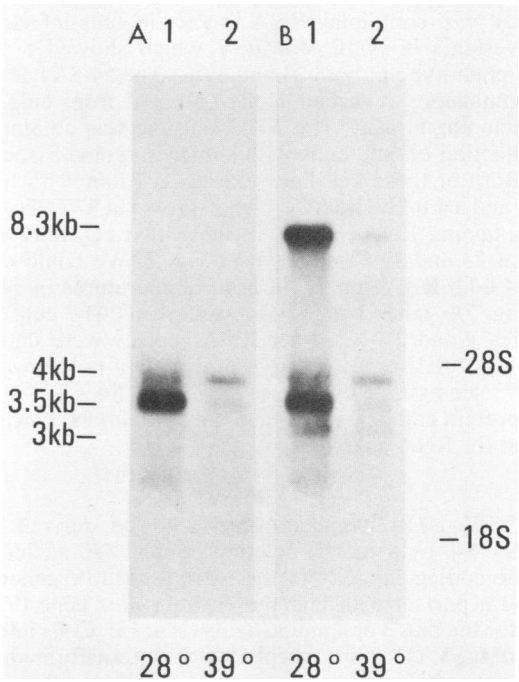

FIG. 4. Virus-specific RNA in the ts110 MoMuSV producer 206-2IC cell line. Poly(A)-containing RNA was analyzed by electrophoresis as described in Fig. 1 and hybridized to a *mos*-specific DNA probe (panel A) or a MoMuLV-specific DNA probe (panel B). Lanes: 1A and B, RNA extracted from 206-2IC cells grown at 28°C; 2A and B, RNA extracted from 206-2IC cells grown at 39°C.

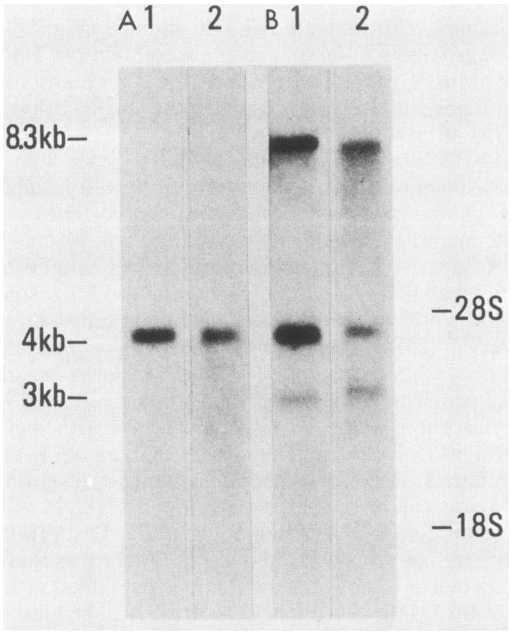

FIG. 5. Virus-specific RNA in 6m2 cells acutely superinfected with MoMuLV. 6m2 cells growing at 33°C were superinfected with MoMuLV and shifted to 39°C for 3 days before the extraction of RNA. Poly(A)-containing RNA was analyzed by agarose gel electrophoresis as described for Fig. 1 and hybridized to a *mos*-specific DNA probe (panel A) or a MoMuLV-specific DNA probe (panel B). Lanes: 1A and B, RNA extracted from 6m2 cells grown at 39°C for 3 days after superinfection with MoMuLV; 2A and B, RNA extracted from 6m2 cells 15 days after superinfection with MoMuLV.

Viral RNA species in two cell lines infected with revertants of ts110 MoMuSV. In addition to 6m2 and 206-2IC cells, we examined *mos*-containing RNA species in cells infected by two revertants of ts110 MoMuSV which showed a transformed phenotype at both 33 and 39°C. The 54-5A4 cell line is a spontaneous revertant clone obtained from 6m2 cells after recloning in agar. The 204-3 cell line was obtained by superinfection of 6m2 cells with simian sarcoma-associated virus. Both of these cell lines express a P100$^{gag\text{-}mos}$ fusion protein and a 4.0-kb viral RNA when grown at 37°C (20). The *mos*-containing RNA species in these two cell lines when grown at 28 and 39°C is shown in Fig. 7. We could detect only a 4.0-kb RNA species at both temperatures in 54-5A4 cells (Fig. 7A, lanes 1 and 2), as well as in 204-3 cells (Fig. 7B, lanes 1 and 2). No other RNA species were detected with a MoMuLV-representative probe (data not shown). It therefore seems that in the revertant cell lines a *gag-mos* fusion protein can be expressed from a 4.0-kb RNA without the need for RNA splicing.

DISCUSSION

MoMuSV ts110 has been shown to be derived from MoMuSV 349 by a central deletion of ca. 1,480 nucleotides (10). The coding capacity of the ts110 MoMuSV genome is p15, pp12, part of p30, and the entire v-*mos* gene (9, 14), except for the first 3 or 4 amino acids (14a, 15). Cells infected with MoMuSV ts110 are morphologically transformed at 28 to 33°C but rapidly resume a normal morphology after a shift to 39°C (4, 5a). These cells contain two viral proteins, P58^{gag} and P85$^{gag\text{-}mos}$, at temperatures permissive for transformation (28 to 33°C) but contain only P58^{gag} at 39°C (5a, 8). Nash et al. have previously shown by S1 nuclease mapping that RNA from 6m2 cells grown at 33°C will protect DNA fragments specific for the 4.0-kb RNA coding for P58^{gag} and the 3.5-kb RNA coding for P85$^{gag\text{-}mos}$ (15). However, only the 4.0-kb RNA-specific DNA species is protected by RNA from 6m2 cells grown at 39°C (15). Temperature shift experiments with the S1 nuclease assay have shown that the 3.5-kb RNA species is nearly completely absent from 6m2 cells shifted from 33 to 39°C for 4 h and undetectable by 8 h after such a shift (15). In the present study, we present direct evidence by blot hybridization that the 3.5-kb RNA is greatly reduced in 6m2 cells after a 6-h shift from 28 to 39°C and is no longer detected after a 24-h shift. This situation can be reversed by a shift back from 39 to 28°C. The 3.5-kb RNA species will reappear within 6 h of a reshift to 28°C and reaches its normal level in 6m2 cells after 24 h. In summary, both the more indirect S1 assays and the direct blot hybridization experiments show convincingly that the 4.0-kb RNA species is always present in 6m2 cells, whereas the 3.5-kb RNA is only present at temperatures at which the cells are morphologically transformed. Moreover, the appearance or disappearance of the 3.5-kb RNA is readily observed within 4 to 6 h of the temperature shift, correlating well with the documented temperature-dependent phenotypic changes observed in this cell line.

It seems clear that expression of the 3.5-kb RNA is generated by posttranscriptional modification of the 4.0-kb RNA, since Southern blot analyses of 6m2 cellular DNA

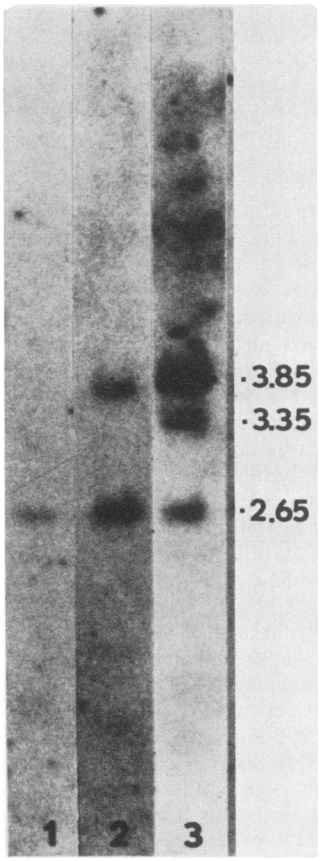

FIG. 6. *Mos*-containing proviral DNA in NRK-2, 6m2, and 206-2IC cellular DNA. Cellular DNA from NRK-2, 6m2, or 206-2IC cells was digested with *Sst*I, analyzed on a 0.8% agarose gel, blotted onto nitrocellulose, and hybridized to a ^{32}P-labeled *mos* probe. Lanes: 1, *Sst*I-digested NRK-2 DNA; 2, *Sst*I-digested 6m2 DNA; 3, *Sst*I-digested 206-2IC DNA.

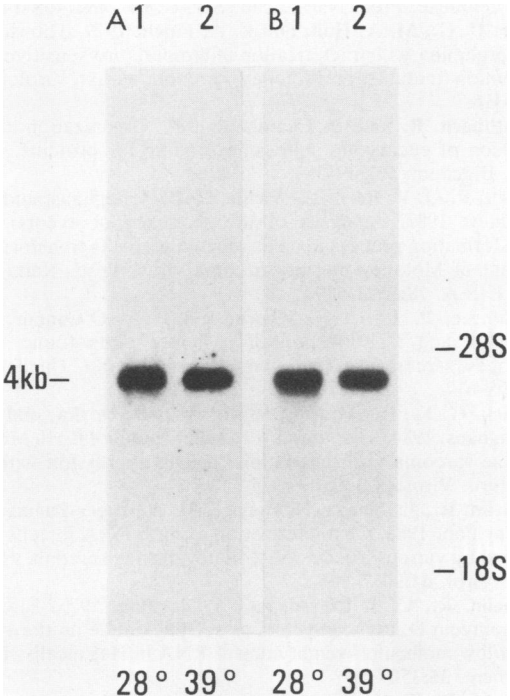

FIG. 7. Virus-specific RNA in two cell lines infected with revertants of ts110 MoMuSV. Poly(A)-containing RNA from 54-5A4 or 204-3 cells was analyzed by agarose gel electrophoresis as described for Fig. 1 and hybridized to a ^{32}P-labeled *mos*-specific probe. Panel A, RNA extracted from 54-5A4 cells grown at 28°C (lane 1) or at 39°C (lane 2); panel B, RNA extracted from 204-3 cells grown at 28°C (lane 1) or at 39°C (lane 2).

have shown that there is a single ts110 MoMuSV proviral DNA in this cell line. Thus, the 4.0-kb and the 3.5-kb RNA species appear to be different transcripts of the same proviral DNA in 6m2 cells. Our previous work has already suggested strongly that the 3.5-kb RNA is derived posttranscriptionally from the 4.0-kb RNA by a temperature-sensitive splicing event, since Nash et al. (15) have shown by S1 nuclease mapping analysis that the *gag-mos* junction point in the 3.5-kb RNA was in the vicinity of splice consensus donor and acceptor sites (3, 17). In addition, recent nucleotide sequencing experiments (14a) have shown directly that the *gag* and *mos* genes are joined out of frame in the 4.0-kb RNA and that this RNA can only be translated into a truncated *gag* gene precursor polypeptide. These same sequencing experiments have also established that the 3.5-kb RNA is derived from the 4.0-kb RNA by the excision of 431 bases bounded exactly by splice donor and acceptor sites. Taken together, these results indicate that the 3.5-kb RNA species is a splicing product of the 4.0-kb genomic RNA. The splicing reaction occurs optimally at 28°C and partly at 33 and 37°C and is inhibited at 39°C.

Another interpretation that could explain the low level of the 3.5-kb RNA at 33°C and its rapid disappearance at 39°C is that it is heat labile and that the lack of the 3.5-kb RNA at restrictive temperatures is due to rapid degradation of the RNA. Such specific instability of the 3.5-kb RNA seems unlikely, since it is readily detected at 39°C (although at a lower amount than at 28°C) in the 206-2IC cell line (Fig. 4A and B, lanes 2). It must be emphasized that 206-2IC cells are 6m2 cells mass infected with MuLV. The producer cell was not recloned.

In nonproducer 6m2 cells, the production of the 3.5-kb RNA is tightly controlled by the growth temperature. However, in contrast to this situation, 6m2 cells chronically infected by MoMuLV (the 206-2IC cell line) produce the 3.5-kb RNA species at 39°C as well as at 28°C. It therefore initially seemed that MoMuLV superinfection could perhaps complement the transcriptional defect in 6m2 cells by supplying certain viral factors absent in MoMuSV ts110-infected cells. Our findings presented here clearly indicate, however, that this is not the case. In 6m2 cells acutely infected with MoMuLV and monitored for 2 weeks, the 3.5-kb RNA species was not produced in cells grown at 39°C. It therefore appears that after acute superinfection, the 4.0-kb ts110-specific RNA cannot be spliced at 39°C and that the presence of the 3.5-kb RNA species in chronically superinfected 6m2 cells grown at that temperature cannot be readily explained by MoMuLV complementation. However, a comparison of the proviral DNAs in 6m2 and chronically infected 206-2IC cells provides a possible explanation for the temperature independence of 3.5-kb RNA production in chronically infected 206-2IC cells. As noted above, *Sst*I-digested 6m2 DNA appears to contain a single 3.85-kb MuSV ts110-related proviral DNA and a 2.65-kb c-*mos* fragment. In contrast, 206-2IC DNA digested with *Sst*I contains three DNA fragments hybridizing to the *mos*-specific probe. In addition to the 2.65-kb rat c-*mos* and the 3.85-kb candidate ts110 DNA fragment detected in 6m2 cells, a unique DNA fragment of about 3.35 kb in size is present. As the *Sst*I enzyme is known to cut once in the MoMuSV long terminal repeat, generating fragments of the exact size (minus one long terminal repeat) of the integrated proviruses, our interpretation is that the 3.35-kb DNA fragment represents an integrated DNA copy of the 3.5-kb RNA species. This interpretation is supported by very recent data (14a), which show that the 3.85-kb DNA possesses a restriction map consistent with a 1.5-kb deletion between the *gag* and *mos* genes and that the 400 to 500 bases present in the 3.85-kb DNA and absent from the 3.35-kb DNA correspond very closely to the 430 bases spliced from the 4.0-kb RNA to form the 3.5-kb RNA. We theorize that, during the numerous cycles of infection undergone by the 206-2IC cell line, viruses containing the 3.5-kb RNA species could have given rise to reverse transcripts which became integrated into the 206-2IC genome. (The same phenomenon seems to have happened to the 3.85-kb proviral DNA, which is much more intense in 206-2IC cells than in 6m2 cells.) Given such an event, for which there is direct evidence in the 206-2IC cell line, the 4.0 kb and the 3.5 kb would be then transcribed independently from their respective proviral DNAs at 39°C as well as at 28°C. The 3.5-kb RNA species detected in 206-2IC cells grown at 39°C is therefore a direct transcript rather than a splicing product of the 4.0-kb RNA.

The use of 6m2 cells both acutely and chronically superinfected with MoMuLV has provided some additional information on the thermosensitive splicing of ts110 MoMuSV RNA. Since we have observed that the 3.0-kb *env* mRNA is still spliced from the 8.3-kb genomic MoMuLV RNA in acutely superinfected 6m2 cells grown at 39°C, it is clear that the thermosensitive splicing event is not host mediated. Moreover, as described above, the splicing defect cannot be complemented by MoMuLV superinfection. Hence, it appears that the 4.0-kb genomic RNA itself or a protein encoded by the 4.0-kb RNA is responsible for the thermosensitivity of the splicing reaction.

It has previously been shown that the 4.0-kb RNA is translated into a P58^{gag} protein which contains p15, pp12,

and about 75% of p30 (9, 14). It was proposed that P58^{gag} was terminated at a stop codon within out-of-frame *mos* sequences (14). Recent evidence referred to above (14a) has shown clearly that this is in fact the case. The 4.0-kb RNA is transcribed from a provirus containing a 1,488-base deletion resulting in the out-of-frame junction of wild-type nucleotides 2404 and 3892. The effect of this deletion is to allow the translation of a P58^{gag} polypeptide containing 14 C-terminal amino acids derived from an alternative *mos* reading frame. Splicing produces the 3.5-kb RNA, which can be translated into a P85$^{gag\text{-}mos}$ protein containing p15, pp12, and 25% of p30 fused to the *mos* gene polypeptide product lacking about 17 N-terminal amino acid residues. Thus, the principal effect of the splicing reaction is to restore the original *mos* reading frame by joining *mos* sequences in frame with *gag* sequences, bypassing the stop codon in the 4.0-kb RNA alternate *mos* reading frames.

As noted earlier, revertants of the 6m2 cell line which appear transformed at all growth temperatures from 28 to 39°C have been isolated. In these cells, it appears that the transforming protein is made by a completely different mechanism than in 6m2 cells. For example, the 54-5A4 and 204-3 revertant cell lines express a P100$^{gag\text{-}mos}$ fusion protein (20) translated from a 4.0-kb viral RNA in which the *mos* sequences appear to be in frame with the *gag* gene without the need for splicing. We have shown in this work that at 28°C as well as at 39°C there is no spliced 3.5-kb RNA detected in these cells. A deletion or an insertion of some nucleotides within the splice donor or acceptor sequences would explain both the absence of splicing of the 4.0-kb RNA species and the synthesis of P100$^{gag\text{-}mos}$ instead of P58^{gag} from the 4.0-kb RNA in 204-3 and 54-5A4 cells. In fact, preliminary results involving the use of the primer extension method (P. Cizdziel, M. Nash, and E. C. Murphy, unpublished data) suggest that there is a short deletion in the 54-5A4 4.0-kb RNA splice acceptor site which would preclude splicing and restore the original *mos* reading frame.

The MoMuSV ts110 system is interesting for several reasons. The increased quantity of P85$^{gag\text{-}mos}$ synthesized in ts110 MoMuSV-infected cells compared with the small quantity of P37^{mos} synthesized in MoMuSV 124-infected cells should facilitate studies on the v-*mos* protein and its mode of action. Moreover, as shown above, ts110 MoMuSV-infected cells contain two structurally different RNAs transcribed from the same proviral DNA. The 3.5-kb RNA is thought to be a splicing product of the 4.0-kb RNA species, and the splicing itself appears to be thermosensitive. Interestingly, the splicing defect appears to be an inherent property of the viral RNA itself. In vitro splicing experiments are in progress to understand the defect of the ts110 MoMuSV genomic RNA and to provide direct information on the splicing mechanism.

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