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# Temperature-Sensitive Splicing Defect of *ts*110 Moloney Murine Sarcoma Virus Is Virus Encoded†

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**ts110 Moloney murine sarcoma virus (Mo-MuSV)-nonproductively infected cells (6m2) have a transformed phenotype at 28 to 33°C and a normal phenotype at 39°C. At temperatures permissive for transformation, 6m2 cells contain P58$^{gag}$ produced from the 4.0-kilobase (kb) viral RNA genome and P85$^{gag\text{-}mos}$ translated from a 3.5-kb spliced mRNA. At 39°C, only the 4.0-kb RNA and its product P58$^{gag}$ are detected. Two temperature-sensitive defects have been observed in *ts*110-infected 6m2 cells: (i) the splicing of the 4.0-kb RNA to the 3.5-kb RNA; and (ii) the thermolability of P85$^{gag\text{-}mos}$ and its kinase activity relative to the wild-type revertant protein, termed P100$^{gag\text{-}mos}$ (R. B. Arlinghaus, J. Gen. Virol. 66:1845–1853, 1985). In the present study, we examined the *mos* gene products of two cell lines (204-2F6 and 204-2F8) obtained by infection of normal rat kidney cells with *ts*110 Mo-MuSV as a simian sarcoma-associated virus pseudotype to see whether the temperature-sensitive splicing defect could be transferred by viral infection. Southern blot analysis of these two cell lines showed that viral DNAs containing restriction fragments from cellular DNA are different from those in 6m2 cells, indicating that 204-2F6 and 204-2F8 cells have different *ts*110 provirus integration sites from those of 6m2 cells. Northern blots, S1 mapping analyses, and immunoprecipitation experiments showed unequivocally that the splicing defect of *ts*110 Mo-MuSV is virus encoded and is independent of host cell factors.**

The Moloney strain of murine sarcoma virus (Mo-MuSV) is a recombinant between Moloney murine leukemia virus (Mo-MuLV) and a cellular oncogene termed c-*mos*. The acquired cellular sequence (v-*mos*; 38) is responsible for in vitro transformation of fibroblasts and tumor production in animals (1). An *env-mos* protein of 37,000 daltons (p37$^{mos}$) encoded by the v-*mos* gene within the 5.3-kilobase (kb) RNA genome of Mo-MuSV 124 has been detected in virus-transformed cells (Fig. 1). p37$^{mos}$ is apparently translated from full-size viral RNA by internal initiation at the beginning of the *mos* gene reading frame situated near the 3′ end of the viral RNA (29). Further characterization and functional studies on p37$^{mos}$ have proven to be difficult because of the trace levels present in chronically transformed cells (29, 30).

A temperature-sensitive (*ts*) mutant of Mo-MuSV (*ts*110), producing higher quantities of a *gag-mos* transforming protein, was obtained by mutagenesis (5). Normal rat kidney (NRK) cells nonproductively infected with this virus (the 6m2 cell line) are morphologically tranformed when grown at 28 and 33°C but not at 39°C (9, 19). Two *ts*110 Mo-MuSV-specific proteins of 58,000 (P58$^{gag}$) and 85,000 (P85$^{gag\text{-}mos}$) daltons are produced in 6m2 cells grown at permissive temperatures (14, 40) (Fig. 1). P58$^{gag}$ is made from the 4.0-kb viral RNA genome, and P85$^{gag\text{-}mos}$ is translated from a 3.5-kb spliced mRNA (17, 24). At 39°C only the 4.0-kb RNA and its product, P58$^{gag}$, can be detected (9). Tryptic peptide mapping and immunoprecipitation experiments have shown that P58$^{gag}$ is the product of a truncated *gag* region and contains p15, p12, and about 75% of p30 (15, 24). P85$^{gag\text{-}mos}$ is composed of p15, p12, and about 15% of p30 fused to v-*mos* protein sequences (10, 24, 36). DNA sequencing of the *gag-mos* junctions (25) within the 4.0-kb RNA genome of *ts*110 MuSV has confirmed these findings. These sequencing studies have established that the *gag* and *mos* genes within the 4-kb RNA are fused as a result of a 1,488-base deletion of sequences present in the 5.3-kb RNA genome of the parental virus. In 4.0-kb RNA, the long, open *mos* reading frame is not in frame with the *gag* gene. A second consequence of the 1,488-base deletion is the removal of the *env*-coded ATG initiation codon used to form p37$^{mos}$ (Fig. 1). The 3.5-kb mRNA was found to contain an even shorter form of the v-*mos* gene lacking approximately 60 bases of the 5′ end of the v-*mos* gene (25). In contrast to the 4.0-kb RNA, the *gag* and *mos* genes in the 3.5-kb RNA are fused in a way that allows a continuous *gag-mos* open reading frame coding for P85$^{gag\text{-}mos}$. Previous experiments have provided evidence indicating that the 3.5-kb RNA is a splicing product of the 4.0-kb genomic RNA (11, 17, 26).

We have previously shown that the *ts* defects of *ts*110 Mo-MuSV in 6m2 cells are manifested at two levels. (i) The splicing of the 4.0-kb RNA to the 3.5-kb RNA is inhibited at or above 37°C (9, 11). (ii) P85$^{gag\text{-}mos}$ and its associated serine kinase activity are thermolabile (15, 18, 19, 35). An important question remaining is whether these defects, and particularly the splicing defect, are host or virus related. To answer this question, we analyzed two cell lines (204-2F6 and 204-2F8), selected because like 6m2 cells, they were morphologically transformed at 33°C but not at 39°C. We analyzed their *ts*110 Mo-MuSV-specific RNAs and proteins and compared them with their 6m2 counterparts to see whether the *ts* splicing defect was transferable by virus infection.

## MATERIALS AND METHODS

**Cells.** *ts*110 Mo-MuSV was produced by UV irradiation of Mo-MuSV 349, a subclone of Mo-MuSV 124. Virus

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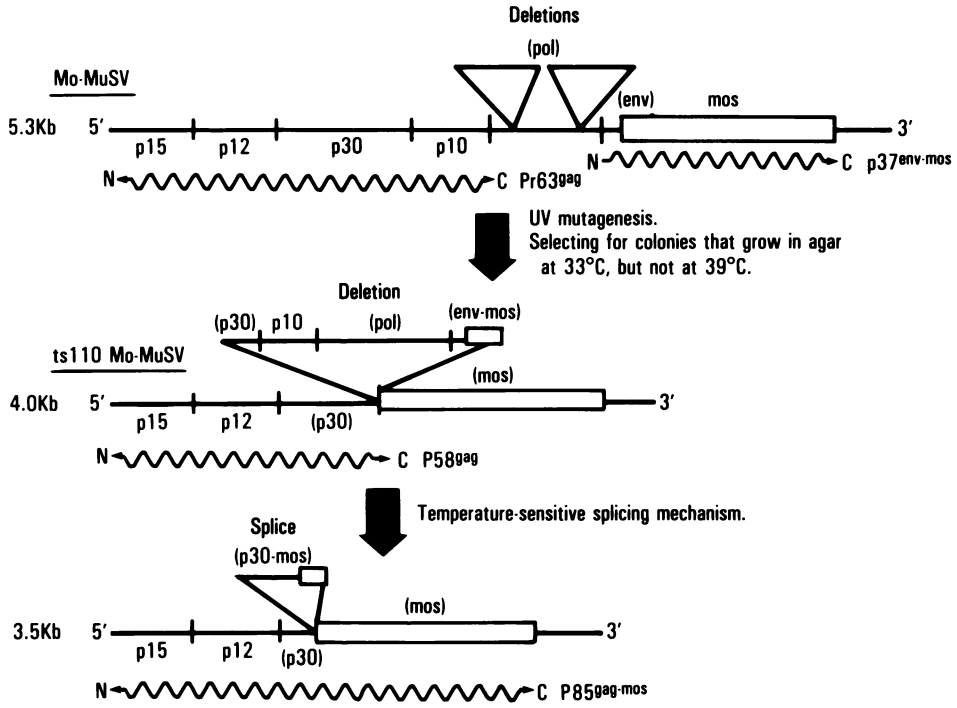


FIG. 1. Diagram of the viral RNAs and proteins produced in Mo-MuSV 124- and *ts*110 MuSV-transformed cells.

nonproducer 6m2 cells were selected by a procedure involving killing of agar clones maintained at 39°C (5).

The 204-2F6 and 204-2F8 cell lines were obtained by two cycles of soft agar cloning of NRK cells infected with the simian sarcoma-associated virus (SSAV) pseudotype of *ts*110 Mo-MuSV rescued from 6m2 cells. After 24 h of infection, NRK cells infected with *ts*110 Mo-MuSV (SSAV) were placed in 0.2% agar and grown at 33°C. Individual isolated colonies were picked into liquid medium and grown to confluency at 33°C. For the second cycle, cells in liquid culture were again plated in soft agar. Agar clones (F6 and F8) were picked from cultures which grew at 33°C but not at 39°C. These *ts* properties were found to be stable upon continuous passage in culture at 33°C. Cell lines were maintained at 33°C in McCoy 5a medium containing 15% (vol/vol) fetal calf serum and transferred to the experimental temperature 2 days before processing.

**DNA extraction and Southern blot analysis.** Cells were rinsed with an isotonic buffer and incubated for 1 h at 37°C in 20 mM Tris (pH 7.5)–2.5 mM EDTA–2% sodium dodecyl sulfate (SDS)–200 μg of proteinase K per ml. The cell lysate was extracted once with an equal volume of phenol-chloroform (1:1) and once with chloroform, and DNA was precipitated with ethanol.

About 20 μg of cellular DNA was digested by the appropriate restriction enzyme, subjected to electrophoresis on 0.7% agarose gel in 90 mM Tris borate–90 mM boric acid–2 mM EDTA at 90 V for 16 h, and transferred to nitrocellulose as described by Southern (34). The prehybridization, hybridization (with a $^{32}$P-labeled *mos*-containing probe), and washing procedures were as described previously (39).

**RNA extraction and Northern blot analysis.** Total RNA was extracted by the hot-phenol procedure (13), and poly(A)$^+$ RNA was selected twice on oligo(dT)-cellulose (3). Poly(A)$^+$ RNA was then denatured by heating for 20 min at 50°C in 50% dimethyl sulfoxide–1 M glyoxal–10 mM sodium phosphate buffer (pH 7) (23) and subjected to electrophoresis in 1% agarose in 10 mM sodium phosphate buffer (pH 7) at 50 V for 10 h. Approximately 5 μg of poly(A)$^+$ RNA was loaded per lane. RNA was transferred to nitrocellulose sheets by the procedure of Thomas (37), and virus-specific RNA

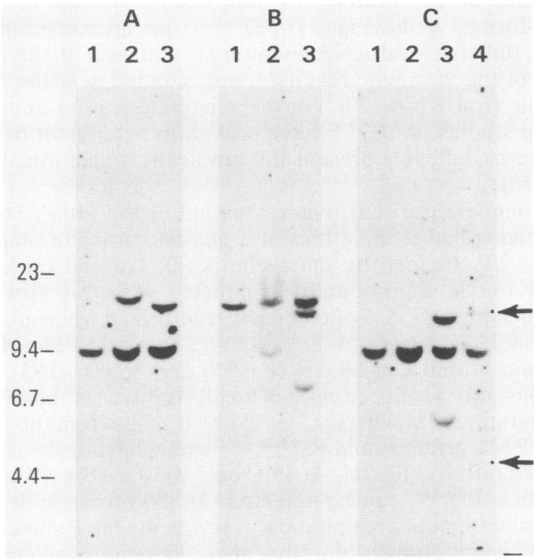


FIG. 2. *mos*-containing DNA in *ts*110 Mo-MuSV-infected cells. High-molecular-weight DNA from NRK, 6m2, 204-2F6, and 204-2F8 cells was digested, analyzed on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to a $^{32}$P-labeled *mos* probe. A, Cellular DNA digested with *Eco*RI. B, Cellular DNA digested with *Bam*HI. C, Cellular DNA double digested with *Eco*RI and *Bam*HI. The DNAs were extracted from NRK cells (lanes 1), 6m2 cells (lanes 2), 204-2F6 cells (lanes 3), or 204-2F8 cells (lane 4).

species were detected by hybridization with a $^{32}$P-labeled DNA probe (31). The prehybridization, hybridization, and washing procedures were as described previously (12, 37). Ribosomal 28S and 18S RNA were run in a parallel lane as size markers and stained with acridine orange (23).

**DNA probes.** Plasmid pK101*mos*, provided by E. Murphy, University of Texas Cancer Center, Houston, Tex., is a pKC7 plasmid into which a 1.3-kb *Bgl*II-*Hin*dIII *mos*-containing DNA fragment of Mo-MuSV 124 has been inserted (17). The insert was excised by a double digestion with *Xba*I and *Hin*dIII, fractionated on agarose gel, and purified by electroelution and absorption to Elutip-D columns (Schleicher & Schuell, Inc.). Nick translation was done by the method of Rigby et al. (31). Approximately $10^6$ cpm of $^{32}$P-labeled DNA were used per lane of RNA or DNA for hybridization (Fig. 2 and 3).

For the S1 mapping of *ts*110 Mo-MuSV RNA at the 5′ side (Fig. 4), we used a 3′-end-labeled 290-bp *Dde*I fragment extending from positions 1481 to 1771 in the Mo-MuLV sequence (33) (from positions 1902 to 2192 in the Mo-MuSV 124 sequence [38]). This fragment was obtained from pMLV$A_3$ (kindly provided by E. Murphy) containing 1.85 kb of *gag* sequence information from a *Sma*I site in the 5′ long terminal repeat to a *Bgl*II site 2.35 kb from the 5′ end of Mo-MuLV DNA into *Sma*I-*Bgl*II-digested pKC7. The insert was removed by *Sma*I-*Bgl*II double digestion, purified by electroelution, and double digested by *Dde*I and *Pst*I. The 290-bp fragment was separated by electrophoresis on a 4% acrylamide gel, purified with an Elutip D column, and 3′ end labeled with the Klenow fragment of DNA polymerase in the presence of unlabeled TTP and [α-$^{32}$P]dATP (3,200 Ci/mmol; New England Nuclear Corp.).

For the S1 mapping of *ts*110 Mo-MuSV RNA at the 3′ side (Fig. 5), we used a 5′-end-labeled *Bgl*II-*Kpn*I 683-bp fragment. This fragment, extending from position 3698 to position 4381 in the Mo-MuSV 124 sequence (38), was obtained from pK101*mos* by a *Bgl*II-*Kpn*I double digestion. It was 5′ end labeled with the 5′ DNA terminus labeling system from Bethesda Research Laboratories, Inc., as recommended by the supplier.

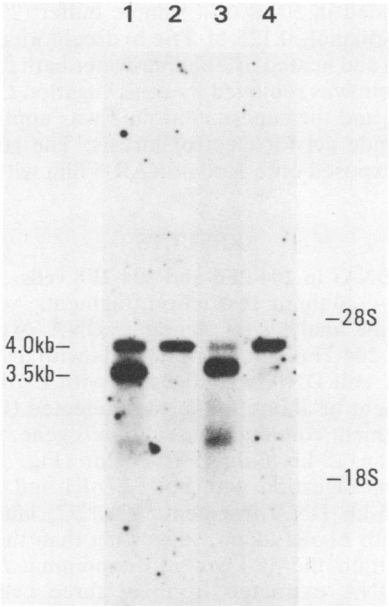


FIG. 3. Virus-specific RNA in 6m2 and 204-2F6 cells. Poly(A)$^+$ RNA from each cell preparation was analyzed by electrophoresis on 1% agarose gels, transferred to nitrocellulose,and hybridized to a $^{32}$P-labeled *mos*-specific probe. Lanes: 1, RNA from 204-2F6 cells grown at 28°C; 2, RNA from 204-2F6 cells grown at 39°C; 3, RNA from 6m2 cells grown at 28°C; 4, RNA from 6m2 cells grown at 39°C.

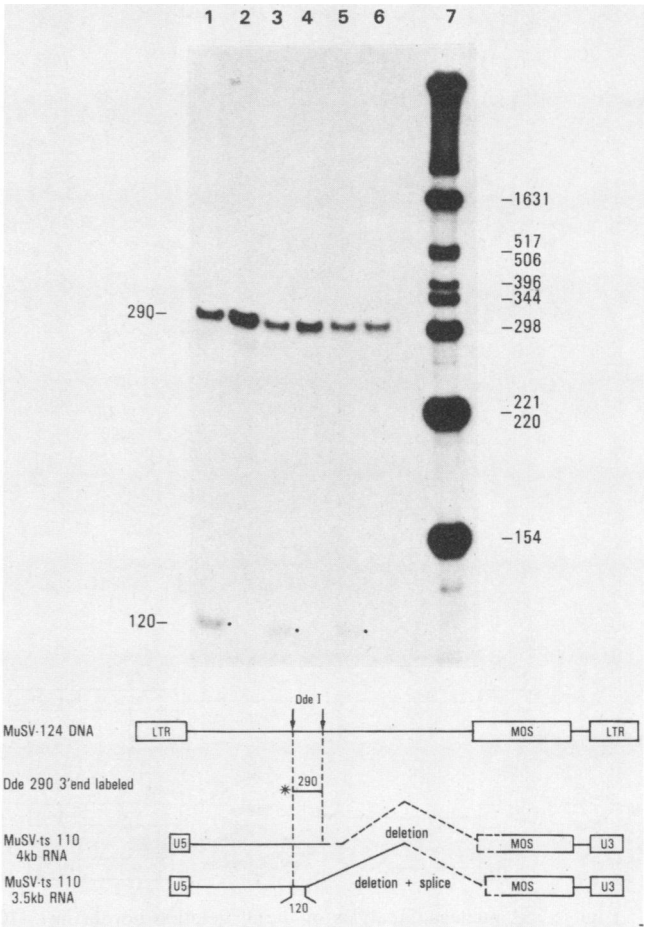


FIG. 4. S1 nuclease analysis of the 5′ deletion border in *ts*110 Mo-MuSV RNA. The 3′-end-labeled *Dde*I 290-bp DNA fragment was hybridized to total cellular RNA, digested with S1 nuclease, and electrophoresed on a 4% polyacrylamide gel containing 8 M urea for 16 h at 100 V. The cellular RNAs were extracted from 6m2 cells grown at 28°C (lane 1) or 39°C (lane 2), 204-2F6 cells grown at 28°C (lane 3) or 39°C (lane 4), 204-2F8 cells grown at 28°C (lane 5) or 39°C (lane 6); lane 7 contains a 5′-end-labeled pBR322-*Hin*fI digest. The diagram shows the deletion in the 4.0-kb *ts*110 RNA relative to Mo-MuSV 124 5.3-kb RNA. It also illustrates the result of the deletion and splicing that produces the 3.5-kb mRNA.

**S1 nuclease analysis.** S1 nuclease analyses were done as described previously (26). Briefly, the 5′- end- or the 3′-end-labeled DNA probe was coprecipitated with 50 μg of total cellular RNA. The pellets were dissolved in 80% formamide–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.8)–400 mM NaCl–1 mM EDTA, heated at 85°C for 15 min, and hybridized at 56°C for 3 h. Single-stranded nucleic acids were digested with S1 nuclease, and the digests were extracted once with phenol-chloroform (1:1) and ethanol precipitated. They were then dissolved in 20 μl of 10 M urea–1 mM EDTA–0.1 N NaOH–0.015% bromocresol green, heated to 50°C for 5 min, and subjected to electrophoresis on a 4% polyacrylamide gel containing 8 M urea at

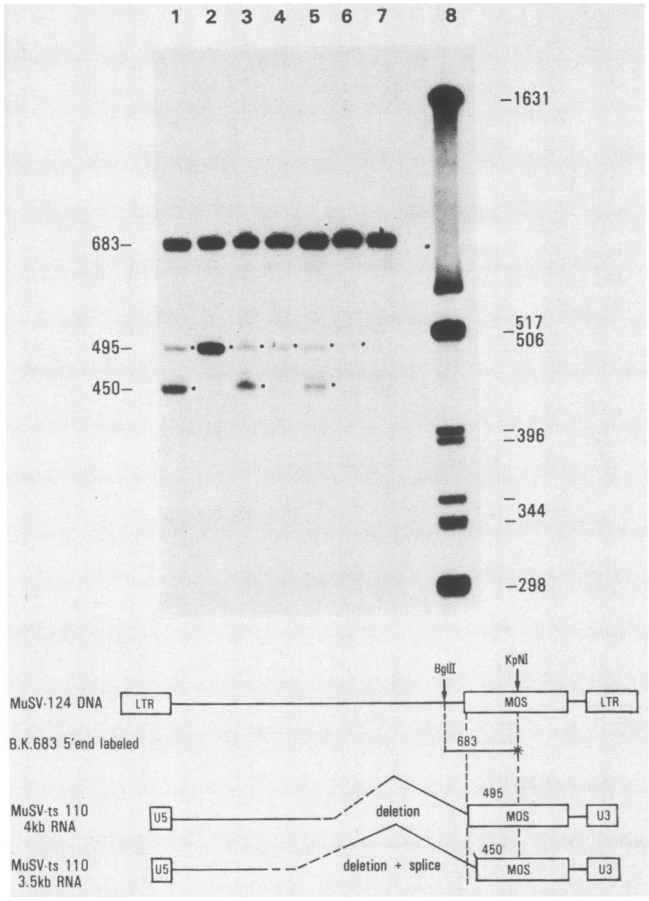


FIG. 3. S1 nuclease analysis of the 3′ deletion border in *ts*110 Mo-MuSV RNA. The 5′-end-labeled *Bgl*II-*Kpn*I 683-bp DNA fragment was hybridized to total cellular RNA, digested with S1 nuclease, and electrophoresed on a 4% polyacrylamide gel containing 8 M urea for 20 h at 150 V. The cellular RNAs were extracted from 6m2 cells grown at 28°C (lane 1) or 39°C (lane 2), 204-2F6 cells grown at 28°C (lane 3) or 39°C (lane 4), 204-2F8 cells grown at 28°C (lane 5) or 39°C (lane 6); lane 7 was done without added RNA, and lane 8 contains a 5′-end-labeled pBR322-*Hin*fI digest. The diagram shows the deletion in the 4.0-kb *ts*110 RNA relative to Mo-MuSV 124 5.3-kb RNA. It also illustrates the result of the deletion and splicing that produces the 3.5-kb mRNA.

100 to 150 V for 16 to 20 h. After electrophoresis, the gels were fixed with 50% methanol–7% acetic acid, dried, and autoradiographed.

**Protein labeling and immunoprecipitations.** The cells maintained at various temperatures for 2 days were pulse-labeled with L-[$^3$H]leucine (40 to 60 Ci/mmol; New England Nuclear) at 500 μCi/ml in Earle balanced salt solution for 20 min. After cell lysis, the cytoplasmic extracts were immunoprecipitated with Rauscher murine leukemia virus anti-p10, anti-p15, and anti-p30 goat sera or anti-SSAV sera obtained from the Logistics Program, National Cancer Institute. The sera were absorbed with uninfected mouse cell extracts as previously described (27). Anti-*mos*(37–55) serum was prepared from a 19-amino-acid cyclic peptide as described previously (10). The immunoprecipitates were washed and fractionated on 8% acrylamide-SDS gels (2), and the dried gels were developed by fluorography as described previously (16).

**Protein kinase assay.** The protein kinase assay was performed as described by Maxwell and Arlinghaus (22). The medium was thoroughly decanted from the cell flasks and immediately placed on ice. Lysis buffer (1% Nonidet P-40 [NP-40], 150 mM NaCl, 1 mM EDTA, 100 Kallikrein inactivation units [KIU] of aprotinin [Trasylol; FBA Pharmaceuticals] per ml in 20 mM sodium phosphate [pH 7.2]) was added (0.5 ml per 25-cm$^2$ cell flask), and the mixture was allowed to incubate on the cell sheet on ice for 15 min with occasional swirling of the lysis buffer. Cells were scraped into the lysis buffer, and the lysate was clarified by centrifuging at 60,000 × *g* for 30 min. The clarified cell lysate was then preabsorbed with 200 μl of pansorbin (10% [wt/vol] suspension of fixed *Staphylococcus aureus*, Cowan strain, purchased from Calbiochem-Behring and washed once with lysis buffer) per 0.5 ml of cell extract. After the pansorbin was pelleted (5000 × *g* for 10 min), 20 μl of anti-*mos*(37–55) (heat inactivated at 56°C for 30 min) was added, and the immune reaction was allowed to proceed for 1 to 2 h. In blocking experiments, an excess of 37–55 cyclic *mos* peptide was added to the sera (2 μg/20 μl of serum). Immune complexes were precipitated with an equal volume of pansorbin, and the immunoprecipitates were washed twice in NP-40–phosphate-buffered saline (0.1% NP-40 and 150 mM NaCl in 10 mM sodium phosphate [pH 7.2]). Immune complexes were drained thoroughly, and each was suspended in 50 μl of NP-40–PBS containing 1 mM pyrophosphate. The kinase reaction was initiated by adding 50 μl of NP-40–PBS containing 20 μM ATP, 10 μCi of [γ-$^{32}$P]ATP (4,000 to 5,000 Ci/mmol; ICN Pharmaceuticals, Inc.), 15 mM $MnCl_2$, and 1 mM sodium pyrophosphate. The reaction was allowed to proceed for 10 min at 22 to 23°C and was then terminated by addition of 3 ml of RIPA buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 100 KIU of Trasylol per ml in 20 mM sodium phosphate [pH 7.2]) containing 2 mM ATP and 1 mM pyrophosphate. The immune complexes were washed twice in RIPA-ATP-pyrophosphate and drained thoroughly. The samples were then suspended in 50 μl of a sample buffer (2% SDS, 10% β-mercaptoethanol, 0.125 M Tris hydrochloride [pH 6.8], 1 mM EDTA) and heated in a boiling-water bath for 3 to 5 min. The pansorbin was removed by centrifugation (5,000 × *g* for 3 to 5 min), and the supernatant fluid was applied to an 8% polyacrylamide gel for electrophoresis. The gel was fixed, dried, and exposed onto Kodak XAR-5 film with an enhancing screen.

## RESULTS

**Proviral DNAs in 204-2F6 and 204-2F8 cells.** For comparison of *mos*-containing restriction fragments, we performed Southern blot analysis of genomic DNA extracted from NRK, 6m2, 204-2F6, and 204-2F8 cells with a v-*mos* probe. When NRK cell DNA was digested with *Eco*RI, a unique DNA fragment of about 9.5 kb was detected (Fig. 2A, lane 1). This fragment contained the rat c-*mos* gene, which is also present in an 18-kb *Bam*HI fragment (Fig. 2B, lane 1). Simultaneous digestion with both *Eco*RI and *Bam*HI produced a 9.5-kb DNA fragment (Fig. 2C, lane 1), as did digestion with *Eco*RI alone, suggesting than the two *Eco*RI sites are within the 18-kb c-*mos*-containing *Bam*HI fragment. In DNA extracted from all three cell types, the c-*mos*-containing restriction fragments were the same sizes, whether digested with *Eco*RI (Fig. 2A), *Bam*HI (panel B), or both enzymes (Fig. 2C).

Since *Eco*RI and *Bam*HI do not cut within the Mo-MuSV 124 genome (38), every additional *mos*-containing fragment should contain a *ts*110 Mo-MuSV provirus, unless the mu-

tant generates internal restriction sites. As expected in 6m2 cells, the *Eco*RI digestion produced an 18-kb DNA fragment (Fig. 2A, lane 2), whereas a 9.5-kb DNA fragment was detected with *Bam*HI-digested (Fig. 2B, lane 2) or *Eco*RI-*Bam*HI double-digested (Fig. 2C, lane 2) DNA. In this case the *Bam*HI sites are within the *Eco*RI fragment, and the v-*mos*- and c-*mos*-containing fragments are indistinguishable when 6m2 genomic DNA is double digested.

Lanes 3 of Fig. 2A and B show the hybridization of the *mos*-specific probe with 204-2F6 DNA digested with *Eco*RI and *Bam*HI, respectively. Besides the 18-kb c-*mos*-containing DNA fragment, two fragments, of 16.5 and 7 kb, were detected with *Bam*HI-digested 204-2F6 DNA (Fig. 2B, lane 3), although only one additional 16.5-kb DNA fragment was seen in *Eco*RI-digested 204-2F6 DNA (Fig. 2A, lane 3). Since 204-2F6 DNA contained two v-*mos*-containing *Bam*HI fragments (Fig. 2B, lane 3), one of the DNA bands seen in Fig. 2A, lane 3 (the c-*mos*-containing 9.5-kb *Eco*RI fragment, which is more intense), should be composed of two different *mos*-containing DNA fragments. *Eco*RI-*Bam*HI double digestion shows two v-*mos*-containing DNAs, of 15.5 and 6 kb, respectively (Fig. 2C, lane 3). The first derives from the 16.5-kb *Eco*RI and the 16.5-kb *Bam*HI fragment, and the second derives from the 9.5-kb *Eco*RI and the 7-kb *Bam*HI fragment. Finally, the genomic DNA extracted from 204-2F8 and double digested with *Eco*RI and *Bam*HI shows two v-*mos*-containing DNA fragments, of 17 and 5 kb (Fig. 2C, lane 4, arrows). The lower intensity of the two v-*mos*-containing fragments compared with that of the c-*mos*-containing fragment in 204-2F8 cell DNA suggests that this cell line is not monoclonal. We conclude from the results shown in Fig. 2 that the 204-2F6 and the 204-2F8 cell lines each contain at least one *ts*110 Mo-MuSV provirus different from the unique *ts*110 provirus in 6m2 cells.

***ts*110-specific viral RNAs in 204-2F6 and 204-2F8 cells.** We compared the *mos*-containing RNA species in 6m2, 204-2F6, and 204-2F8 cells by Northern blot analysis and S1 mapping. Figure 3 presents a Northern blot of poly(A)$^+$ RNA extracted from 204-2F6 cells grown at 28 and 39°C as compared with the RNA extracted from 6m2 cells grown at the same temperatures. Two RNA species, of 4.0 and 3.5 kb, were detected at 28°C in 204-2F6 cells (Fig. 3, lane 1) as well as in 6m2 cells (lane 3). However, at 39°C only the 4.0-kb RNA species was detected in both cell lines (lanes 2 and 4, respectively).

Thus, it appears that 6m2 cells and 204-2F6 cells each have similar-sized v-*mos*-containing viral RNAs. However by this test, it is difficult to know whether the 204-2F6 *mos*-containing viral RNA species are exactly the same as those in 6m2 cells. Therefore, we performed S1 mapping analysis with two end-labeled DNA fragments known to discriminate between the 4.0- and the 3.5-kb RNA species in 6m2 cells (26). Any major differences in sequences covered by the probes would be detected by this methodology.

For the experiment shown in Fig. 4, we used a 3′-end-labeled 290-bp *Dde*I fragment extending from positions 1902 to 2192 in the Mo-MuSV 124 wild-type DNA sequence (38). This fragment is known to fully hybridize with the 4.0-kb *ts*110 Mo-MuSV genomic RNA (Fig. 4, bottom) (26). The 3.5-kb RNA species, however, protects 120 bases from digestion by S1 nuclease. Before S1 hydrolysis, this *Dde*I 290 fragment was hybridized with total RNA extracted from 6m2 cells grown at 28°C (Fig. 4, lane 1) or 39°C (lane 2), and as expected, the 120-base protected fragment was detected only at 28°C. With RNA extracted from either 204-2F6 cells grown at 28°C (lane 3) and 39°C (lane 4) or 204-2F8 cells grown at 28°C (lane 5) and 39°C (lane 6), a 120-base protected fragment was seen only with RNA extracted from cells grown at 28°C.

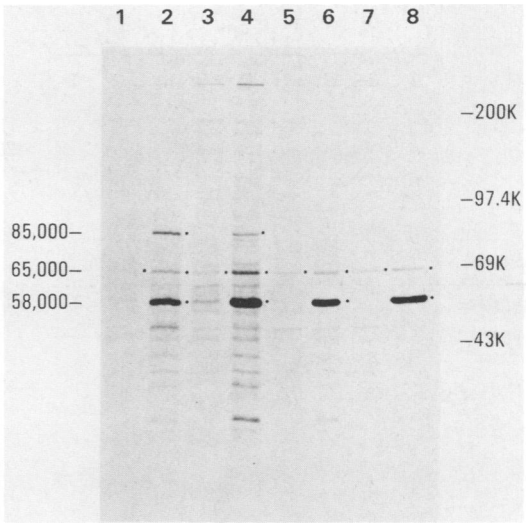


FIG. 6. Immunoprecipitation of virus-specific proteins in 240-2F6 cells. 204-2F6 cells were maintained at various temperatures for 48 h and pulse-labeled with L-[$^3$H]leucine for 20 min. Cytoplasmic extracts were immunoprecipitated with either anti-p10 (lanes 1, 3, 5, and 7) or anti-p30 (lanes 2, 4, 6, and 8). The washed immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis, dried, and processed for fluorography. Lanes: 1 and 2, 204-2F6 cells grown at 28°C; 3 and 4, 204-2F6 cells grown at 33°C; 5 and 6, 204-2F6 cells grown at 37°C; 7 and 8, 204-2F6 cells grown at 39°C.

For studying the 3′ side of the deletion in the *ts*110 Mo-MuSV RNA species, we used a 5′-end-labeled *Bgl*II-*Kpn*I 683-bp fragment. This fragment spans the *env-mos* junction of Mo-MuSV 124 from positions 3698 to 4381 (38). In 6m2 cells 495 and 450 bases, respectively, were protected by the 4.0- and the 3.5-kb RNA species from S1 digestion (Fig. 5, bottom) (26). These fragments can be seen when RNA extracted from 6m2 cells grown at 28°C was used to perform the S1 mapping analysis (Fig. 5, lane 1). Only the 495-base protected fragment was detected with 6m2 cells grown at 39°C (lane 2), showing that the 4.0-kb RNA species is the only *mos*-containing RNA present at this temperature. The situation is quite similar with RNA extracted from 204-2F6 cells grown at 28°C (lane 3) and 39°C (lane 4) or from 204-2F8 cells grown at the same temperatures (lanes 5 and 6). Some additional DNA fragments can be seen in Fig. 5. They correspond to slight contamination of the DNA probe, as they are equally present in an S1 mapping experiment performed without cellular RNA (lane 7).

The results of Northern blot and S1 mapping analyses show unequivocally that the 204-2F6 and 204-2F8 cells contain very similar *mos* specific RNA species as in 6m2 cells at 28°C as well as at 39°C. Moreover, the *gag-mos* junctions have exactly the same sequences as judged by S1 mapping.

***ts*110-specific proteins in 204-2F6 and 204-2F8 cells.** Viral proteins present in 204-2F6 cells grown at different temperatures are shown in Fig. 6. The viral proteins were detected by pulse-labeling cells with [$^3$H]leucine; the cells were lysed, and the cytoplasmic extracts were processed by immunoprecipitation with anti-p10 and anti-p30 sera. Three proteins of 85,000, 65,000, and 58,000 daltons were detected with anti-

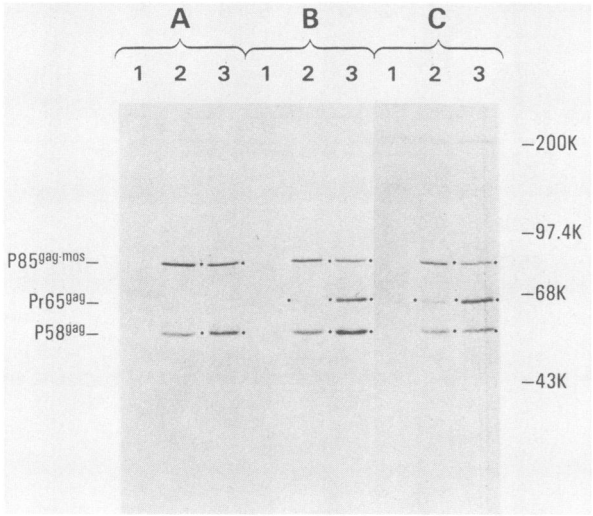


FIG. 7. Comparison of virus-specific proteins in 6m2, 204-2F6, and 204-2F8 cells. 6m2 cells (panel A), 204-2F6 cells (panel B), and 204-2F8 cells (panel C) were grown at 28°C for 48 h and immunoprecipitated, as described for Fig. 6, with anti-p10 (lanes 1), anti-p15 (lanes 2), or anti-p30 (lanes 3).

p30 serum when the cells were grown at 28 or 33°C (Fig. 6, lanes 2 and 4). However, the 85,000-dalton protein was not detected when 204-2F6 cells were grown at 37 or 39°C (lanes 6 and 8). Neither the 85,000- nor the 58,000-dalton protein was detected by anti-p10 sera (lanes 1, 3, 5, and 7), although some background proteins comigrated with each of these proteins. Like the 58,000-dalton protein, the 65,000-dalton protein was detected at all temperatures with anti-p30 (lanes 2, 4, 6, and 8). Similar results were obtained with 204-2F8 cells.

To compare the sizes and antigenic content of viral proteins present in 204-2F6 and 204-2F8 cells with $P85^{gag\text{-}mos}$ and $P58^{gag}$ found in 6m2 cells (14, 40), we performed parallel immunoprecipitations of extracts from cells grown at 28°C with anti-p10, anti-p15, and anti-p30 sera (Fig. 7). The 85,000-dalton protein immunoprecipitated from 204-2F6 and 204-2F8 cells comigrated with $P85^{gag\text{-}mos}$ immunoprecipitated from 6m2 cells. The 58,000-dalton protein also comigrated with $P58^{gag}$ of 6m2 cells. Both proteins contained p15 and p30 antigenic determinants but lacked p10 determinants. We then tested the 85,000-dalton protein of 204-2F6 and 204-2F8 cells for *mos* sequences by using an antiserum raised against a synthetic 37-55 v-*mos* peptide (10). The 85,000-dalton protein was immunoprecipitated by this antiserum from extracts of 204-2F6 cells (Fig. 8, panel A, lane 3). The immunoprecipitation was specific, as antisera blocked with excess polypeptide did not immunoprecipitate the 85,000-dalton protein from 204-2F6 cells (Fig. 8A, lane 4). Identical results were obtained when 204-2F8 cells were analyzed. Thus 204-2F6, 204-2F8, and 6m2 cells contain the same *ts*110 Mo-MuSV-specific proteins, $P58^{gag}$ being present at all temperatures and $P85^{gag\text{-}mos}$ being present only at temperatures permissive for transformation.

In addition to $P85^{gag\text{-}mos}$, several smaller v-*mos*-related proteins were specifically recognized by our anti-*mos* serum in 204-2F6 and 2F8 cell lines. Their origin is unknown, but all are larger than $p37^{mos}$. These smaller *mos*-containing proteins probably represent either degraded or processed forms of $P85^{gag\text{-}mos}$.

Finally, the 65,000-dalton protein, present in both 204-2F6 and 204-2F8 cells, was not detected in 6m2 cells with anti-p10 (Fig. 7A, lane 1), anti-p15 (lane 2), or anti-p30 (lane

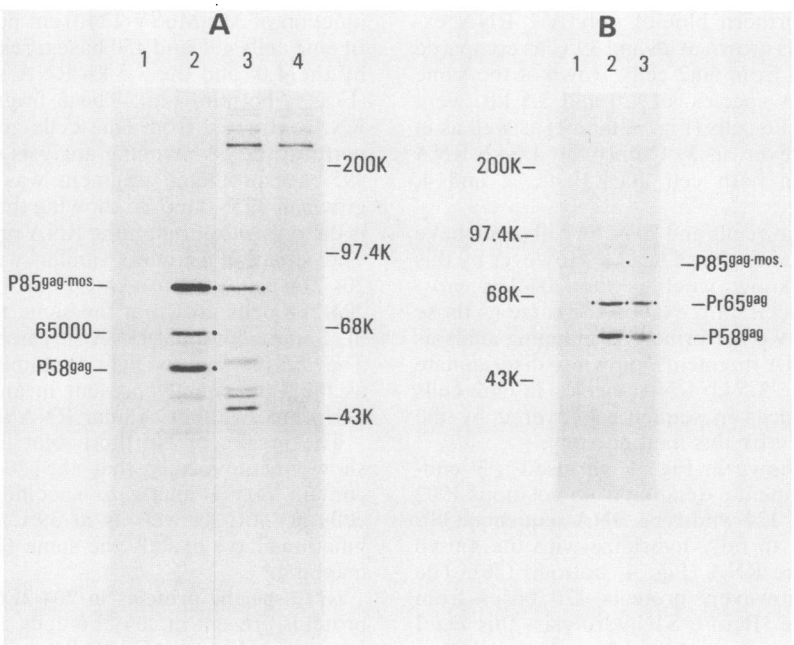


FIG. 8. A. Immunoprecipitation of *mos* containing proteins in 204-2F6 cells. 204-2F6 cells were grown at 28°C for 48 h and immunoprecipitated as described for Fig. 6 with anti-p10 (lane 1), anti-p30 (lane 2), anti-*mos*(37–55) (lane 3), and anti-*mos*(37–55) sera preincubated with an excess of synthetic peptide (lane 4). B. Immunoprecipitation of proteins in 204-2F6 cells with anti-SSAV serum. 204-2F6 cells were grown at 28°C for 48 h and immunoprecipitated as described for Fig. 6 with anti-p10 (lane 1), anti-p30 (lane 2), or anti-SSAV (lane 3).

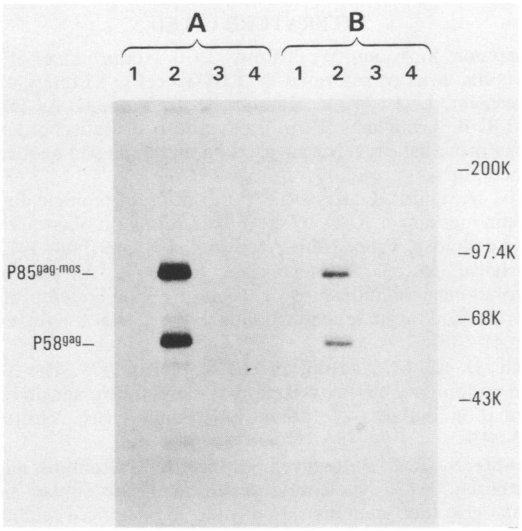


FIG. 9. In vitro kinase activity associated with *ts*110 Mo-MuSV proteins in 6m2 and 204-2F6 cells. 6m2 and 204-2F6 cells were grown at 28 or 39°C for 48 h and immunoprecipitates were subjected to the immune complex kinase assay with [$\gamma$-$^{32}$P]ATP. 6m2 cells (panel A) or 204-2F6 cells (panel B) grown at 28°C (lanes 1 and 2) or 39°C (lanes 3 and 4) were immunoprecipitated with anti-p10 (lanes 1 and 3) or anti-p30 (lanes 2 and 4).

3). Since 204-2F6 and 204-2F8 cells were obtained by infection of NRK cells with *ts*110 Mo-MuSV pseudotyped by SSAV, SSAV-specific components could be present in these cell lines. The 65,000-dalton protein absent in 6m2 cells but present in 204-2F6 and 204-2F8 cells could be a SSAV product. In Fig. 8, panel B shows immunoprecipitation experiments with anti-SSAV sera. The 65,000-dalton protein can be seen in 204-2F6 (Fig. 8B, lane 3) and 204-2F8 cells. Note that the anti-SSAV sera more easily detected the 65,000-dalton protein than either of the P85$^{gag\text{-}mos}$ or P58$^{gag}$ proteins. Because of the expected homology of the p30 group-specific antigens of simian and murine type C retroviruses (4, 6), one would expect it to be recognized more efficiently by anti-p30 than anti-p15 or anti-p10 sera (Fig. 6–8). On the basis of its size and antigenicity, we believe that this 65,000-dalton protein is the SSAV *gag* precursor, but more work is needed to prove this.

***gag-mos* kinase activity in 204-2F6 and 204-2F8 cells.** We have previously shown that P85$^{gag\text{-}mos}$ has a closely associated kinase activity phosphorylating itself and P58$^{gag}$ at serine residues (18, 19). A similar activity was found to be associated with p37$^{mos}$ (22). To determine whether this kinase activity was present in 204-2F6 cells, we performed immune complex kinase assays. Since anti-p10 serum does not immunoprecipitate P85$^{gag\text{-}mos}$, no kinase activity was detected with anti-p10 sera in either 6m2 cells or 204-2F6 cells grown at 28 or 39°C (Fig. 9A and B, lanes 1 and 3). Anti-p30 immunoprecipitates, which contain P85$^{gag\text{-}mos}$ when cells are grown at 28°C, were able to phosphorylate P85 and P58 in immune complex kinase assays derived from 6m2 (Fig. 9A, lane 2) as well as from 204-2F6 cells (Fig. 9B, lane 2). When cells were grown at 39°C (where we have shown that there is no detectable P85$^{gag\text{-}mos}$ but the anti-p30 immunoprecipitates contained P58$^{gag}$), there was no kinase activity in the immune complex kinase assay derived from both 6m2 (as previously described in reference 18) and 204-2F6 lines tested (Fig. 9A and B, lanes 4). It must be emphasized that the putative SSAV-specific Pr65$^{gag}$ is not phosphorylated by the P85$^{gag\text{-}mos}$-associated kinase activity in 204-2F6 cells (Fig. 9B, lane 2). We have previously shown that Pr65$^{gag}$ of Mo-MuLV is also not phosphorylated by the P85$^{gag\text{-}mos}$ kinase in immunoprecipitates derived from MuLV-infected 6m2 cells (18). The results of kinase activities in 204-2F8 cells were exactly the same as those obtained with 204-2F6 cells (results not shown).

## DISCUSSION

In this work we present evidence that a structural defect in the 4.0-kb *ts*110 Mo-MuSV genomic RNA prevents its splicing at restrictive temperatures. The 204-2F6 and 204-2F8 cell lines used here were obtained by two cycles of soft agar cloning following infection of NRK cells with *ts*110 Mo-MuSV pseudotyped with SSAV. We compared viral components in these cells with the well-characterized *ts*110-specific RNA and proteins in 6m2 cells. At temperatures permissive for transformation (e.g., 28°C), the 4.0- and 3.5-kb RNA species as well as P58$^{gag}$ and P85$^{gag\text{-}mos}$ (and its associated kinase activity) could be detected in all three cell lines. The 3.5-kb RNA and P85$^{gag\text{-}mos}$ as well as its kinase, however, were absent at 39°C in 204-2F6 and 204-2F8 cells as well as in 6m2 cells. Therefore, we can assume that in

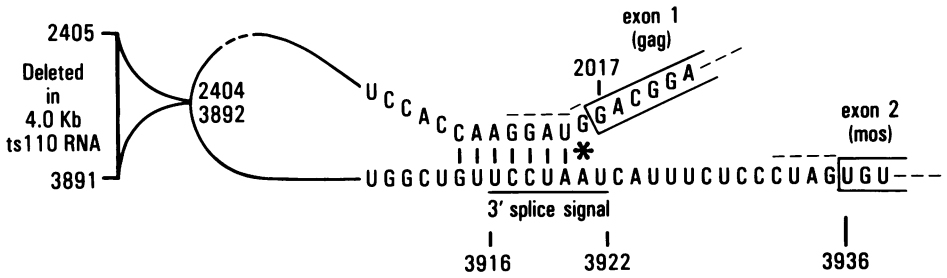


FIG. 10. Possible base-pairing interactions involved in the formation of the 3.5-kb *ts*110 RNA by RNA splicing of the 4.0-*kb* RNA genome. The sequence of Mo-MuSV 124 and the numbering system of Van Beveren et al. (38) has been used for this diagram. The model shows a portion of the *ts*110 Mo-MuSV 4.0-kb RNA genome. The 4.0-kb genome was formed as a result of a 1,488-base deletion of sequences present in the 5.3-kb genome of the parental virus. *, Possible site for a 2'-5' phosphodiester bond (a branch point) between the adenosine residue of the 3' splice signal and the guanosine residue at the 5' end of the intron. This bond would result in a lariat-like structure found in RNA splicing intermediates. The nucleotides overlined at the 5' and 3' intron borders could base pair with the 5' side of U1 RNA (including a possible G-U pairing between *ts*110 RNA and U1 RNA). Exon 1 ends inside p30 at base 2017; exon 2 begins inside of v-*mos* at base 3936. The 3' splice signal underlined in the figure corresponds to the branch point consensus sequence as described by Ruskin et al. (32).

204-2F6 and 204-2F8 cells the 3.5-kb RNA species is, as in 6m2 cells, a splicing product of the 4.0-kb genomic RNA and that the splicing reaction remains thermosensitive after infection of new cells with *ts*110 Mo-MuSV.

Another possible interpretation of our results is that the 3.5-kb RNA is unstable at 39°C in 6m2 cells as well as in the new cell lines. Such specific instability of the 3.5-kb RNA seems unlikely, since it is readily detected at 39°C in the 206-2IC cell line, which is a 6m2 cell line productively infected with Mo-MuLV (Fig. 4A and B, lanes 2, in reference 11). We have shown previously that 206-2IC cells, unlike 6m2 cells, contain two proviral DNAs that could code for the 4.0- and 3.5-kb RNAs, respectively (11). Considering all the available information, we cannot eliminate the unlikely possibility that in 204-2F6 and 204-2F8 cells the 3.5-kb RNA is less stable than in 6m2 cells.

In previous work we have shown that superinfection of 6m2 cells with Mo-MuLV did not promote *ts*110 Mo-MuSV splicing at the restrictive temperature, although the Mo-MuLV-specified 3.0-kb *env*-mRNA was still spliced at this same temperature (11). This finding provided indirect evidence for a virus defect responsible for the splicing temperature sensitivity of the 4.0-kb RNA. The fact that the splicing defect can be transferred by viral infection, as shown in this paper, provides direct evidence supporting this conclusion.

The more obvious explanation for the thermosensitivity of the splicing reaction in *ts*110 Mo-MuSV is that the RNA-RNA structures bringing together the sequences to be spliced are somehow heat unstable. Since the *ts*110 genome has not yet been cloned, only the sequence of Mo-MuSV 124 can be examined (38). We have found a sequence (14 to 20 nucleotides upstream from the proposed acceptor splice sequence) that is homologous to the globin consensus 3′ splice branch point signal (32) which has been involved in lariat formation in in vitro splicing experiments (20, 28, 32) (Fig. 10). It should be pointed out, that this particular region of *ts*110 Mo-MuSV was not sequenced by Nash et al. (25) in their primer extension sequencing of *ts*110 4.0-kb RNA, since their primer covered this branch point site. Moreover, upstream of this 3′ splice signal there are a 7-base sequence complementary with the 5′ border of the intron at the proposed donor splice sequence (Fig. 10, see base-paired sequence) and a 9-base sequence (5 + 4) in the donor and acceptor sites of the intron complimentary to the 5′ portion of the U1 small nuclear RNA (see overlined bases in Fig. 10), according to the model first proposed by Lerner et al. for the general splicing reaction (21). However, no subgenomic *mos*-containing RNA species with these splicing signals nor the other donor and acceptor splice sites has been unequivocally detected in Mo-MuSV 124-infected cells (7, 8, 12). Splicing intermediates containing lariat structures have not yet been described in retroviruses. It seems likely, however, that the sequences described above should play a role in the 4.0-kb RNA splicing, and a complete sequence analysis of *ts*110 should provide more information on the splicing defect.

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