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Murine Sarcoma Virus ts110 RNA Transcripts: Origin from a Single Proviral DNA and Sequence of the *gag-mos* Junctions in Both the Precursor and Spliced Viral RNAs

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Our previous studies have argued persuasively that in murine sarcoma virus ts110 (MuSVts110) the *gag* and *mos* genes are fused out of frame due to a ~1.5-kilobase (kb) deletion of wild-type murine sarcoma virus 349 (MuSV-349) viral information. As a consequence of this deletion, infected cells grown at 39°C appear morphologically normal, producing a 4-kb viral RNA and a truncated *gag* gene product, P58^{gag}. At 33°C, however, MuSVts110-infected cells appear transformed, producing two viral RNAs, about 4 and 3.5 kb in length, and two viral proteins, P58^{gag} and P85$^{gag\text{-}mos}$. Recent S1 nuclease analyses (Nash et al., J. Virol. 50:478–488, 1984) suggested strongly that at 33°C about 430 bases surrounding the out-of-frame *gag-mos* junction and bounded by consensus splice donor and acceptor sites are excised from the 4-kb RNA to form the 3.5-kb RNA. As a result of this apparent splicing event, the *gag* and *mos* genes seemed to be fused in frame and allowed the translation of P85$^{gag\text{-}mos}$. In the present study, DNA primers hybridizing to the MuSVts110 4- and 3.5-kb RNAs just downstream of the *gag-mos* junction points were used to sequence these junctions by the primer extension method. We observed that, relative to wild-type MuSV-349 5.2-kb RNA, the MuSVts110 4-kb RNA had suffered a 1,488-base deletion as a result of the fusion of wild-type *gag* gene nucleotide 2404 to wild-type *mos* gene nucleotide 3892. This *gag-mos* junction is out of frame, containing both TAG and TGA termination codons in the reading frame 42 and 50 bases downstream of the *gag-mos* junction, respectively. Thus, the MuSVts110 4-kb RNA can only be translated into a truncated *gag* precursor containing an additional C-terminal 14 amino acid residues derived from an alternate *mos* gene reading frame. Similar analyses of the MuSVts110 3.5-kb RNA showed a further loss of both *gag* and *mos* sequences over those deleted in the original 1,488-base deletion. In the MuSVts110 3.5-kb RNA, we found that *gag* nucleotide 2017 was fused to *mos* nucleotide 3936 (nucleotide 2449 in the MuSVts110 4-kb genome). This 431-base excised fragment is bounded exactly by in-frame consensus splice donor and acceptor sequences. As a consequence of this splice event, the TAG codon is excised and the restoration of the original *mos* gene reading frame allows the TGA codon to be bypassed. As a complement to the above sequence data, blot hybridization studies showed unequivocally that MuSVts110-infected nonproducer 6m2 cells contain a single, approximately 4.4-kb MuSVts110-related viral genome. The restriction map of this provirus was consistent with a relationship to wild-type MuSV-349 viral DNA by way of a ~1.5-kb deletion between the *gag* and *mos* genes. No 3.5-kb provirus could be detected in 6m2 cells, necessitating that the 3.5-kb RNA be derived from the transcription product of the 4.4-kb genome. In MuSVts110 producer 206-2IC cells chronically superinfected with Moloney murine leukemia virus, however, an integrated 3.9-kb MuSVts110-related genome was readily apparent. From its restriction map, this 206-2IC unique viral DNA seems to have arisen by integration of a helper virus reverse transcriptase-mediated cDNA copy of the spliced 3.5-kb RNA.

Transformation of cells by wild-type Moloney murine sarcoma virus (Mo-MuSV) is mediated by P37^{mos}, the full-size polypeptide product of the v-*mos* gene. 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 (4, 8, 12). MuSVts110, a temperature-sensitive (ts) transformation mutant of MuSV (2), differs from wild-type MuSV in that its transforming polypeptide is a *gag-mos* fusion protein, designated P85$^{gag\text{-}mos}$, which is produced in infected cells grown at 33°C but absent when these cells are grown at 39°C (6, 21). Studies in our laboratory have centered on the mechanism by which the differential expression of P85$^{gag\text{-}mos}$ is governed. It was first observed that, in infected cells grown at the nonpermissive temperature of 39°C, a truncated *gag* gene precursor polypeptide, P58^{gag}, and a 4-kilobase (kb) viral RNA were readily observed (6). Within 2 to 3 h of a shift to the permissive growth temperature of 33°C, however, P85$^{gag\text{-}mos}$ together with a 3.5-kb viral RNA could be found in infected cells (6, 11). Their appearance correlated well with the induction of the transformed phenotype (3). Initial heteroduplexing experiments performed to determine the structure of the MuSVts110 viral RNA species established that, relative to wild-type viral RNA, both the 4- and 3.5-kb MuSVts110 RNAs contained central deletions which spanned the region between a point within the p30 coding sequence and the 5′ end of the v-*mos* gene (7). In further experiments, using S1 nuclease mapping (11), it was established that the MuSVts110 4-kb RNA contained a deletion of about 1,475 bases which joined the 3′ end of the p30 coding region to a joint just downstream of the first *mos* gene initiation codon. Similar S1 analyses on the MuSVts110 3.5-kb RNA revealed that a consensus splice donor site in the 5′ end of the p30 coding region (at nucleotide 2017) seemed to be joined in frame to a splice acceptor site in the 5′ end (nucleotide 3936) of the v-*mos* gene.

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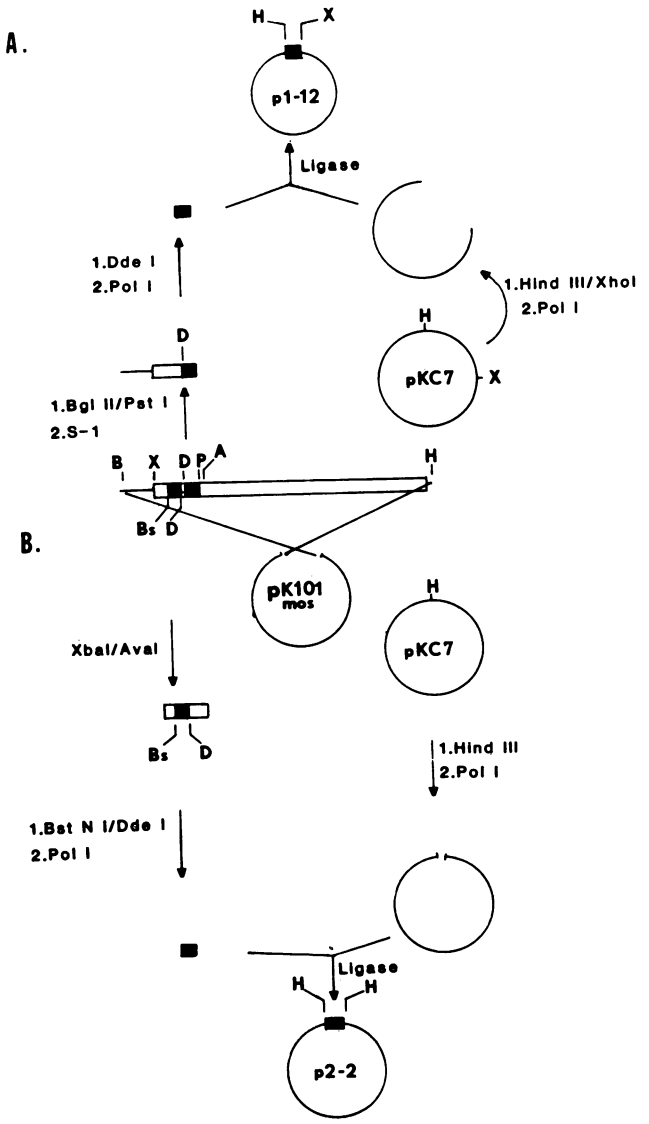

FIG. 1. Construction of primers for DNA sequencing. As described in detail in the text, primer 1 (A) was constructed by insertion of a 52-bp *Dde*I fragment of the wild-type MuSV-124 *mos* gene into *Hin*dIII-digested pKC7 DNA. Before ligation, all restriction sites were blunt ended by "filling in" with the Klenow fragment of *E. coli* DNA polymerase I. Primer 2 (B) was constructed by insertion of a 56-bp *Dde*I/*Pst*I fragment of the wild-type MuSV-124 *mos* gene into *Hin*dIII/*Xho*I-digested pKC7 plasmid DNA. Before ligation, all restriction sites were blunt ended by filling in with the Klenow fragment or were trimmed by S1 nuclease digestion.

Thus, our previous studies in this system suggested that P58^{gag} was the product of an out-of-frame fusion of the *gag* and *mos* genes and that a possibly temperature-sensitive splicing event permitted the alignment of these two genes in a continuous open reading frame. These suggestions have awaited the resolution of two points. First, it was necessary that the *gag* gene-*mos* gene junctions in the two MuSVts110 RNAs be sequenced to test the assumptions regarding their proposed compositions. In the present study we have shown by primer extension nucleotide sequencing that the primary transcript of MuSVts110 viral DNA is a 4-kb RNA in which the *gag* and *mos* genes are, in fact, joined out of frame such that termination of translation would occur shortly after the *gag-mos* junction. The polypeptide product of this RNA is predicted to be a truncated *gag* precursor polypeptide containing a short C terminus translated from an alternative *mos* gene reading frame. Further sequencing experiments showed that the MuSVts110 3.5-kb RNA is derived from the 4-kb RNA by a temperature-dependent splicing mechanism which excises 431 bases surrounding the out-of-frame *gag* gene-*mos* gene junction in the 4-kb RNA and rejoins the *gag* and *mos* genes in frame to allow the translation of P85$^{gag\text{-}mos}$.

The second point addressed in this study is the important question of the number of MuSVts110 proviruses in 6m2 cells. We present evidence here that MuSVts110-infected 6m2 cells contain a single 4.4-kb viral DNA which, relative to wild-type MuSV-349 DNA, contains an approximately 1.5-kb central deletion between the *gag* and *mos* genes. Thus, separate origins for the MuSVts110 4- and 3.5-kb RNAs seem extremely unlikely and the proposed splicing mechanism seems all the more attractive.

MATERIALS AND METHODS

Cells and viruses. Mo-MuSVts110 was derived from Mo-MuSV-349, a subclone of Mo-MuSV-124 (1), as described by Blair et al. (2). Nonproducer normal rat kidney (NRK) cells infected with Mo-MuSVts110, termed 6m2, were maintained at 33°C in McCoy 5a medium containing 15% (vol/vol) fetal calf serum. MuSVts110 producer cells, designated 206-2IC, were generated by superinfection of 6m2 cells with the IC strain of Moloney murine leukemia virus (Mo-MuLV) (6). These cells were maintained in the above culture medium and used for virus production in 2-quart (ca. 1.9-liter) roller bottles. A spontaneous revertant of the 6m2 cell line, designated 54-5A4, was also maintained as above at 37°C. The 54-5A4 cells exhibited a transformed phenotype at both 33 and 39°C.

DNA primer construction. The construction of DNA primers from wild-type MuSV-124 DNA is depicted in Fig. 1. Briefly, primer 1 was constructed by excision of a 342-base pair (bp) DNA fragment between an upstream *Bgl*II site (nucleotide 3698) and a *Pst*I site (nucleotide 4040) within the wild-type *mos* gene of MuSV-124 DNA (generously given to us by Dino Dina). This fragment was blunt ended by digestion with S1 nuclease and further digested with *Dde*I. The 56-bp *Dde*I/*Pst*I fragment was filled in with the Klenow fragment of *Escherichia coli* DNA polymerase I, ligated to agarose gel-purified pKC7 (13) plasmid DNA doubly digested with *Hin*dIII/*Xho*I and then filled in with the Klenow fragment, and used to transform *E. coli* RRI. The 56-bp insert in the plasmid, designated p1-12, could be released by *Hin*dIII/*Xho*I double digestion.

Primer 2 was constructed exactly as described by Donoghue and Hunter (5). A DNA fragment extending from an *Xba*I site 10 bp upstream (nucleotide 3863) of the beginning of the *mos* gene to an *Ava*I site 194 bp downstream (nucleotide 4057) was excised from wild-type MuSV-124 DNA. This fragment was doubly digested with *Bst*NI/*Dde*I, filled in with the DNA polymerase I Klenow fragment, ligated to *Hin*dIII-digested/Klenow-filled-in pBR322 DNA, and used to transform *E. coli* RRI. By using this subcloning strategy, the 56-bp insert in the plasmid, which we designated p2-12, could be excised by *Hin*dIII digestion. This plasmid is identical to pDD39 described by Donoghue and Hunter (5).

Purification of plasmid DNA. The various inserts and fragments described above were excised by digestion with the appropriate restriction enzyme(s), size fractionated on a 0.8% agarose gel, and stained with ethidium bromide. DNA

was purified from agarose by adsorption to glass powder (18).

In cases where small DNA fragments were purified from polyacrylamide gels, gel slices were crushed to a paste in 2 ml of TNE buffer (10 mM Tris [pH 8], 100 mM NaCl, 1 mM EDTA) and agitated overnight. Acrylamide granules were removed by centrifugation, and the supernatants were filtered through Isolab QS-P columns to remove the remaining acrylamide. The column effluent containing the DNA was phenol extracted, ethanol precipitated, and dissolved in water.

Isolation of cellular RNA. Intracellular RNA was isolated from tissue culture cells by a guanidine hydrochloride extraction procedure (20). Briefly, cellular pellets were dissolved in solution A, 8 M guanidine hydrochloride–100 mM sodium acetate (NaOAc; pH 5), and subjected to vigorous Dounce homogenization. After centrifugation of the extracts at 10,000 rpm for 15 min, nucleic acids were precipitated from the supernatant by addition of 0.5 volume of ethanol at −20°C for 30 min and collected by centrifugation at 7,000 rpm for 20 min. The nucleic acid pellets were dissolved in 0.25 to 0.50 the original volume of solution B (solution A plus 0.1 volume of 200 mM EDTA) and again precipitated with 0.5 volume of ethanol. This last step was repeated twice more, decreasing the volume of solution B by half each time. The final pellet was dissolved in 20 mM EDTA (pH 7.5) and extracted with chloroform-isobutanol (4:1). The aqueous phase was mixed with 2 volumes of 4.5 M NaOAc (pH 6), and the RNA was precipitated at −20°C overnight. The RNA was collected by centrifugation, washed once with 3 M NaOAc and once with 70% ethanol, and dissolved in water.

Preparation of cellular DNA (13). Cells growing in roller culture were rinsed with an isotonic buffer and gently lysed in 2% sodium dodecyl sulfate (SDS)–8 M urea–0.35 M NaCl–1 mM EDTA buffered with 10 mM Tris (pH 8). The cell lysate was extracted with an equal volume of phenol-chloroform (1:1) saturated with the above SDS-urea buffer and ethanol precipitated. Nucleic acids from each roller culture were dissolved in 3 to 5 ml of water, digested sequentially with 50 μg of RNase per ml at 37°C for 60 min and 100 μg of proteinase K per ml at 37°C for 120 min, re-extracted with phenol-chloroform, precipitated with ethanol, and dissolved in water.

Blot hybridization of DNA. Routinely, 15 μg of cellular DNA, digested with a given restriction enzyme as recommended by the supplier, was analyzed on a 0.8% agarose gel in 50 mM Tris (pH 8.3)–40 mM NaOAc–2 mM EDTA at 40 V for 16 to 18 h and transferred to nitrocellulose essentially as described by Southern (15) and modified by Wahl et al. (19). The nitrocellulose was prehybridized at 37 to 42°C in 50% formamide–5× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt solution (0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone)–0.1% SDS, containing 100 μg of sheared salmon sperm DNA and 2 mg of base-hydrolyzed yeast RNA per ml. Hybridization was carried out at 37 to 42°C in the same solution containing 1× Denhardt solution and 2×10^6 cpm of ^{32}P-labeled probe (1×10^8 to 3×10^8 cpm/μg) per ml. After hybridization, the filters were washed four times with 3× SSC–0.1% SDS and twice with 0.1× SSC–0.1% SDS at 37°C, dried, and autoradiographed.

5′ End labeling. Restriction fragments were first dephosphorylated with calf intestinal phosphatase (Boehringer Mannheim Corp.). Briefly, DNA was adjusted to 50 mM Tris (pH 9), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 1 mM spermidine containing 10 U of calf intestinal phosphatase at 37°C. After 30 min, an additional 10 U of calf intestinal phosphatase was added for another 30 min. The digest was mixed with 0.1 volume of 10× TNE and 0.05 volume of 10% SDS for 15 min at 68°C to halt the reaction. After phenol-chloroform (1:1) extraction, the nucleic acids were ethanol precipitated. For 5′ end labeling, dephosphorylated DNA was adjusted to 100 mM Tris (pH 7.6), 20 mM $MgCl_2$, 10 mM dithiothreitol, 0.2 mM spermidine, and 0.2 mM EDTA, to which was added 10 to 20 U of T4 kinase and 150 μCi of [γ-^{32}P]ATP for 1 h at 37°C. After the addition of 20 μg of carrier DNA, the reaction mix was desalted by passage over a G-50 column equilibrated in TNE buffer. Radioactivity in the void volume was pooled, ethanol precipitated, and dissolved in TE buffer (10 mM Tris [pH 8], 1 mM EDTA).

S1 nuclease analysis. S1 nuclease analyses were done essentially as described previously (11). Briefly, the 5′ end-labeled 359-bp insert from pBA.36 was hybridized to 10 μg of total cellular RNA from 6m2 cells grown at 39, 33, or 28°C, as indicated in the text, in 80% formamide–40 mM PIPES [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% HOAc, dried, and autoradiographed.

Primer extension. 5′ end-labeled primer 1 or 2 was mixed individually with 10 μg of total cellular RNA from 6m2 cells grown at 39, 33, or 28°C, as indicated in the text, and ethanol precipitated. The pellets were thoroughly dissolved in 30 μl of 80% formamide–40 mM PIPES (pH 6.8)–400 mM NaCl–1 mM EDTA, immersed in an 85°C water bath for 10 min, transferred to a water bath at 65°C, and allowed to hybridize during cooling to room temperature overnight (5). After hybridization, the reaction mixes were ethanol precipitated and dissolved in 25 μl of 10 mM Tris (pH 7.4). Primer extension in the presence of dideoxynucleoside triphosphates (ddNTPs) was carried out essentially as described by Donoghue and Hunter (5). A 5-μl portion of the hybrid mix was dispensed into four separate tubes, to each of which was added 1 μl of a 200 μM dNTP mix (dATP, dCTP, dGTP, dTTP), 1 μl of a ddNTP mix calculated to produce the ratios given in parentheses (ddATP/dATP = 1.3; ddCTP/dCTP = 0.4; ddGTP/dGTP = 0.3; ddTTP/dTP = 0.9), and 1 μl of avian myeloblastosis virus reverse transcriptase (Seikagaku; 10 U/μl). These mixtures were incubated at 40 to 42°C for 60 min to extend the primers, after which they were ethanol precipitated after the addition of 10 μg of carrier yeast RNA and 10 mM $MgCl_2$.

Sequence analysis. The extended primers were dissolved in 75% formamide–20 mM EDTA (pH 8.0)–0.01% bromophenol blue-xylene cyanol, heated to 100°C for 2 min, and analyzed on a 6% polyacrylamide–8 M urea gel in TBE buffer (50 mM Tris [pH 8.3], 50 mM boric acid, 2 mM EDTA) at 2,000 V for 105 min. After electrophoresis, the gel was either subjected to autoradiography directly, using Kodak XAR-2 film and Dupont Cronex Lightning-plus intensifying screens, or fixed with 10% HOAc–10% methanol for 20 min, dried, and autoradiographed.

RESULTS

Relative abundance of MuSVts110-related mRNA species. As reported previously (11), we have developed a sensitive assay for the detection of both the 4.0- and the 3.5-kb

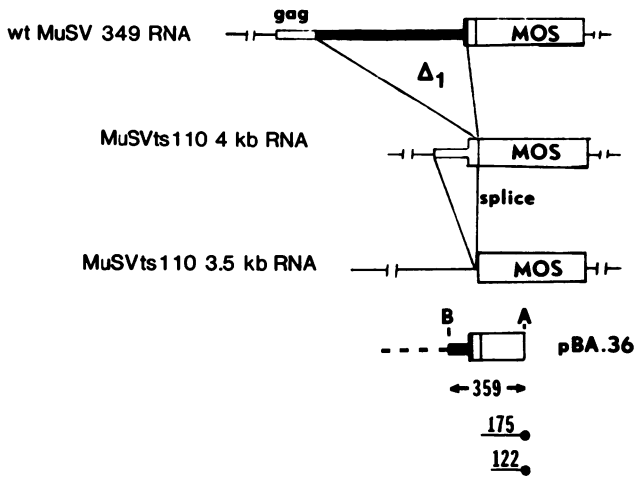

FIG. 2. S1 nuclease assay for MuSVts110 viral RNA. The assay depends on the differential hybridization of the 359-bp insert in pBA36 to wild-type MuSV-349 RNA, MuSVts110 4-kb RNA, and MuSVts110 3.5-kb RNA. As shown, wild-type MuSV-349 RNA, MuSVts110 4-kb RNA, and MuSVts110 3.5-kb RNA, respectively, will protect 359, 175, and 122 bp of the 5′ end-labeled pBA36 359-bp insert from digestion by S1 nuclease.

MuSVts110 RNAs in the presence of each other. The assay relies on the incomplete hybridization of a 359-bp wild-type viral DNA fragment (designated pBA.36) to both MuSVts110 RNAs (Fig. 2). Because of the ~1.5-kb central deletion, the MuSVts110 4-kb RNA will protect approximately 175 bases of the pBA.36 insert from digestion with S1 nuclease. Since the 3.5-kb RNA has a larger deletion in its *mos* gene than does the 4-kb RNA, hybrids between it and pBA36 can protect only 122 bases of pBA36 from S1 digestion. Thus, as shown previously (11), and in the accompanying paper (5a), both the 4- and the 3.5-kb RNAs can readily be detected in 6m2 cells grown at 33°C. Within 2 h of a shift to 39°C, however, only the 4-kb RNA can be detected in 6m2 cells. Interestingly, if 6m2 cells are shifted to 28°C, the spliced 3.5-kb RNA becomes predominant at the expense of the unspliced 4-kb RNA (5a; G. E. Gallick, R. Hamelin, S. Maxwell, D. Duyka, and R. B. Arlinghaus, Virology, in press). Thus, by simple temperature shifts, we can select conditions under which either the 4.0- or the 3.5-kb RNA predominates in 6m2 cells.

***gag-mos* junctions in the MuSVts110 RNA species.** In Fig. 3 are illustrated the regions of the MuSVts110 4- and 3.5-kb RNAs to which primers 1 and 2 will hybridize. The approximate positions of the *gag-mos* junctions in both of these RNAs, as determined by S1 nuclease mapping experiments such as the one described above, are also indicated (nucleotide 3883 in the 4-kb RNA and nucleotide 3936 in the 3.5-kb RNA). Note that primer 2 will hybridize along its entire length only to the 4-kb RNA; hybridization of primer 2 to the 3.5-kb RNA is interrupted at its 3′ end because of the deletion in the *mos* gene, and thus this hybrid cannot be extended with reverse transcriptase. Hence, an unambiguous sequence for the *gag-mos* junction in the 4-kb RNA can be obtained with primer 2. In contrast, primer 1 will hybridize completely with both RNAs. Hence, its extension will result in two superimposed and thus uninterpretable sequences if one uses RNA from 6m2 cells grown at 33°C. As discussed below, however, we were able to circumvent this problem by the extension of primer 1 hybridized to RNA from 6m2 cells grown at 28°C. In these cells, the 3.5-kb RNA is the dominant MuSVts110 species (5a). Extension of these primer 1 hybrids produced a clear sequence of the *gag-mos* junction in the 3.5-kb RNA.

As a control on the method and as a check on whether the wild-type MuSV-349 sequence was the same as the published sequence of MuSV-124 in the region under analysis, primers 1 and 2 were individually hybridized to MuSV-349 RNA and extended in the presence of dideoxynucleotides. An example of the sequence data obtained from these two experiments is shown in Fig. 4. Using both primers, we found that the MuSV-349 RNA sequence was identical to the published MuSV-124 sequence in the region analyzed (nucleotides 3815 to 3972 of the MuSV-124 sequence; data not completely shown).

We next hybridized 5′ end-labeled primer 2 to cellular RNA from 6m2 cells grown at either at 33 or 39°C and extended the primer as was done with MuSV-349 RNA. From the nucleotide sequence obtained (Fig. 5), we could ascertain that the MuSVts110 4-kb RNA had suffered a 1,488-base deletion relative to wild-type RNA (Fig. 5B), resulting in the fusion of *gag* gene nucleotide 2404, located 71 bases upstream of the p30-p10 junction in wild-type viral RNA, to *mos* gene nucleotide 3892, found 17 bases downstream from the 5′ end of the *mos* gene. The position of this deletion is in good agreement with our data, using the S1 nuclease method (11), and with the results of an earlier heteroduplexing study (7). By using S1 nuclease, the deletion was predicted to join nucleotide 2409 to nucleotide 3883, located 25 bases upstream of the 3′ end of the primer 1-RNA hybrid (nucleotide 3908). The actual junction point was found to be only 12 to 13 nucleotides upstream of nucleotide 3908 (Fig. 5). This 1,488-base deletion has resulted in the removal of the first *mos* gene initiation codon (cf. Fig. 7A and B) and would cause the *mos* gene to be translated out of frame for 14 amino acids before reaching a TAG and then a TGA stop codon 42 and 54 bases downstream of the *gag-mos* junction point. Interestingly, the 1,488-base deletion is bracketed by the 6-base direct repeat AACGCC found at nucleotides 2399 to 2404 and 3886 to 3891 in the wild-type *gag* and *mos* genes, respectively. One of these repeats was retained after the deletion. The calculated size for this deleted RNA is 3,820 nucleotides (wild-type 5,320 bases minus a 1,488-base deletion), in good agreement with the 4-kb size previously estimated by blot hybridization (6, 11, 16).

To obtain an interpretable *gag-mos* junction sequence for the MuSVts110 3.5-kb RNA, we hybridized primer 1 to RNA from 6m2 cells grown at 28°C. The bulk of the viral RNA in 6m2 cells grown under these conditions is 3.5 kb in size. The sequence obtained from such an extended primer 1 hybrid is shown in Fig. 6. From this sequence it could be ascertained (Fig. 6B) that nucleotide 3936 in the wild-type *mos* gene (2488 in the MuSVts110 4-kb RNA), located 44 bases downstream of the *gag-mos* junction in the 4-kb RNA, was fused to nucleotide 2017 in the *gag* gene, located 387 bases upstream of the *gag-mos* junction in the 4-kb RNA. As expected, this fusion point is about 40 bases upstream of the 3′ end of the primer 1/viral RNA hybrid. The sequence of the 3.5-kb RNA is unambiguous up to the intron/exon border (Fig. 6). Upstream of this border, however, the presence of low levels of 4.0-kb RNA in the RNA preparation decreased the resolution obtainable. If, however, one considers only the major bands in the sequence ladder, it is clear that, as suggested by our previously published work (11), the splice donor site (9) surrounding nucleotide 2017 and the splice

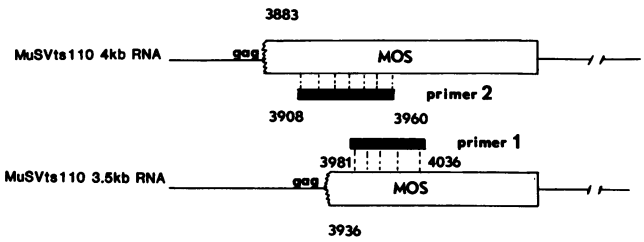

FIG. 3. Hybridization of primers 1 and 2 to the MuSVts110 4- and 3.5-kb RNAs. Primer 1 will hybridize to a 56-base stretch of both the 4.0- and 3.5-kb MuSVts110 RNAs about 50 bases downstream of the proposed splice site. Primer 2 was constructed to hybridize completely to a 52-base stretch of the MuSVts110 4-kb RNA approximately 25 bases downstream of the proposed *gag* gene-*mos* gene junction at nucleotide 3883. Because its upstream terminus extends across the proposed splice junction in the MuSVts110 3.5-kb RNA, the hybrid formed with this particular RNA cannot be extended.

acceptor surrounding nucleotide 3936 (9) are joined in frame as (GCA G/TG TCT). The fusion of nucleotides 2017 and 3936 (2448 in the 4.0-kb RNA), removing 431 bases from the 4-kb RNA, excises the TAG stop codon found at the intron/exon border and restores the original *mos* reading frame, allowing the bypass of the TGA stop codon (Fig. 7B). Thus, an in-frame *gag-mos* fusion protein, presumably P85$^{gag\text{-}mos}$, can be translated from the resulting 3.5-kb RNA. Calculating from the established MuSV-124 sequence, this fusion protein would have a theoretical molecular weight of about 72,000 composed of 325 *gag* gene amino acids and 353 *mos* gene amino acids. The RNA from which this protein would be translated is 3,389 bases, 431 bases shorter than the 3,820-base RNA, in good agreement with the 3.5-kb size previously estimated by blot hybridization (6, 11, 16).

MuSVts110-related proviruses in cells. It was of fundamental importance to determine the number and structural organization of MuSVts110-related proviruses in 6m2 cells since the validity of our interpretation of our data rests on the assumption that there should be only one transcriptionally active MuSVts110 provirus in 6m2 nonproducer cells and that transcription of this viral DNA should produce only the unspliced 4-kb RNA as the primary transcript. The predicted size of this DNA should be about one long

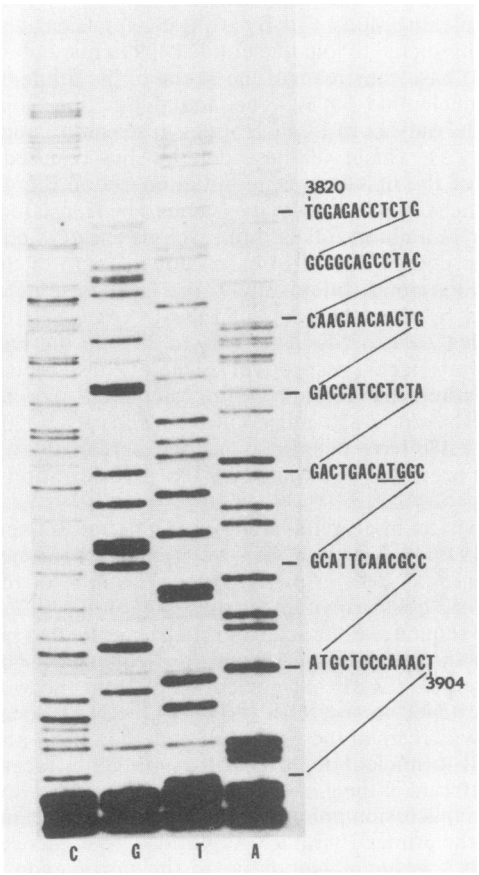

FIG. 4. Nucleotide sequencing of wild-type MuSV-349 RNA by use of primer 2. Primer 2 was 5′ end labeled, hybridized to MuSV-349 RNA, and extended in the presence of ddNTPs, and the product was analyzed on a 6% polyacrylamide sequencing gel. The sequence obtained was identical to that published by Van Beveren et al. (17).

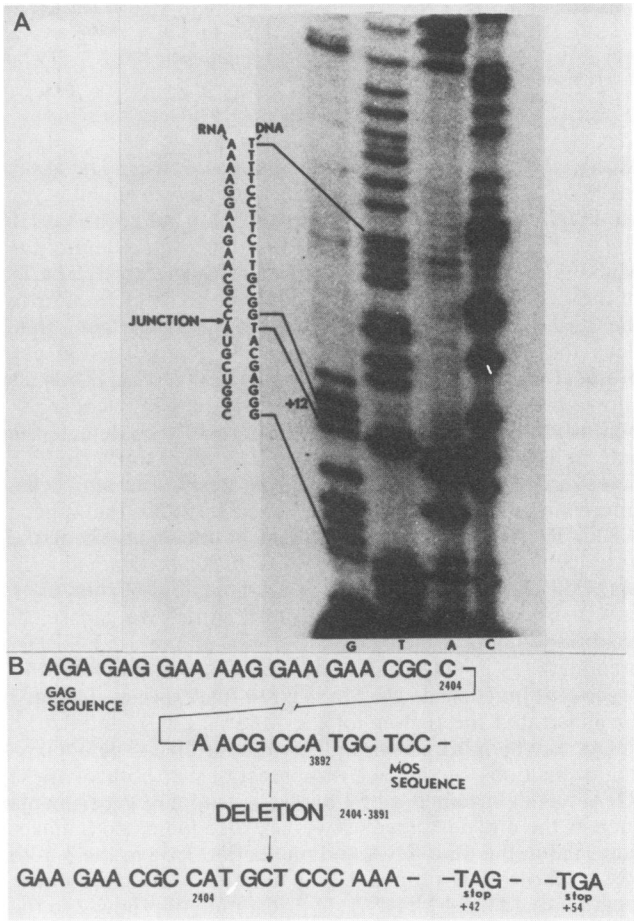

FIG. 5. Nucleotide sequence of the *gag* gene-*mos* gene junction in MuSVts110 4-kb RNA. 5′ end-labeled primer 2 was hybridized to 10 μg of cellular RNA from 6m2 cells grown at 33°C and extended by reverse transcriptase in the presence of ddNTPs, and the product was analyzed on a 6% polyacrylamide sequencing gel. (A) Nucleotide sequence of extended primer 2. Alongside the sequence is shown the actual DNA sequence read from the gel (complementary to the RNA plus strand) and the plus-strand RNA sequence. In (B) the *gag* gene-*mos* gene junction in the MuSVts110 4-kb DNA is shown diagrammatically.

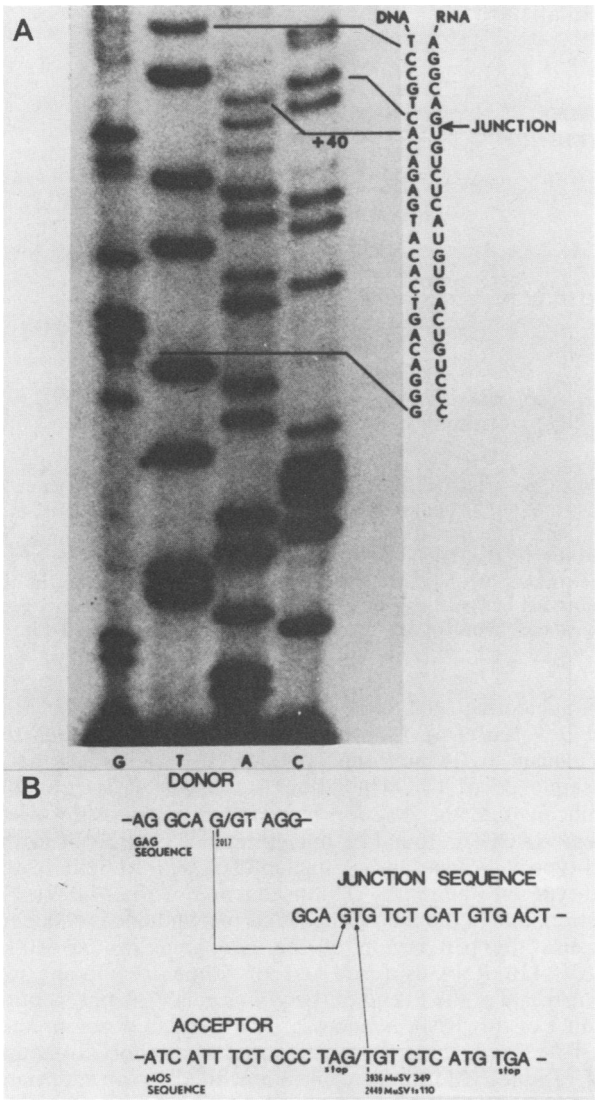

FIG. 6. Nucleotide sequence of the splice site in the MuSVts110 3.5-kb RNA. 5′ end-labeled primer 1 was hybridized to 10 μg of cellular RNA from 6m2 cells grown at 28°C, extended in the presence of ddNTPs, and analyzed on a 6% polyacrylamide sequencing gel. (A) Nucleotide sequence of extended primer 1. Alongside the sequence is shown the actual DNA sequence read from the gel and the complementary plus-strand RNA sequence. In (B), the *gag* gene-*mos* gene splice junction in the MuSV-349 3.5-kb RNA (as DNA) is shown diagrammatically.

terminal repeat (LTR) (~590 bases) larger than the 3,820-base RNA transcript, or about 4.4 kb.

Previously we have presented preliminary evidence that 6m2 cells contain a single candidate MuSVts110 provirus residing in an approximately 12-kb *mos*+ *Bam*HI DNA fragment (11). In the data presented below, we reaffirm and extend the previous evidence. Digestion of 6m2 cellular DNA with *Bam*HI yields two *mos*+ DNA fragments, approximately 21 and 12 kb in size (Fig. 8A, lane 2). Since *Bam*HI-digested DNA from uninfected NRK-2 cells contains only the 21-kb *mos*+ species (Fig. 8A, lane 1), the 21-kb DNA represents the c-*mos* gene and the MuSVts110 provirus probably resides in the 12-kb fragment. DNA from other MuSVts110-infected cell lines, such as producer 206-2IC and revertant 54-5A4 cells, as expected, also contain a 12-kb *mos*+ DNA (Fig. 8A, lanes 3 and 4). As the 21-kb rat c-*mos* gene is a single-copy gene, the equivalent hybridization intensity observed with the 12-kb viral DNA suggests that it also is present at the level of one copy per genome. In contrast to this simple situation, *Bam*HI-digested DNA from wild-type MuSV-349-infected cells shows a more complex pattern of *mos*+ proviral DNAs, ranging from 8 to 20 kb in size (Fig. 8A, lane 5). If 6m2 cellular DNA is digested with *Bam*/*Sst*I or *Sst*I alone (*Sst*I is expected to cut once within each viral LTR, yielding a DNA exactly the size expected for the primary transcript), the 12-kb *mos*+ *Bam*HI fragment is reduced to about 3.85 kb in size in 6m2, 54-5A4, and 206-2IC cellular DNA (Fig. 8B, lanes 2, 3, and 4). In contrast, *Sst*I digestion of DNA from cells infected with wild-type MuSV-349 yielded a major 5.2-kb *mos*+ DNA fragment (Fig. 8B, lane 5). In uninfected NRK-2 cells, the 21-kb *Bam*HI c-*mos* fragment is cut by *Sst*I to 2.65 kb in size (Fig. 8B, lane 1). Thus, the 3.85-kb viral DNA is exactly the size expected for the MuSVts110 viral DNA, about 1.5 kb shorter than 5.2-kb wild-type DNA and distinct from the c-*mos* gene.

A partial restriction map of wild-type MuSV-349 DNA is shown at the top of Fig. 9. From this map it can be seen that the 1.5-kb deletion in the MuSVts110 genome should have removed the unique *Sal*I and *Xba*I sites present in the wild-type DNA. A comparison of 6m2 DNA and wild-type MuSV-349 DNA cut with *Sst*I, *Sst*I/*Sal*I, and *Sst*/*Xba*I showed that, as predicted, the 3.85-kb provirus was lacking the *Sal*I and *Xba*I restriction sites (Fig. 10A, lanes 1, 3, and 5) and that the wild-type MuSV-349 5.2-kb viral DNA contained the unique *Sal*I and *Xba*I restriction sites (Fig. 10B, lanes 2 and 3) at the positions established by other investigators (14, 17). To establish the approximate limits of the deleted region in the 3.85-kb viral DNA, double digests were performed with *Sst*I/*Xho*I and *Sst*/*Hin*dIII. The size of the virus-derived *mos*+ fragments produced by these digests, 2.4 and 3.4 kb, respectively (shown in Figure 10A, lanes 7 and 9), were about 1.5 kb shorter than the 3.7- and 4.6-kb fragments produced by *Sst*I/*Xho*I or *Sst*I/*Hin*dIII digests of wild-type MuSV-349 DNA (Fig. 10B, lanes 4 and 5). The 2.65-kb fragments seen in Fig. 10A, lanes 1, 2, 3, 4, 7, 8, 11, and 12, are derived from the rat c-*mos* gene, as are the ~1.6-kb fragments seen in Fig. 10A, lanes 5, 6, 9, and 10. Taken together, these data suggest that the 3.85-kb viral DNA contains a ~1.5-kb deletion mapping roughly between the *Xho*I site and the *Hin*dIII site. However, since an *Sst*I/*Bgl*I digest of the 3.85-kb viral DNA yielded a ~1.6-kb *mos*+ fragment exactly the size observed in *Bgl*I-digested wild-type viral DNA (cf. Fig. 10A, lane 11, with Fig. 10B, lane 6), the deletion in the 3.85-kb DNA could be further localized to the region between the *Xho*I site (located at wild-type nucleotide 1982) and the *Bgl*I site in the 5′ end of the v-*mos* gene (nucleotide 4120). By S1 mapping and primer extension sequencing, the actual MuSVts110 deletion maps between wild-type nucleotides 2404 and 3892 (see above). Thus, the organization of the 3.85-kb viral genome in 6m2 cells as estimated by restriction mapping correlates quite closely with its predicted structure based on these previous data.

Viral genomes in 206-2IC MuSVts110 producer cells. In the course of these experiments we were initially surprised to find that the 206-2IC producer cell line chronically superinfected with Mo-MuLV, unique among all MuSVts110-infected cell lines examined, contained a second virus-related *mos*+ DNA fragment. In an *Sst*I digest of 206-2IC

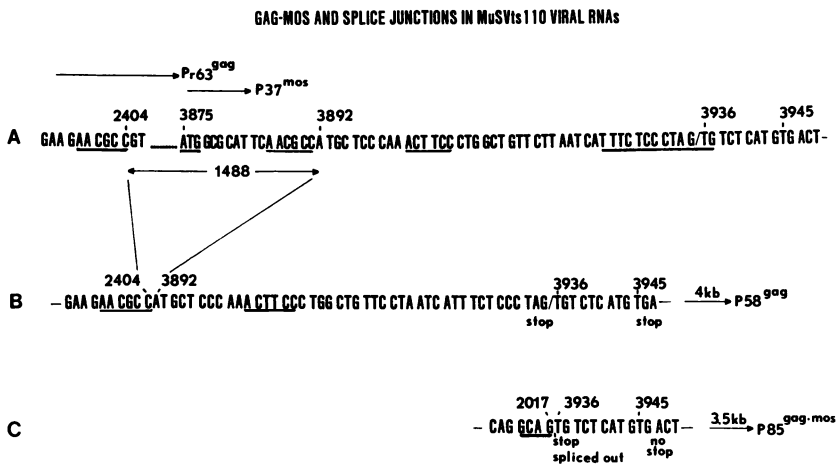

FIG. 7. Comparison of the MuSV-349 5.2-kb RNA, MuSVts110 4-kb RNA, and MuSVts110 3.5-kb RNA sequences. (A) Sequence of MuSV-349 RNA between nucleotides 2395 and 3949, showing nucleotides 2404 and 3892 bounded by the AACGCC repeats, the first *mos* initiation codon at nucleotide 3875, the intron/exon border at nucleotide 3936, and the out-of-frame TAG and TGA termination codons. (B) Sequence of the MuSVts110 4-kb RNA at the *gag* gene-*mos* gene junction, showing the deletion of 1,488 nucleotides including one of the AACGCC repeats, the out-of-frame *mos* sequence, and the in-frame stop codons at nucleotides 3933 and 3945. (C) Sequence of the MuSVts110 3.5-kb RNA at the *gag-mos* splice junction, showing the splice junction between *gag* gene donor nucleotide 2017 and *mos* gene acceptor nucleotide 3936, which restores the *mos* gene to its original reading frame, avoiding the TGA stop codon at nucleotide 3945.

DNA, this second fragment was estimated to be about 3.35 kb in size (Fig. 8B, lane 4; Fig. 10A, lane 2). To characterize this 3.35-kb DNA, a series of double digests similar to those done on 6m2 DNA (Fig. 10A) was performed, with the following results. As did the 3. 85-kb viral DNA in both 6m2 and 206-2IC cells, the 3.35-kb viral DNA lacked *Sal*I and *Xba*I restriction sites (Fig. 10A, lanes 4 and 6). Also, *SSt*I/*Xho*I and *Sst*I/*Hin*dIII double digests of the 3.35-kb DNA generated *mos*+ DNA fragments 400 to 500 bp shorter than those produced by digestion of the 3.85-kb viral DNA (cf. lanes 7 and 8 and 9 and 10, Fig. 10A). However, the *Sst*I/*Bgl*I digests of both the 3.8- and 3.35-kb DNAs (Fig. 10B, lanes 11 and 12) produced *mos*+ DNA fragments of identical size (1.6 kb), suggesting that both DNAs have an identical structure from a position very near the 5′ end of the v-*mos* gene downstream through the 3′ LTR. From these mapping data, it seems clear that the 400 to 500 bases present in the 3.85-kb DNA and missing from the 3.35-kb DNA must be located in the approximately 650-base region between the *Xho*I and *Bgl*I sites in the 3.85-kb DNA. Since this is approximately the region which is spliced out of the 4-kb DNA to form the 3.5-kb RNA, we favor the interpretation that the 3.35-kb DNA represents a DNA copy of the spliced 3.5-kb RNA which was reverse transcribed by the helper virus-associated reverse transcriptase and integrated into the genomic DNA of the 206-2IC cell line.

DISCUSSION

The data presented in this study argue forcefully that a single, centrally deleted, 4.4-kb (calculated size including both LTRs) viral DNA is transcriptionally active in MuSVts110-infected nonproducer 6m2 cells. When 6m2 cells are grown at 39°C, this 4.4-kb viral DNA is transcribed to form an approximately 3.8-kb viral RNA. If, as is reasonable, one assumes the polyadenylate tail on this RNA to be 100 to 150 nucleotides, the full size of this RNA should be 3.9 to 4.0 kb, in very close agreement with the 4.0-kb size estimated in our previous work (6, 11, 16). This 4.0-kb RNA is related to wild-type 5.2-kb MuSV-349 RNA by way of a 1,488-base deletion which had as its result the fusion of *gag* gene nucleotide 2404 to *mos* gene nucleotide 3892 (using the Van Beveren et al. [17] numbering system). Although the mechanism by which this deletion was generated as a consequence of UV irradiation is unclear, it is probably significant that the deletion is bounded by the 6-bp direct repeat AACGCC found at nucleotides 2399 to 2404 in the wild-type *gag* gene and at nucleotides 3886 to 3891 in the wild-type *mos* gene. As as consequence of the MuSVts110 deletion, one of the 6-base repeats is lost and one is retained. Because the p30 region of the *gag* gene in the 4.0-kb MuSVts110 RNA is fused out of frame to a point just downstream of the 5′ end of the v-*mos* gene, the polypeptide product of this RNA is necessarily a truncated *gag* precursor, $P58^{gag}$, which very probably contains a short (14-amino acid residue) C-terminal peptide encoded by an alternate, apparently normally unused, v-*mos* reading frame. Termination of the translation of this polypeptide is dictated by two closely spaced stop codons found in this particular *mos* reading frame 42 and 54 bases downstream from the *gag-mos* junction point. A further consequence of this deletion is the removal of the first *mos* gene initiation codon located at wild-type nucleotide 3875, a point 17 bases upstream of the 3′ end of the deletion. Thus, the MuSVts110 4.0-kb RNA cannot be translated to form wild-type $P37^{mos}$. Previously published in vitro translation experiments (10) have shown unequivocally that, in contrast to similarly translated MuSV-349 5.2-kb RNA, the MuSVts110 4.0-kb RNA does not give rise to $P37^{mos}$ but instead is quite capable of supporting the translation of $P33^{mos}$ and $P23^{mos}$ from the initiation codons found further downstream.

Our previous data obtained by S1 nuclease mapping strongly suggested that the *gag-mos* junction in the smaller MuSVts110 3.5-kb RNA was an in-frame junction formed by the union of consensus splice donor and acceptor sites found surrounding nucleotides 2017 and 3936 (11). The present sequence data support this previous suggestion. Hence, it now seems clear that in 6m2 cells grown at 33°C 431 bases surrounding the out-of-frame *gag-mos* junction in the 4.0-kb RNA can be spliced out, joining *gag* nucleotide 2017 to *mos* nucleotide 3936 (nucleotide 2448 in the 4-kb RNA sequence).

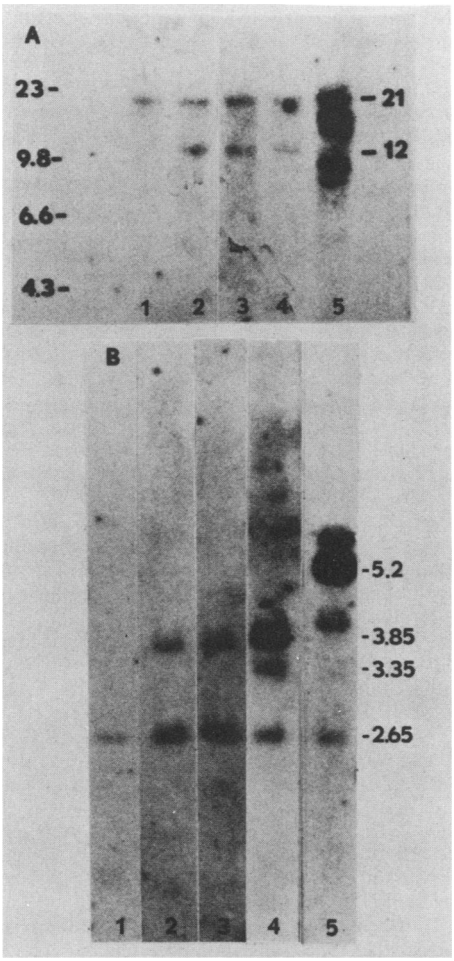

FIG. 8. Proviral DNA in MuSVts110-infected cells. High-molecular-weight DNA from 6m2 cells, 54-5A4 revertant cells, 206-2IC producer cells, MuSV-349-infected mouse TB cells, and uninfected NRK-2 cells was digested with *Bam*HI or doubly digested with *Bam*HI and *Sst*I, analyzed on a 0.8% agarose gel, transferred to nitrocellulose, and hybridized to a ^{32}P-labeled v-*mos*-specific probe. (A) v-*mos* hybridization to *Bam*HI digests of (lane 1) NRK-2, (lane 2) 6m2, (lane 3) 54-5A4, (lane 4) 206-2IC, and (lane 5) MuSV-349 DNAs. (B) v-*mos* hybridization to *Bam*HI/*Sst*I double digests of (lane 1) NRK-2, (lane 2) 6m2, (lane 3) 54-5A4, (lane 4) 206-2IC, and (lane 5) MuSV-349 DNAs.

This splice event excises an in-frame TAG stop codon and restores the original *mos* gene reading frame 9 bases upstream of the previously in-frame TGA termination codon. The resulting 3.35-kb RNA (originally estimated as 3.5 kb in length) could code for a fusion protein containing 325 *gag* gene amino acids and 353 *mos* gene amino acids.

The validity of our interpretation of the above data rests on the fundamental point that there should be only one transcriptionally active MuSVts110-related provirus in 6m2 cells. The blot hybridization experiments presented in this study establish that this is almost surely the case. Using enzymes that cut once within each viral LTR, we found a single 3.85-kb viral DNA (about 4.4 kb with the other LTR added in) in 6m2 cells. The intensity of the signal observed was equivalent to that of the single-copy rat c-*mos* gene. Also, we found that enzymes which would cut wild-type viral DNA within the expected 1,488-bp deletion (nucleotides 2404 to 3892) do not cut the 3.85-kb DNA. Moreover, by means of enzymatic double digests, it was found that the 3.85-kb viral DNA was related to wild-type DNA by way of a deletion roughly spanning the region between an *Xho*I site at *gag* gene nucleotide 1982 and a *Bgl*I site at *mos* gene nucleotide 4120. These nucleotide addresses are within a few hundred bases of the nucleotide 2404 to 3892 deletion mapped by S1 nuclease assays (11) and the primer extension sequencing in the present study. Thus, the 3.85-kb viral DNA in 6m2 cells is almost surely the source of the MuSVts110 4.0-kb RNA.

Although there is no trace in 6m2 cells of a viral DNA from which the MuSVts110 3.5-kb RNA could be transcribed, producer 206-2IC cells chronically superinfected with Mo-MuLV contain a 3.35-kb DNA whose organization very closely resembles the known structure of the approximately 3.35-kb RNA. Since this viral DNA can be readily detected in 206-2IC DNA, this observation reinforces our finding that 6m2 cells lack such a viral DNA. Our present interpretation of this finding is that this 3.35-kb DNA represents an integrated cDNA copy of the 3.5-kb RNA transcribed by the helper virus reverse transcriptase present in the 206-2IC cell line. The presence of a transcriptionally active provirus producing the 3.5-kb RNA at any temperature without the need for splicing is an attractive possibility in this one cell line since 206-2IC cells appear to produce the 3.5-kb RNA (5a) and P85$^{gag\text{-}mos}$ at both 33 and 39°C (6). The production of the 3.5-kb RNA at 39°C by 206-2IC cells was originally thought to be possibly due to a complementation of the splicing defect in MuSVts110 mediated by viral factors provided by superinfecting Mo-MuLV. Recent data, however (5a), argue against this possibility since 6m2 cells acutely infected with Mo-MuLV cannot produce the 3.5-kb

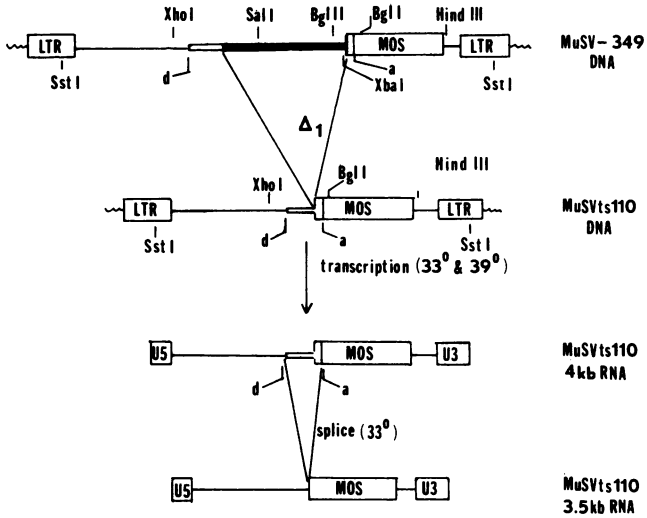

FIG. 9. Partial restriction maps showing the structural relationship of wild-type MuSV-349 viral DNA to MuSVts110 viral DNA and its transcription products. Based on the analyses shown in Fig. 8 and 10, the proposed structure of wild-type MuSV-349 viral DNA is shown in the topmost drawing. According to our data, MuSVts110 was generated as a consequence of the deletion of 1,488 bases of wild-type sequence information (▬). Transcription of MuSVts110 DNA at either 33 or 39°C yields a 4-kb RNA in which the *gag* and *mos* genes are fused out of frame. At 33°C, splicing of 431 bases of MuSVts110 4-kb RNA information (═) between in-frame splice donor (d) and acceptor (a) sites produces a 3.5-kb RNA from which a *gag-mos* fusion protein can be translated.

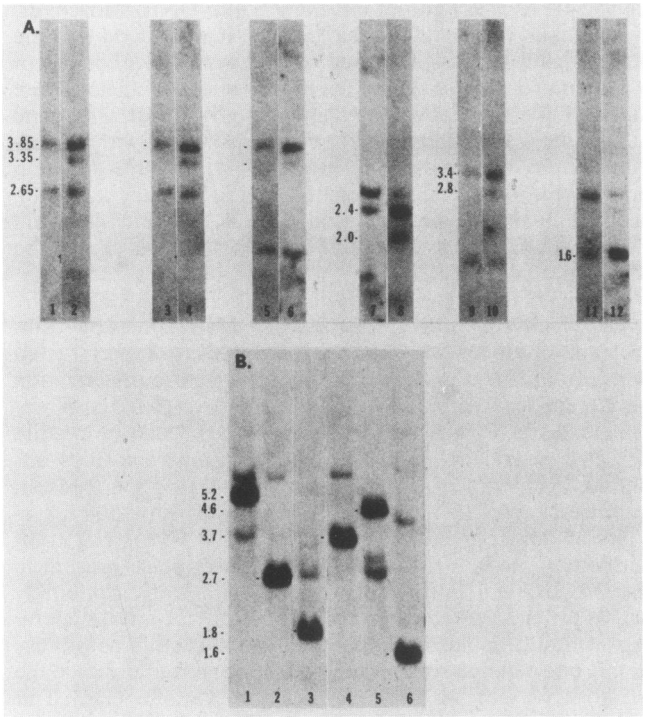

FIG. 10. Structure of proviral DNA in MuSVts110 nonproducer 6m2 cells, MuSVts110 producer 206-2IC cells, and wild-type MuSV-349-infected mouse TB cells. DNA from 6m2, 206-2IC, or MuSV-349-infected TB cells was digested with various restriction enzymes, analyzed on a 0.8% agarose gel, and hybridized to a ^{32}P-labeled v-*mos*-specific probe. (A) 6m2 DNA is in lanes 1, 3, 5, 7, 9, and 11. 206-2IC DNA is in lanes 2, 4, 6, 8, 10, and 12. (Lanes 1, 2) *Sst*I-digested DNA; (lanes 3, 4) *Sst*I/*Sal*I-digested DNA; (lanes 5, 6) *Sst*I/*Xba*I-digested DNA; (lanes 7, 8) *Sst*I/*Xho*I-digested DNA; (lanes 9, 10) *Sst*I/*Hin*dIII-digested DNA; (lanes 11, 12) *Sst*I/*Bgl*I-digested DNA. (B) MuSV-349 DNA digested with (lane 1) *Sst*I, (lane 2), *Sst*I/*Sal*I, (lane 3) *Sst*I/*Xba*I, (lane 4) *Sst*I/*Xho*I, (lane 5) *Sst*I/*Hin*dIII, or (lane 6) *Sst*I/*Bgl*I.

RNA at 39°C, although the spliced Mo-MuLV envelope mRNA can be formed at both 33 and 39°C. Thus, Mo-MuLV superinfection cannot promote splicing of the MuSVts110 4.0-kb RNA at 39°C. Instead, production of the 3.5-kb RNA at 39°C in chronically superinfected 206-2IC cells seems to be due to direct transcription from a new proviral DNA.

Molecular cloning experiments are presently under way with the object of obtaining MuSVts110 viral DNA clones to answer some of the remaining important questions in this system. It will be of great interest to determine whether the temperature-dependent splicing is an inherent property of the viral RNA or whether other as yet unappreciated host factors are involved.

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