Activation of Thermosensitive RNA Splicing and Production of a Heat-Labile P85^{*gag-mos*} Kinase by the Introduction of a Specific Deletion in Murine Sarcoma Virus-124 DNA

MARYELLEN DE MARS, PAUL E. CIZDZIEL,[†] AND EDWIN C. MURPHY, JR.*

Department of Tumor Biology, Section of Virology, University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Houston, Texas 77030

Received 28 December 1987/Accepted 10 March 1988

Murine sarcoma virus *ts110* (MuSV*ts110*) is a conditionally transformation-defective MuSV mutant lacking 1,487 bases found in its wild-type parent, MuSV-349 (MuSV-124). Expression of the MuSV*ts110* *v-mos* gene product, P85^{*gag-mos*}, requires splicing of the viral transcript to align the *gag* and *mos* genes in frame. However, this splice event is restricted to growth temperatures of 33°C or lower. No splicing of the viral RNA, no production of P85^{*gag-mos*}, and, hence, no cell transformation is observed at growth temperatures above 33°C. To determine whether thermosensitive splicing is an intrinsic property of MuSV*ts110* RNA specified by the 1,487-base deletion or a result of a cellular defect, we examined an "equivalent" or MuSV*ts110* DNA (designated *ts32* DNA) constructed by combining wild-type MuSV-124 DNA fragments with a synthetic oligonucleotide to yield an otherwise wild-type viral DNA containing the same 1,487-base deletion as authentic MuSV*ts110*. As observed in control cells (6m2 cells) infected with the authentic MuSV*ts110* virus, NIH 3T3 cells transfected with *ts32* DNA appeared morphologically transformed when grown at 33°C, but were converted to a more normal, flattened shape within a few hours of a shift to 39°C. In concert with these morphological changes, both the processing of the *ts32* RNA transcripts and the production of *ts32* p85^{*gag-mos*} kinase were found to be optimal at growth temperatures from 28 to 33°C, but dramatically reduced at 37 to 41°C. Like authentic P85^{*gag-mos*}, the *ts32* P85^{*gag-mos*} kinase activity was rapidly inactivated by brief exposure to 39°C. These results suggested that the MuSV*ts110* equivalent is functionally indistinguishable from authentic MuSV*ts110* and that the novel temperature-sensitive splicing of MuSV*ts110* transcripts is specified by an intrinsic property of the viral RNA.

Murine sarcoma virus (MuSV) *ts110* (MuSV*ts110*) is a conditionally transformation-defective retrovirus (4) with an unusual pattern of viral gene expression. If grown at 33°C or lower, MuSV*ts110*-infected cells (6m2 cells) assume the transformed phenotype, but revert rapidly to a normal morphology when shifted to 39°C (4, 13, 15). In these cells, the transformed phenotype is maintained by a virus-encoded protein, P85^{*gag-mos*}, whose mRNA is produced by splicing of the full-length viral transcript (13, 23, 24). However, this splice event and, hence, the transformed phenotype in MuSV*ts110*-infected cells are observed only at growth temperatures of 33°C or lower. Above 33°C, viral RNA splicing is markedly diminished, becoming virtually undetectable at 39°C and above. Our previous studies have shown that, relative to wild-type MuSV-349, MuSV*ts110* has sustained a 1,487-base frameshift deletion between the *gag* gene and the 5' end of the *v-mos* gene (15, 23, 24). (Previous reports [24] erroneously indicated that the deletion in MuSV*ts110* DNA relative to wild-type MuSV-124 was 1,488 bases in length.) Because the deletion included the *v-mos* gene initiation codon and fused the *gag* and *mos* genes out of frame, no *v-mos* polypeptide can be translated (24). Translation of the full-length MuSV*ts110* RNA is terminated just downstream of the *gag-mos* junction, yielding a truncated polypeptide, P85^{*gag*}. At 33°C, however, a 431-base intron surrounding the *gag-mos* junction can be removed by activation of previ-

ously cryptic 5' and 3' splice sites. The net effect of this splice event is to cause a frameshift that places the *gag* and *mos* genes in a continuously open reading frame, allowing the translation of P85^{*gag-mos*}.

It has been of considerable interest to explore the structural basis underlying thermosensitive splicing of MuSV *ts110* RNA with the hope of exploiting this knowledge for the study of general mechanisms in RNA splicing (reviewed in reference 25). As outlined above, the single known structural difference between MuSV*ts110* and wild-type MuSV-349 is a 1,487-base deletion between cryptic splice sites (24). Since no splicing has ever been observed between these sites in MuSV-349 RNA, the deletion alone might well explain the activation of these cryptic sites. However, because MuSV*ts110* is a UV-induced mutant selected for a single biological property, that of growth temperature-dependent cell transformation (4), it remains possible that other as yet undetected structural alterations, some of which might influence splicing, may have occurred. To test this possibility, we constructed a viral DNA (designated *ts32* DNA) differing from wild-type MuSV-124 DNA only in the 1,487-base deletion characteristic of MuSV*ts110*. Assessment of the biological properties of *ts32* suggests that this deletion is the primary determinant that defines the biological behavior of MuSV*ts110*.

MATERIALS AND METHODS

Cell culture. The 6m2 cell line, NRK cells infected with the MuSV*ts110* retrovirus (4), was maintained in complete McCoy medium supplemented with 10% fetal bovine serum.

* Corresponding author.

[†] Present address: Laboratory of Pulmonary Pathobiology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709.

FIG. 1. Comparative structure of wild-type MuSV-124 DNA and MuSV_{ts110} DNA. The structure of each viral DNA is given between the *Sst*I sites in either long terminal repeat sequence. Restriction sites relevant to this study are noted, and their nucleotide addresses are given. The long terminal repeats and internal *v-mos* region of the viral DNA are designated by a boldface line. The structure of MuSV_{ts110} DNA is given below that of wild-type MuSV-124. Restriction sites, nucleotide addresses, long terminal repeats, and *v-mos* sequences are indicated as for MuSV-124. The exact position of the 1,487-base deletion in MuSV_{ts110} relative to MuSV-124 is indicated.

NIH 3T3, Psi-2, and NRK cells were maintained in Dulbecco high-glucose minimal essential medium.

Isolation of cellular RNA. RNA was isolated from tissue culture cells by a previously described guanidine thiocyanate-guanidine hydrochloride extraction procedure (3).

Blot hybridization of cellular RNA. Total cellular RNA (3 to 5 μ g) in water was denatured in 20 mM MOPS [3-(*N*-morpholino)propanesulfonic acid] buffer (pH 7.0) containing 2.2 M formaldehyde–50% formamide at 55°C for 15 min and analyzed on 0.8 to 1.1% agarose gels containing 20 mM MOPS (pH 7) and 2.2 M formaldehyde. Electrophoresis was performed at 50 V in 20 mM MOPS (pH 7). After electrophoresis, the RNA was directly transferred to nitrocellulose in 20 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and hybridized to a ³²P-labeled *v-mos*-specific probe in 5 $\times$ SSC–50% formamide–0.2% sodium pyrophosphate–0.1% sodium dodecyl sulfate–100 μ g of heparin per ml (27, 28) as described previously (3, 7).

Construction of synthetic MuSV_{ts110} DNA. To construct an equivalent of the MuSV_{ts110} genome, we took advantage of the comparative structure of MuSV_{ts110} and wild-type MuSV-124 DNA (29) (refer to Fig. 1). The “synthetic” MuSV_{ts110} DNA (designated *ts32*), containing a 1,487-base deletion identical to that found in authentic MuSV_{ts110}, was constructed by combining the following three wild-type MuSV-124 DNA fragments and a synthetic oligonucleotide (Fig. 2).

(i) A fragment containing the 5' splice site was prepared (Fig. 2, steps 1 and 2) by digestion of wild-type MuSV-124 DNA sequentially with *Xho*I–*Sal*I and *Nci*I. The resulting 326-base fragment, extending from MuSV-124 nucleotides 1982 to 2351, contains the MuSV_{ts110} 5' splice site at nucleotide 2017 and terminates at an *Nci*I site 53 bases upstream from the *gag* gene–*mos* gene junction (2404/3892) in MuSV_{ts110} (refer to Fig. 2).

(ii) A fragment containing the 3' splice site and the *v-mos* gene was prepared (Fig. 2, steps 3 and 4) by digestion of wild-type MuSV-124 DNA first with *Bgl*II–*Hind*III and then partially with *Bst*N-I to obtain an 1,114-base fragment extending from MuSV-124 nucleotides 3909 to 5023. This fragment contains the 3' splice site at nucleotide 3936 and

has a *Bst*N-I terminus at nucleotide 3909, 17 bases downstream from the MuSV_{ts110} *gag* gene–*mos* gene junction (refer to Fig. 2).

(iii) The “acceptor” DNA, containing the balance of the wild-type MuSV-124 viral DNA cloned into the *Pst*I site of pBR322, was prepared by linearization of the MuSV-124 plasmid p101 (a gift of Dino Dina) with *Xho*I, followed by partial digestion with *Hind*III (Fig. 2). A 7.4-kilobase (kb) fragment from the partial digest, lacking only the MuSV-124 sequence between the *Xho*I site at 1982 and the *Hind*III site at 5022, was used as the acceptor DNA.

(iv) The 5' and 3' splice site fragments lacked 70 bases of known sequence (24) between the *Nci*I and *Bst*N-I sites surrounding the *gag*–*mos* junction in MuSV_{ts110}. To obtain this junction fragment, we synthesized two partially complementary 46-base oligonucleotides (refer to Fig. 2B). The fragments were hybridized and filled in with DNA polymerase, resulting in a 92-base duplex containing *Nci*I and *Bst*N-I sites 70 bases apart. Adequate amounts of the junction fragment were prepared by subcloning the 70-base *Nci*I–*Bst*N-I digestion product of the 92-mer into pKC7 DNA (26) that had been previously digested with *Xho*I–*Hind*III and then modified with *Xho*I–*Nci*I and *Hind*III–*Bst*N-I linkers (refer to Fig. 2B).

The above fragments were assembled as follows. The 3' splice site fragment (the product of step 4 in Fig. 2) was ligated to the *Hind*III terminus of *Xho*I–*Hind*III-digested pKC7 DNA and purified on an agarose gel (step 5 in Fig. 2). Separately, the *Nci*I termini of the 5' splice site and junction fragments were first ligated (step 6 in Fig. 2), purified on a polyacrylamide gel, and then ligated to the free *Xho*I and *Bst*N-I termini of the pKC7 vector already linked to the 3' splice site fragment (step 7 in Fig. 2). The resulting subclone, pINT (designated “intron” in Fig. 2), was digested with *Xho*I–*Hind*III, and the 1.55-kb intron-containing insert was ligated into the *Xho*I and *Hind*III termini of the MuSV-124 acceptor plasmid (step 8 in Fig. 2). *Escherichia coli* RRI was transformed with this DNA, and transformants were selected by hybridization to a *v-mos* probe and analyzed by restriction digestion.

DNA ligation and bacterial transformation. Ligation of

FIG. 2. Construction of the synthetic MuSV $ts110$ DNA. (A) As detailed in the text, wild-type MuSV-124 was digested with restriction enzymes to yield (i) a 5' splice site fragment (steps 1 and 2), (ii) a 3' splice site fragment (steps 3 and 4), and (iii) an acceptor fragment. The 3' splice site fragment was ligated to *XhoI*-*HindIII*-digested pKC7 vector DNA (step 5). (B) A synthetic oligonucleotide designed to span the region between the *NciI* site in the 5' splice site fragment and the *BstN-I* site in the 3' splice site fragment was made and subcloned. The subcloned oligonucleotide, designated LINK, was ligated to the 5' splice site fragment (step 6) and then to the pKC7 DNA containing the 3' splice site fragment. The insert from the resulting subclone, designated INTRON, was ligated to the acceptor fragment to yield the synthetic MuSV $ts110$ DNA, designated $ts32$. ltr, Long terminal repeat.

vectors and inserts, usually a total of 1 μ g, was carried out under conditions similar to those described by King and Blakesley (16). For transformation, competent *E. coli* RRI, HB101, or DH5 (Bethesda Research Laboratories, Inc.) cells were used under conditions suggested by the supplier.

Transfection and G-418 selection. Cotransfection of NIH 3T3 cells with linearized $ts32$ DNA and pSV2neo was carried out by a standard calcium phosphate transfection protocol (10). G-418-resistant cells were selected by growth in 400 μ g of G-418 per ml (Geneticin; GIBCO).

S1 nuclease analysis. DNA probes for S1 nuclease analysis were either 3' or 5' end labeled as described previously (22, 23). Hybridization of cellular RNA with these probes and determination of viral gene expression by S1 nuclease analysis were performed as described previously (22, 23).

DNA sequencing by primer extension. The 5'-end-labeled *Pmos1* primer (5'-GAAGGCATTACACCCTCAGA-3') complementary to MuSV-124 nucleotides 3956 to 3976 was mixed with 5 μ g of *Saccharomyces cerevisiae* tRNA and approximately 1 μ g of the linear double-stranded DNA to be sequenced, and was precipitated with ethanol. The centrifuged pellets were dissolved in 50 mM Tris (pH 7.5)–10 mM $MgCl_2$ –50 mM NaCl–1 mM dithiothreitol, heated in boiling

water for 10 min, rapidly quenched, hybridized at 20°C for 45 min, and sequenced by primer extension (6, 7, 24).

Immune complex kinase assay. Kinase assays (17, 18) were performed on anti-p15-immunoprecipitated cell extracts according to the method of Maxwell and Arlinghaus (22) in a final volume of 100 μ l containing 10 μ M ATP, 7.5 mM $MnCl_2$, and 10 μ Ci of [γ - 32 P]ATP, as modified by Biggart et al. (3). Phosphorylated proteins were analyzed on an 8% polyacrylamide gel containing 0.1% sodium dodecyl sulfate and detected by autoradiography (3, 22).

RESULTS

Characterization of the MuSV $ts110$ equivalent *gag* gene-*mos* gene junction. Confirmation of $ts32$ DNA structure was done by analysis of the pattern of *NciI* and *BstN-I* sites in the *gag* gene-*mos* gene junction region between the *XhoI* and *HindIII* sites (shown in Fig. 3C). Deletion of nucleotides 2404 to 3892 in MuSV-124 DNA should yield DNA with a reduced number and diagnostic pattern of *NciI* and *BstN-I* sites (Fig. 3C). In particular, the 70-base *NciI*-*BstN-I* fragment located near the 3' splice site was considered most diagnostic since its presence would confirm the precise insertion of the synthetic oligonucleotide.

FIG. 3. Structural analysis of the *ts32* DNA (A) Organization of the 1.55-kb *Xho*I-to-*Hind*III intron region of *ts32* DNA, characterized with respect to diagnostic *Nci*I and *Bst*N-I restriction sites (shown in panel C). The digestion products were analyzed on a nondenaturing 4% polyacrylamide gel. The *ts32* intron was digested with either (lane 2) *Nci*I, (lane 3) *Bst*N-I, or (lane 4) *Nci*I and *Bst*N-I. Lanes 1 and 5 contain, respectively, the undigested 1.55-kb intron fragment and a standard of pBR322 DNA digested with *Hinf*I. (B) Sequence of the *ts32* intron across the *gag* gene-*mos* gene junction. The 1.55-kb intron of *ts32* DNA was isolated and hybridized to the *Pmos*1 primer. The hybrid was extended with DNA polymerase I (Klenow fragment) in the presence of dideoxynucleotide triphosphates, and the products of the reaction were analyzed on an 8% polyacrylamide gel containing 8 M urea. The photograph shown is a composite of two autoradiographs because separate loadings were necessary to achieve adequate resolution of the upstream region around the *Nci*I site. Underneath the figure, the plus-strand DNA sequence is illustrated in the region around the *Nci*I site (5' end of the junction fragment), the *gag* gene-*mos* gene junction, the *Bst*N-I site (3' end of the junction fragment), and the MuSV*ts*110 splice site. (C) Comparison of the *Nci*I and *Bst*N-I restriction sites between the *Xho*I and *Hind*III sites in MuSV-124 and *ts32* DNA. The position of the synthetic oligonucleotide in the *ts32* DNA is represented in boldface.

Digestion of the *ts32 Xho*I-*Hind*III fragment yielded the pattern predicted for MuSV*ts*110. *Nci*I digestion released fragments approximately 920, 370, and 270 bases in length (Fig. 3A, lane 2). *Bst*N-I digestion yielded products approximately 900, 220, 190, 175, and 50 bases in length (Fig. 3A, lane 3). Double digestion with *Nci*I-*Bst*N-I released the diagnostic 70-base *Nci*I-*Bst*N-I fragment from the 270-base *Nci*I and 220-base *Bst*N-I fragments (Fig. 3A, lane 4).

To ensure that *gag* and *mos* frames in *ts32* DNA were aligned as they are in authentic MuSV*ts*110, we sequenced *ts32* DNA in the region upstream from the 3' splice site. Extension of the *Pmos*1 primer from a point 20 nucleotides downstream of the 3' splice site yielded a sequence in this reconstructed region identical to that of authentic MuSV*ts*110 RNA in 6m2 cells (Fig. 3B) (24; P. E. Cizdziel and E. C. Murphy, Jr., unpublished data). Regions of interest in the sequence shown are the 3' splice site (24), the *Bst*N-I terminus of the synthetic oligonucleotide, the *gag*-*mos* junction sequence characteristic of MuSV*ts*110 (24), and the *Nci*I terminus of the synthetic oligonucleotide (Fig. 3B). We conclude that, at least with respect to the 1,487-base deletion and the relative placement of the 5' and 3' splice sites, the *ts32* and authentic MuSV*ts*110 DNA sequences are identical.

Biological activity of *ts32* DNA. The hallmark of MuSV*ts*110-infected cells is a growth temperature-regulated morphology. As a function of the conditional defect in viral

RNA splicing, MuSV*ts*110-infected cells appear transformed at 33°C and morphologically normal at 39°C (1, 4, 5, 13, 23, 24). To determine whether cells into which the *ts32* DNA was introduced would behave similarly, we cotransfected *ts32* and pSV2neo DNA into both NIH 3T3 cells and the virus-packaging Psi-2 cell line (21). G-418-resistant colonies were selected and tested for morphologic phenotype, for P85^{*gag-mos*} production and kinase activity, and for conditionally defective viral RNA splicing. For some experiments, *ts32* virus was harvested from the transfected Psi-2 cells and used to infect NRK cells.

Morphology of *ts32*-transfected cells. The morphology of single-cell clones of the G-418-resistant *ts32* transfectants was examined at growth temperatures of 33 and 39°C. NIH 3T3 cells transfected with *ts32* DNA (3T3/*ts32* cells) and grown at 33°C were refractile and somewhat rounded and grew in a disorganized, piled-up fashion (Fig. 4A). After a 4-h shift to 39°C, 3T3/*ts32* cells had significantly changed in morphology, having shifted to a flattened, more organized, contact-inhibited pattern of growth (Fig. 4B). Thus, 3T3/*ts32* cells acquired a temperature-dependent morphologic phenotype very similar to that established for cells infected with authentic MuSV*ts*110 (3a-5).

P85^{*gag-mos*} kinase activity in cells containing *ts32* DNA. To determine whether the morphologic changes observed in *ts32*-transfected cells correlated with the production of

FIG. 4. Morphological phenotype of $ts32$ -transfected NIH 3T3 cells (3T3/ $ts32$ cells). NIH 3T3 cells cotransfected with $ts32$ DNA and pSV2neo were selected on the basis of G-418 resistance. Antibiotic-resistant clones were then grown in mass culture in the absence of G-418. The phenotype of 3T3/ $ts32$ cells (A) grown at 33°C or (B) shifted from 33 to 39°C is shown.

transforming P85^{*gag-mos*} kinase, as has been well established for 6m2 cells (1, 9), extracts were prepared from transfected 3T3/ $ts32$ cells grown at 33 or 39°C as well as from NRK cells infected with a viral harvest obtained from Psi-2/ $ts32$ transfectants (NRK/ $ts32$ cells) grown at both 33 and 39°C. The extracts were immunoprecipitated with anti-p15 serum, and an immune complex kinase assay (17, 18) was performed on the immunoprecipitates. Control 6m2, 3T3/ $ts32$, and

FIG. 5. P85^{*gag-mos*} kinase activity in NIH 3T3 and NRK cells containing $ts32$ DNA. Extracts prepared from 3T3/ $ts32$ cells, NRK cells infected with a viral harvest from $ts32$ -transfected Psi-2 cells (NRK/ $ts32$ cells), control 6m2 cells, and uninfected NIH 3T3 cells grown at either 33 or 39°C were immunoprecipitated with anti-p30. The kinase activity in the immunoprecipitates was estimated by the immune complex kinase assay. The phosphorylated products were analyzed on an 8% polyacrylamide gel containing 0.1% sodium dodecyl sulfate. Gel shows kinase activity in (lane 1) NIH 3T3 cells at 33°C, (lane 2) NIH 3T3 cells at 39°C, (lane 3) 6m2 cells at 33°C, (lane 4) 6m2 cells at 39°C, (lane 5) 3T3/ $ts32$ cells at 33°C, (lane 6) 3T3/ $ts32$ cells at 39°C, (lane 7) NRK/ $ts32$ cells at 33°C, and (lane 8) NRK/ $ts32$ cells at 39°C.

NRK/ $ts32$ cells grown at 33°C produced an anti-p15-precipitable, 85-kilodalton kinase (P85^{*gag-mos*}) capable of both autophosphorylation and the phosphorylation of P58^{*gag*} (Fig. 5, lanes 3, 5, and 7). Based on its size, immunoreactivity with anti-*gag* sera, kinase activity, and growth temperature-dependent pattern of expression, this polypeptide was assumed to be P85^{*gag-mos*}. Uninfected NIH 3T3 extracts contained a nonspecific 65-kilodalton kinase activity at both 33 and 39°C, but no 85-kilodalton kinase at all (Fig. 5, lanes 1 and 2). If 6m2, 3T3/ $ts32$, or NRK/ $ts32$ cells were shifted to 39°C overnight, the 85-kilodalton kinase was no longer detectable (Fig. 5, lanes 4, 6, and 8). Thus, 3T3/ $ts32$ and NRK/ $ts32$ cells are indistinguishable from 6m2 cells in this regard; the transformed phenotype correlates positively with P85^{*gag-mos*} kinase production.

Authentic MuSV $ts110$ has been reported to have two conditional defects. The first defect involves conditional splicing of the viral transcript (23, 24), and the second defect has been inferred from the fact that P85^{*gag-mos*} kinase activity is rapidly inactivated by exposure to 39°C as compared to wild-type P37^{*mos*} kinase (17, 18). The molecular basis for the heat lability of the kinase activity is unknown. However, it seems likely either that the substitution of *gag* sequences for 5' *mos* sequences in P85^{*gag-mos*} could be responsible or that a second, as yet uncharacterized mutation in the MuSV $ts110$ v-*mos* gene has conferred instability on the kinase activity. A test of the heat sensitivity of the P85^{*gag-mos*} kinase in $ts32$ -infected cells should allow a discrimination between these possibilities since $ts32$ is presumably identical to wild-type MuSV-124 except for the engineered deletion. To compare the 6m2 and $ts32$ P85^{*gag-mos*} kinase activities, an immune complex kinase assay was performed on cell extracts prepared from 6m2 and NRK/ $ts32$ cells grown at 33°C and transferred to a 39°C water bath for

FIG. 6. Heat sensitivity of P85^{gag-mos} kinase activity. Immune complex kinase assays were performed on anti-p15 immunoprecipitates of 6m2 or NRK/*ts32* cell extracts from cells grown at 33°C or grown at 33°C and heated to 39°C for 30 min. (A) On the left is an autoradiograph of P85^{gag-mos} kinase activity in 6m2 cells at 33°C (33°) and 6m2 cells heated at 39°C for 30 min (39°shift). At the right is a gel scan of the autoradiograph. (B) On the left is an autoradiograph of P85^{gag-mos} kinase activity in NRK/*ts32* cells at 33°C (33°) and NRK/*ts32* cells heated at 39°C for 30 min (39°shift). At the right is a gel scan of the autoradiograph.

30 min. The brief heat treatment dramatically inactivated the P85^{gag-mos} kinase present in both 6m2 and NRK/*ts32* cells at 33°C (Fig. 6, compare the lanes designated "33°" and "39°shift"). The degree of inactivation was quantitated by scanning the kinase profiles shown in Fig. 6. For both the 6m2 and *ts32* P85^{gag-mos} kinases, 30 min at 39°C reduced their activities by 80 to 90%. These data suggest strongly that the *ts32* and MuSV*ts110* P85^{gag-mos} kinases are enzymatically equivalent and, further, that the heat lability of the kinase is solely a function of the replacement of the N terminus of the *v-mos* gene with *gag* sequences.

Viral RNA splicing in cells containing *ts32* DNA. In 6m2 cells, P85^{gag-mos} is translated from the spliced viral RNA. Since in 6m2 cells this splicing even takes place only at 33°C

or below, it was clearly of great interest to determine whether RNA transcribed from *ts32* DNA retained this unique property. Two S1 nuclease probes have previously been used to estimate 5' splice site cleavage and exon ligation in 6m2 cells (24). The DD.29 probe extends across the 5' splice site, and the pBA.36 probe extends across the 3' splice site (Fig. 7G). Unspliced 3.8-kb MuSV*ts110* RNA will protect 290 and 175 bases of DD.29 and pBA.36, respectively, from S1 nuclease digestion, whereas the spliced 3.3-kb RNA will protect only 120 bases of DD.29 and 122 bases of pBA.36 (Fig. 7G) (23, 24). S1 nuclease analysis of RNA obtained from both 6m2 (Fig. 7A and B) and 3T3/*ts32* (Fig. 7C and D) cells grown at 28 to 41°C showed that at all growth temperatures in both cell lines, 290 bases of DD.29 and 175 bases of pBA.36 were protected (Fig. 7A through D, lanes 1 through 4, respectively), reflecting the presence of the unspliced MuSV*ts110* RNA. In both 6m2 and 3T3/*ts32* cells, accurate splicing of this transcript, as estimated by the protection of 120 bases of DD.29 and 122 bases of pBA.36, was observed at 28 and 33°C (Fig. 7A through D, lanes 1 and 2, respectively). However, as expected, no 5' splice site cleavage or exon ligation was observed in 6m2 cells at 39 and 41°C (Fig. 7A and B, respectively, lanes 3 and 4). Similarly, in the 3T3/*ts32* cells, cleavage at the 5' splice site as well as exon ligation was diminished at 39°C and reduced to trace amounts at 41°C (Fig. 7C and D, respectively, lanes 3, 4, and 4'). Neither probe was protected by RNA from uninfected NRK cells (Fig. 7A through D, lanes designated NRK) or NIH 3T3 cells (data not shown). Thus, *ts32* RNA splicing has retained the growth temperature sensitivity characteristic of authentic MuSV*ts110* RNA. This argues strongly that thermosensitive splicing is specified by some *cis*-acting property of the viral transcript.

Some differences, however, were observed between 6m2 and 3T3/*ts32* cells. Most notably, the viral RNA levels in 3T3/*ts32* cells were about fivefold lower than those in 6m2 cells, and, in contrast to 6m2 cells, a small amount of viral RNA splicing was detectable at 39°C in 3T3/*ts32* cells (compare Fig. 7A and B, lanes 3, with Fig. 7C and D, lanes 3). To explore the possibility that the transfection of *ts32* DNA in a plasmid environment might have contributed to the differences observed between the transcription and splicing of *ts32* and authentic MuSV*ts110* transcripts, we tested viral RNA splicing in NRK cells infected with a viral harvest from *ts32*-transfected Psi-2 cells. S1 nuclease analysis showed clearly that these *ts32*-infected cells (NRK/*ts32* cells) produced high levels of viral RNA (compare the signal intensity in Fig. 7D and F; in both cases, 50 µg of either 3T3/*ts32* [Fig. 7D] or NRK/*ts32* RNA [Fig. 7F] was used) whose splicing was strictly governed by growth temperature. In these cells, cleavage and ligation of the *ts32* transcripts were limited to 28 to 33°C (Fig. 7E and F, respectively, lanes 1 and 2); no splicing at all was detectable at 39 to 41°C (Fig.

TABLE 1. Cleavage at the 5' splice site and exon ligation^a

Cells	5' Splice site cleavage (%)		Exon ligation (%) at temp:	
	33°C	39°C	33°C	39°C
6m2	45	1	20	0
NRK/ <i>ts32</i>	47	0	27	0

^a Cleavage at the 5' splice site was estimated by scanning lanes 2 and 3 in Fig. 7A and E, using a Beckman DU-8 spectrophotometer. Exon ligation was similarly estimated by scans of lanes 2 and 3 in Fig. 7B and F. The results are expressed as the percent of the total MuSV*ts110* RNA.

FIG. 7. Splicing of $ts32$ RNA transcripts. Total cellular RNA was prepared from 6m2, 3T3/ $ts32$, and NRK/ $ts32$, grown at 33°C and shifted overnight to 28, 33, 37, or 41°C, or from control NIH 3T3 and NRK cells. Samples (10 μ g) of 6m2, NRK, or NRK/ $ts32$ RNA, or 50 μ g of 3T3/ $ts32$ RNA, were hybridized either to 5'-end-labeled pBA.36 or 3'-end-labeled DD.29 DNA, and the hybrids were digested with S1 nuclease. The protected fragments were analyzed on a 5% polyacrylamide gel containing 8 M urea. (A, C, and E). Analysis of 5' splice site cleavage in 6m2, 3T3/ $ts32$, and NRK/ $ts32$ cells, respectively, at various growth temperatures: lanes 1, 28°C; lanes 2, 33°C; lanes 3, 37°C; lanes 4, 41°C. Lanes designated Probe, NRK, Psi-2, or 6m2 are S1 control assays containing probe only (+10 μ g of yeast tRNA), NRK, or 6m2 33°C RNA. (B, D, and F) Analysis of exon ligation in 6m2, 3T3/ $ts32$, and NRK/ $ts32$, respectively, at various growth temperatures: lanes 1, 28°C; lanes 2, 33°C; lanes 3, 37°C; lanes 4, 41°C. Lane 4', Overexposure of lane 4. Lanes designated Probe or NRK are control S1 assays performed as described for panels A and E. For all gels, the protected fragments were analyzed on a 5% polyacrylamide gel containing 8 M urea, using a 5'-end-labeled *Hinf*I digest of pBR322 as a size standard (STD).

7E and F, respectively, lanes 3 and 4). The results provide a strong indication that retrovirally integrated $ts32$ DNA can generate transcripts whose fate is indistinguishable from that of authentic MuSV $ts110$ RNA.

A quantitative estimate of the cleavage and ligation reactions in 6m2 and NRK/ $ts32$ cells confirmed that both cleavage and ligation were limited to 33°C (Table 1). At 33°C, an average of about 25% of the MuSV $ts110$ RNA was found as the spliced species, while about 75% remained unspliced. However, the fraction of MuSV $ts110$ RNA cleaved at the 5' splice site (about 45%) was about twice that of RNA spliced at the usual 3' splice site (about 25%). Alternate splicing probably accounts for the 20% of MuSV $ts110$ RNA cleaved at the 5' splice site but not ligated to the usual 3' site, since recent experiments have revealed alternate splicing of the 5' splice site to a 3' splice site near nucleotide 2242 (8). Any

remaining cleaved but unligated MuSV $ts110$ RNA probably exists as splicing intermediates, cleaved but not ligated to either 3' splice site.

A final experiment was performed to confirm that the DD.29 and pBA.36 probe fragments protected by RNA from 6m2 and $ts32$ -containing cells in the S1 analyses actually reflected the presence of the intact 3.8- and 3.3-kb MuSV $ts110$ RNAs. Total cellular RNA from 6m2, NRK/ $ts32$, and control NRK cells was analyzed on an agarose-formaldehyde gel and hybridized to a *v-mos* probe. At 39°C, both 6m2 and NRK/ $ts32$ cells expressed a 3.8-kb viral RNA (Fig. 8, lanes 3, 5, and 7). At 28°C, as expected from the results of all the previous experiments, the spliced 3.3-kb viral RNA was clearly present in each cell line (Fig. 8, lanes 2, 4, and 6). RNA from control NRK cells did not produce a signal (Fig. 8, lane 1).

FIG. 8. Blot hybridization of cellular RNA. Total cellular RNA (3 to 5 μ g) or RNA from 6m2, NRK/*ts32*, or control NRK cells grown at 28 or 39°C was analyzed in a 1% agarose gel containing 2.2 M formaldehyde, transferred to nitrocellulose, and hybridized to a 32 P-labeled *v-mos*-specific probe.

DISCUSSION

The MuSV $ts110$ equivalent DNA constructed for this study was made to further our understanding of the molecular basis for growth temperature-dependent fluctuations in the levels of unspliced and spliced MuSV $ts110$ RNAs. Previously, we have established that, relative to parental MuSV-349 (2), MuSV $ts110$ had sustained a 1,487-nucleotide deletion that allowed splicing of previously cryptic 5' and 3' splice sites in the MuSV-124 *gag* and *mos* genes, respectively (15, 23, 24). This splice event was novel in that accumulation of the spliced species was observed only at growth temperatures of 33°C or lower (11, 24). No splicing, or at least no accumulation of the spliced viral RNA, was observed at 39°C (11, 24). We have recently shown that the selectively enhanced accumulation of the spliced species of MuSV $ts110$ RNA observed at 33°C and below (11, 13, 15, 23, 24) can be directly ascribed to selective changes in the efficiency of MuSV $ts110$ RNA splicing as a function of growth temperature, rather than to selectively increased degradation of the spliced RNA species at higher temperatures or to any general thermosensitivity of RNA splicing in 6m2 cells (7, 11). These observations, suggesting that temperature-sensitive splicing is an MuSV $ts110$ -encoded property, are supported by previous evidence in the literature. For example, the NRK cells infected with the original MuSV $ts110$ stock were otherwise untreated, arguing that any novel property acquired would most probably be due to some intrinsic property of the virus (3a). Moreover, NRK cells newly infected with mixtures of MuSV $ts110$ and helper murine leukemia virus (MuLV) acquire a temperature-sensitive phenotype and exhibit thermosensitive splicing of the viral RNA (12). However, the fact that 6m2 cells superinfected with helper MuLV eventually acquire the ability to make P85^{*gag-mos*} at 39°C as well as at 33°C from a viral RNA indistinguishable from the MuSV $ts110$ 3.3-kb spliced RNA (11, 24) left open the possibility that thermosensitive splicing might not be totally determined by the MuSV $ts110$ virus.

Subsequent work has shown that the helper MuLV-mediated instability of MuSV $ts110$ -characteristic gene expression is very probably due to MuLV-directed reverse transcription and subsequent integration of a cDNA copy of the spliced MuSV $ts110$ RNA, thus allowing transcription and translation of a P85^{*gag-mos*} mRNA without the need for splicing (24). To sidestep this instability and to evaluate the effect of the 1,487-nucleotide deletion on otherwise parental MuSV-124 DNA, we constructed the *ts32* DNA and tested its biological

activity, reasoning that if RNA transcribed from this DNA when introduced into a variety of cell lines were spliced in a temperature-sensitive fashion, it would be convincing evidence that thermosensitive splicing is due to a *cis*-acting feature of the viral transcript determined exclusively by the 1,487-nucleotide deletion. In the work presented here, we have shown that the MuSV $ts110$ equivalent DNA (*ts32* DNA), when transfected into NIH 3T3 cells, confers on these cells a temperature-dependent morphologic phenotype similar to that observed for 6m2 cells. As for 6m2 cells (13, 17, 18), morphologic transformation of *ts32*-transfected cells at 33°C correlates positively with the synthesis of a heat-labile viral kinase, P85^{*gag-mos*}. The retention of heat lability by the *ts32* P85^{*gag-mos*} kinase activity is especially interesting since it suggests strongly that this lability is solely a function of the loss of the 5' end of the *mos* gene and its replacement with *gag* sequences rather than the effect of some as yet undetected mutation unique to the *v-mos* sequence in MuSV $ts110$.

As would be expected from the presence of P85^{*gag-mos*} in *ts32*-transfected cells only at low growth temperatures, the *ts32* viral RNA is spliced in a temperature-sensitive fashion, demonstrating unequivocally that a *cis*-acting function peculiar to the viral RNA must specify thermosensitive splicing. As yet, however, we have no explanation for why viral RNA splicing in cells infected with *ts32* virions seems more strictly dependent on growth temperature than that of RNA transcribed from transfected *ts32* DNA, except to note that the transfected versus retrovirally integrated *ts32* DNAs are presumably in different microenvironments. These presumptive differences in viral integration sites probably also explain the fivefold higher level of viral transcripts in the *ts32*-infected cells as compared with the *ts32* transfectants, since it has previously been documented that the long terminal repeat portions of retroviral DNAs introduced into cells by infection are substantially less methylated than those introduced by transfection, resulting in much higher RNA transcription from the viral DNAs integrated during infection (14). The splicing competence of RNA transcribed from the *ts32* DNA, irrespective of the means used to introduce the DNA into cells, suggests that the 1,487-base deletion is the sole alteration required to form an RNA with this novel splicing property. However, since some differences were noted in the splicing efficiency of authentic MuSV $ts110$ and *ts32* transcripts, it is possible that other, more subtle differences exist between these MuSV $ts110$ and *ts32* transcripts that are not involved in conversion of wild-type MuSV-124 to the splicing-competent state, but which affect splicing efficiency.

It is of great interest to determine how the deletion converted the intron from an inactive to an active yet thermosensitive form. In wild-type MuSV-124, authentic MuSV $ts110$, and *ts32*, and 5' and 3' splice site sequences (and presumably the branch-point sequence) are identical (8, 24; Cizdziel and Murphy, unpublished data). Thus, activation for thermosensitive splicing would not seem to involve these sequences directly. Our most recent experiments favor the possibility that alterations in nonconserved (i.e., non-splice site, non-branch-point sequences) intron sequences were instrumental in activation of the cryptic splice sites in wild-type MuSV-124. For example, removal of 1,103 bases of nonconserved MuSV-124 intron sequences (the intron is defined here as those sequences between nucleotides 2017 and 3936), resulting in an 815-base intron, will activate accurate, temperature-sensitive splicing between the MuSV $ts110$ splice sites. Moreover, a shorter (662-base) deletion

will activate temperature-sensitive splicing between the MuSVts110 5' splice site and the MuLV *env* mRNA 3' splice site (19, 20) retained in this particular mutant (M. de Mars, P. E. Cizdziel, and E. C. Murphy, Jr., manuscript in preparation).

Regardless of the splice sites used, in each case the MuSV splicing event, once activated, appears to be temperature sensitive. While we do not currently understand which structures in the MuSVts110 RNA transcript are most important for thermosensitive splicing, the use of the *env* 3' splice site in one of our mutants argues that the selection of a particular 3' splice site and branch-point sequence is not critical. Moreover, alternate, thermosensitive splicing can occur between the MuSVts110 5' splice site and a 3' splice site 225 nucleotides downstream, suggesting that no more than 225 nucleotides of nonconserved sequence at the 5' end of the MuSVts110 intron may govern thermosensitive splicing (7). These 225 nucleotides also encode part of the p30 region found in P58^{gag}. Thus, we are considering the possibility that P58^{gag} may participate in some way in thermosensitive splicing. We are constructing a potentially splicing-competent P58^{gag}-defective viral mutant to test this possibility.

Finally, on the basis of recent experiments, it seems most likely that very restricted changes within the intron sequence can render the thermosensitive splicing event constitutive. We have characterized a series of stable revertant cell lines (cells having acquired the ability to maintain the transformed state of 39°C) derived by treatment of 6m2 cells with nitrosomethyl urea, a carcinogen known to cause G:C-to-A:T transitions in DNA, and nickel chloride, a carcinogen whose mode of DNA damage is poorly understood (3, 3a). In all of these revertants, the MuSVts110 RNA can be spliced efficiently at both 33 and 39°C, arguing that single base changes in regions critical to splicing had occurred (3, 3a). Experiments are in progress to localize the structural changes in the viral DNA in these revertants.

ACKNOWLEDGMENTS

The order of the first two authors is arbitrary.

We are grateful for the excellent technical assistance of Sandra A. Kinney, Jade L. Wong, and James J. Syrewicz and indebted to our colleagues Neal W. Biggart, Elizabeth A. Putnam, Deborah A. Sterner, and Susanna M. Chiocca for numerous helpful discussions. Nancy Edwards and Tania Busch provided skillful help in manuscript typing and photography, respectively.

This work was supported in part by grants CA-34734 from the National Cancer Institute and G-854 from the Robert A. Welch Foundation. Synthetic oligonucleotides were provided by the University of Texas System Cancer Center Macromolecular Synthesis Facility (supported by Core Grant CA-16672). Both M. de Mars and P. E. Cizdziel hold predoctoral fellowships awarded by the Rosalie B. Hite Foundation.

LITERATURE CITED

- Arlinghaus, R. B. 1985. The ts110 Moloney mouse sarcoma virus system. *gag-mos* gene products and cellular transformation. *J. Gen. Virology* **66**:1845-1853.
- Ball, J. K., J. A. McCarter, and S. M. Sunderland. 1982. Evidence for helper-independent murine sarcoma virus. I. Segregation of replication-defective viruses. *Virology* **56**:268-284.
- Biggart, N. B., G. E. Gallick, and E. C. Murphy, Jr. 1987. Nickel-induced heritable alterations in retroviral transforming gene expression. *J. Virol.* **61**:2378-2388.
- Biggart, N. B., and E. C. Murphy, Jr. 1988. Analysis of metal-induced mutations altering the expression or structure of a retroviral gene in a mammalian cell line. *Mutat. Res.* **198**: 115-129.
- Blair, D. G., M. A. Hull, and E. A. Finch. 1979. The isolation and preliminary characterization of temperature-sensitive transforming mutants of Moloney sarcoma virus. *Virology* **95**: 303-316.
- Brown, R., J. P. Horn, L. Wible, R. B. Arlinghaus, and B. R. Brinkley. 1981. Analyses of the sequences of events in the transformation process in cells infected with a ts transformation mutant of Moloney murine sarcoma virus. *Proc. Natl. Acad. Sci. USA* **78**:5593-5597.
- Chen, F. Y., and P. H. Seeberg. 1985. Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. *DNA* **4**:165-170.
- Cizdziel, P. E., M. de Mars, and E. C. Murphy, Jr. 1988. Use of pre-mRNA splicing in vivo to study a thermosensitive splicing event. *Mol. Cell. Biol.* **8**:1558-1569.
- Cizdziel, P. E., M. A. Nash, D. G. Blair, and E. C. Murphy, Jr. 1986. Molecular basis underlying phenotypic revertants of Moloney murine sarcoma virus MuSVts110. *J. Virol.* **57**:310-317.
- Gallick, G. E., R. Hamelin, S. Maxwell, D. Duyka, and R. B. Arlinghaus. 1984. The *gag-mos* hybrid protein of ts110 Moloney murine sarcoma virus: variation of gene expression with temperature. *Virology* **139**:366-374.
- Graham, R., and A. Van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456-467.
- Hamelin, R., B. L. Brizzard, M. A. Nash, E. C. Murphy, Jr., and R. B. Arlinghaus. 1985. Temperature-sensitive viral RNA expression in Moloney murine sarcoma virus ts110-infected cells. *J. Virol.* **53**:616-623.
- Hamelin, R., K. Kabat, D. Blair, and R. B. Arlinghaus. 1986. Temperature-sensitive splicing defect of ts110 Moloney murine sarcoma virus is virus encoded. *J. Virol.* **57**:301-309.
- Horn, J. P., T. G. Wood, E. C. Murphy, Jr., D. G. Blair, and R. B. Arlinghaus. 1981. A selective temperature-sensitive defect in viral RNA: expression in cells infected with a transformation mutant of murine sarcoma virus. *Cell* **25**:37-46.
- Hwang, L.-H. S., and E. Gilboa. 1984. Expression of genes introduced into cells by retroviral infection is more efficient than that of genes introduced into cells by DNA transfection. *J. Virol.* **50**:417-424.
- Junghans, R. P., E. C. Murphy, Jr., and R. B. Arlinghaus. 1982. Electron microscopic analysis of ts110 Moloney mouse sarcoma virus, a variant of wild-type virus with two RNAs containing large deletions. *J. Mol. Biol.* **161**:229-255.
- King, P. V., and R. W. Blakesley. 1986. Optimizing DNA ligations for transformation. *Focus (Bethesda Research Laboratories)* **8**:1-3.
- Kloetzer, W. S., S. A. Maxwell, and R. B. Arlinghaus. 1983. P85^{gag-mos} encoded by ts110 Moloney murine sarcoma virus has an associated protein kinase activity. *Proc. Natl. Acad. Sci. USA* **80**:412-416.
- Kloetzer, W. S., S. A. Maxwell, and R. B. Arlinghaus. 1984. Further characterization of the P85^{gag-mos}-associated protein kinase activity. *Virology* **138**:143-155.
- Lazo, P. A., V. Prasad, and P. N. Tsichlis. 1987. Splice acceptor site for the *env* message of Moloney murine leukemia virus. *J. Virol.* **61**:2038-2041.
- Mann, R., and D. Baltimore. 1985. Varying the position of a retrovirus packaging sequence results in the encapsidation of both unspliced and spliced RNAs. *J. Virol.* **54**:401-407.
- Mann, R., R. C. Mulligan, and D. Baltimore. 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* **33**:153-159.
- Maxwell, S. A., and R. B. Arlinghaus. 1985. Use of site-specific antipeptide antibodies to perturb the serine kinase catalytic activity of p37^{mos}. *J. Virol.* **55**:874-876.
- Nash, M., N. V. Brown, J. L. Wong, R. B. Arlinghaus, and E. C. Murphy, Jr. 1984. S1 nuclease mapping of viral RNAs from a temperature-sensitive transformation mutant of murine sarcoma virus. *J. Virol.* **50**:478-488.
- Nash, M. A., B. L. Brizzard, J. L. Wong, and E. C. Murphy, Jr. 1985. 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. *J. Virol.* **53**:624–633.
25. **Padgett, R. A., P. J. Grabowski, M. M. Konarska, S. Seiler, and P. A. Sharp.** 1986. Splicing of messenger RNA precursors. *Annu. Rev. Biochem.* **55**:1119–1150.
26. **Rao, R. N., and S. G. Rogers.** 1979. Plasmid pKC7: a vector containing ten restriction endonuclease sites suitable for cloning DNA segments. *Gene* **7**:79–82.
27. **Singh, L., and K. W. Jones.** 1984. The use of heparin as a simple cost-effective means of controlling background in nucleic acid hybridization procedures. *Nucleic Acids Res.* **12**:5627–5638.
28. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503–517.
29. **Van Beveren, C., F. Van Straaten, J. A. Galleshaw, and I. M. Verma.** 1981. Nucleotide sequence of the genome of a murine sarcoma virus. *Cell* **27**:97–108.