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Construction of a Mammalian Transducing Vector from the Genome of Moloney Murine Leukemia Virus

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A 0.9-kilobase DNA fragment from the genome of Moloney murine leukemia virus, including the viral long terminal repeat, was covalently linked to the herpes simplex virus I thymidine kinase (*tk*) gene whose promoter was previously removed. The hybrid DNA structure was introduced into the chromosome of tk^- mouse cells at single copy numbers, via transfection procedures. Cells expressing the newly introduced *tk* gene were identified by the HAT selection procedure and analyzed for *tk*- and moloney murine leukemia virus-specific DNA and RNA sequences by blot hybridization procedures. Expression of the *tk* gene is dependent on function(s) provided in *cis* by the viral DNA fragment. Vectors derived from this region are termed rGag (rG) vectors.

The development of DNA-mediated gene transfer systems (8, 20, 39) permits the introduction of a number of DNA sequences into mammalian cells. As a consequence, a variety of DNA vectors have been developed which will propagate and express covalently linked genes. Most of these vectors were derived from simian virus 40, an animal virus of monkey origin (10, 11, 25, 26). The hybrid DNA structures constructed in vitro with recombinant DNA technologies are usually propagated in a monkey cell line specially developed for this purpose (6). The foreign genes carried by the simian virus 40 vector are expressed at high level, albeit transiently, from the autonomously replicating vector DNA for 1 to 3 days followed by cell death.

The genome of retroviruses is another source of mammalian vectors. Upon infection, the genomic RNA of retroviruses is reverse transcribed, and the resulting double-stranded DNA integrates into the host cell from where the viral genes are expressed without any adverse effects on cell viability (for a review, see reference 3). Recently, a variety of vectors were constructed from the genome of retroviruses. Blair et al. (4) used a murine sarcoma virus vector to express the cellular *mos* gene. Huang et al. (15) constructed a vector from the genome of mouse mammary tumor virus to express the *HaSV* p21 gene. Lee et al. (18) used a similar vector to express the mouse dihydrofolate reductase gene, and Joyner et al. (17) used the left end of the spleen focus-forming virus to express the herpes thymidine kinase gene. In contrast to the simian virus 40 transduction system, the hybrid retrovirus vectors were introduced into the chromosome of the recipient cells and propagated and expressed in this form indefinitely. Shimotono and Temin (29), Wei et al. (38), and Tabin et al. (35) have shown that retrovirus vectors containing foreign genes can be also packaged into viral particles by phenotypic mixing with a helper virus.

The use of mammalian vectors for the transduction and expression of genes in mammalian cells provides a powerful experimental approach for the study of the mechanism of gene expression in mammalian cells. Effects of specific modifications introduced in vitro can be studied by these methods (9, 24). The inherent flexibility afforded by the recombinant DNA technologies offers a systematic approach for identification and characterization of *cis*-acting sequences required for gene expression and regulation (10–12, 25). The nature of the promoter, splicing, and polyadenylation signals are examples of such *cis*-acting functions which are currently under investigation. Signals for other functions (e.g., ribosome binding) have yet to be identified.

We have begun a systematic study to identify the *cis*-acting functions governing the expression of the Moloney murine leukemia virus (M-MuLV) genome. In addition to understanding regulation of the M-MuLV gene expression, we hope that this approach will enable us to construct efficient vectors for gene transfer in mammalian cells. As a first step in this direction, we report the identification of a DNA fragment from the left end of the viral genome containing several, if not all, *cis*-acting functions required for the expression of an adjacent gene. The M-MuLV long terminal repeat (LTR) and additional 5′ end sequences were covalently linked to the

herpes simplex virus (HSV) thymidine kinase (*tk*) gene, whose promoter was previously removed. The chimeric molecule was introduced into mouse L-cells by transfection. We show that the chimeric molecule is functional by biological and biochemical criteria. In addition, we describe a new strategy for the isolation of specific ligation products which results in a substantial simplification of current procedures.

MATERIALS AND METHODS

Cells. Mouse LMTK$^-$ cells (*tk*$^-$ L-cells) were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum or horse serum.

DNA transfections. DNA transfections were conducted by a modification (13) of a method described by Wigler et al. (39). *tk*$^+$ colonies were selected by the HAT selection system (34), and colonies were isolated by the cloning ring method.

DNA isolation and blot hybridization. DNA was isolated from cell monolayers by sodium dodecyl sulfate as described previously (14). The DNA was digested with the appropriate restriction endonuclease, fractionated on 1% agarose gels, denatured, and transferred to nitrocellulose by the method of Southern (33). Appropriate DNA was nick translated by the method of Rigby et al. (28) to a specific activity of 1×10^8 to 2×10^8 cpm/g. After hybridization and washing the filters were exposed to X-ray film, and autoradiographs were obtained.

RNA isolation and blot hybridization. Total cellular RNA was isolated by the hot phenol method (32), fractionated on formaldehyde-agarose gels (19), and transferred to nitrocellulose (36). After hybridization and washing the filters were exposed to X-ray film, and autoradiographs were obtained.

RESULTS

The objective of this series of experiments is to identify the *cis*-acting sequences governing the expression of the M-MuLV *gag* gene. The *gag* gene is expressed from the left end of the viral genome. The 5′ end of the *gag* mRNA (the unspliced [?] 35S RNA species) maps in the left LTR 449 nucleotides from its end. (The M-MuLV LTR is 594 nucleotides long [30].) The putative initiation codon for the M-MuLV *gag* gene product lies 476 nucleotides outside the LTR (27). Thus a 621-nucleotide untranslated sequence is present at the 5′ end of the M-MuLV *gag* mRNA. This region, though, contains three AUG triplets out of frame with the *gag*-coding sequence (30). Accordingly we have isolated several different DNA fragments from the 5′ end of the M-MuLV genome and begun to test their ability to direct the expression of a viral gene (HSV *tk*) whose 5′ end was removed. Results describing one such M-MuLV sequence are presented here.

The various DNA sequences used in the construction of the chimeric plasmid and the different steps used to accomplish the goal are presented in Fig. 1. We have isolated a 0.9-kilobase (kb) BalI fragment from the M-MuLV DNA (small circular form cloned into pBR322 containing one copy of LTR [31]). This fragment contains the LTR, 61 base pairs of DNA to its 5′ end, and 310 base pairs at the 3′ end (30). No AUG triplets are present between the putative cap site and the *Bal*I site (30). We have covalently linked the HSV *tk* gene to this end of the M-MuLV DNA fragment. To facilitate the joining of the two DNA segments, we have modified the ends of M-MuLV *Bal*I fragment with synthetic *Eco*RI DNA linkers (1, 21).

The structure of the DNA fragment carrying the HSV *tk* gene is also shown in Fig. 1. The DNA sequence we have used is derived from a 3.4-kb *Bam*HI fragment which contains all the information necessary for *tk* gene expression (5, 23, 39). The complete nucleotide sequence of the *tk* gene-coding region as well as a portion of its 5′ and 3′ ends has been determined (22, 37). This gene contains a 105-base-pair sequence corresponding to the 5′ untranslated region of the mRNA, including a unique *Bgl*II site 50 nucleotides downstream from the cap site. We have isolated the *Bgl*II-*Eco*RI fragment, which contains all of the coding sequences of the *tk* gene along with the putative polyadenylation signal and modified its end by the addition of synthetic *Eco*RI linkers (Fig. 1).

Covalent joining of the M-MuLV and HSV *tk* DNA fragments: a simplified procedure for the isolation of a desired ligation product. The M-MuLV and HSV *tk* DNA fragments were covalently joined in a ligation reaction by using T4 DNA ligase, through the artificially introduced *Eco*RI ends. Covalent joining of two DNA fragments by enzymatic ligation through a common restriction site results in several combinations requiring a step of molecular cloning to isolate and amplify the desired structure. The standard approach involves two cycles of molecular cloning. For example, to ligate two *Eco*RI fragments, first one DNA component is cloned in the *Eco*RI site of pBR322 and partially digested with *Eco*RI, followed by the introduction of the second DNA fragment; the resulting clones are analyzed for the desired configuration (one of four among recombinant structures carrying both DNA components). We have introduced a considerable simplification, limiting the procedure to one cloning event and avoiding the necessity for partial restriction enzyme digestion.

A schematic diagram of this procedure is shown in Fig. 1. (i) The M-MuLV DNA fragment was incubated with calf intestine phosphatase, preventing self ligation and its subsequent incorporation into pBR322 by itself (see below).

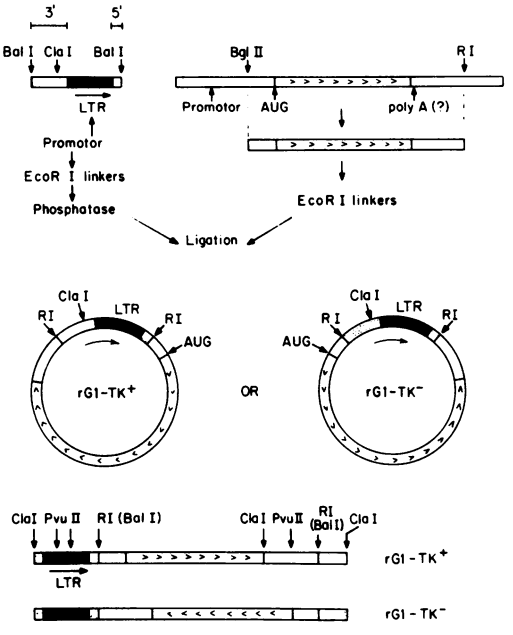

FIG. 1. Covalent joining of the M-MuLV and HSV *tk* DNA fragments. The small circular DNA form of M-MuLV cloned in pBR322 (from the *Hin*dIII site) was digested with *Bal*I, and the 0.9-kb fragment, including the viral LTR and additional 61 nucleotides from the 5′ end and 310 nucleotides from the 3′ end of the viral genome, was purified on acrylamide gels. *Eco*RI DNA linkers were enzymatically added (1, 21), and the modified DNA fragment was cloned into the *Eco*RI site of pBR322, digested with *Eco*RI, purified on acrylamide gels, and incubated with calf intestine phosphatase (Boehringer Mannheim) to remove terminal phosphates. The HSV type I *tk* *Bam*HI DNA fragment cloned in the *Bam*HI site of pBR322 (pHSV106) (23) was digested with *Eco*RI and *Bgl*II, modified enzymatically with *Eco*RI DNA linkers, recloned into the *Eco*RI site of pBR322, and digested with *Eco*RI, and the *tk* DNA fragment was purified on acrylamide gels. The phosphatase-treated M-MuLV fragment and the *tk* DNA fragment were incubated with T4 DNA ligase at a final concentration of 10 to 20 μg/ml and subsequently digested with *Cla*I. The *Cla*I digest (10 to 50 ng) was ligated with 200 ng of pBR322 (digested with *Cla*I and incubated with calf intestine phosphatase) in a volume of 10 μl. The reaction mixture was used to transform *E. coli* (strain C600), and colonies were selected on ampicillin plates and screened for M-MuLV-and *tk* DNA-containing bacteria by the Benton and Davis procedure (2). Over 60% of colonies were of recombinant nature, all (over 100) carrying both M-MuLV and *tk* sequences. The majority of resulting plasmids analyzed with restriction enzymes carried the M-MuLV and *tk* DNA fragments covalently joined in either orientation as shown, designated rG1-tk$^+$ and rG1-tk$^-$. (Note that the use of *Cla*I resulted in a permutation of the original M-MuLV DNA fragment, involving the 3′ end of the viral genome. This is not expected to effect the biological activity of the hybrid structure.) A minority of the plasmids analyzed (5%) had a more complex structure, either containing two *tk* DNA fragments or resulting from longer concatameric structures, a byproduct of the ligation reaction performed under the conditions described above.

(ii) The phosphatase-treated M-MuLV DNA fragment was ligated to the *tk* DNA fragment. (iii) The ligation products were digested with *Cla*I. This enzyme has no recognition site in the *tk* DNA fragment, which eliminates its incorporation into pBR322 by itself. *Cla*I, however, has one recognition site in the M-MuLV DNA fragment, outside the LTR in the unique 3′ end region (Fig. 1). Thus cleavage of the M-MuLV fragment with *Cla*I will not interrupt the LTR and the adjacent 5′ end DNA sequences. (iv) The *Cla*I digest was cloned into pBR322 which had been previously digested with *Cla*I and incubated with calf intestine phosphatase. The resulting DNA was used to transform *Escherichia coli* K-12 strain C600. Over 60% of the bacterial colonies recovered after transfection were of recombinant nature, all containing both the M-MuLV and the *tk* DNA insert in one of two configurations (Fig. 1).

In summary, the cloning procedure described above resulted only in recombinant plasmids carrying both DNA components of the ligation reactions. This is so because neither DNA component can be inserted into pBR322 by itself either because it lacks the appropriate restriction site used for insertion (*tk* DNA, *Cla*I site) or because it is incubated with phosphatase (the M-MuLV DNA fragment).

We have isolated the two possible combinations between the M-MuLV and the *tk* DNA fragments. In rG1-*tk*$^+$ the direction of transcription of the M-MuLV and *tk* DNA fragments is the same, whereas in rG1-*tk*$^-$ the transcriptional orientations of the two DNA segments are opposite each other.

M-MuLV-mediated expression of the *tk* gene product. The virus-dependent expression of the HSV *tk* gene was analyzed by transfection of tk$^-$ mouse cells with the recombinant DNA structures described above. Cells in which a biologically active *tk* gene product is expressed in a stable fashion were identified and isolated by the HAT selection procedure (34). When *tk*$^-$ L-cells were transfected with rG1-*tk*$^+$ DNA, HAT-resistant colonies appeared (Table 1). In this plasmid the transcription orientation of both the M-MuLV and the *tk* gene are the same. Transfection with the *Eco*RI linker-modified *Bgl*II-*Eco*RI fragment of the *tk* gene alone does not elicit the appearance of HAT-resistant colonies. Most important, no HAT-resistant colonies appeared after transfection with rG1-tk$^-$, in which the M-MuLV and *tk* sequences are in opposite transcriptional orientations. These re-

TABLE 1. Transfection of LMTK$^-$ cells with chimeric plasmids[a]

Plasmid	HAT-resistant colonies	
	Expt I	Expt II
pHSV106	212	ND
pHSV106$_{del}$	0	0
rG1-*tk*$^+$	155	312
rG1-*tk*$^-$	1	0

[a] DNA transfections were performed as described in the text. Samples (0.1 to 0.2 μg) of each DNA were transfected on five 10-cm plates (10^6 cells per plate), and colonies were scored 14 days after HAT application. pHSV106 is pBR322 containing a 3.4 kb HSV DNA fragment bearing the complete *tk* gene (23). pHSV106$_{del}$ consists of the *Bgl*II-*Eco*RI HSV *tk* DNA fragment (Fig. 1) modified by *Eco*RI DNA linkers and cloned in pBR322. The structures of rG1-*tk*$^+$ and rG1-*tk*$^-$ are shown in Fig. 1. ND, Not determined.

sults strongly suggest that the *tk* gene is expressed as a biologically active product through the *cis*-acting functions present on the M-MuLV-derived vector.

Analysis of DNA and RNA in HAT-resistant cells. To directly correlate the appearance of HAT-resistant cells with the expression of the *tk* gene of the newly acquired recombinant DNA we analyzed the DNA and RNA content of cells derived from several independently isolated HAT-resistant colonies obtained by transfection of LMTK$^-$ cells with rGl-tk$^+$.

DNA from 13 HAT-resistant colonies was analyzed by the Southern blot-hybridization procedure (33). The DNA was digested with each of a number of restriction endonucleases and probed with labeled M-MuLV LTR or the *tk* *Bgl*II-*Eco*RI DNA fragment. Digestion of the DNA with *Bam*HI or *Hin*dIII (enzymes which have no recognition sites in the M-MuLV or *tk* DNA) permitted us to deduce that the DNA is integrated into a high-molecular-weight form and that each cell line contained 1 to 4 copies of the input DNA. The integrity of the input DNA can be ascertained unambiguously only in cell lines containing a single copy of the donor DNA. We have analyzed two such cell lines in detail (Fig. 2, clones 6a and 6b). Digestion of the DNA of the two cell lines with *Hin*dIII has shown that both cell lines contain one integrated vector DNA per genome. Digestion with *Pvu*II generated a 2.0-kb DNA fragment comigrating with a 2.0-kb DNA fragment generated by digestion of the original plasmid, rG1-*tk*$^+$, with *Pvu*II (Fig. 1). The presence of this 2.0-kb band confirms the intactness of the integrated vector DNA. As predicted, the newly integrated DNA can be detected with either the *tk* or the M-MuLV probe. Cells containing one copy of integrated DNA per genome are the appropriate cells for analyzing the structure of RNA species from the newly acquired DNA because they provide an unambiguous structural correlation between the DNA template and its RNA transcript. Accordingly we have analyzed the RNA from cell lines 6a and 7a.

Total polyadenylated RNA from the two cell lines was fractionated on denaturing agarose gels (19), transferred to nitrocellulose paper (38), and probed with the appropriate labeled DNA (Fig. 3).

With the *tk* DNA as a hybridization probe, a single polyadenylated RNA species was detected. It corresponded to the size of RNA expected to be made from the chimeric plasmid. A similar size RNA species was synthesized in clone 7a (data not shown). When M-MuLV DNA was used as a hybridization probe, the same size RNA was barely detectable after a considerably longer exposure (Fig. 3), properly reflecting the hybrid nature of the RNA transcribed from the rG1-*tk*$^+$ DNA, consisting of 1,460 nucleotides of *tk* DNA and 204 nucleotides of M-MuLV DNA. Note that hybridization with M-MuLV detected two abundant RNA species in clone 6a as well as control LMTK$^-$ cells. They were endogenous retrovirus RNA species comigrating with the M-MuLV RNA species synthesized in viral infected NIH/3T3 cells (data not shown).

DISCUSSION

We have demonstrated the use of a retrovirus-derived vector (rG1) for the transduction and expression of the HSV *tk* gene in mammalian cells. Similar reports describing the use of retrovirus-derived vectors for the expression of covalently linked genes have been recently published (4, 15, 17, 18). The M-MuLV vector, rG1, mediates the expression of the HSV *tk* gene. This is clearly demonstrated in Table 1. Only when both the M-MuLV and the *tk* DNA fragments were covalently linked in the same transcriptional orientation was the *tk* gene expressed to a biologically active gene product (see also reference 17).

The M-MuLV DNA fragment in rG1 appears to contain all of the *cis*-acting functions required for the expression of the covalently linked *tk* gene. It consists of the viral LTR and 61 additional nonrepeated nucleotides (to the *Bal*I site) from the 5′ end of the genome (30) (Fig. 1). This region contains the function(s) required for the normal expression of the viral *gag* (and *pol*) gene (hence vectors derived from this region were termed rGag or rG vectors).

One obvious function required for the expression of the viral genes is a promoter for RNA transcription localized in the viral LTR. Because

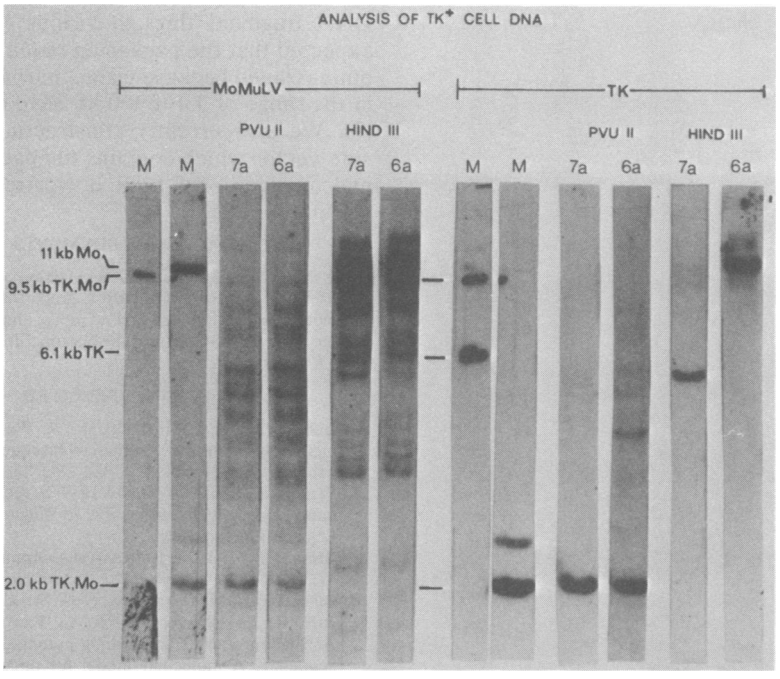

FIG. 2. Analysis of DNA from HAT-resistant cells obtained by transfection with rG1-tk^+ DNA. Samples (5 μg) of DNA from two HAT resistant clones were digested with 5 U of *Hin*dIII or *Pvu*II, fractionated on 1% agarose gels, transferred to nitrocellulose paper, hybridized with a ^{32}P-labeled, *tk*-specific probe and subjected to autoradiography. The sema filter was hybridized to a ^{32}P-labeled, M-MuLV-specific probe after removal of the *tk* probe by NaOH. rG1-tk$^+$ 6a and 7a are two clones previously shown to contain single copies of vector DNA integrated into the cell genome. *Hin*dIII has no recognition site in either the M-MuLV or the *tk* DNA fragment. *Pvu*II has one recognition site in HSV *tk* DNA and two closely spaced sites in the M-MuLV DNA fragment as shown in Fig. 1. M, Unlabeled DNA markers carrying M-MuLV, *tk*, or both sequences. The 2.0-*kb* DNA fragment is derived from the rG1-tk^+ plasmid digested with *Pvu*II. It serves as a marker for the integrity of the rG1-tk^+ DNA integrated in the genome of clones 6a and 7a. The purity of the *tk* and M-MuLV probes and especially the efficiency of removal of the *tk* probe before hybridization with the M-MuLV probe is reflected by the pattern of bands in the marker (M) lanes. tk^- cells digested with *Hin*dIII or *Pvu*II do not hybridize to the *tk* probe (data not shown). The multiple bands appearing after hybridization with the M-MuLV represent the endogeneous retrovirus-related sequences present also in tk^- cell DNA (data not shown). (*Hin*dIII-digested 6a DNA hybridized with the M-MuLV probe contained an additional band comigrating with the one observed after hybridization with the *tk* probe, but was largely obscured by the presence of endogeneous bands.)

the *tk* gene lacks its own promoter (Fig. 1 and above) clearly this is one function provided by the viral vector for the expression of the *tk* gene. As predicted, the RNA transcribed of the rG1-tk^+ vector is of a hybrid nature (Fig. 3 and above). Are there additional functions encoded in the M-MuLV genome required for the efficient expression of its genes? The construction of rG1 and similar rGag vectors (E. Gilboa, unpublished results) is an appropriate starting point for investigation of this question. In addition, we are currently conducting in vitro mutagenesis experiments on rG1 and other rG vectors to address this question.

Is rG1 of general value, can it express any gene in the form of a DNA fragment carrying its coding sequences? It is possible that the expression of the *tk* gene in rG1-tk^+ is a special case. For example, the remaining 58 nucleotides at the 5′ untranslated region (between *Bgl*II and the AUG condon) may specify a yet unidentified function (ribosomal binding site?) absent from the M-MuLV vector (37). An indication that the expression of the *tk* gene is not a special case is provided by the observation that the bacterial Neo^r gene (16) can also be expressed if linked to the rGag vector (E. Gilboa, unpublished results). The bacterium-derived gene is not expected to contain any *cis*-acting elements that can be recognized by a mammalian system. Although further studies are required to establish the efficiency of expression of rG1 linked genes, it appears that this M-MuLV-derived vector can be used to express the genetic infor-

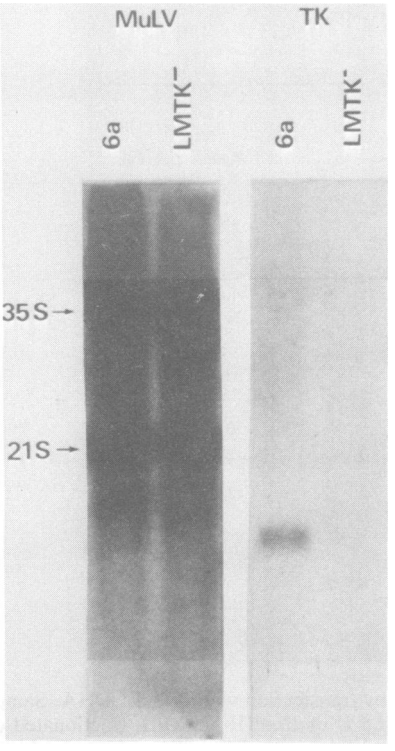

FIG. 3. Analysis of RNA from a HAT-resistant clone obtained by transfection with rG1-*tk*$^+$ DNA. Total cellular RNA was prepared from untransfected LMTK$^-$ and clone 6a cells (Fig. 2), and polyadenylated RNA was selected on oligodeoxythymidylic acid-cellulose columns. RNA (1 μg) was subjected to electrophoresis in a 1% agarose formaldehyde gel, transferred to nitrocellulose paper, and hybridized with either a ^{32}P-labeled, *tk*-specific probe or an M-MuLV-specific probe. The M-MuLV-specific probe detects also endogeneous viral RNA species. The two major species comigrate with the two M-MuLV-specific RNA species synthesized in infected mouse cells and are present in comparable amounts (data not shown). The M-MuLV portion of the hybrid RNA synthesized in clone 6a is barely seen because the M-MuLV-specific sequences comprise around 10% of the hybrid RNA and because of the background generated by the high abundance of cross-hybridizing L-cell RNA species.

mation of any DNA fragment carrying the coding sequence of a given gene. In the retrovirus transducing system described here, the transduced DNA is introduced into the chromosome of the recipient cells from which it is expressed constitutively without having adverse effects on cell viability. It is possible, with reasonable frequency (Fig. 2 and above), to introduce not more than one copy of DNA into the chromosomes of recipient cells, enabling a direct correlation between the structure of the DNA template and its expression. Retrovirus-derived vectors can also be used to transmit the foreign DNA fragment through virions (29, 38). It is expected that the packaging requirement will be quite relaxed because viable murine retroviruses in the range of 3.4 to 9.0 kb have been detected (7). We are currently constructing a transmission vector which contains the packaging signal and an additional LTR downstream from the selectable gene.

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