EXPRESSION OF KIRSTEN MURINE SARCOMA VIRUS SEQUENCES IN BEAGLE DOG TISSUES

PRINCIPAL INVESTIGATORS
P. R. Kerkof
G. Kelly

Abstract — Labeled cDNA synthesized from RNA extracted from $^{238}PuO_2$-, $^{239}PuO_2$-, and ^{90}Sr-induced lung tumors in Beagle dogs, from nontumor tissue from $^{239}PuO_2$-exposed dogs, and from unexposed dog lung and liver tissue produces strong hybridization signals with a plasmid (pKSma) that contains Kirsten murine sarcoma virus (KMSV) sequences. At least 90 percent of the KMSV sequences are expressed in these dog tissues, including sequences corresponding to p21 K-ras, gp70 envelope glycoprotein, and at least one other proviral sequence. The expression of Kirsten ras and other sarcoma virus sequences may have important implications for the interpretation of carcinogenesis studies in these dogs.

We are employing a procedure that allows a battery of cloned oncogene DNA, immobilized on nylon filters, to be simultaneously probed with radiolabeled cDNA synthesized from polyadenylated mRNA extracted from radiation-induced lung tumors in Beagle dogs[1] (1986-87 Annual Report, LMF-120, pp. 291-297). Using labeled cDNA, we have detected RNA sequences in $^{239}PuO_2$-induced lung tumors, in nontumor tissue from $^{239}PuO_2$-exposed dogs, in a ^{90}Sr-induced lung tumor, and in unexposed dog lung tissue all of which yield strong hybridization signals to a K-ras-containing plasmid, designated pKSma. Out of the 22 cloned oncogene plasmid DNAs used in the battery, labeled cDNA synthesized from tumor or nontumor lung tissue from every Beagle dog used thus far in these studies has hybridized most strongly with the pKSma DNA. The high level of expression of sequences hybridizing to the pKSma sequences in both exposed and unexposed Beagle dog tissues has prompted a further investigation to determine which of the murine sarcoma virus sequences in the pKSma plasmid are expressed in these tissues.

METHODS

Source of Tissue

The tissue samples used in this study were obtained at necropsy from Beagle dogs exposed to ^{90}Sr (FAP), $^{238}PuO_2$, or $^{239}PuO_2$ as part of ongoing studies at this Institute (Table 1). The tissues consisted of lung tumors from exposed dogs, nontumor lung tissue from exposed dogs, and lung and liver tissue from unexposed dogs.

Nucleic Acid Procedures

Genomic DNA and total RNA from the tissue samples, and cloned plasmid DNA, were prepared by established techniques.[2,3] Poly A(+) mRNA was selected for by oligo-(dT)-cellulose chromatography of total RNA.[4] Plasmid DNA was digested using restriction endonucleases, and the digestion fragments were separated and sized by Agarose gel electrophoresis.[2,3] The digestion fragments were transferred from the Agarose gel to nylon filters by the procedure of Southern.[5]

Labeled cDNA was synthesized from poly A(+) mRNA by using AMV reverse transcriptase, an oligo-(dT) primer, dATP, dGTP, dTTP, and alpha ^{32}P-dCTP.[2,3] Labeled probes were synthesized from plasmid DNA or from plasmid digestion fragments by the random-primed DNA synthesis procedure, again using alpha ^{32}P-dCTP as the labeled nucleotide.[6] Southern transfers and slot-blots of RNA

Table 1

Tissues Used in This Study Obtained

at Necropsy From the Beagle Dogs Listed

Dog Tattoo	Inhaled Radionuclide	Dose to Lung at Death (rads)	Age at Death (days)
1271A	Control	0	2669
1191A	Control	0	2986
859C	Control	0	4620
689U	Control	0	4235
770S	Control[a]	0	4850
1025B	$^{239}PuO_2$	473	3713
1041B	$^{239}PuO_2$	489	3377
1057S	$^{239}PuO_2$	569	3267
1100B	$^{239}PuO_2$	595	3429
1060S	$^{239}PuO_2$	612	3466
1134B	$^{239}PuO_2$	835	3094
1061T(N)	$^{239}PuO_2$	883	3465
1061T(T)	$^{239}PuO_2$	883	3465
1145T	$^{239}PuO_2$	978	2563
1222T	$^{239}PuO_2$	1001	2773
1220B	$^{239}PuO_2$	1033	2974
1072B	$^{239}PuO_2$	1221	3354
1364S	$^{239}PuO_2$	1376	1981
1320A	$^{239}PuO_2$	2323	1834
724S	$^{238}PuO_2$	440	4415
762U	^{90}Sr (FAP)	6400	4274

[a]Spontaneous lung tumor in unexposed, control dog.

and DNA were prehybridized and hybridized with the labeled probes in formamide-containing solutions at 42°C, washed under various stringency conditions,[3] and autoradiographed.[2,3]

RESULTS

Restriction Map of Proviral KMSV DNA and of Cloned pKSma DNA

The pKSma plasmid clone, which was obtained from the American Type Culture Collection, is composed of a 4.5-Kbp Bam HI/Sma I fragment of Kirsten murine sarcoma proviral DNA inserted into the Bam HI site of pAT153.[7] A partial restriction map of the Kirsten murine sarcoma virus proviral DNA and the 4.5-Kbp insert into pAT153 is shown in Figure 1. Both the proviral DNA and the pKSma plasmid clone DNA contain two sequences of particular interest for this study. The first of these sequences is a 0.60-Kbp, Sst II/Hinc II fragment containing the entire sequence encoding the p21 Kirsten *ras* protein. The second is a 0.73-Kbp, Hind III/Sma I (Bam HI) segment containing at least a portion of the encoded sequence for the gp70 viral envelope glycoprotein.

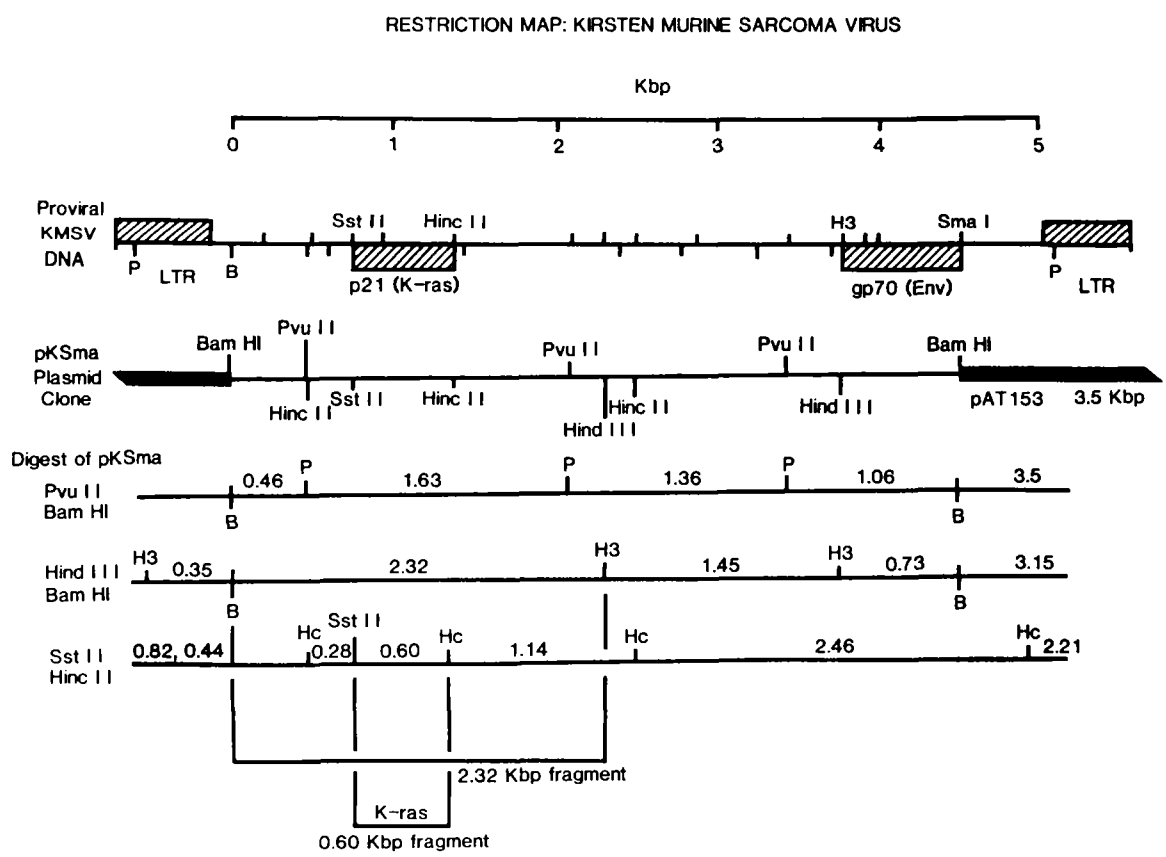


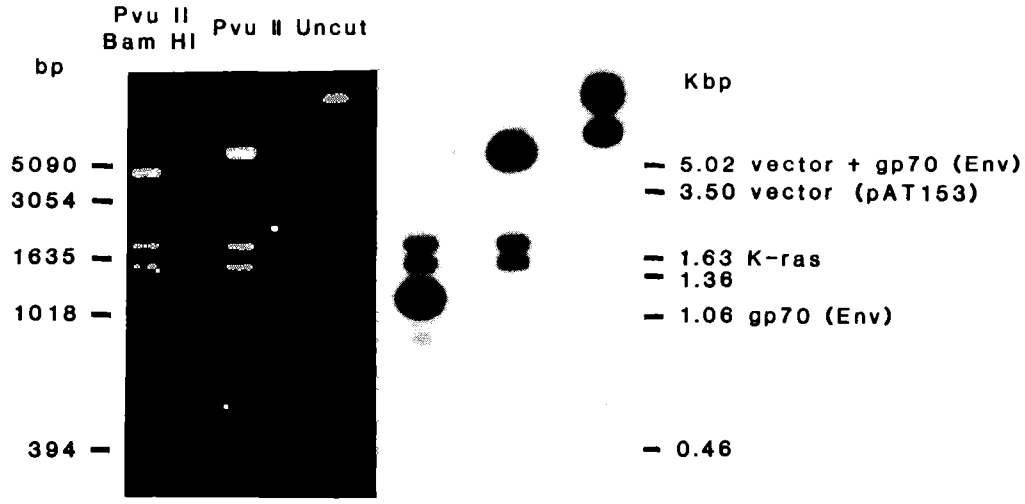
Figure 1. A restriction map is shown of Kirsten murine sarcoma virus proviral DNA and of the 4.5-Kbp insert in pKSma (adapted from references 7 and 8). Digestion of pKSma with three restriction enzyme combinations results in the fragment sizes indicated. Restriction enzyme abbreviations are as follows: P, Pvu II, B, Bam HI; H3, Hind III; and Hc, Hinc II. LTR stands for Long Terminal Repeat.

Southern Blot of Uncut and Restriction-Digested pKSma

Uncut, Pvu II-digested, and Pvu II/Bam HI-digested pKSma DNA were electrophoretically separated and transferred to nylon filters. The DNA on the filters was hybridized with ^{32}P-cDNA synthesized from poly A(+) mRNA extracted from a lung tumor in animal 12221. An autoradiograph of

the filter indicated strong hybridization to both bands of the uncut pKSma. Pvu II cuts the plasmid DNA into three fragments, all of which hybridized with sequences in the labeled cDNA population. The 1.63-Kbp, K-ras-containing fragment and the 1.36-Kbp middle fragment yielded approximately equal hybridization signals, whereas the strongest hybridization signal was obtained from the 5.02-Kbp segment (Fig. 2). The combined digestion of pKSma with Pvu II and Bam HI results in cutting the 5.02-Kbp, Pvu II fragment into segments corresponding to the 3.5-Kbp pAT153 vector, a 1.06-Kbp fragment containing the gp70 region, and a 0.46-Kbp fragment originating upstream from the K-ras sequence. No hybridization signal was obtained from either the pAT153 vector or the 0.46-Kbp fragment, but a strong signal was seen with the gp70-containing fragment (Fig. 2). Even though the strongest hybridization signal was seen with the putative, gp70-containing segments in each lane of the autoradiograph, sequences corresponding to the p21 K-ras, and to at least one other proviral sequence, are also represented in the total cDNA population (Fig. 2).

SOUTHERN BLOT OF pKSma AND RESTRICTION DIGESTS.



HYBRIDIZED WITH 32P-cDNA SYNTHESIZED FROM TUMOR 1222T POLY (A) + mRNA.

Figure 2. The pKSma plasmid digested with Pvu II/Bam HI, Pvu II only, or undigested (uncut) was electrophoresed in a 1% Agarose gel, transferred to a nylon filter and hybridized with ^{32}P-labeled cDNA synthesized from poly A(+) mRNA from a lung tumor in dog 1222T. Strong hybridization signals are seen with uncut pKSma and with pKSma fragments containing proviral sequences corresponding to the gp70 envelope glycoprotein, to p21 K-ras, and to at least one other proviral sequence.

Slot-Blot Analysis of RNA from Beagle Dog Tissues

Total RNA was extracted from lung tumor or nontumor tissues and from liver tissue of one unexposed, control dog. The RNA from these tissues was applied in 10, 2, and 0.4 µg amounts to a nylon filter in a commercial slot-blot apparatus. A ^{32}P-labeled probe synthesized from the 2.32-Kbp fragment from a Bam HI/Hind III digest of pKSma was hybridized to the RNA on this filter. The probe, which contained K-ras sequences, but no gp70 sequences, hybridized to the RNA

from every tissue, including the RNA from the liver of the unexposed, control dog (1271A) (Fig. 3, panel A). The signals, although reduced in some cases, persisted after a higher stringency wash of the filter (data not shown).

The probe was stripped from the filter and the filter rehybridized with a ^{32}P-labeled probe for the β-actin gene. The relative intensities of the signals obtained using the β-actin probe were essentially the same as those obtained using the K-*ras*-containing probe, indicating that the variation in signal intensities of the different RNA samples is most likely due to discrepancies in the estimation of the amounts of RNA applied to the filter (compare Fig. 3, panels A and B).

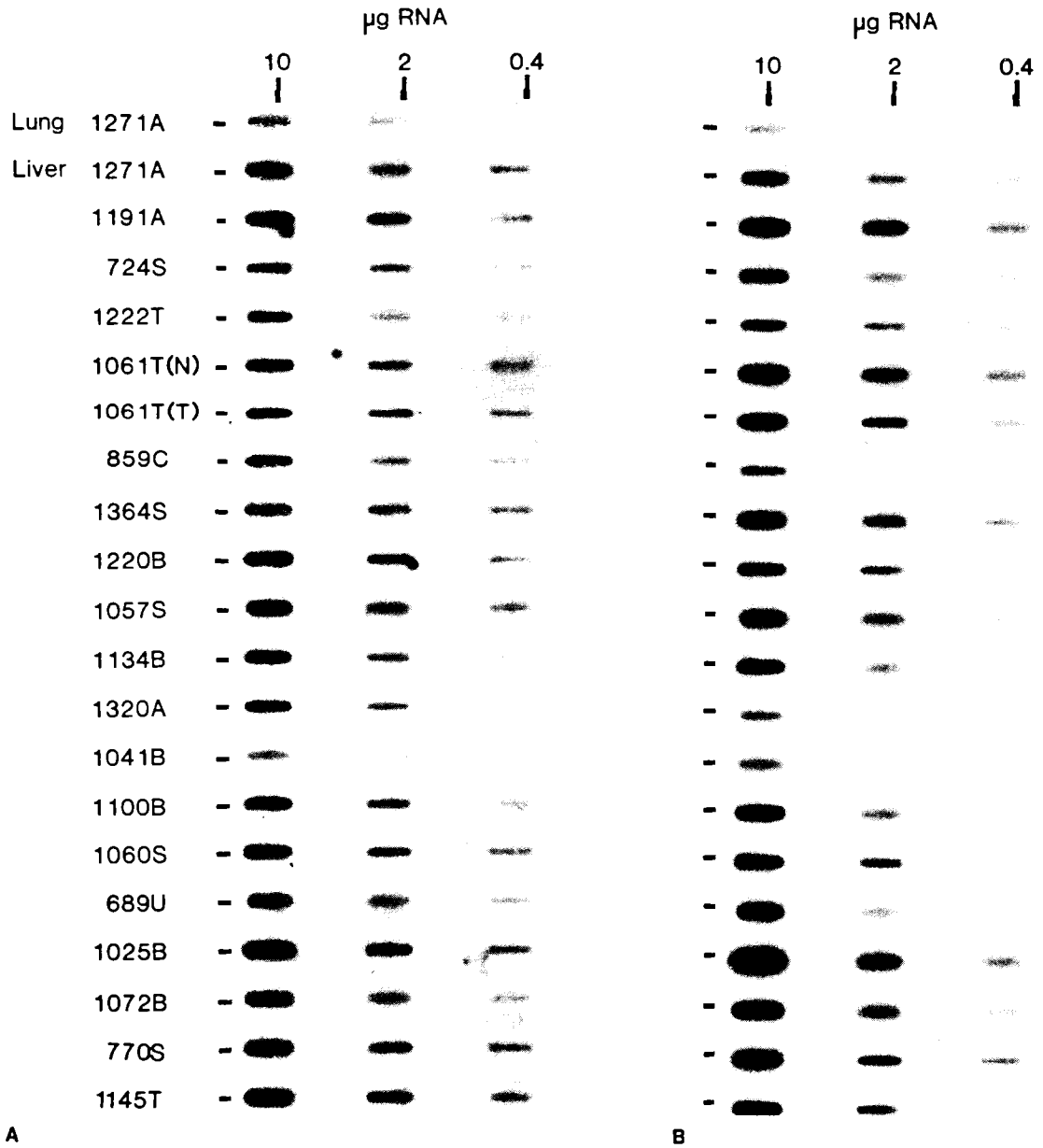


Figure 3. Panel A: RNA was extracted from tumor and nontumor lung tissue from Beagle dogs and immobilized on a nylon filter by the slot-blot procedure. RNA extracted from liver tissue of animal 1271A was also used. The filter was hybridized in a formamide solution containing a ^{32}P-probe synthesized from the 2.32-Kbp fragment from a Bam HI/Hind III digest of pKSma, washed and autoradiographed. Panel B: The filter was stripped of labeled probe and rehybridized with a ^{32}P-probe synthesized from the 0.7-Kbp fragment of a β-actin-containing plasmid. The stringency in both cases was 2X SSC, 0.1% SDS, 22°C, 0.5 h. (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate.)

Slot-Blot Analysis of DNA from Beagle Dog Tissues

A slot-blot of DNA extracted from some of the dog tissues used in this study was hybridized with a ^{32}P-labeled probe synthesized from the 2.32-Kbp fragment. Strong hybridization signals were observed from all of the DNA samples (Fig. 4, panel A). The labeled probe was stripped from this filter and the filter was reprobed with labeled β-actin sequences (Fig. 4, panel B). The DNA from the unexposed, control dog 1271A produced a stronger hybridization signal with the K-ras-containing, 2.32-Kbp probe than with the β actin probe, indicating a possible increased number of copies of the 2.32-Kbp sequence in the DNA of this dog (Fig. 4).

Because the 2.32-Kbp fragment from the Bam HI/Hind III digestion of pKSma includes sequences on either side of the p21 K-ras region, it was desirable to use a probe which would be more specifically localized to the p21 K-ras region. Therefore, the pKSma was digested with Sst II/Hinc II restriction enzymes. The products of this digestion include a 0.60-Kbp fragment containing a 567-bp sequence that encodes the entire 189 amino acid sequence of the p21 K-ras protein.[8] A ^{32}P-labeled probe was synthesized from this 0.60-Kbp fragment, and was used to probe slot-blots of DNA and RNA extracted from tumor and nontumor tissues of Beagle dogs. The results of this analysis with the ras-specific probe confirm that p21 K-ras sequences are present in the DNA and are expressed in all of the tissues studied (data not shown). Similar analyses with probes synthesized from the other regions of the proviral sequences in pKSma are needed to establish the level of expression of each of the murine sarcoma virus sequences in these dogs.

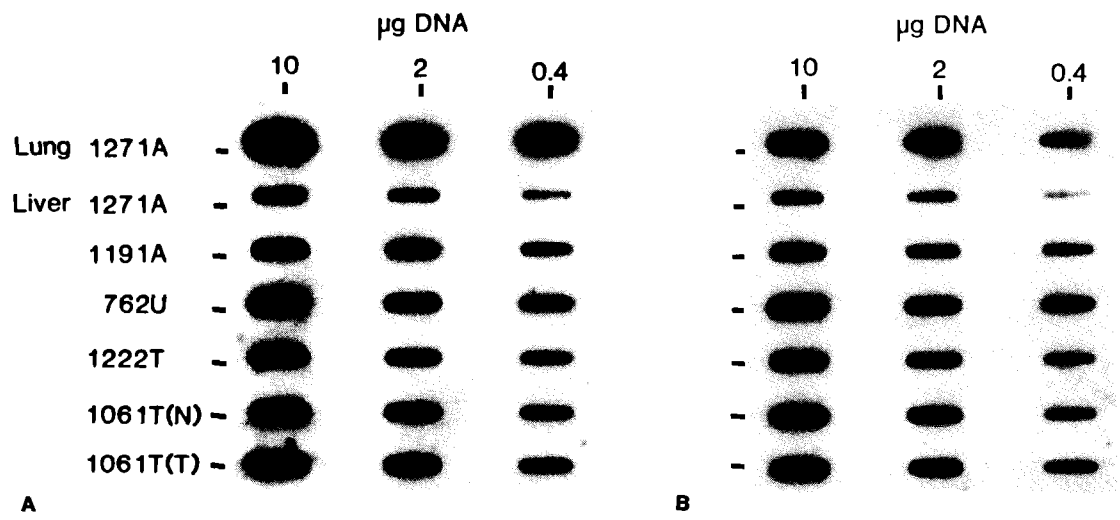


Figure 4. Panel A: DNA extracted from tumor and nontumor tissues from Beagle dogs was slot-blotted onto a nylon filter. The filter was hybridized with a ^{32}P-probe synthesized from the 2.32-Kbp fragment from a Bam HI/Hind III digest of pKSma, washed and autoradiographed. Panel B: The filter was stripped of labeled probe and rehybridized with a ^{32}P-labeled, β actin probe. Stringency in both cases was 2X SSC, 0.1% SDS, 22°C, 0.5 h.

DISCUSSION

Because ^{32}P-cDNA failed to hybridize strongly to the human K-ras specific plasmid, p640 (1986-87 Annual Report, LMF-120, pp. 291-297), we initially thought that the signal obtained by hybridizing labeled cDNA to pKSma DNA was not due to hybridization to the K-ras gene. This initial observation was confirmed in later experiments, which revealed a minimal signal when labeled p640 was used to probe tissue RNA (data not shown). The reason for the weak signal with p640 is not known, but it is possible that the sequences in the Beagle dog may lack sufficient

homology to hybridize strongly with the human K-ras sequences. Nevertheless, the data presented here demonstrate clearly that lung tumor tissue and nontumor tissue from the lung and liver of Beagle dogs in this study contain DNA sequences that are expressed in the RNA population, and which hybridize strongly to Kirsten murine sarcoma proviral sequences present in the pKSma plasmid. Sequences in the labeled cDNA population synthesized from poly A(+) mRNA from the 12221 tumor produce as intense a hybridization signal with the 1.36-Kbp, Pvu II fragment as with the p21 K-ras region of the pKSma plasmid, both of which were weaker than the signal obtained with the putative gp70 envelope region of the pKSma.

Blot analysis to detect expression of sequences that hybridize to pKSma fragments has been carried out only with labeled cDNA synthesized from poly A(+) mRNA prepared from the lung tumor of dog 12221. However, strong hybridization signals to pKSma have been observed with labeled cDNA synthesized from RNA extracted from tissues of every Beagle dog examined thus far in the study. These strong signals are most likely due to sequences in the cDNA that hybridize to the gp70 and other regions of the murine sarcoma virus sequences in pKSma, in addition to the K-ras region, which yields a weaker signal when assayed alone.

The translation of the sequences hybridizing to the K-ras and envelope glycoprotein regions of pKSma into p21 and gp70 proteins in the Beagle dog tissues has not been investigated, but could be determined by analysis with anti-p21 and anti-gp70 antibodies.

The Southern blot of a Pvu II/Bam HI digest of pKSma probed with labeled tumor cDNA showed no hybridization to the 3.5-Kbp, pAT153 vector or to the 0.46-Kbp fragment upstream of the K-ras-containing fragment. However, labeled cDNA sequences hybridized to fragments representing the remaining 90 percent of the 4.5-Kbp Kirsten murine sarcoma proviral sequences present in the pKSma insert. Whether these sequences represent expression of endogenous proviral sequences present in the dog genome or are due to active virions is not clear at the present time, and must await further investigation.

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