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Biochemistry

"Retroposon" insertion into the cellular oncogene c-*myc* in canine transmissible venereal tumor

(rearranged c-*myc*/repetitive DNA/transposable element/*Kpn* I family)

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ABSTRACT **We examined by Southern blotting the state of the cellular oncogene c-*myc* in the dog transmissible venereal tumor. The tumor DNA contains a 16.8-kilobase pair (kbp) rearranged c-*myc* fragment in addition to the normal 15-kbp and 7.5-kbp fragments. We compared the structure of the cloned rearranged c-*myc* (rc-*myc*) with that of a cloned normal c-*myc* and found that the rearrangement was due to the insertion of a 1.8-kbp DNA upstream to the first exon of c-*myc*. The inserted DNA is flanked by 10-base-pair direct repeats and contains a dA-rich tail, suggesting its origin from mRNA. Partial sequence of the inserted element showed 62% homology with the primate interdispersed *Kpn* I repetitive element. These results provide an example for the behavior of repetitive DNA sequences like the *Kpn* I family, as movable elements that can transpose nearby to oncogenes or other structural genes and perhaps affect their activity.**

A growing body of evidence indicates that the activity of cellular oncogenes can be influenced in a number of ways, as a result of which the oncogene may acquire the capacity to contribute to the transformation of normal cells (1). Major mechanisms implicated in the induction of the transforming activity of cellular oncogenes are (*i*) integration of a retroviral genome in the vicinity of a cellular oncogene and consequent increase in the oncogene's level of expression (2–4); (*ii*) generation of a point mutation in the oncogene coding region leading to the formation of an altered gene product (5–8); (*iii*) gene amplification, by which the oncogene copy number may increase as much as 60-fold (9–12); and (*iv*) oncogene translocation to another chromosome (13–16). Our work has provided an example of oncogene activation by yet a different process: the integration of an endogenous retrovirus-like DNA element (identified as an intracisternal A particle or IAP genome) within the coding region of the oncogene c-*mos* in mouse plasmacytomas XRPC 24 and NSI (17–19). The rearranged c-*mos* gene of XRPC 24 is actively transcribed and has transforming activity (17), suggesting some role for activated c-*mos* in the progression of XRPC 24 tumor. IAP genes are present in numerous copies in the mouse genome. Our work as well as that of others demonstrates their behavior as movable elements and brings out their potential to profoundly alter cellular gene expression (17, 20).

The endogenous IAP genome, which behaves like a transposable element, probably represents a defective retrovirus and it is of great interest to extend this example and to provide evidence for the transposition of cellular DNA elements into cellular genes in mammals and to investigate their effect on these genes. Mammalian DNA contains several families of highly repeated sequences, some of which have been suggested to be mobile elements (21–23). Part of the evidence for this notion is based on the finding that these elements, such as SINE and LINE (24), are bounded by direct repeats of 7–20 base pairs (bp), similar to the integrated provirus of the retroviruses. On the other hand, in contrast to IAP or proviruses, the SINE or LINE elements do not contain symmetrical termini at their ends (terminal repeats) but contain in one of their ends a poly(A)-rich tail, suggesting their origin in mRNA (25). For this reason *Alu* and related sequences as well as processed genes were recently defined as "retroposons" (26).

Since we found that IAP can activate the oncogene c-*mos* by DNA transposition it seems reasonable to look for transposition of other DNA elements in tumor tissues. Somatic DNA rearrangement of the oncogene suggests a change in the cellular gene by DNA transposition. Here we describe the analysis of c-*myc* in canine transmissible venereal tumor (TVT) and demonstrate the transposition of a retroposon-like element upstream to the c-*myc*.

Canine TVT is a naturally occurring neoplastic disease of uncertain histologic origin that affects the external genitalia of both sexes and is transmitted during coitus (for review see ref. 27). In addition to the natural mode of transmission, TVT can also be transplanted experimentally to adult, immunocompetent, allogeneic dogs by subcutaneous inoculation of living tumor cells. TVT is antigenic in the dog, and the tumor regresses spontaneously in most experimentally transplanted animals within several months after transplantation. The etiologic agent that originally induced this tumor is unknown, but cytogenetic and immunologic studies strongly suggest that the tumor is transmitted from one animal to another by the transplantation of cells. TVT is characterized by extensive and specific chromosome aberrations (28–30). Immunological studies showed that the anti-TVT immune response is directed, at least in part, against antigens of the major histocompatibility complex (MHC) (31). Since MHC antigens that are expressed on the tumor cells are of donor origin, these findings also suggest that TVT is transmitted by transplantation of cells. The uniqueness of TVT lies, therefore, in the fact that this is the only naturally occurring tumor that is transmitted by cell transplantation.

In this paper we describe a rearranged c-*myc* in TVT due to the insertion of a 1.8-kbp DNA upstream to the first exon of c-*myc*. A 10-bp sequence of c-*myc* was found as direct repeats at the boundary of the insert, indicating duplication of the target site. In addition, the insert contains approximately 60 bp of dA-rich tail. The inserted element was found to be represented in a high number of copies in canine DNA and to cross-hybridize also with a repetitive element in human DNA. A partial DNA sequence of the inserted element showed approximately 60% homology to the primate *Kpn* I repetitive element, suggesting that some repetitive DNA se-

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Abbreviations: IAP, intracisternal A particle(s); TVT, transmissible venereal tumor; bp, base pair(s).

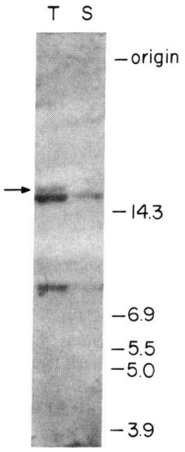

FIG. 1. Southern blot analysis of c-*myc* sequences in canine TVT and spleen DNA. Aliquots (10 μg) of high molecular weight DNA were digested with *Eco*RI, electrophoresed on 0.7% agarose gels, and, after blotting onto nitrocellulose filters, hybridized to the radiolabeled *myc* probe pM104BH. Numbers at the right side are in kbp according to DNA markers of λ Charon 4A digested with *Eco*RI/*Bam*HI. T, tumor DNA; S, spleen DNA. Both tumor and spleen were from the same dog (dog A), but Southern blots of tumor DNA from two other dogs (B and C) showed the same results.

quences may activate structural genes when transposed into their vicinity.

MATERIALS AND METHODS

Tumors. TVTs were maintained by transplantation in adult dogs and removed surgically as previously described (32, 33). Representative normal tissues (spleen and liver) were taken from the same dogs. Tissues were frozen in liquid nitrogen and kept at −70°C until used.

Restriction Enzyme Analysis. High molecular weight cellular DNA and phage DNA were digested with restriction enzymes (New England Biolabs) in various combinations. Electrophoresis of agarose gels, blotting, and hybridization to nick-translated probes were performed as described (17–19). Poly(A)-rich RNA was electrophoresed and blotted as described by Thomas (34). The c-*myc* probes used were pM104BH, corresponding to exons 2 and 3 of mouse c-*myc* (13) and kindly donated by M. Cole, and the *Pvu* II/*Xho* I fragment corresponding to exon 1 of human *myc*, kindly donated by C. Croce.

Molecular Cloning. c-*myc* and rc-*myc* *Eco*RI DNA fragments from TVT DNA of dog A were enriched by electrophoresing 500 μg of digested DNA on a 0.7% agarose gel. After identification of the fragments by blotting and hybridization of a guide strip of the gel, the DNA was eluted from the corresponding regions on the remainder of the gel by absorption to glass beads (35). The eluted DNA was cloned in phage λ Charon 4A DNA arms, and after packaging the *myc*-containing plaques were identified and purified as described (36).

DNA Sequence Analysis. Fragments containing the region that differs between c-*myc* and rc-*myc* were subcloned and subjected to sequence analysis. The *Pst* I/*Pst* I fragments of c-*myc* and rc-*myc* (see Fig. 2) were cloned in phage M13mp8 and sequences by the dideoxy method (37). Additional sequence was also determined by end labeling and sequencing by the Maxam and Gilbert method (38).

RESULTS

Rearranged c-*myc* in Canine TVT. The analysis of the c-*myc* in TVT and normal canine tissue (spleen) obtained from the same dog (A) is presented in Fig. 1. DNA (10 μg) was digested with *Eco*RI, separated by electrophoresis on 0.7% agarose, transferred to nitrocellulose, and hybridized to the *myc* probe. Both tumor and spleen DNA showed two *myc* bands, 7.5 and 15 kbp. The tumor DNA contains an additional band of approximately 16.8 kbp, which we designate rearranged c-*myc* (rc-*myc*). This band was present also in tumor DNA taken from two other dogs (B and C). However, the spleen DNA did not contain this band even after a longer exposure. The difference in intensity of the hybridizing bands between tumor and normal tissue may be due to chromosomal polyploidy in the tumor tissue. To compare c-*myc* (the 15-kbp band) with rc-*myc* (the 16.8-kbp band) we cloned the two genes in λ Charon 4A after enrichment of the respective bands by preparative agarose gel electrophoresis.

Fig. 2 depicts the physical map of canine c-*myc* and rc-*myc* as determined by restriction enzyme analysis and hybridization with the two *myc* probes, which hybridize either to exon 1 or to exons 2 and 3 of c-*myc*.

The map demonstrates that c-*myc* and rc-*myc* share identical restriction sites downstream and upstream to the two *Pst* I sites. However, rc-*myc* differs from c-*myc* by the insertion of approximately 1.8 kbp of DNA between the two *Pst* I sites. Further evidence for the identity between the 5′ regions of the two clones was obtained by hybridizing an *Eco*RI/*Kpn* I fragment of c-*myc* with an *Eco*RI/*Kpn* I digest of rc-*myc*. The results (not shown) showed strong hybridization with the 2.2-kbp *Eco*RI/*Kpn* I fragment of rc-*myc*, as expected from the map (Fig. 2).

rc-*myc* in TVT Contains a Retroposon-like Element. To locate repetitive DNA in the cloned c-*myc* and rc-*myc* we hybridized the cloned phages or a subcloned region of rc-*myc* (pRCM1, containing the *Hin*dIII fragment of rc-*myc*; Fig. 2)

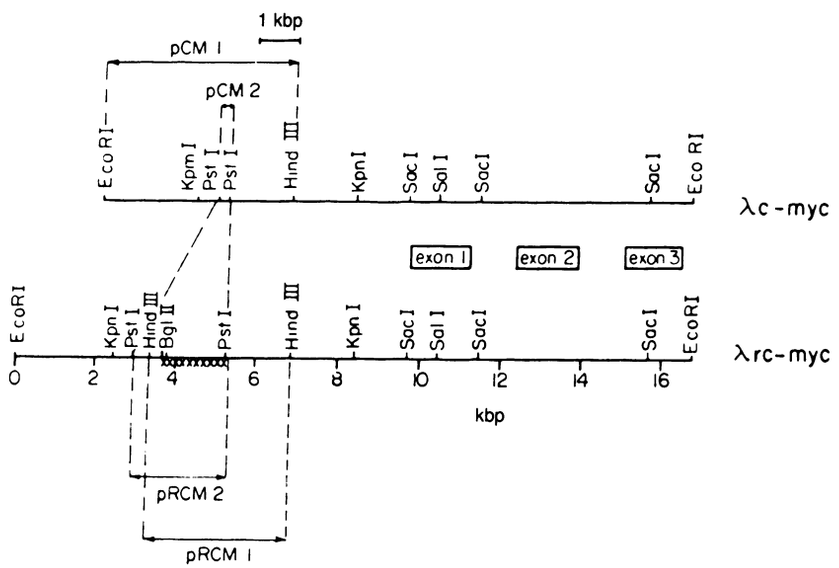

FIG. 2. Physical maps of c-*myc* and rc-*myc* inserts in cloned phages. Phage DNA was digested with several restriction enzymes in various combinations, separted on agarose gel, and hybridized to various *myc* probes to map *myc* exons. xxxx, Repetitive DNA (see Fig. 3). Subcloned fragments are named in the corresponding regions.

with nick-translated canine DNA (1 μg). The results (Fig. 3) demonstrate that rc-*myc* contains a highly repetitive DNA fragment located in the 1.5-kbp *Bgl* II/*Pst* I fragment that is contained in the inserted DNA of rc-*myc*. The c-*myc* clone contains a slightly hybridizable fragment (Fig. 3) that can be located to the 5′ *Eco*RI/*Hin*dIII fragment of the clone (Fig. 2). The results suggest that this region of the c-*myc* clone contains a sequence that is slightly repetitive in canine DNA but is certainly represented in the genome in a much smaller copy number than the inserted repetitive sequence in rc-*myc*. It is not clear whether this slightly repetitive sequence in c-*myc* is involved in the insertion in rc-*myc* by homologous recombination. To further verify the repetitive nature of the inserted DNA we hybridized blots of *Eco*RI-digested canine, mouse, and human DNA with a subclone containing the 1.5-kbp *Bgl* II/*Pst* I fragment of rc-*myc*. The results clearly show that the DNA element inserted into rc-*myc* is highly repetitive in canine DNA (Fig. 4, lanes D) irrespective of whether the DNA was from the tumor (Fig. 4, lane C-T) or from a normal liver tissue (Fig. 4, lane C-L). It is noteworthy that the inserted element in rc-*myc* cross-hybridizes also with repetitive sequences in human DNA but not in mouse DNA (Fig. 4, lanes H and M).

We wished to know the nature of the inserted DNA in rc-*myc* and in particular to analyze the junctions between the inserted DNA and the c-*myc* gene. To do this we compared the sequence of the 270-bp *Pst* I fragment of c-*myc* [subcloned and denoted pCM2 (Fig. 2)] with the sequence of the termini of the 2.0-kbp *Pst* I fragment [subcloned as pRCM2 (Fig. 2)] of rc-*myc*.

The results (Fig. 5) showed identity between the sequence of 258 bp and 60 bp of c-*myc* and rc-*myc* at the 5′ and 3′ ends, respectively, of the two *Pst* I fragments. It is also clearly shown that the *Pst* I fragment of rc-*myc* contains an approximately 1.8-kbp insert of DNA that split the *Pst* I fragment of c-*myc*. The sequence data showed that this insert is bounded by a 10-bp repeat (A-T-T-C-T-C-T-G-G-C) of the c-*myc* sequence and the insert itself has a tail of approximately 60 bp of A-rich sequence just before the junction with the cellular DNA. These features of the insert may classify it as a retroposon (26) and suggest that the insertion was in a tail-to-head orientation with respect to c-*myc*, approximately 5 kbp upstream to the first exon of c-*myc* (Fig. 2). Fig. 5 also shows the homology between the partial sequence of the inserted sequence in rc-*myc* and that of the monkey *Kpn* I element. The comparison was done by Maxine Singer, using a consensus sequence obtained from various *Kpn* I fragments (M. Singer, personal communication). The inserted sequence shows 62% homology to a region of the *Kpn* I element beginning approximately 900 bp from the 3′ end of the *Kpn* I element. This is similar to the homology between mouse MIF repetitive element and monkey *Kpn* I element (39). It is of interest that the homology is in the 3′ to 5′ orientation of the *Kpn* I element, suggesting a possibility of inversion of the inserted element.

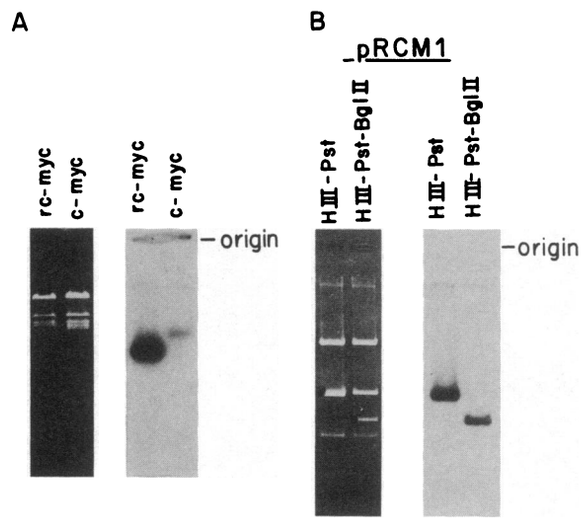

FIG. 3. Hybridization of c-*myc* and rc-*myc* to labeled canine DNA. Canine DNA was sheared, and 1 μg was radiolabeled by nick-translation and used as a probe to detect repetitive DNA in digests of c-*myc* and rc-*myc*. (*A*) Southern blot analysis of c-*myc* and rc-*myc* phage DNA digested with *Hin*dIII and *Eco*RI. (*B*) Southern blot analysis of pRCM1 digested with *Hin*dIII, *Pst* I, and *Bgl* II as indicated. In both *A* and *B*, the ethidium bromide pattern is on the left and the autoradiogram is on the right.

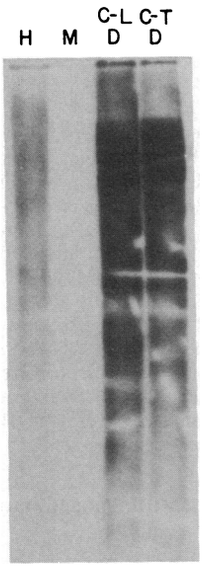

FIG. 4. Southern blot analysis of canine, mouse, and human DNA with the repetitive DNA fragment from rc-*myc*. Aliquots (20 μg) of DNA were digested with *Eco*RI, electrophoresed on a 0.7% agarose gel, and blotted onto nitrocellulose. Hybridization was with the *Bgl* II/*Pst* I fragment from pRCM1 (Fig. 2). D, dog; H, human; M, mouse; C-L, canine liver; C-T, canine TVT.

Another evidence for the homology between the inserted element in rc-*myc* and the primate *Kpn* I element was obtained by hybridization of a pRCM1 digest (Fig. 3) with a cloned monkey *Kpn* I fragment. The replicative form of M13 clone 8/6.1 (a gift of J. Skowronski), which contains a 2.2-kbp *Hin*dIII/*Xho* I fragment derived from the 3′ end of *Kpn* I element, was nick-translated and hybridized to the Southern blot of pRCM1 digested as in Fig. 3. The fragment that hybridized to the nick-translated canine DNA also hybridized to the *Kpn* I probe. The hybridization was stable to a wash with 0.45 M NaCl/0.045 M sodium citrate/0.1% sodium dodecyl sulfate at 60°C for 20 min. This and the sequence homology clearly demonstrate that the inserted sequence in rc-*myc* is the canine homologue of the primate *Kpn* I element.

Elevated Expression of c-*myc* in TVT. Since the inserted retroposon in rc-*myc* was inserted upstream to the *myc* gene it was of great interest to analyze whether it had any effect on the expression of the *myc* gene in TVT. The problem of *myc* activation and its transcriptional level was recently studied extensively in mouse plasmacytomas and Burkitt lymphoma. In these studies it was not always easy to find the normal cellular counterpart to the transformed cell such that the comparison was meaningful (40, 41). This problem is even more significant in TVT because little is known about the cell type of this tumor. We compared the level of *myc* in mRNA from three TVTs obtained from different dogs (A–C), all of them shown to contain the same rc-*myc* DNA band, and from normal canine liver. The results shown in Fig. 6 suggest that c-*myc* transcripts are increased in at least one TVT (C-T). Hence, it is possible that the inserted repetitive sequence has an effect on the transcriptional activity of the rearranged c-*myc*. However, further experiments are necessary to analyze this question.

```
          PstI                                                                70
rc-myc    CTGCAGCATA GGGGTGGGGC AGGGAGACAA CATTTTAAAA ACAATGCATT TTTAAAAATG CACCAAGATT
 c-myc    ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                                                             140
rc-myc    TTCTTCACTG CCTTTTTTTT TTATCATTCC TACGAATGAA TGATTGGCCA GATTTCGTCT GCTCGTCTGC
 c-myc    ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                                                             210
rc-myc    TGAAGAGCTT CCCAGTGTTC CTCTCACTGG GACACATGGT TAGCACAGGA AATACTGGTG AGGCTTTCCC
 c-myc    ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                                                HinIII       280
rc-myc    ATCCTTTAAC ATTCTCTGGC TGTCTTCGAC CCCCGCCGAC TGTATCCTTT GCTGTGCAAA AGCTTCTTAT
 c-myc    ----------
 Kpn I                        --C--GT-CA -T-T-AT-GT A--T--T--- --------G- ----CT---G

                                                                             350
rc-myc    CTTGATGAAG TCCCAATAGT TCATTTTTGC TTTTGTTTCT TTTGCCTTCG TGGATGTATC TTGCAAGAAG
 c-myc
 Kpn I    T--A--T-GA -----T-T-- CA-----G-- --------C-C A----T--T- GA-T-T--GA CATG---TCT

                                                                      417BglII
rc-myc    TTACTATGGC CGAGTTCAAA AAGGGTGTTG CCTGTGTTCT TCTCTAGGAT TTTGATGGAA CCTTGCC...
 c-myc
 Kpn I    --G-CCAT-- -T-TG--CTG --T---A--- ---AG---T- CT------G- ---T----T- TTAG-T-

                                                                             510
rc-myc    TGAAGTGTAT GGTGAAAGAG AGTGGTCTAG TTTCATTCTT TCTGCATGTG GATGTCCAAT TTCCCAGCAC
 c-myc
 Kpn I    -TTT--A--A ----T---GA --G-A--C-- -----G---- ---A-T-A-- -C--C-A-T- ----------

                                                                             580
rc-myc    CATTTATTGA GAGCAATGTC TTTCTTCCAA TGGATAGTCT TTCCTCCTTT ATCGAATATT AGTTGCCCAT
 c-myc
 Kpn I    --------A- ATAGGGAA-- C--TCC---T -TCT-GT--C -CT-AGG--- G--A--G--C --A--GTTG-

                                                                        635
rc-myc    AAAGTTCAGG TCCACTTCTG GATTCTCTAT TCTGTTGGAC TGATCTATGT GTCTG.....~1.4 kbp..
 c-myc
 Kpn I    -G-TG-GT-- -TT-T----- GAAG----G- ------CC-T --G-----A- -----

                                                                             2070
rc-myc    TGTTCATCTG TATGTTAGTA AATTGAACAC CAATAAAAAT AAAATAAAAT AAAATAAAAT AAAATAAAAT
 c-myc

                                                                             2140
rc-myc    AAAAAAAAAA AATAAAATTC TCTGGCCGCC TTGGTTTTGA ATCTCATGGC CCTTCTTTCA AAATGATCTT
c-myc                 ---- ---------- ---------- ---------- ---------- ----------
          PstI
rc-myc    TCTGCAG
 c-myc    -------
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FIG. 5. Partial sequence of the DNA insert in rc-*myc*. The sequence shows the beginning and end of the insert and compares the junction between c-*myc* and the insert in rc-*myc*. The insert of pCM2 (Fig. 2) derived from c-*myc* was subcloned in M13mp8 and sequenced in both orientations. The *Pst* I fragment of pRCM2 (Fig. 2) was subcloned in M13mp8 and the two ends were sequenced by the dideoxy method. The sequence of the left hand *Pst* I/*Bgl* II fragment of pRCM2 was determined by the Maxam and Gilbert method (38) after labeling of the *Bgl* II site. The sequence between the two *Bgl* II sites (downstream from nucleotide 417) was determined for both strands by the Maxam and Gilbert method. A small region (approximately 25 bp) overlapping the *Bgl* II site has not yet been sequenced and is marked by dots after nucleotide 417. Direct repeats at both ends of the insert are boxed. Identical nucleotides are marked by dashes. The sequence of the insert is compared with that of monkey *Kpn* I element (M. Singer, personal communication). The *Kpn* I sequence is of the complementary strand beginning at nucleotide 5079 and ending at nucleotide 4663 of the *Kpn* I element.

DISCUSSION

We have shown previously in two different cases of mouse myeloma that an endogenous repetitive DNA element can behave as a movable element and transpose nearby or into the c-*mos* oncogene (17–19). In these examples the movable element was the genome of the retrovirus-like element IAP. Here we demonstrate the transposition of another type of a repetitive DNA element upstream to the c-*myc* oncogene in canine TVT. In this case the 1.8-kbp insert is flanked by 10-bp direct repeats resulting from duplication of the target site. The insert also contains a 3′ tail of A-rich sequence 60 bp in length, suggesting its origin from a poly(A)-tailed mRNA like other processed genes. These features may classify the insert in rc-*myc* as a retroposon (26).

The inserted DNA was found to be highly repetitive in canine DNA and also cross-hybridized with human DNA. Analysis of mRNA from normal or tumor tissue by hybridization with the cloned insert from rc-*myc* demonstrated heterogeneous transcripts ranging from 0.2 to 6.0 kilobases. These data bear resemblance to the properties of the family of long interspersed DNA elements (LINE) recently described in mammals. The primate *Kpn* I element is 6–7 kbp long, and several examples of these genes were shown to suffer truncation as well as inversions and deletions. All of them terminate with an A-rich tail (25, 41), and recent analysis of their transcripts in HeLa cells showed heterogeneous RNA ranging from 0.2 to over 5 kilobases (42). The primate *Kpn* family is the equivalent of the mouse MIF family, and the sequence of the monkey *Kpn* element shows 60–70% homology to the known sequence of murine MIF element (43). The partial sequence of the insert in rc-*myc* (Fig. 5) also shows 62% homology to that of monkey *Kpn* element. Taken together, all these data strongly suggest that the DNA element inserted upstream to c-*myc* in TVT is the canine equivalent of the primate *Kpn* family. Hence, our work demonstrates that LINE (24) genes can behave like mobile elements that transpose to the vicinity of a structural gene. Recent work by Nomiyama *et al.* (44) showed the insertion of a *Kpn* I element into mitochondrial DNA, again demonstrating that *Kpn* I can be a movable element.

The transposition of the canine LINE element upstream to

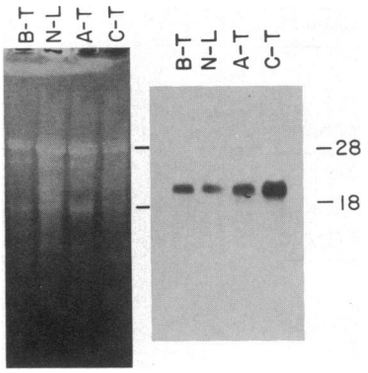

FIG. 6. Analysis of poly(A)$^+$ RNA from canine tissues for the presence of *myc* transcripts. Aliquots (10 μg) of poly(A)-selected RNA from TVT of three dogs (A-T, B-T, C-T) and liver of normal dog (N-L) were heated at 60°C for 10 min and electrophoresed at 100 V for 5 hr on a 1% agarose gel. The RNA was transferred with 1.5 M NaCl/0.15 M sodium citrate to nitrocellulose filters and hybridized to mouse *myc* probe. The ethidium bromide pattern of the RNA after electrophoresis is shown on the left and the autoradiogram is on the right.

c-*myc* may or may not influence the transcriptional activity of c-*myc*. Our analysis of the mRNA shows a definite increase in the *myc* mRNA level in TVT as compared to that of liver mRNA. However, it is not known whether the comparison of TVT RNA with liver RNA is meaningful. The studies on translocated *myc* in Burkitt lymphoma and mouse plasmacytoma demonstrated that a comparison with the correct stage of the cell type, the one that is equivalent to the tumor cell, is essential if one is to understand if the rearranged c-*myc* is transcriptionally activated (39, 40). Since not much is known about the identity of the tumor cell in TVT and of its normal equivalent, it is impossible to arrive at a firm conclusion before other types of experiments will be carried out.

We thank Dr. Maxine Singer for help in comparing the DNA sequences with *Kpn* I sequence and Dr. J. Skowronski for the probe of the *Kpn* I element, and we thank Dr. C. Croce and Dr. M. Cole for the c-*myc* probes. The expert technical assistance of Mrs. S. Hazum is gratefully acknowledged. This work was supported in part by grants from the Richard Cohn Cancer Research Fund, the Leukemia Research Foundation, Inc., Chicago (to D.G.), the Gutwirtz Foundation (to D.G.), and the Israel Cancer Research Fund (to G.R. and D.C.).

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