JOURNAL OF VIROLOGY, Mar. 1988, p. 788–794
0022-538X/88/030788-07$02.00/0

Vol. 62, No. 3

Recombination between Two Integrated Proviruses, One of Which Was Inserted Near c-*myc* in a Retrovirus-Induced Rat Thymoma: Implications for Tumor Progression

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Received 28 September 1987/Accepted 23 November 1987

Of 17 Moloney murine leukemia virus (MoMuLV)-induced rat thymomas, 2 contained rearrangements in c-*myc*. In one of these tumors the observed rearrangement was not due to the insertion of an intact MoMuLV provirus. The rearranged c-*myc* DNA fragment from this thymoma was cloned and examined by restriction endonuclease mapping, hybridization to MoMuLV proviral DNA probes, and DNA sequence analysis. These analyses revealed that the c-*myc* rearrangement in this tumor was due to the presence of a partially duplicated MoMuLV long terminal repeat (LTR) 5′ to c-*myc* exon 1. The orientation of this LTR structure was opposite to the transcriptional orientation of c-*myc*. The sequences at the 3′ flanking side of the LTR structure were derived from a cellular DNA region which maps to the same chromosome as c-*myc* (chromosome 7), although to a site distant from this proto-oncogene. These findings present evidence for a homologous recombination event occurring between sequences of two proviruses integrated on the same chromosome, one of which was inserted near the c-*myc* proto-oncogene. The recombination product contains three copies of the MoMuLV LTR 72-base-pair direct repeat and is associated with a high level of c-*myc* expression. The reciprocal product of this recombination was not detected. We propose that recombination between homologous sequences may play a significant role in the generation of chromosomal rearrangements and therefore in tumor induction and progression.

DNA recombination appears to be associated with tumor induction and progression. Many tumors carry aberrant chromosomes generated by intrachromosomal or interchromosomal DNA recombination leading to deletions, inversions, or translocations (13, 23, 39). These chromosomal rearrangements frequently are tumor specific, occur because of recombination in the vicinity of cellular proto-oncogenes, and probably are involved in oncogenesis (3, 7, 13, 23). In other cases the rearrangements appear to be random and their involvement in tumor induction and progression is uncertain. Another example of DNA recombination occurring during oncogenesis involves the transposition of certain DNA elements within the cellular genome. This has been observed in murine plasmacytomas in which intracisternal A particle DNA sequences were transposed to the immunoglobulin kappa light-chain locus (11) or to the c-*mos* proto-oncogene (5) and in the WEHI-3B murine myelomonocytic leukemia line in which IAP sequences were observed in the vicinity of the gene coding for interleukin-3 (38). Similarly, we have observed the insertion of an *Alu* short interspersed repeated element in the domain of the human homolog of the *Mlvi*-2 locus in a human B-cell lymphoma (9).

In this report we present evidence that in Moloney murine leukemia virus (MoMuLV)-induced rat thymonas DNA recombination may be promoted by homology between the recombining DNA sequences. In the recombination event described here the homologous sequences are two MoMuLV proviruses integrated on the same chromosome, one of which has been inserted 5′ to the c-*myc* proto-oncogene. The result was a rearrangement of c-*myc* associated with deletion of the cellular DNA sequences between the two proviruses. We propose that since approximately one-third of the mammalian genome is composed of repeated DNA, recombination between homologous sequences may occur frequently during oncogenesis, leading to rearrangements that may play a role in tumor induction and progression.

MATERIALS AND METHODS

The origin of the tumors used in these experiments has been described previously (31–33). Briefly, they were derived by intraperitoneal injection of 50,000 XC PFU of MoMuLV into newborn Osborn Mendel rats. Normal tissues were obtained from 4- to 5-month-old uninjected Osborn Mendel rats.

Preparation of cellular DNA. High-molecular-weight cellular DNA was prepared from normal rat and tumor tissues by standard procedures as previously described (30).

Southern blot analysis of cellular or cloned DNA. Cellular DNA (10 μg) or cloned DNA (ca. 0.5 μg) was digested with restriction endonucleases purchased from Bethesda Research Laboratoties, Inc., or New England BioLabs, Inc., and electrophoresed in 0.7% agarose gels. The DNA was transferred onto nitrocellular filters and hybridized to nick-translated cloned DNA probes. Hybridizations took place in 50% formanide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–10% dextran sulfate at 42°C for 16 to 24 h. The dextran sulfate was eliminated in Southern blots of cloned DNA. The filters were washed initially at room temperature in 2× SSC–0.1% sodium dodecyl sulfate. The final washes were done in 0.1× SSC–0.1% sodium dodecyl sulfate at 65°C. When heterologous DNA probes were used the hybridizations took place in 40% formamide–6× SSC at 37°C, and the 65°C wash was done with 1× SSC–0.1% sodium dodecyl sulfate.

Preparation and analysis of cellular RNA. Poly(A)$^+$ tumor and normal cell RNA was prepared by chromatography on oligothymidylate acid-cellulose (1). Denatured poly(A)$^+$

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RNA was electrophoresed in 1 to 1.5% agarose–formaldehyde gels, transferred to nitrocellulose, and hybridized to nick-translated cloned DNA probes.

Molecular cloning. Cellular DNA (ca. 0.5 mg), was digested with *Eco*RI and electrophoresed in a preparative agarose gel. The agarose gel was sliced, and the DNA included in each slice was removed from the agarose by electroelution. The fractionated DNA, enriched for the desired DNA fragments, was ligated to the arms of the bacteriophage lambda vector λgtWES.λB′ (16). The ligation product was in vitro packaged to generate recombinant phage particles. Virus plaques resulting from recombinanat phage infection of *Escherichia coli* were screened by using nick-translated viral or cellular DNA probes. Fragments of the DNA insert of the lambda clone were subcloned into plasmid vector pUC9 by standard procedures.

Restriction enzyme mapping of cloned DNAs. Restriction enzyme maps of the cloned DNAs were generated by using single and double restriction endonuclease digestions and hybridizations to the appropriate nick-translated DNA probes.

DNA sequence analysis. The *Eco*RI-*Bam*HI fragment containing the recombinant long terminal repeat (LTR) was subcloned in M13mp18 and M13mp19 by using the *Eco*RI, *Kpn*I, and *Bam*HI restriction endonuclease sites. The sequence was determined by the dideoxy chain termination method (24). The clones containing direct repeats were also sequenced by using two specific oligonucleotides, 5′-CTG GACCGCATCTGGGGA-3′ and 5′-CATGGTATTGAGGA AAAAGTA-3′, which hybridize, respectively, to the region 5′ and 3′ of the direct repeats in the LTR. The sequences were analyzed by using the Staden (28) and Wilbur and Lipman (37) sequence analysis computer programs implemented by the Fox Chase Cancer Center Computer Facility.

RESULTS

DNA rearrangements in c-*myc* in MoMuLV-induced rat thymomas. Genomic DNA from 17 MoMuLV-induced rat thymomas was digested with *Eco*RI, subjected to agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to a DNA probe derived from exon 3 of the human c-*myc*. The rat c-*myc* locus was detected as an 18-kilobase (kb) *Eco*RI fragment. This analysis revealed rearrangements in c-*myc* in 2 of 17 tumors (Fig. 1A). Unexpectedly, however, the rearranged c-*myc* *Eco*RI fragment in one of the tumors (D_1) was smaller than the fragment generated from the unrearranged c-*myc* allele. If the rearrangement was due to the insertion of an intact MoMuLV provirus the rearranged *Eco*RI fragment would be larger than the nonrearranged one, because MoMuLV lacks an *Eco*RI restriction endonuclease site. The rearranged *Eco*RI fragment from tumor D_1 was cloned in the *Eco*RI site of the bacteriophage lambda vector λgtWES.λB′. A restriction endonuclease map of this clone (D_1myc1) is shown in Fig. 1B and is compared with the map of the unrearranged c-*myc* allele. This comparison revealed that novel sequences were introduced at the 5′ end of the clone, upstream from the *Hin*dIII site.

The c-*myc* rearrangement in tumor D_1 was due to insertion of proviral DNA sequences upstream from exon 1. Southern blot analysis of the insert of the clone D_1myc1 and hybridization to the MoMuLV LTR probe revealed that the c-*myc* rearrangement was due to the insertion of proviral DNA sequences (Fig. 2A, lane a). This conclusion was supported by the comigration of the cloned DNA with one of the cellular DNA fragments that were detected by hybridization of *Eco*RI-digested tumor D_1 DNA to a MoMuLV LTR probe (Fig. 2A, lane b). The LTR sequences were confined to the 5′ end of the clone. Digestion of the DNA of the clone D_1myc1 with *Bam*HI and hybridization to the LTR revealed that only the extreme 5′ 2-kb fragment contained sequences homologous to the probe (Fig. 2B). The position of the LTR sequences relative to the c-*myc* proto-oncogene was shown by the experiment in Fig. 2C. Digestion of the clone D_1myc1 by *Hin*dIII generated an 8.6- and a 3.2-kb DNA fragment. All three exons of the human c-*myc* proto-oncogene hybridized to the 8.6-kb fragment, whereas the MoMuLV LTR hybridized to the 3.2-kb fragment. We conclude that the MoMuLV

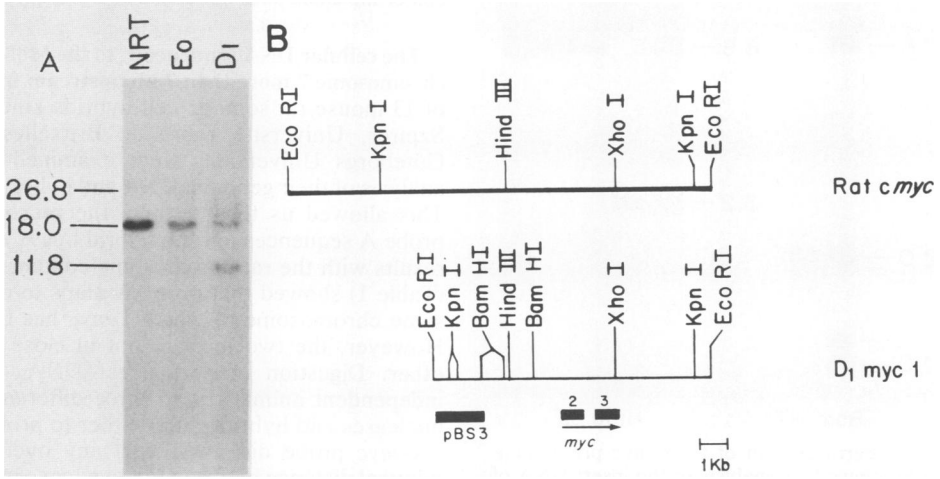

FIG. 1. (A) c-*myc* rearrangements in MoMuLV-induced thymic lymphomas. Southern blot analysis of *Eco*RI-digested DNA from normal rat thymus (lane NRT) and the thymomas E_0 and D_1. Hybridization was performed with a human 3′ exon c-*myc* probe. The numbers on the left indicate the sizes of the corresponding DNA fragments in kilobases. (B) Restriction endonuclease maps of the normal and rearranged alleles of the rat c-*myc* proto-oncogene. The map of the normal allele was generated by restriction endonuclease digestion of normal rat DNA and hybridization to a probe derived from exon 3 of c-*myc* (22). The map of the rearranged allele was generated by restriction endonuclease digestion of clone D_1myc1. The *Bam*HI sites were determined only for the cloned DNA. The arrow represents the direction of transcription of the rat c-*myc* locus. The relative orientation of c-*myc* exons 2 and 3 was determined by digesting the *Eco*RI fragment from clone D_1myc1 with *Xho*I and hybridizing it to c-*myc* probes specific for these two exons (data not shown).

LTR sequences were inserted upstream from exon 1 of the c-*myc* proto-oncogene.

Rearrangement of MoMuLV LTR upstream from the c-*myc* proto-oncogene and cellular origin of sequences at the extreme 5′ end of clone D_1mycl. The *Eco*RI-*Bam*HI DNA fragment at the 5′ end of clone D_1mycl was subcloned into the plasmid vector pUC9. This clone (pBS3; Fig. 1B) was digested with *Kpn*I, *Kpn*I-*Bam*HI, or *Kpn*I-*Eco*RI subjected to agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to the MoMuLV LTR probe. The results (Fig.3A) indicated that the LTR homologous sequences contained two *Kpn*I sites 0.3 kb from each other (Fig. 3C) that were both flanked by LTR-related sequences. Since the MoMuLV LTR contains only one *Kpn*I site, this finding suggested that the detected LTR structure was composed of two contiguous LTR sequences. Furthermore, since the MoMuLV LTR is approximately 0.6 kb long, this finding suggested that the generation of the novel LTR structure was associated with the deletion of approximately 0.3 kb of LTR sequences between the *Kpn*I sites. The *Kpn*I-*Eco*RI DNA fragment from the extreme 5′ end of clone D_1mycl (Fig. 3C) was digested with *Sac*I, subjected to agarose gel electrophoresis, transferred to nitrocellulose, and hybridized either to itself (Fig. 3B, lane a) or to a MoMuLV genomic DNA probe (Fig. 3B, lane b). Since the 0.2-kb fragment at the extreme 5′ end of the clone did not hybridize to MoMuLV, we conclude that it is composed of cellular sequences. A subclone of this DNA fragment will be referred to as probe A in the following experiments.

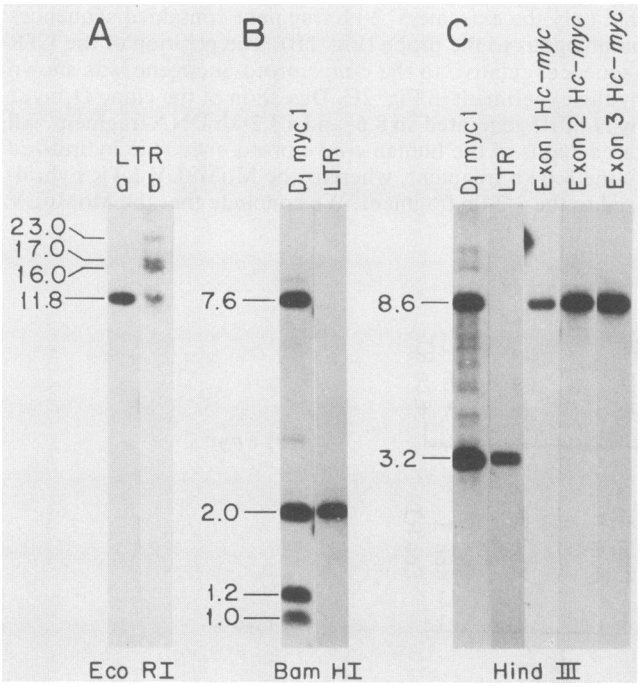

FIG. 2. Nature of the rearrangement of the c-*myc* proto-oncogene in tumor D_1. (A) Southern blot analysis of the insert DNA of the clone D_1mycl (lane a) and *Eco*RI-digested DNA derived from tumor D_1 (lane b). Hybridization was performed with a MoMuLV LTR probe. (B) Southern blot analysis of *Bam*HI-digested insert DNA of clone D_1mycl following hybridization either to itself (D_1mycl) or to the MoMuLV LTR. (C) Southern blot analysis of *Hin*dIII-digested insert DNA of clone D_1mycl. Hybridization was performed with the probes D_1mycl, LTR, exon 1, exon 2, and exon 3 of the human c-*myc* as indicated.

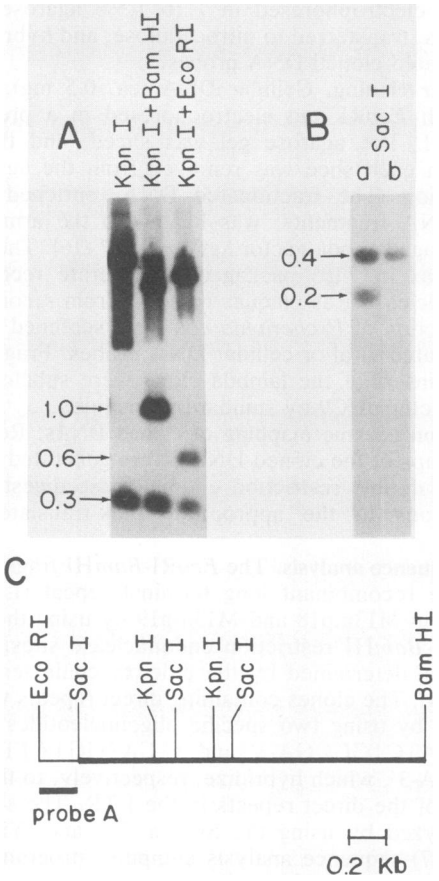

FIG. 3. Analysis of the 5′ end of clone D_1mycl. (A) Southern blot analysis of the DNA of plasmid pBS3 (Fig. 2). The hybridization was performed with the MoMuLV LTR probe. (B) Southern blot analysis of the *Eco*RI-*Kpn*I fragment from the 5′ end of clone pBS3 (Fig. 3C) following digestion with *Sac*I and hybridization to itself (lane a) or to MoMuLV proviral DNA (lane b). (C) Restriction endonuclease map of the insert of plasmid pBS3. Probe A at the 5′ end of the clone does not contain viral sequences.

The cellular DNA sequence 3′ to the MoMuLV LTR maps to chromosome 7 more than 7 kb upstream from c-*myc*. A panel of 13 mouse-rat somatic cell hybrids kindly provided by C. Szpirer, Université Libre de Bruxelles, and G. Levan, Göteborgs Universitet, were examined by Southern blot analysis of their genomic DNA and hybridization to probe A. This allowed us to determine the presence or absence of probe A sequences in each hybrid line. Comparison of these results with the rat chromosome content of the hybrid lines (Table 1) showed that probe A maps to chromosome 7: the same chromosome to which c-*myc* has been assigned (29). However, the two loci are not in close proximity to each other. Digestion of normal rat DNA, derived from two independent animals, with three different restriction endonucleases and hybridization either to probe A or to an exon 3 c-*myc* probe did not reveal any overlaps (Fig. 4). The minimal distance between the two loci can be estimated from the *Eco*RI digest. Since the *Eco*RI fragments detected with the two probes do not overlap (Fig. 4), the map location of probe A in normal rat DNA should be 5′ to the *Eco*RI fragment containing c-*myc* in normal DNA (Fig. 1B). The distance between this *Eco*RI site at the 5′ end and the provirus inserted near the c-*myc* proto-oncogene is approximately 7 kb (Fig. 1B). Therefore, we conclude that the two

proviruses that recombined to generate the recombinant LTR structure had integrated on chromosome 7 at a distance greater than 7 kb.

Sequence analysis of the integrated MoMuLV LTR and its flanking cellular DNA. To determine the sequence of events that led to the complex c-*myc* rearrangement observed in tumor D_1, we undertook a sequence analysis of the LTR and the flanking DNA in clone D_1mycl. The results of this analysis (Fig. 5) provided direct evidence that the double LTR structure detected near c-*myc* in tumor D_1 was generated by recombination between the LTR sequences of two independent proviruses. The definitive evidence that this was a recombination between two independent proviruses and not between the LTR sequences of one provirus was presented by the nucleotide sequence of the 3′ and 5′ cell-LTR junctions. When the MoMuLV provirus integrates into the cellular genome, a short direct repeat (4 base pairs [bp]) flanking the 5′ and 3′ LTR is generated by duplication of a single-copy target site (8, 25). Therefore, if the partly deleted double LTR was generated by recombination between the 5′ and the 3′ LTR of a single provirus, the DNA sequence on either side of the observed LTR structure should be identical. Since this is not the case, we conclude that the recombining LTRs belonged to two independent proviruses. The sequence analysis revealed that the novel recombination product contains three 72-bp direct repeats. The recombination point from the first LTR is located at position 7995 in the MoMuLV genome, which is at the end of the first 72-bp direct repeat. The second LTR was recombined at position 8320 of MoMuLV, which is located within the R region. The location of the recombination site in the novel LTR structure is at position 527 in Fig. 5.

Both U3 sequences in the recombinant molecule carry a mutation in its first nucleotide: a C-to-G nucleotide transversion. This nucleotide is at position 8264 in the genome of MoMuLV (36) and at positions 259 and 585 in the recombinant molecule in Fig. 5. The presence of this mutation suggests that it was present in the virus which gave rise to the two proviruses that underwent recombination.

TABLE 1. Correlation of rat chromosomes and probe A in 13 hybrid clones

Chromosome	No. of hybrid clones with following probe A and chromosome retention[a]:				% Discordant
	+/+	−/−	+/−	−/+	
1	4	3	6	0	46
2	8	1	2	2	30.8
3	8	1	2	2	30.8
4	9	0	1	3	30.8
5	4	1	8	0	61.5
6	5	1	5	2	53.8
7	10	3	0	0	0
9	3	3	7	0	53.8
10	6	2	4	1	38.5
11	6	2	4	1	38.5
12	8	2	2	1	23
13	8	2	2	1	23
14	4	1	6	2	61.5
15	5	2	5	1	46
16	8	0	2	3	38.4
17	7	0	3	3	46
18	8	1	2	2	30.8
19	4	3	6	0	46
20	7	1	3	2	38.4

[a] +/+, Containing probe A and the indicated chromosome; −/−, lacking probe A and the indicated chromosome; +/−, containing probe A and lacking the indicated chromosome; −/+, lacking probe A and containing the indicated chromosome.

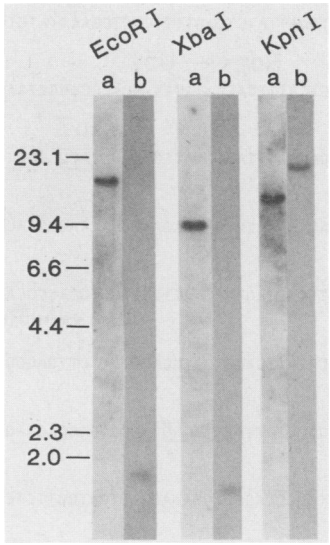

FIG. 4. Localization of the cellular DNA sequences at the 5′ end of clone D_1mycl relative to c-*myc*. Southern blot analysis of *Eco*RI-, *Xba*I-, and *Kpn*I- digested normal rat DNA hybridized to a probe derived from exon 3 of c-*myc* (lanes a) or to probe A (lanes b).

Our interpretation on the origin of this novel LTR recombinant structure is shown in Fig. 6. According to this model, the recombinant LTR is the result of the resolution of the structure produced by the homologous pairing of two misaligned LTRs in which the second direct repeat from one LTR matches the first direct repeat from the other. The reciprocal product of this recombination should be represented by an *Eco*RI band larger than 24 kb which would contain two proviruses minus one LTR (approximately 17 kb) plus the sequences upstream from the c-*myc* provirus (7 kb). This reciprocal product was not detected, leading us to conclude that it may have been deleted.

c-*myc* expression in the thymoma D_1. Poly(A)$^+$ normal and tumor cell RNA were electrophoresed in agarose-formaldehyde gels, transferred to nitrocellulose filters, and hybridized to a probe derived from exon 3 of the rat c-*myc* proto-oncogene. Figure 7 shows a comparison of the c-*myc* mRNA in the normal rat thymus, the normal rat liver, and tumor D_1. The level of the main 2.5-kb mRNA transcript was significantly higher in tumor D_1 than in the normal tissues.

DISCUSSION

Tumor induction and progression depends on two types of processes that affect the quantitative or qualitative expression of cellular proto-oncogenes (3): (i) recombination between cellular DNA sequences leading to genomic DNA rearrangements (7, 23, 39), and (ii) point mutations altering the structure and function of the c-*onc* gene protein products (2).

In this communication we present evidence that in MoMuLV-induced rat thymomas, DNA recombination may be promoted by homology between distantly located DNA sequences. The homologous sequences in the case presented in this report were the 3′ and the 5′ LTR of two independent proviruses on chromosome 7. The fact that this was not an intramolecular recombination event between the 5′ and the 3′ LTR of one provirus was determined by sequence analysis

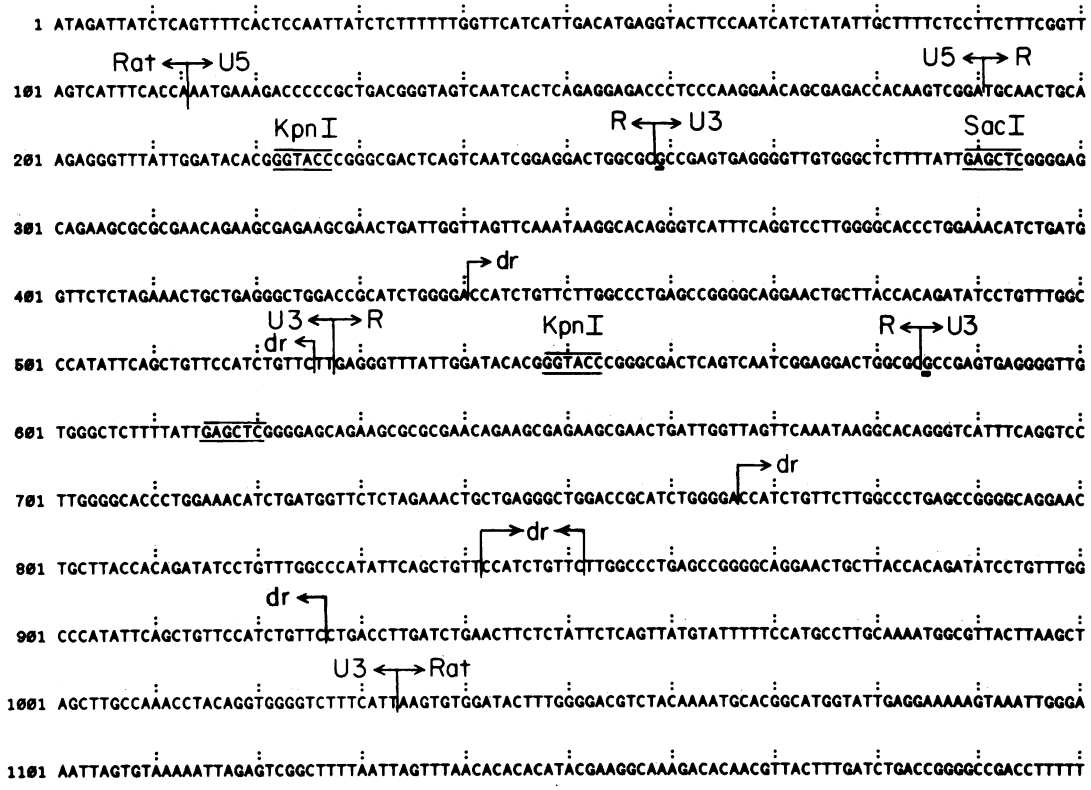

FIG. 5. Nucleotide sequence of the novel LTR recombinant structure detected in tumor D_1. The figure shows the minus strand of the proviral DNA sequences. The recombination site between the two LTRs is at position 527 in this sequence. The mutated nucleotides 259 and 585 are underlined. Symbols: dr, direct repeat; U3, U5, and R, different regions of the LTR structure. The reference sequence used was obtained from Weiss et al. (36).

which revealed that the provirus-host junctions on the two ends of the recombinant LTR structure were unrelated.

The point of recombination was at the 3′ end of the first 72-bp direct repeat of one LTR and in close proximity to the 5′ end of the R sequence of the other LTR. We conclude that this recombination event was the result of the homologous pairing between two LTR direct repeats (Fig. 6). The lack of detection of the reciprocal product of this recombination event argues that it may have been deleted. This would have happened if the two proviruses had integrated in the same orientation on chromosome 7 upstream from c-*myc*. In this case the recombination we observed would generate a

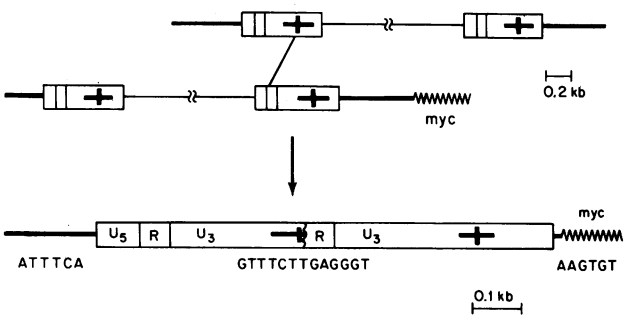

FIG. 6. Recombination between two independent MoMuLV proviruses. Symbols: —⌇ ⌇—, proviruses; ▭, LTR sequences; ——, ⁓⁓, flanking cellular DNA. The bars inside the LTR sequences indicate the 72-bp repeats, and the crossbar represents the overlap between the two direct repeats. The point at which the two LTR structures recombined is indicated by a connecting line (upper panel), and the joining between the two LTR sequences is indicated by a wavy vertical line (lower panel). The nucleotides at the recombination site and the cellular sequences flanking the recombinant molecule are also shown.

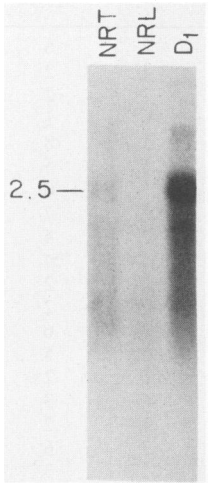

FIG. 7. c-*myc* expression. Northern (RNA) blot analysis of poly(A)$^+$ RNA from normal rat thymus (lane NRT), normal rat liver (lane NRL), and tumor D_1 with a probe specific for exon 3 of the c-*myc* gene.

circular extrachromosomal reciprocal product which would probably lack the ability to replicate and therefore would be lost. Other, less likely, interpretations of these results were considered. The second provirus could be located in the allelic chromosome 7 either 5′ or 3′ of the c-*myc* proto-oncogene. Recombination between the two proviruses in that case would preserve the reciprocal product, which was not detected. Alternatively, the second provirus could be located in the same chromosome and in opposite orientation to the c-*myc* provirus. This interpretation is also unlikely, again because recombination would preserve the reciprocal product. The final possibility, that the second provirus was located 3′ to c-*myc* in the same orientation, can be excluded because recombination would be associated with the deletion of the rearranged c-*myc*.

The rearranged c-*myc* DNA band representing the product of recombination was present in the tumor DNA in equimolar amount with the DNA band representing the unrearranged c-*myc* allele. In the absence of selection, the frequency of spontaneous recombination between two low-copy-number homologous sequences located on the same chromosome occurs in approximately 10^{-6} to 10^{-7} cells per generation (19–21, 34, 35). In that context the detection of the recombination event described here indicates that it was selected because it may have provided the cells with a significant growth advantage. It is indeed interesting that the level of c-*myc* expression in the thymoma D_1 is elevated. The deregulation of c-*myc* expression in tumors carrying a provirus near c-*myc* is probably due to the insertion of the retrovirus enhancer in the vicinity of the gene (6, 12). This suggests that in tumor D_1 the recombinant LTR structure upstream from the c-*myc* proto-oncogene may function as a strong enhancer element for initiation of transcription at the normal c-*myc* promoter. The strength of this enhancer may depend on the tandem repetition of the LTR sequences in the generated recombinant LTR. This is supported by previous work showing that the tandem repetition of a DNA sequence may have profound effects on its ability to function as an enhancer element (15). Alternatively, the growth advantage of the cells of tumor D_1 may result from the deletion of the cellular sequences between the two recombining proviruses. Such a deletion may be removing regulatory sequences from the 5′ region of the c-*myc* proto-oncogene. Karyotypically detectable deletions of certain chromosomal regions have been seen reproducibly in a variety of neoplasms (23), and it is thought that they play a significant role in tumor induction and progression (14).

In the recombination discussed in this report, the homologous DNA sequences are represented by two integrated MoMuLV proviruses. However, similar phenomena may take place between a variety of endogenous homologous sequences. Approximately one-third of the mammalian genome is composed of repeated DNA sequences (26) that could provide the DNA homology required for such recombination events. Although the frequency of this type of recombination for any single locus is low, the overall frequency of homologous recombination in tumor cells is likely to be high owing to the high copy number of the different families of repeated sequences in the mammalian genome. In some cases these homologous recombination events may be selected because they provide the cells with a growth advantage (4, 10, 18, 27). Recombination between repeated DNA sequences has been observed previously in germ line cells. The outcome of the recombination was the generation of heritable genetic mutations. This was shown for a human subject with familial hypercholesterolemia in whom an *Alu* sequence present in exon 3 and an additional *Alu* sequence present in the 3′ untranslated region of the gene for the low-density lipoprotein receptor recombined to generate a truncated gene (17). The data presented in this report, however, indicate that such phenomena can occur as somatic events and that such events may play a role in tumor induction and progression. Owing to the high copy number of repeated sequences in the mammalian genome, we suggest that this type of recombination may occur with high frequency during oncogenesis, leading to chromosomal rearrangements that may or may not be detectable cytogenetically.

ACKNOWLEDGMENTS

We thank C. Szpirer and G. Levan for providing the mouse-rat somatic cell hybrids; T. Yeung and W. Dinehart for the synthesis of oligonucleotides; S. Bear, W. Mason, J. Summers, and J. Taylor for useful comments on the manuscript; Beverly Schafer for technical assistance; and Pat Bateman for typing the manuscript.

This work was supported by Public Health Service grants CA-38047 (to P.N.T.), CA-06927, and RR-05539 from the National Institutes of Health and an appropriation from the Commonwealth of Pennsylvania (to the Fox Chase Cancer Center). P.A.L. is a Fellow of the Cancer Research Institute of New York, and P.N.T. is a scholar of the Leukemia Society of America, Inc.

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