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Concerted DNA Rearrangements in Moloney Murine Leukemia Virus-Induced Thymomas: a Potential Synergistic Relationship in Oncogenesis

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Rat thymic lymphomas induced by Moloney murine leukemia virus carry DNA rearrangements due to provirus integration in at least five independent cellular DNA domains (*Mlvi-1*, *Mlvi-2*, *Mlvi-3*, RMo*Int-1*, and c-*myc*). We had previously shown that rearrangements in more than one of these domains could occur in the same tumor. In this report we extend these findings by showing that, with one exception, tumors containing provirus insertions in *Mlvi-1* always contained provirus insertions in a second locus, *Mlvi-2*. To determine whether both events occurred in the same population of tumor cells, we examined the clonal nature of these tumors by taking advantage of allelic polymorphisms that occur naturally in both *Mlvi-1* and *Mlvi-2*. Tumors with provirus insertions in both *Mlvi-1* and *Mlvi-2* arising in rats heterozygous at one of these loci were identified. DNA from these tumors was analyzed by restriction endonuclease digestion and hybridization to DNA probes derived from both *Mlvi-1* and *Mlvi-2*. Thus, we determined the clonal nature of three thymomas and showed that in these tumors both insertion events occurred in the same population of tumor cells. The concomitant appearance of provirus insertions in *Mlvi-1* and *Mlvi-2* suggests a synergism of these two events that may be important in tumor induction and progression.

Retroviruses that lack a transforming (*onc*) gene induce clonal tumors after a long latency period following inoculation into the appropriate animal host (for a review, see reference 39). The DNA provirus generated by reverse transcription of retrovirus RNA appears to integrate randomly in the genome of virus-infected nontumor cells (for a review, see reference 46). However, in tumors induced by retroviruses that lack an *onc* gene, the DNA provirus is reproducibly detected in the same cellular DNA region in many tumors (3, 4, 6, 8, 11, 18, 20–27, 29, 38, 43–45). It is believed that provirus integration in these regions plays an important role in tumor induction and progression because: (i) in some cases these regions represent known cellular protooncogenes, such as c-*myc* (3, 11, 20–23, 26, 38) or c-*erbB* (8); (ii) in other cases, although the relationship of these regions to known cellular protooncogenes has not been established, provirus integration has been associated with transcriptional activation of the neighboring cellular sequences (4, 6, 24). This activation is mediated by transcription control elements in the U_3 region of the proviral long terminal repeat which represents the major determinant of the virus oncogenic potential (1, 5, 16, 19, 40–42); and (iii) provirus integration is random in tumors induced by retroviruses, such as human T cell leukemia virus (33) or bovine leukemia virus (9, 13), which may induce disease by producing *trans*-acting factors that modify the expression of other genes (10, 31, 36, 47). This would be unlikely to occur if the detection of the provirus in common regions of integration in retrovirus-induced tumors was simply the result of specificity in the selection of the region of provirus insertion.

Moloney murine leukemia virus (MoMuLV), a retrovirus lacking a transforming gene, induces thymic lymphomas in mice and rats (44). The rat thymomas carry genomic DNA rearrangements, due to provirus integration, in at least five cellular DNA regions: *Mlvi-1* (44), *Mlvi-2* (43, 45), *Mlvi-3* (P. Tsichlis, unpublished data), RMo*Int-1* (18), and c-*myc* (38; P. Tsichlis, unpublished data). *Mlvi-1*, *Mlvi-2*, and *Mlvi-3* are three independent loci whose DNAs have no homology, detectable by hybridization, and which map on different rat chromosomes. Furthermore, none of them is related to c-*myc* by hybridization (P. N. Tsichlis, M. A. Lohse, C. Szpirer, J. Szpirer, and G. Levan, submitted for publication).

We have previously shown that certain tumors carry rearrangements in more than one of these cellular DNA domains (43). We now show that rearrangements in *Mlvi-1* occur in concert with rearrangements in *Mlvi-2*. The concomitant appearance of *Mlvi-1* and *Mlvi-2* rearrangements in a single tumor, however, may be due to the facts that the tumor is composed of diverse populations of transformed cells and that each population carries a provirus in a single locus. Alternatively, provirus insertions in both loci may take place in the same population of tumor cells. To distinguish between these two alternatives, we undertook a study to elucidate the clonal nature of these tumors. We reasoned that if these thymomas were monoclonal with regard to provirus insertion in *Mlvi-1* or *Mlvi-2*, the two independent events should coexist in the same population of tumor cells. In this study, we took advantage of naturally occurring polymorphisms in *Mlvi-1* and *Mlvi-2* detected by restriction endonuclease digestion. DNA from tumors arising in *Mlvi-1* or *Mlvi-2* heterozygous rats and carrying a provirus in *Mlvi-1* and *Mlvi-2* was examined by Southern blot analysis and hybridization to *Mlvi-1* and *Mlvi-2* DNA probes. These analyses allowed us to determine the clonal nature of three

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TABLE 1. Distribution of *Mlvi-1*, *Mlvi-2*, *Mlvi-3*, and c-*myc* rearrangements among MoMuLV-induced rat thymic lymphomas

Thymomas	Locus[a]			
	Mlvi-1	*Mlvi-2*	*Mlvi-3*	c-*myc*
C_3	+	+	−	−
A_2	+	+	−	−
A_7	+	+	−	−
B_2	+	+	−	−
B_2b	+	−	−	+
B_8L	+	+	+	−
B_{1-1}	+	+	−	−
A_3b	−	−	−	−
B_{0-1}	−	−	−	−
A_3	−	−	−	−
B_4R	−	−	−	−
D_1b	−	−	+	−
D_0	−	−	−	−
A_5	−	−	−	−
F_1	−	−	−	−
D_1	−	−	−	+
E_0	NT	NT	NT	+

[a] +, Locus rearranged because of the insertion of a provirus; −, absence of a detectable rearrangement; and NT, not tested.

thymomas and conclude that in these tumors *Mlvi-1* and *Mlvi-2* rearrangements coexist in the same population of cells.

MATERIALS AND METHODS

Thymomas. The generation of the MoMuLV-induced rat thymomas used in this study has been described previously (43–45). Briefly, newborn Osborn-Mendel rats were injected intraperitoneally with 50,000 XC PFU of MoMuLV. Thymomas developed 4 to 6 months after virus inoculation.

Southern blot analysis of cellular and cloned DNA. Normal rat thymus and tumor cell DNA as well as cloned DNA were digested with restriction endonucleases purchased from New England BioLabs, Inc., or Bethesda Research Laboratories, Inc., and electrophoresed in 0.7 to 1% horizontal agarose gels. The DNA was transferred onto nitrocellulose filters (37) and hybridized to the appropriate ^{32}P-labeled DNA probes at 42°C in 50% formamide and 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for approximately 16 to 20 h. The filters were then washed several times in 2× SSC–0.1% sodium dodecyl sulfate at room temperature, followed by one or two 30-min washes at 65°C in 0.1× SSC–0.1% sodium dodecyl sulfate.

Molecular cloning. Cellular DNA (0.2 to 0.3 mg) digested by *Bam*HI was fractionated by preparative agarose gel electrophoresis. Cellular DNA from individual fractions enriched for the desired DNA fragments was ligated to the *Bam*HI arms of the bacteriophage lambda vector λ1059 (12). The product of the ligation was in vitro packaged, and the resulting phage particles were plated on the *Escherichia coli* K-12 strain LE392. Phage plaques were screened with the appropriate cellular DNA probes.

RESULTS

Out of 16 MoMuLV-induced rat thymomas we examined, 7 carried DNA rearrangements due to provirus integration in multiple loci. Furthermore, rearrangements in two of these loci (*Mlvi-1* and *Mlvi-2*) occurred in concert (Table 1).

Concerted appearance of *Mlvi-1* and *Mlvi-2* rearrangements in MoMuLV-induced rat thymomas. Approximately 25 kilobases (kb) of *Mlvi-1* and 30 kb of *Mlvi-2* cellular DNA sequences were cloned, and their restriction endonuclease maps were determined (Fig. 1). Rearrangements in both *Mlvi-1* and *Mlvi-2* due to provirus insertion were observed in 6 of 16 thymomas. A single thymoma carried a rearrangement only in *Mlvi-1*. The sites of provirus integration in each individual tumor (Fig. 1) were determined by restriction endonuclease digestion and hybridization to *Mlvi-1* or *Mlvi-2* probes (data not shown). Some of these data were confirmed by molecular cloning of the rearranged *Mlvi-1* fragments in tumors C_3, A_2, and A_7 and the rearranged *Mlvi-2* fragments in tumors C_3, A_2, A_7, and B_8L (43, 44; this report).

A selected fraction of these data showing the presence of concerted *Mlvi-1* and *Mlvi-2* rearrangements is shown in Fig. 2. These data demonstrate that six of the seven tumors with an *Mlvi-1* rearrangement also contain a rearrangement in *Mlvi-2*. In fact, tumor A_7 carries two independent proviruses in both *Mlvi-1* and *Mlvi-2*. The presence of two independent rearrangements in *Mlvi-1* in this tumor was determined by Southern blot analysis of *Sac*I- or *Kpn*I-digested tumor DNA and hybridization to pTS25E/P and pE28 probes, respectively (Fig. 2A). The rearranged band detected in both experiments cannot be explained by the insertion of a single provirus, given the restriction endonuclease map of *Mlvi-1* (Fig. 1). The insertion of two independent proviruses in *Mlvi-2* was originally suggested by the detection of two (10.5- and 5-kb) *Sac*I fragments hybridizing to the pTS6 probe (Fig. 2B). This was confirmed by the experiments that will be presented in Fig. 6.

The only exception to the rule of concerted *Mlvi-1* and *Mlvi-2* rearrangements was tumor B_2b, which showed only a rearrangement in *Mlvi-1*. This, however, was an atypical tumor in that it carried an *Mlvi-1* rearrangement only in a fraction of the tumor cells, as determined by the light intensity of the rearranged *Mlvi-1* band (Fig. 2A). Furthermore, it may be significant that this tumor also carried a rearrangement in c-*myc* (Table 1).

Significance of the concerted *Mlvi-1* and *Mlvi-2* rearrangements in MoMuLV-induced thymomas. The significance of the concerted *Mlvi-1* and *Mlvi-2* rearrangements depends on whether the two events occurred in the same or different populations of tumor cells. If both events occurred in the same cell population, our data would strongly suggest synergism between *Mlvi-1* and *Mlvi-2* during oncogenesis. To determine whether both events indeed occurred in the same cells, we examined the clonal nature of these tumors by taking advantage of allelic polymorphisms that occur naturally in *Mlvi-1* and *Mlvi-2*. Our strategy was to search for tumors derived from rats that were heterozygous for *Mlvi-1* (or *Mlvi-2*) and which contained an integrated provirus in one of the two alleles of this locus. Since the two alleles could be distinguished, the allele that contained the provirus could be determined. The clonal nature of such tumors was examined by asking the question whether the unrearranged counterpart of the provirus-containing allele was detectable by Southern blot analysis of the tumor DNA. If it could not be detected we could conclude that the tumor was monoclonal with regard to provirus insertion in this locus. If such a tumor contained rearrangements in both *Mlvi-1* and *Mlvi-2*, naturally both rearrangements coexisted in the same population of tumor cells. Variations of this strategy were utilized to determine the clonal nature of three tumors (A_2, B_{1-1}, and A_7).

DNA polymorphism in the *Mlvi-1* and the *Mlvi-2* loci. Normal Osborn-Mendel rat DNA was digested with *Kpn*I,

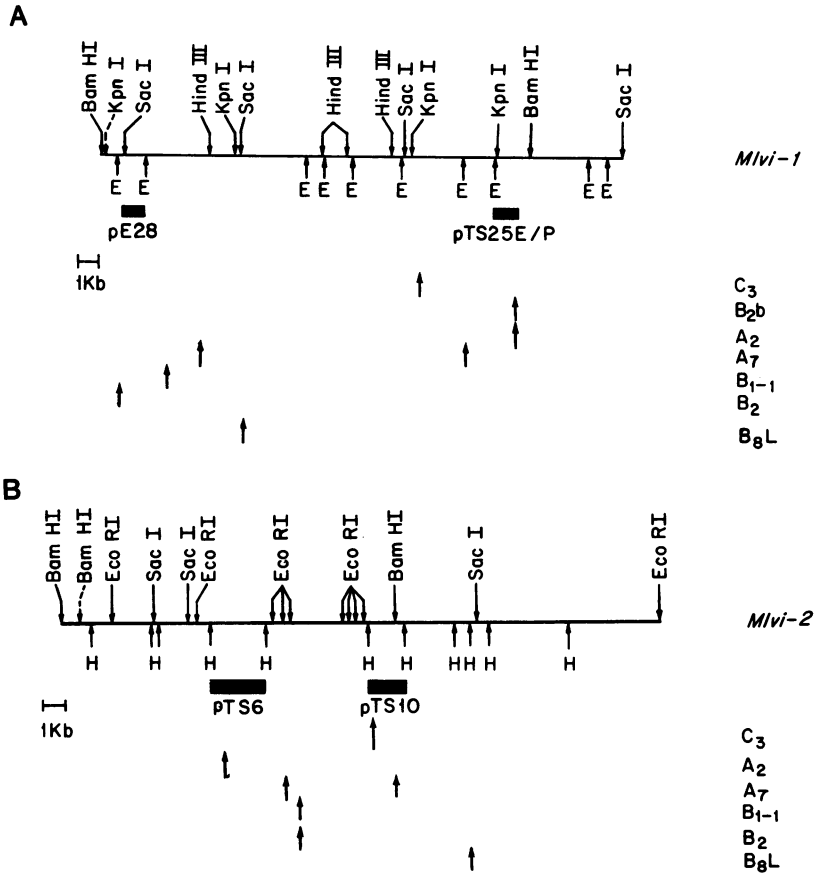

FIG. 1. Restriction endonuclease maps and sites of provirus integration in *Mlvi-1* (A) and *Mlvi-2* (B). The solid black bars indicate the origin of the DNA probes. The arrows indicate the sites of provirus integration in *Mlvi-1* and *Mlvi-2* in the tumors shown in the right-hand side of the figure. The dashed arrows, indicating the *Kpn*I site in *Mlvi-1* and the *Bam*HI site in *Mlvi-2*, represent restriction sites that are present only in one of the two *Mlvi-1* or *Mlvi-2* alleles. The *Eco*RI sites in *Mlvi-1* and the *Hin*dIII sites in *Mlvi-2* are designated by the letters E and H, respectively.

and after agarose gel electrophoresis and transfer to nitrocellulose filters, it was hybridized to the *Mlvi-1* DNA probe pE28 (Fig. 3). The results indicate that individual Osborn-Mendel rats are polymorphic in the *Mlvi-1* locus. Two alleles were observed: *Mlvi-1*^{a}, giving rise to a 22-kb *Kpn*I DNA fragment, and *Mlvi-1*^{b}, giving rise to a 6.6-kb *Kpn*I DNA fragment. Polymorphism was also observed in the *Mlvi-2* locus. After *Bam*HI digestion of normal Osborn-Mendel rat DNA, two *Mlvi-2* alleles were detected: *Mlvi-2*^{a}, represented by a 16.5-kb *Bam*HI DNA fragment, and *Mlvi-2*^{b}, represented by a 15.5-kb *Bam*HI DNA fragment.

The *Mlvi-2* polymorphism described here is due to the insertion of a long interspersed repeated element (LINE) in the *Mlvi-2*^{a} allele. This gave rise to the LINE containing *Mlvi-2*^{b} allele (7). The nature of the *Mlvi-1* polymorphism has not yet been determined.

***Mlvi-2* rearrangements in tumor A_2.** DNA from a normal *Mlvi-2* heterozygous rat and tumor A_2 was digested with *Bam*HI, and after agarose gel electrophoresis and transfer to nitrocellulose, it was hybridized to the *Mlvi-2* probe pTS6. In tumor A_2, the *Mlvi-2*^{b} allele was absent, while a novel 11-kb band was observed (Fig. 4A). A possible explanation for these data is that A_2 is a monoclonal tumor derived from an *Mlvi-2* heterozygous rat and that the novel 11-kb band was generated by provirus integration in the *Mlvi-2*^{b} allele. This is indeed the case. The 16.5-kb and the 11-kb *Mlvi-2* *Bam*HI fragments detected in tumor A_2 were cloned into the lambda vector λ1059. Restriction endonuclease maps of the two clones [λCl228$A_{2\text{-}5}$ (*Mlvi-2*^{a}) and λCl228$A_{2\text{-}7}$ (*Mlvi-2*$^{b \cdot v}$)] were generated (Fig. 4F). One end of λCl228$A_{2\text{-}7}$ contains MoMuLV *gag-pol* sequences. Sequences at the opposite end differ between the two clones. In fact, λCl228$A_{2\text{-}7}$ was derived from the LINE containing *Mlvi-2*^{b} allele (7) which was rearranged as a result of provirus insertion. The *Mlvi-2*^{b} allele that carried the provirus was designated *Mlvi-2*$^{b \cdot v}$.

A selected fraction of the data that allowed us to draw these conclusions is shown in the top portion of Fig. 4. Both λCl228$A_{2\text{-}5}$ and λCl228$A_{2\text{-}7}$ hybridize to the pTS6 probe (Fig. 4B). However, only λCl228$A_{2\text{-}7}$ hybridizes to an MoMuLV probe containing portions of the *gag* and *pol* viral genes (Fig. 4C). The presence of heterologous sequences in the opposite end of the two clones was demonstrated by hybridization of *Hin*dIII-digested DNA to pTS39H/E and pTS44H/S probes. pTS39H/E hybridized to different-size *Hin*dIII fragments derived from the two clones. pTS44H/S hybridized only to λCl228$A_{2\text{-}5}$ (Fig. 4D and E).

These data demonstrate that tumor A_2 was derived from an *Mlvi-2* heterozygous rat. The 11-kb *Bam*HI band was the result of provirus integration in the *Mlvi-2*^{b} allele that is no longer detected in the tumor A_2 DNA. These data indicate that this thymoma is indeed monoclonal with regard to provirus integration in the *Mlvi-2* locus. Since tumor A_2 also

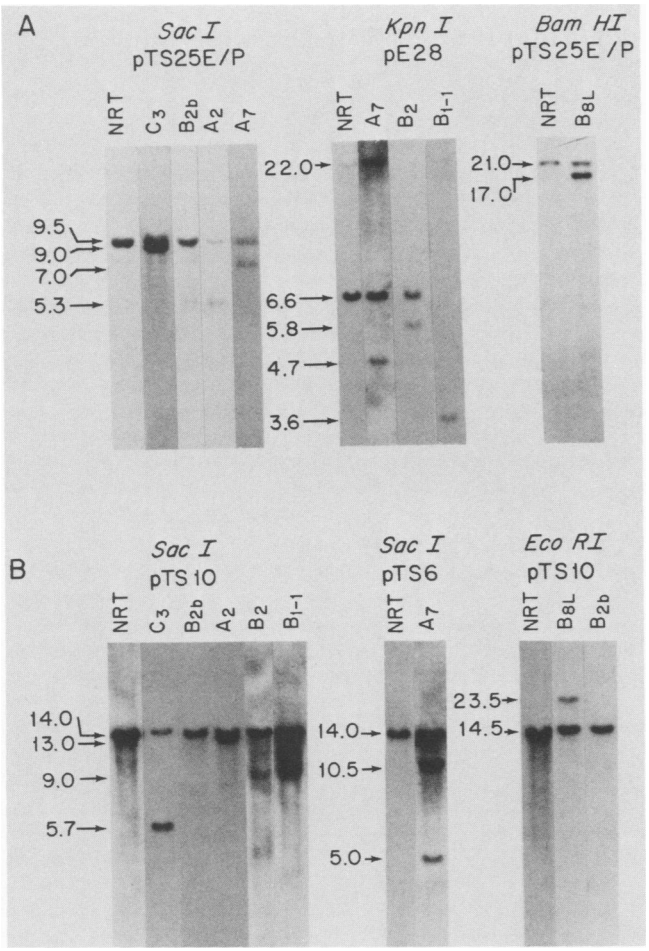

FIG. 2. DNA rearrangements due to provirus insertions in *Mlvi-1* (A) and *Mlvi-2* (B). (A) Left panel: southern blot analysis of normal rat thymus (NRT) and tumor DNA from tumors C_3, B_2b, A_2, and A_7 digested with *Sac*I and hybridized to pTS25E/P. The rearranged band in tumor B_2b is faint, indicating that the rearrangement is present only in a fraction of the tumor cells. Middle panel: NRT and tumor DNA from tumors A_7, B_2, and B_{1-1} digested with *Kpn*I and hybridized to pE28. Tumors A_7 and B_{1-1} were derived from rats heterozygous for the *Mlvi-1* locus. The rearranged *Kpn*I band in tumor A_7 is independent of the rearranged *Sac*I *Mlvi-1* band in the same tumor shown in the left panel. Right panel: NRT and tumor B_8L DNA digested with *Bam*HI and hybridized to pTS25E/P. The molecular sizes in all the figures were determined by comparison with the migration pattern of the DNA fragments generated by *Hin*dIII digestion of wild-type phage lambda DNA. (B) Left panel: NRT and tumor C_3, B_2b, A_2, A_7, B_2, and B_{1-1} DNA digested with *Sac*I and hybridized to the *Mlvi-2* probe pTS10. No rearrangement was detected in tumor B_2b. Middle panel: NRT and tumor A_7 DNA digested with *Sac*I and hybridized to the *Mlvi-2* probe pTS6. Right panel: NRT and tumor B_8L and B_2b DNA digested with *Eco*RI and hybridized to pTS10. No rearrangement was detected in tumor B_2b.

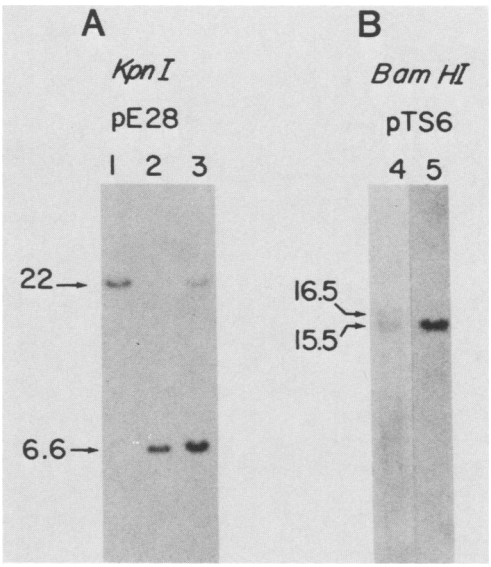

FIG. 3. Restriction-fragment-length polymorphism in the *Mlvi-1* (A) and the *Mlvi-2* (B) locus. (A) Southern blot analysis of normal rat DNA digested with *Kpn*I and hybridized to the *Mlvi-1* probe pE28. Lanes: 1, rat homozygous for the *Mlvi-1*^{a} allele; 2, rat homozygous for the *Mlvi-1*^{b} allele; 3, heterozygous rat. (B) Normal rat DNA digested with *Bam*HI and hybridized to the *Mlvi-2* probe pTS6. Lanes: 4, heterozygous rat; 5, rat homozygous for the *Mlvi-2*^{b} allele. This polymorphism is due to the insertion of a member of the family of LINEs (see the text).

contains a provirus in *Mlvi-1*, provirus integration in the two loci must coexist in the same population of cells.

***Mlvi-1* rearrangement in tumor B_{1-1}.** DNA from a normal rat and tumor B_{1-1} was digested with *Kpn*I, and after agarose gel electrophoresis and transfer to nitrocellulose, it was hybridized to the *Mlvi-1* probe pE28. In tumor B_{1-1}, the *Mlvi-1*^{b} allele was absent, whereas a novel 3.6-kb band was observed (Fig. 2A, middle panel). A possible explanation for these data is that B_{1-1} is a monoclonal tumor derived from an *Mlvi-1* heterozygous rat and that the rearranged 3.6-kb band was generated by provirus insertion in the *Mlvi-1*^{b} allele. This is indeed the case. The site of provirus insertion was determined by digestion of normal rat and tumor B_{1-1} DNA with *Sac*I or *Hin*dIII and hybridization to the *Mlvi-1* probe pE28. A rearranged band was detected after digestion with both enzymes (Fig. 5A). The size of the band detected after *Sac*I digestion was 2.7 kb, while the size of the band detected after *Hin*dIII digestion was approximately 15 kb. The position of *Sac*I and *Hin*dIII cleavage sites in *Mlvi-1* allowed us to determine the position of these sites in the rearranged locus. The resulting restriction map agreed with that predicted by the insertion of a MoMuLV provirus (35) in the site indicated by the solid black arrow (Fig. 5B).

The insertion of the provirus in the *Mlvi-1*^{b} (as opposed to the *Mlvi-1*^{a}) allele at this site was determined by the novel 3.6-kb *Kpn*I band hybridizing to the pE28 probe (Fig. 2A, middle panel). Provirus insertion at this site in *Mlvi-1*^{a} would give rise to a >18-kb *Kpn*I band (Fig. 5B). The *Mlvi-1*^{b} allele containing the provirus was designated *Mlvi-1*$^{b \cdot v}$. Since the unrearranged *Mlvi-1*^{b} allele was no longer detected in tumor B_{1-1} DNA (Fig. 2A), we conclude that this tumor was monoclonal with regard to provirus integration in *Mlvi-1*. Tumor B_{1-1} carried an additional rearrangement in the *Mlvi-2* locus (Fig. 2B). Therefore, the rearrangements observed in *Mlvi-1* and *Mlvi-2* must coexist in the same population of tumor cells.

***Mlvi-2* rearrangements in tumor A_7.** Tumor A_7 is particularly interesting since it appears to be composed of two populations of tumor cells, both of which contain rearrangements in *Mlvi-1* and *Mlvi-2*. This tumor, therefore, underlines the significance of the concerted appearance of *Mlvi-1* and *Mlvi-2* rearrangements in MoMuLV-induced rat thymomas.

Figure 6A shows a *Bam*HI digest of tumor A_7 DNA

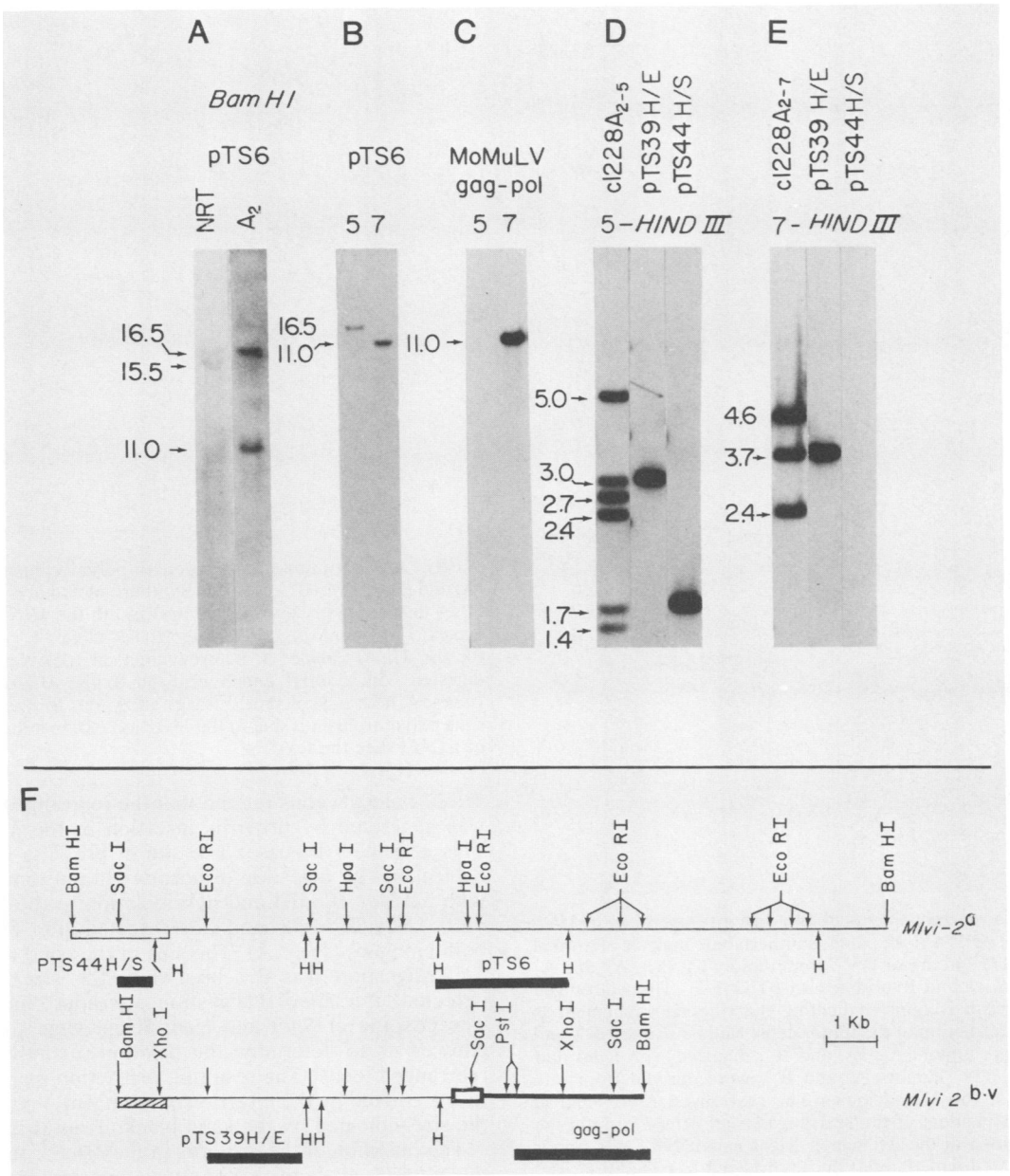

FIG. 4. Clonal nature of tumor A_2 with regard to provirus insertion in *Mlvi-2*. (A) Southern blot analysis of normal rat thymus (NRT) and tumor A_2 DNA digested with *Bam*HI and hybridized to the *Mlvi-2* probe pTS6. Since the provirus was inserted within the sequences represented by the probe, two rearranged *Mlvi-2* bands should be detected (Fig. 1B). However, the two comigrate at 11 kb. The lack of detection of the 15.5-kb band in tumor A_2 indicates that if cells containing this *Mlvi-2* band are present in the tumor they should represent less than 2% of the tumor cells. This was determined by DNA mixing experiments (data not shown). (B and C) λCl228$A_{2\text{-}5}$: *Mlvi-2*^{a} (lane 5) and λCl228$A_{2\text{-}7}$: *Mlvi-2*$^{b\cdot v}$ (lane 7) insert DNA hybridized to the pTS6 and the MoMuLV *gag-pol* probes. (D) *Hin*dIII-digested λCl228$A_{2\text{-}5}$ (*Mlvi-2*^{a}) DNA hybridized to itself, pTS39H/E, and pTS44H/S. (E) *Hin*dIII-digested λCl228$A_{2\text{-}7}$ (*Mlvi-2*$^{b\cdot v}$) DNA hybridized to itself, pTS39H/E, and pTS44H/S. (F) Restriction endonuclease maps of λCl228$A_{2\text{-}5}$ (*Mlvi-2*^{a}) and λCl228$A_{2\text{-}7}$ (*Mlvi-2*$^{b\cdot v}$), indicating the site of provirus insertion (heavy line on the right-hand side of the figure) and the map position of the heterologous sequences between the two cloned DNAs (open, as opposed to shaded, bars on the left side of the figure). The region indicated by the shaded bar in the left-hand side of λCl228$A_{2\text{-}7}$ represents LINE sequences which were inserted in the *Mlvi-2*^{a} allele (see the text).

hybridized to the *Mlvi-2* probe pTS6. Four *Mlvi-2* bands were detected in tumor A_7, two of which comigrated with the two *Bam*HI bands derived from the known *Mlvi-2* alleles. Therefore, tumor A_7 was derived from an *Mlvi-2* heterozygous rat. The two novel *Bam*HI bands were generated by provirus integration in the *Mlvi-2*^{a} allele (20-kb band) or the *Mlvi-2*^{b} allele (14-kb band). Restriction endonuclease maps of the two rearranged alleles indicating the sites of provirus integration and the map position on the heterologous sequences identifying the two alleles are shown in Fig. 6D. Some of the data that allowed us to draw these conclusions are shown in Fig. 6 (A through C).

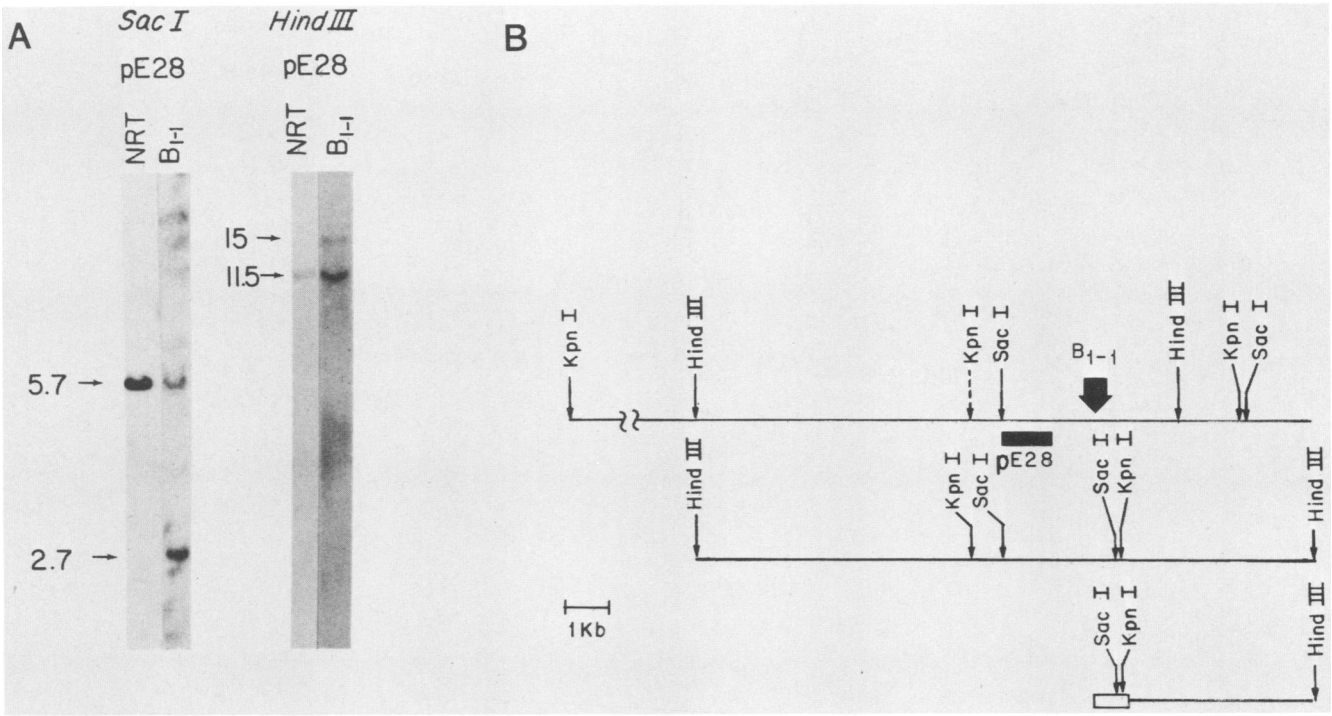

FIG. 5. Clonal nature of tumor B_{1-1} with regard to provirus insertion in *Mlvi-1*. (A) Southern blot analysis of normal rat thymus (NRT) and tumor B_{1-1} DNA digested with *Sac*I or *Hin*dIII and hybridized to the *Mlvi-1* probe pE28. (B) Restriction endonuclease map of *Mlvi-1* and site of provirus integration in tumor B_{1-1}. The linear map of *Mlvi-1* was interrupted to include the *Kpn*I site on the left-hand side which is located at a distance of 22 kb from the *Kpn*I site on the right-hand side of the map. The *Kpn*I site indicated by the dotted arrow is absent in *Mlvi-1*^{a} or present in *Mlvi-1*^{b}. The solid black arrow indicates the site of provirus insertion in the *Mlvi-1*^{b} allele. The middle line represents the restriction endonuclease map of the rearranged *Mlvi-1* band. The bottom line shows the map position of the *Sac*I, *Kpn*I, and *Hin*dIII sites in the MoMuLV proviral long terminal repeat (open box) and the 5′ half of the proviral genome.

The novel 14-kb *Bam*HI band was cloned into the lambda vector λ1059 (λCl228A$_7$). The cloned DNA hybridized to both the *Mlvi-2* pTS6 probe and the MoMuLV *gag-pol* probe (Fig. 6B). Therefore, the 14-kb band was generated by MoMuLV provirus insertion. *Hin*dIII digestion of λCl228A$_7$ DNA and hybridization to the pTS39H/E probe revealed a 3.7-kb band as expected if the clone was derived from the *Mlvi-2*^{b} allele. This was confirmed by the lack of hybridization of the *Mlvi-2* probe pTS44H/S to the λCl228A$_7$ DNA (Fig. 6B).

To determine whether the novel 20-kb *Bam*HI band was generated because of provirus integration in *Mlvi-2*, normal rat thymus and tumor A$_7$ DNA were digested with *Sac*I, which cleaves the MoMuLV long terminal repeat, and hybridized to the *Mlvi-2* probe pTS6 (Fig. 2B, middle panel). In addition to the 14-kb *Sac*I fragment detected in both normal and tumor DNA, two additional bands were detected in A$_7$ DNA. The 5-kb band was expected by the insertion of the provirus detected in λCl228A$_7$ (Fig. 6D). The 10.5-kb band could be generated by the insertion of a provirus at the second *Mlvi-2* provirus integration site (Fig. 1 and 6D). Provirus insertion at this site could also explain the generation of the 20-kb *Bam*HI band detected by hybridization to pTS6. The MoMuLV provirus lacks *Eco*RI cleavage sites, and its genome is approximately 8.8 kb long. Therefore, if the 10-kb *Sac*I band (or the 20-kb *Bam*HI band) is due to the insertion of a provirus at this site, digestion of tumor cell DNA with *Eco*RI and hybridization to the pTS10 probe should detect a tumor-specific DNA fragment approximately 8.8 kb longer than that observed in normal rat DNA. This is indeed the case (Fig. 6C). We conclude that both rearranged bands in tumor A$_7$ were generated by the insertion of a provirus.

To determine whether the rearranged 20-kb *Bam*HI DNA fragment was derived from the *Mlvi-2*^{a} (as opposed to the *Mlvi-2*^{b}) allele, tumor A$_7$ DNA was digested with *Bam*HI and fractionated by agarose gel electrophoresis. A DNA fraction enriched for the 20-kb *Bam*HI *Mlvi-2* fragment was electrophoresed in a 0.7% agarose gel either without or after digestion with *Sac*I and hybridized to the pTS44H/S probe. A 20-kb fragment was detected, as expected, in the undigested DNA fraction. *Sac*I digestion generated a 3.8-kb *Mlvi-2* fragment which comigrates with a 3.8-kb *Sac*I fragment derived from the *Mlvi-2*^{a} allele (Fig. 6A). We conclude that the 20-kb rearranged band was derived by provirus integration in *Mlvi-2*^{a}.

Therefore, tumor A$_7$ is composed of at least two populations of tumor cells, both of which contain a provirus in *Mlvi-2*. Although these data do not formally exclude the possibility of more than two tumor cell populations, this is unlikely. The four *Bam*HI *Mlvi-2* fragments can be grouped in two pairs of bands of approximately equal intensity: (i) the 20-kb band of the *Mlvi-2*$^{a \cdot v}$ allele and the 15.5-kb band of the *Mlvi-2*^{b} allele and (ii) the 14-kb band of the *Mlvi-2*$^{b \cdot v}$ allele and the 16.5-kb band of the *Mlvi-2*^{a} allele. The similarity of intensities between each rearranged band and the band from the heterologous unrearranged allele strongly suggests that this tumor is indeed composed of two populations of cells, both of which carry a provirus in *Mlvi-2*.

Since tumor A$_7$ also carries two proviruses in *Mlvi-1* (Fig.

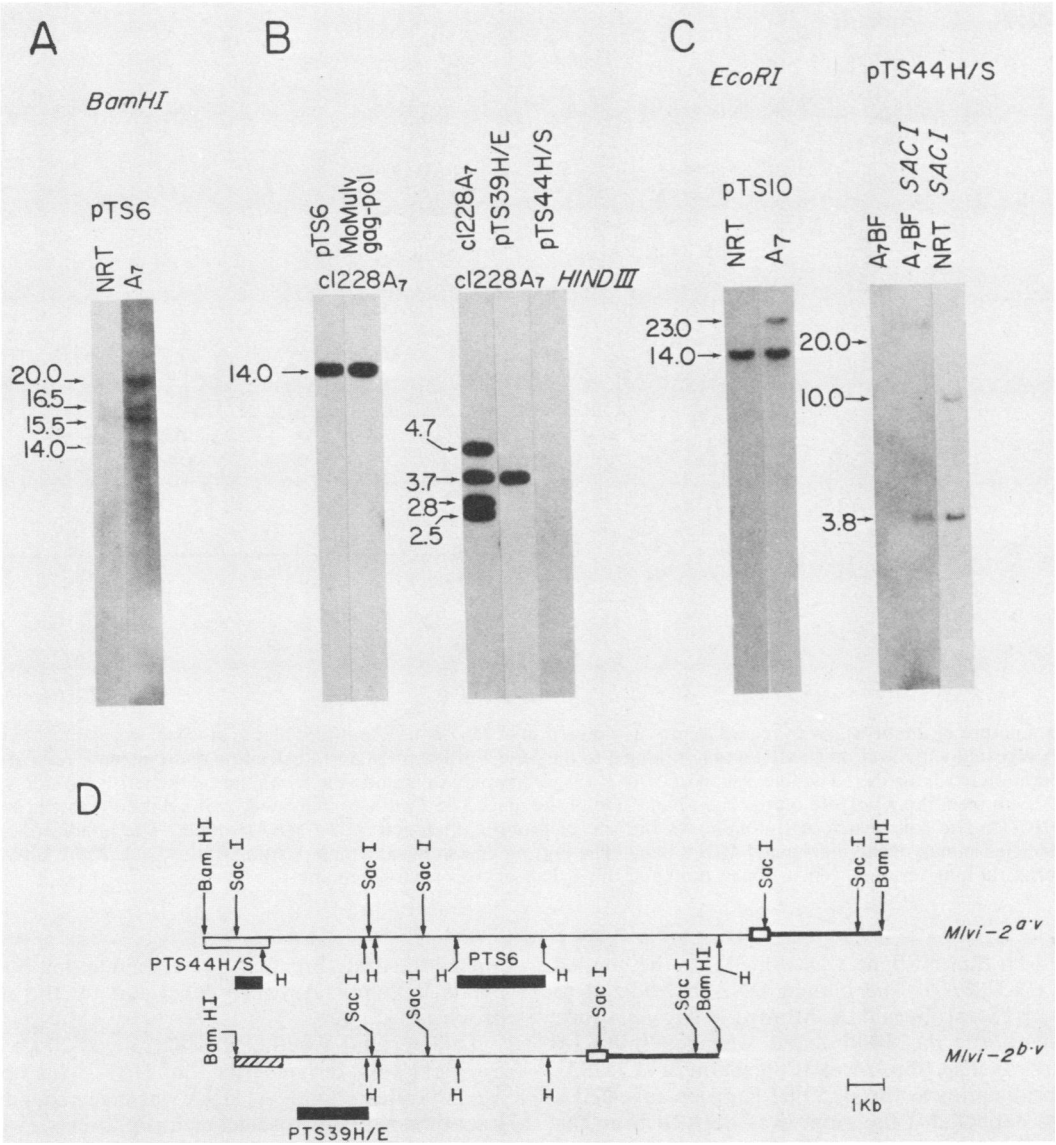

FIG. 6. Clonal nature of tumor A_7 with regard to provirus insertion in *Mlvi-2*. (A) Southern blot analysis of normal rat thymus (NRT) and tumor A_7 DNA digested with *Bam*HI and hybridized to the *Mlvi-2* probe pTS6. (B) Left panel: λCl228A_7 (*Mlvi-2*$^{b \cdot v}$) insert DNA hybridized to the pTS6 and the MoMuLV *gag-pol* probes. Right panel: *Hin*dIII-digested λCl228A_7 DNA hybridized to itself, pTS39H/E, and pTS44H/S. (C) Left panel: NRT and tumor A_7 DNA digested with *Eco*RI and hybridized to the *Mlvi-2* probe pTS10. Right panel: *Bam*HI-digested tumor A_7 DNA was fractionated by agarose gel electrophoresis, and the fraction enriched for the rearranged 20-kb *Mlvi-2* fragment was electrophoresed in parallel with the *Sac*I-digested NRT DNA either undigested (A_7 BF) or after *Sac*I digestion (A_7 BF *Sac*I). The *Mlvi-2* DNA fragment pTS44H/S was utilized as the hybridization probe. (D) Restriction endonuclease maps of the 20-kb rearranged *Bam*HI fragment (*Mlvi-2*$^{a \cdot v}$) and of λCl228A_7 (*Mlvi-2*$^{b \cdot v}$) indicating the sites of provirus insertion and the map position of the heterologous sequences distinguishing the two alleles. The shaded bar of the left-hand side of λCl228A_7 indicates the map position of the inserted LINE sequences.

1 and 2), we conclude that rearrangements in *Mlvi-1* and *Mlvi-2* in tumor A_7 coexist in the same populations of tumor cells.

DISCUSSION

It has been proposed that the induction and progression of neoplasia is a complex multiple-step process (2, 17, 32). However, although intuitively logical, this hypothesis lacks strong experimental support. In many tumors studied to date, oncogenesis has been associated with a single genetic change leading to the activation of a known or putative cellular protooncogene (8, 14, 15, 21, 22, 28, 30, 34). Furthermore, in tumors in which two genetic events have been proposed (for a review, see reference 2), the necessity for both events during tumor induction and progression has not been determined.

Rat thymomas induced by MoMuLV offer a unique opportunity to determine whether oncogenesis indeed involves multiple steps and to study the nature of these processes. Work by us and others has demonstrated five genetic changes occurring in these tumors as a result of provirus insertion at five independent cellular DNA regions: *Mlvi-1*

(44), *Mlvi-2* (43, 45), *Mlvi-3* (P. Tsichlis, unpublished data), RMo*Int-1* (18), and c-*myc* (38; P. Tsichlis, unpublished data). Data presented in this report indicate that two of these events, i.e., provirus insertion in *Mlvi-1* and *Mlvi-2*, occur in concert during oncogenesis. Furthermore, since at least some of these tumors were shown to be monoclonal, both events take place in the same population of tumor cells. Rearrangements in *Mlvi-2* were detected in six of seven tumors carrying rearrangements in *Mlvi-1*, and the single discordant *Mlvi-1*(+)/*Mlvi-2*(−) thymoma carried the *Mlvi-1* rearrangement only in a fraction of the tumor cells. Furthermore, this tumor carries a rearrangement in c-*myc* which may have been an alternative to provirus insertion in *Mlvi-2*. One tumor (A_7) appears to be composed of two populations of tumor cells, both of which carry rearrangements in *Mlvi-1* and *Mlvi-2*.

The strong association between *Mlvi-1* and *Mlvi-2* rearrangements in MoMuLV-induced rat thymomas cannot be explained by chance alone ($P < 0.0023$, Fisher exact two-tail test). We suggest therefore that the concomitant appearance of these rearrangements represents the phenotypic expression of a functional association between these two events. It appears very likely, for example, that *Mlvi-1* and *Mlvi-2* interact in a synergistic manner during oncogenesis and that this interaction is crucial for tumor induction and progression. This would suggest that neither of the two events is independently sufficient for tumor induction, although a combination of the two may be. Such synergism between oncogenes has been described before, although only in studies of transformation of cells in culture. In these experiments it was shown that established cell lines, such as NIH 3T3 and Rat-1, can be transformed efficiently by the T24 Harvey *ras-1* oncogene (17, 32). However, the same oncogene induces only abortive transformation in primary rat embryo fibroblasts or primary baby rat kidney cells. Transfection of these cells with cloned DNA of *myc*, polyoma large T antigen (17), or the adenovirus gene coding for the E1A protein (32) renders them susceptible to transformation by *ras*. These experiments provided strong evidence for the necessity of oncogene interaction during induction of transformation of cells in culture. However, they did not necessarily indicate that such interactions occurred during oncogenesis. We suggest that the evidence presented in this report indicates that such interactions may indeed take place during induction and progression of naturally occurring neoplasms. A second formal possibility along the lines of a functional association between *Mlvi-1* and *Mlvi-2* would be that provirus insertion in one of these loci renders the cell susceptible to provirus insertion in the other. If this was the case, one of the two events would always be present only in a small fraction of the tumor cells unless both events occurred almost simultaneously. Our data, however, do not support this prediction, and we suggest that this explanation is unlikely.

Contrary to the concerted appearance of the *Mlvi-1* and *Mlvi-2* rearrangements, provirus integration in *Mlvi-3* and c-*myc* occurs in a small fraction of tumors and does not appear to be associated with the rearrangement of another locus. The contrast provided by this finding provides further support to the hypothesis that *Mlvi-1* and *Mlvi-2* act synergistically during oncogenesis. Furthermore, it indicates that *Mlvi-3* and c-*myc* operate either independently or in concert with other loci not yet identified. The existence of other unidentified loci is further supported by the finding that although some of the MoMuLV-induced rat thymomas carry multiple genomic DNA rearrangements, no detectable alterations were identified at known loci in 7 of 16 tumors. These tumors are now being tested for provirus insertion in RMo*Int-1* (18) and *pim-1* (4), a putative oncogene identified in an analysis of retrovirus-induced murine thymomas.

An interesting feature that distinguishes the MoMuLV-induced thymomas from other retrovirus-induced neoplasms is the presence of multiple integrated proviruses in a single tumor (18, 38, 43, 44). In this paper, we showed that multiple proviruses may be present not only in the same tumor but in the same population of cells in a single thymoma. This is rather surprising because virus-infected cells normally exhibit superinfection resistance (48). Two possible explanations may be given for this finding. First, the expression of the provirus in the target cells for thymic oncogenesis could be low due to the possibly undifferentiated nature of these cells. This would allow superinfection of the already infected cells, since superinfection resistance depends on the expression of the viral envelope gene (48). Second, the reintegration of the provirus does not require virus production and superinfection. In this case, reintegration may simply require reverse transcription of the cytoplasmic viral RNA. Differentiating between these two possibilities will be very important in our understanding of the interactions between the virus and its host.

In summary, we presented evidence that MoMuLV-induced rat thymomas carry multiple genomic DNA rearrangements mediated by provirus integration and that rearrangements in two loci (*Mlvi-1* and *Mlvi-2*) occur in concert during tumor induction and progression. Furthermore, we have shown that at least in some tumors, *Mlvi-1* and *Mlvi-2* rearrangements occur together in the same population of tumor cells. These data indicate that the two loci may act in a synergistic fashion during oncogenesis and that their interaction may be crucial for tumor induction and progression.

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