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Tumor Progression in Murine Leukemia Virus-Induced T-Cell Lymphomas: Monitoring Clonal Selections with Viral and Cellular Probes

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Clonal selections occurring during the progression of Moloney murine leukemia virus (MuLV)-induced T-cell lymphomas in mice were examined in primary and transplanted tumors by monitoring various molecular markers: proviral integration patterns, MuLV insertions near c-*myc* and *pim-1*, and rearrangements of the immunoglobulin heavy chain and β-chain T-cell receptor genes. The results were as follows. (i) Moloney MuLV frequently induced oligoclonal tumors with proviral insertions near c-*myc* or *pim-1* in the independent clones. (ii) Moloney MuLV acted as a highly efficient insertional mutagen, able to activate different (putative) oncogenes in one cell lineage. (iii) Clonal selections during tumor progression were frequently marked by the acquisition of new proviral integrations. (iv) Independent tumor cell clones exhibited a homing preference upon transplantation in syngeneic hosts and were differently affected by the route of transplantation.

Oncogenic transformation is a multistep process in which the various steps generally enhance the malignancy (14, 15, 28, 45). The early stages in this process are characterized by the repeated clonal outgrowth of rarely occurring cells, which are selected on the basis of reduced growth control. This ultimately results in highly malignant cells, as was found in rapidly metastasizing tumors (3, 4, 16). The multistep model is experimentally supported by studies in which the progression of precancerous cells to overt malignancy was monitored by observing (reproducible) alterations in phenotypic behavior, differentiation of specific protein markers, and karyotype. Recently, highly specific changes involving chromosomal translocations and activation of oncogenes were associated with the biology of transformation in distinct malignancies (10, 13, 21, 27, 33, 51, 60, 61).

In this study we examined series of primary and transplanted lymphomas, induced by Moloney murine leukemia virus (MoMuLV) with respect to differentiation-specific and transformation-related DNA markers, to obtain insight into the clonal composition and sequential selection processes that are active during progressive oncogenic transformation.

Lymphomagenesis in AKR and BALB/Mo mice or in mice infected as newborns with murine leukemia virus (MuLV) is accompanied by somatic amplification and integration of MuLV proviruses in new chromosomal sites of tumor tissues (1, 8, 11, 22, 46, 50, 55, 63, 64). It was frequently claimed that these lymphomas are monoclonal in origin (5, 46). This was based upon the observation that the pattern of integrated mink cell focus-forming recombinant proviruses in tumor tissues was identical for tumor tissue obtained from different anatomical sites of a single animal, but different among individual animals. Another argument in favor of the monoclonal nature of spontaneous lymphomas in strain AKR mice came from studies with the X-linked enzyme phosphoglycerate kinase (7). Lymphomas arising in BALB/Mo mice also seemed largely monoclonal (24, 63). However, the possibility that tumor tissues are in fact composed of a few clonal cell populations was left open (H. van der Putten, Ph.D. thesis, Catholic University of Nijmegen, The Netherlands). The oligoclonal composition of stage II and III lymphomas induced by intrathymic injection of MCF69L1 virus in AKR/J mice (42) was convincingly shown (41). Detection of multiple rearrangements of the diversity DNA segment (D) from the heavy-chain immunoglobulin gene to the joining (J) region (D-J heavy-chain immunoglobulin rearrangement) in AKR/J thymic leukemias (23) also suggested an oligoclonal nature of these tumors.

Insertional mutagenesis by somatically acquired MuLV genomes seems to be the predominant mechanism by which retroviruses, lacking transforming genes, contribute to transformation. In approximately 15% of the spontaneous lymphomas induced by endogenous MuLVs, proviral insertion in the vicinity of the *pim-1* or c-*myc* loci is observed (8, 11, 34, 41, 50). In MoMuLV-induced BALB/c and C57BL T-cell lymphomas, somatically acquired MuLV proviruses are found in 50% of the primary tumors in the vicinity of *pim-1* and in 45% in the vicinity of c-*myc* (11, 50). Proviral insertion is invariably associated with the presence of high levels of c-*myc* or *pim-1* mRNA (8, 49, 50).

In 25% of the primary T-cell lymphomas induced by MoMuLV in BALB/c and C57BL mice, we observed proviral integration in the vicinity of both *pim-1* and c-*myc*. In a few cases it was proven that these tumors were oligoclonal (50). To obtain further insight into the heterogeneity and clonal selection mechanisms which were operational during the development of these tumors, we analyzed a series of primary and transplanted T-cell lymphomas with respect to the proviral integration pattern and the rearrangements of the T-cell receptor β-chain and immunoglobulin heavy-chain genes. The results show that the clonal selection can be

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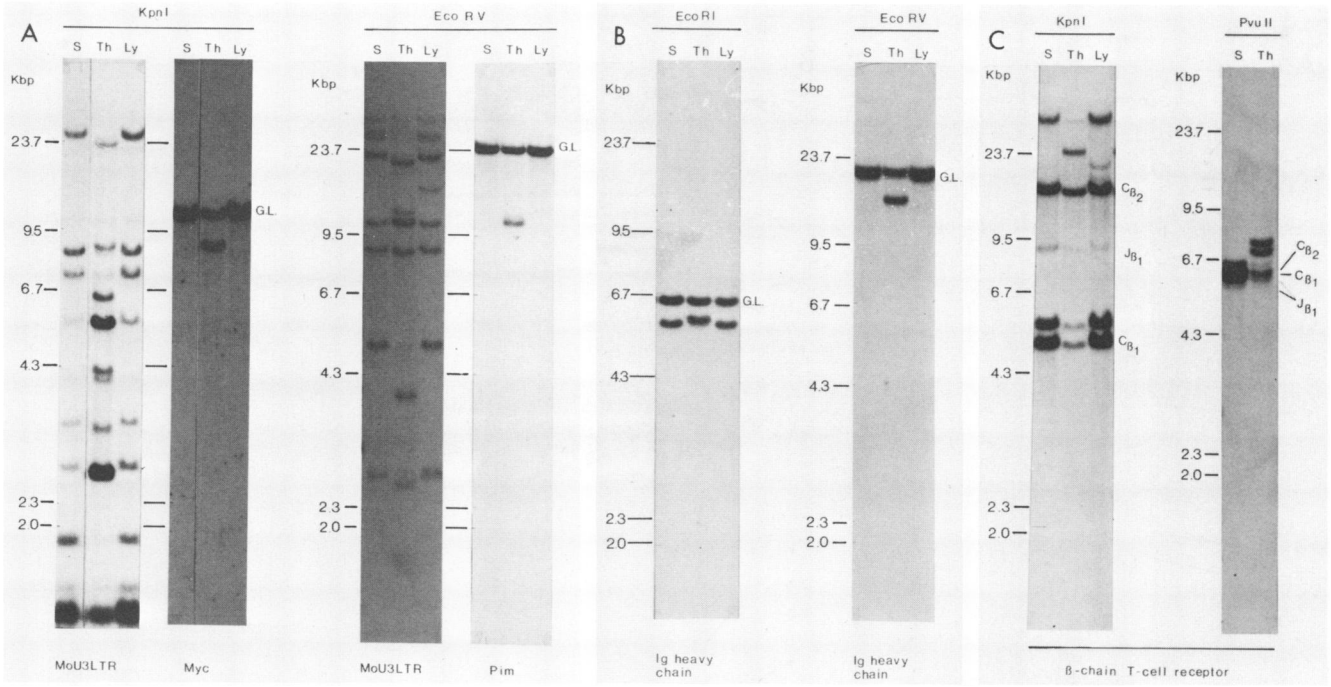


FIG. 1. DNA analyses of lymphoma from BALB/c mouse 54 induced by newborn infection with MoMuLV. Ten-microgram samples of lymphoma DNA obtained from spleen (S), thymus (Th), and mesenteric lymph nodes (Ly) of mouse 54 were digested with restriction endonucleases *Kpn*I and *Eco*RV (A), *Eco*RI and *Eco*RV (B), and *Kpn*I and *Pvu*II (C), separated on 0.6% agarose gels, transferred to nitrocellulose filters, and annealed to probes representative for MoU3LTR, c-*myc*, and *pim-1* (A), for immunoglobulin heavy-chain J region (pJ11) (B), and for the β-chain of the T-cell receptor (86T5 cDNA clone) (C). The left and right autoradiographs of the *Kpn*I and *Eco*RV digests (A) were obtained from the same filter. Abbreviations used for the indicated fragments: G.L., germ line; $C\beta_1$, $C\beta_2$, and $J\beta_1$ are the germ line fragments recognized by the 86T5 or J15 probe of the β-chain of the T-cell receptor, as previously described (35, 53).

examined by monitoring alterations with respect to these markers. The transplantation of T-cell lymphomas was used to record the outgrowth of cell populations already present in the primary tumor. This approach allowed us to define the steps in oncogenic transformation which contributed to an improved transplantability or increased metastatic potential of the tumor cells. Tumor progression was frequently associated with the acquisition of additional proviruses. The viruses, which continued to reinfect cells, seemed to act as permanently active mutagens, capable of subsequently activating series of genes, each of which may contribute to a more malignant phenotype.

MATERIALS AND METHODS

Mice, cell lines, and viruses. The origin of mice, cell lines, and the isolation procedure of MoMuLV clone 1A were described previously (64, 68).

Source of lymphomas and high-molecular-weight DNA preparation. The injection of newborn BALB/c, 129, and C57BL mice with MoMuLV clone 1A and the preparation of lymphomas were performed as described by Jaenisch et al. (25). For the transplantation experiments, 5×10^6 to 10×10^6 viable primary lymphoma cells were injected intraperitoneally (i.p.) or subcutaneously (s.c.) into syngeneic recipient mice, which developed gross lymphomas in 2 to 4 weeks. After autopsy the following organs were removed: thymus, spleen, and lymph nodes. Suspensions of viable cells obtained from primary and transplanted lymphomas were cryopreserved in liquid nitrogen in 10% dimethyl sulfoxide as previously described (68). High-molecular-weight DNA from lymphoma and nonlymphoma tissues was prepared as described previously (64).

Restriction enzyme analyses. DNA samples (10 μg) were digested in a volume of 50 μl under conditions recommended by the suppliers (Amersham Corp., Arlington Heights, Ill.; Bethesda Research Laboratories, Inc., Gaithersburg, Md.; Boehringer GmbH, Mannheim, Federal Republic of Germany). In addition, bovine serum albumin was added to 100 μg/ml. Gel electrophoresis, transfer to nitrocellulose filters, and hybridization with ^{32}P-labeled probes were carried out as described previously (11, 46). Fragments of bacteriophage lambda, generated by restriction endonuclease *Hin*dIII, were used as molecular weight markers.

Hybridization probes. The hybridization probes, specific for the U3 region of the MoMuLV long terminal repeat (LTR) (MoU3LTR) and for *pim-1* (probe A), were described previously (11). For detection of c-*myc* sequences, the *Xba*I-*Hin*dIII fragment, covering exon 2, intron 2, and the major part of exon 3, was used (51). For detection of the D-J immunoglobulin heavy-chain rearrangements, the 1.7-kilobase-pair *Eco*RI-*Bam*HI fragment containing the J3 and J4 gene segments of the mouse (pJ11 probe) was used (36). Somatic rearrangements of the β-chain gene of the T-cell receptor were detected with the 86T5 cDNA clone (20) and with the probes 4.1 and J15 (53).

RESULTS

Proviral integrations in the vicinity of both the *pim-1* and c-*myc* gene were observed in 25% of primary MoMuLV-induced T-cell BALB/c and C57BL lymphomas (50). In most of these lymphoma DNAs a considerable difference in ratio of hybridization intensity was observed between the modified and germ-line alleles of the c-*myc* and *pim-1* genes,

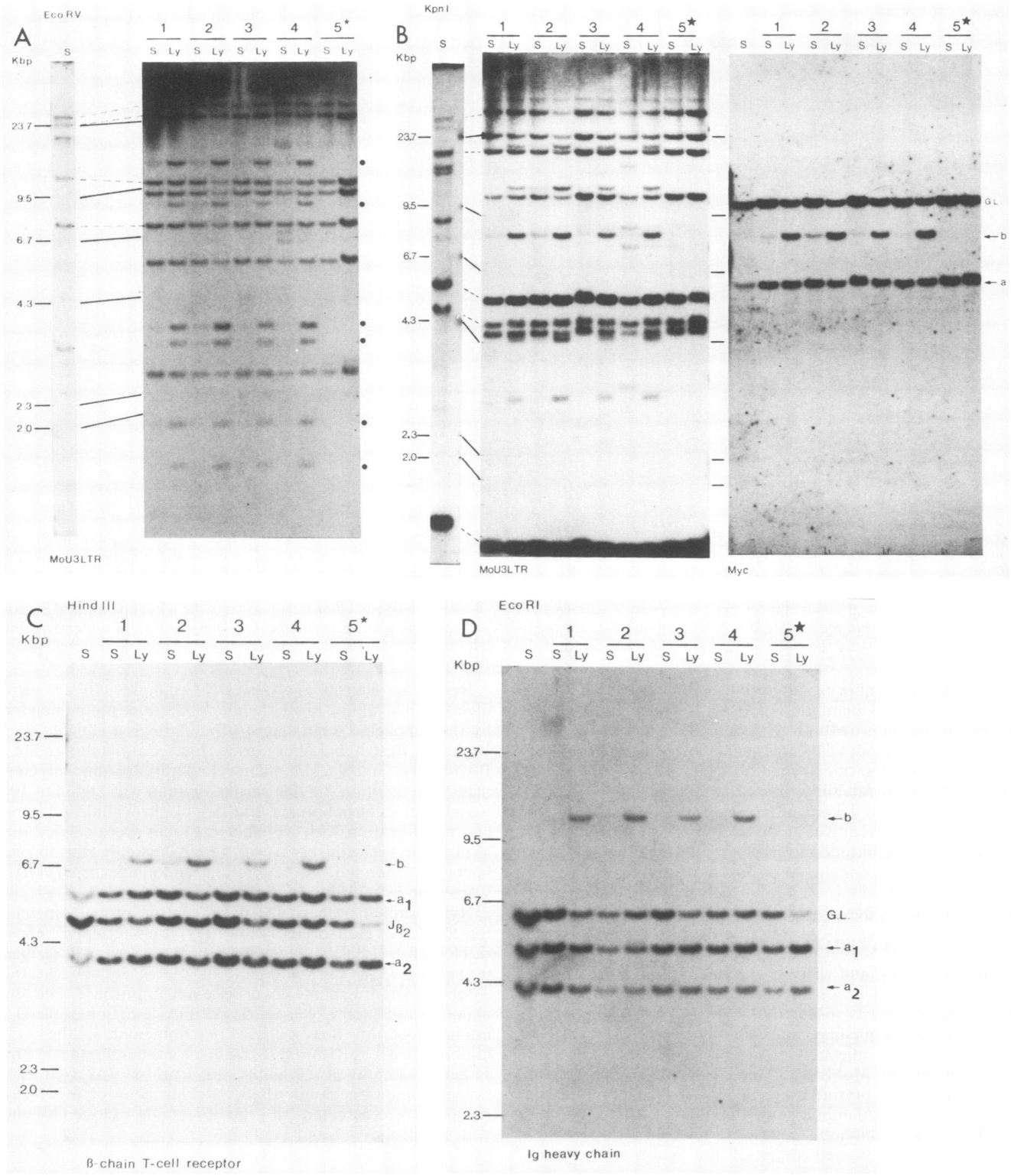


FIG. 2. DNA analyses of lymphoma from BALB/c mouse 36 induced by newborn infection with MoMuLV and of lymphomas caused by transplantation of the primary spleen tumor into recipient mice. Ten-microgram samples of lymphoma DNA obtained from spleen (S) of mouse 36 and from spleen (S) and mesenteric lymph nodes (Ly) of recipient mice 1 through 5 were digested with restriction endonucleases *Eco*RV (A), *Kpn*I (B), *Hin*dIII (C), and *Eco*RI (D), separated on 0.6% agarose gels, transferred to nitrocellulose filters, and annealed to probes representative for MoU3LTR (A and left autoradiograph of B), for c-*myc* (right autoradiograph of B), for the β-chain of the T-cell receptor (J15 probe) (C), and for the immunoglobulin heavy-chain J region (pJ11 probe) (D). The left and right autoradiograph of B were obtained from the same filter, with the exception of the lane representing the primary spleen tumor. The number of the recipient mouse is shown above the autoradiographs. Unnumbered lanes, Primary tumor tissue. Transplantation of lymphomas into mice 1 through 4 was performed by i.p.

respectively. Usually the unaltered allele showed a much higher hybridization intensity, even in those tumors which consisted of nearly pure lymphomatous tissue (11, 50; unpublished results). Analysis of the proviral integration pattern of a large number of lymphomas by Southern blotting also revealed significant variations in the hybridization intensity of the distinct proviral integrations (unpublished results). This was not expected in monoclonal tumors, consisting of virtually pure lymphoma tissue, in which the new fragments resulting from proviral integration in the c-*myc* and *pim-1* region should have exhibited nearly the same hybridization intensity as the unaltered germ-line allele. In addition, the independent proviral integrations of a monoclonal lymphoma were expected to show equal hybridization intensities on Southern blots upon cleavage of the DNA with *Eco*RV and use of the U3LTR probe (see below).

To evaluate whether these primary T-cell lymphoma tissues are composed of oligoclonal cell populations and to monitor tumor progression, primary BALB/c and C57BL T-cell lymphomas were transplanted into syngeneic recipient mice, and the tumors, which developed after 2 to 4 weeks, were analyzed for monoclonality or oligoclonality with respect to the T-cell receptor β-chain and immunoglobulin heavy-chain D-J rearrangements, as well as *pim-1* and c-*myc* rearrangements, all in relation to the MoMuLV proviral integration patterns (detailed below). For transplantation, lymphoma cells were either injected i.p. or s.c.

Viral and cellular gene analysis. All lymphoma DNAs were analyzed in the following way. (i) The MoMuLV proviral integration pattern was examined. Southern blots containing DNAs cleaved with various restriction endonucleases were hybridized with the MoU3LTR probe (11). The analyses with *Eco*RV were of special interest, since this enzyme cleaves in the LTR 0.14 kbp from the 5′ boundary of the U3LTR region, leaving 140 base pairs of U3LTR covalently linked to the flanking cellular fragment, independent of the type of enhancer duplication which almost invariably is observed in the LTR of the integrated MCF proviruses (2, 62). Therefore, the hybridization intensities of the junction fragment do not depend on proviral enhancer duplications. (ii) Proviral integration in the vicinity of the c-*myc* and *pim-1* genes was examined. Southern blots containing *Kpn*I digests (for c-*myc*) and *Eco*RI or *Eco*RV digests (for *pim-1*) were hybridized with the appropriate probes (8, 11, 49, 50). To confirm that the alterations were caused by integrated proviruses, the hybridization patterns obtained with c-*myc* and *pim-1* probes were superimposed upon the patterns obtained with the viral probe. In all instances in which changes in c-*myc*, *pim-1*, or both regions were observed, proviral sequences could be shown to hybridize to the same fragment, although coincidence of fragments could not be excluded in some cases. (iii) Somatic D-J rearrangements of the immunoglobulin heavy-chain gene were examined. This type of rearrangement is observed in T-cell clones (30) and in T-cell lymphomas (23, 69). We used pJ11 (36), containing the J3 and J4 segments, as a probe to analyze Southern blots of *Eco*RI- or *Eco*RV-digested lymphoma DNA. (iv) Somatic rearrangements of the β-chain of the T-cell receptor were examined. Expression of the β-chain of the T-cell receptor in T cells and in T-cell lymphomas is the result of genomic rearrangements through which the separate DNA segments, encoding the β-chain of the T-cell receptor, are assembled into a functional gene (6, 18, 20, 35, 52). Hybridizations were performed with the 86T5 cDNA probe, which recognizes the $J\beta_1$ and Cβ regions of the β-chain for the T-cell receptor (20), and with the probes 4.1 and J15, which recognize the $C\beta_1$ and $C\beta_2$ regions, and the region situated between $J\beta_2$ and $C\beta_2$ (specific for the β_2 region), respectively (53). Hybridizations of Southern blots containing lymphoma DNAs cleaved with *Kpn*I, *Pvu*II, and *Hin*dIII with these probes allowed us to resolve the structure of the rearranged alleles. These four parameters were used to define the clonality of the lymphomas that were studied. It is possible that these parameters could be supplemented with others which might further subdivide the different tumor clones.

Multiple tumor development. Infection of BALB/c and C57BL newborn mice with MoMuLV frequently gave rise to multiple focal T-cell lymphomas, resulting in the outgrowth of clones both at different anatomical sites and in one organ. Multiple tumor development in newborn mice after infection with MoMuLV was most strikingly seen in the different lymphomas of BALB/c mouse 54. Lymphomas developed at different anatomical sites in this mouse. The proviral integration pattern in the thymus was completely different from the pattern observed in spleen and mesenteric lymph nodes. This difference is best illustrated by probing a Southern blot containing *Kpn*I- and *Eco*RV-cleaved DNA with the MoU3LTR probe (Fig. 1A). The difference was further substantiated by the analysis of additional markers. (i) The proviral integrations observed in the vicinity of the *pim-1* and c-*myc* genes were present in the lymphomatous tissue of the thymus but absent in tumor tissues of spleen and mesenteric lymph nodes (Fig. 1A). (ii) The immunoglobulin heavy-chain D-J rearrangement in the thymus tumor was completely different from the rearrangement seen in the spleen and mesenteric lymph node tumor tissue (Fig. 1B). (iii) The rearrangements of the β-chain of the T-cell receptor that were detected in the thymus lymphoma differed from the somatic rearrangements in the spleen and mesenteric lymph node tissue (Fig. 1C). Oligoclonal tumor development within the same organ was illustrated in BALB/c mouse 36. i.p. transplantation of primary lymphoma cells from the spleen of mouse 36, which was infected as a newborn with MoMuLV, gave rise to a biclonal tumor cell population in the mesenteric lymph nodes of four recipients (Fig. 2A and B). One of the cell populations, which was abundantly present in some of the outgrown mesenteric lymph tumor grafts and which was present only in minor amounts in the splenic outgrowths of the mice transplanted i.p., was hardly detectable in the primary tumor used for transplantation. The biclonal character of the mesenteric lymph node tumors, which arose after transplantation of cells from the primary tumor, was unambiguously established as follows. (i) The proviral integration pattern in Southern blot analysis after cleavage with *Eco*RV showed that one of the clones was characterized by the fragments shown in Fig. 2A (solid circles). (ii) Both clones which could be discerned in these tumors had a provirally activated c-*myc* gene, indicated by the hybridization of fragments a and b (Fig. 2B) with the c-*myc* probe. The lymphomatous mesenteric lymph node

injection of spleen cells of mouse 36, and transplantation of lymphomas into mouse 5 (marked with star) was performed by s.c. injection of the same cells. a, a_1, and a_2, Fragments representative for the cell population, which is present in all the lymphomas of the recipient mice; b, fragments representative for the cell type, which settled almost exclusively in the lymph nodes of the i.p. transplanted recipient mice; ●, proviral integration fragments (A). Abbreviations are as in the legend to Fig. 1.

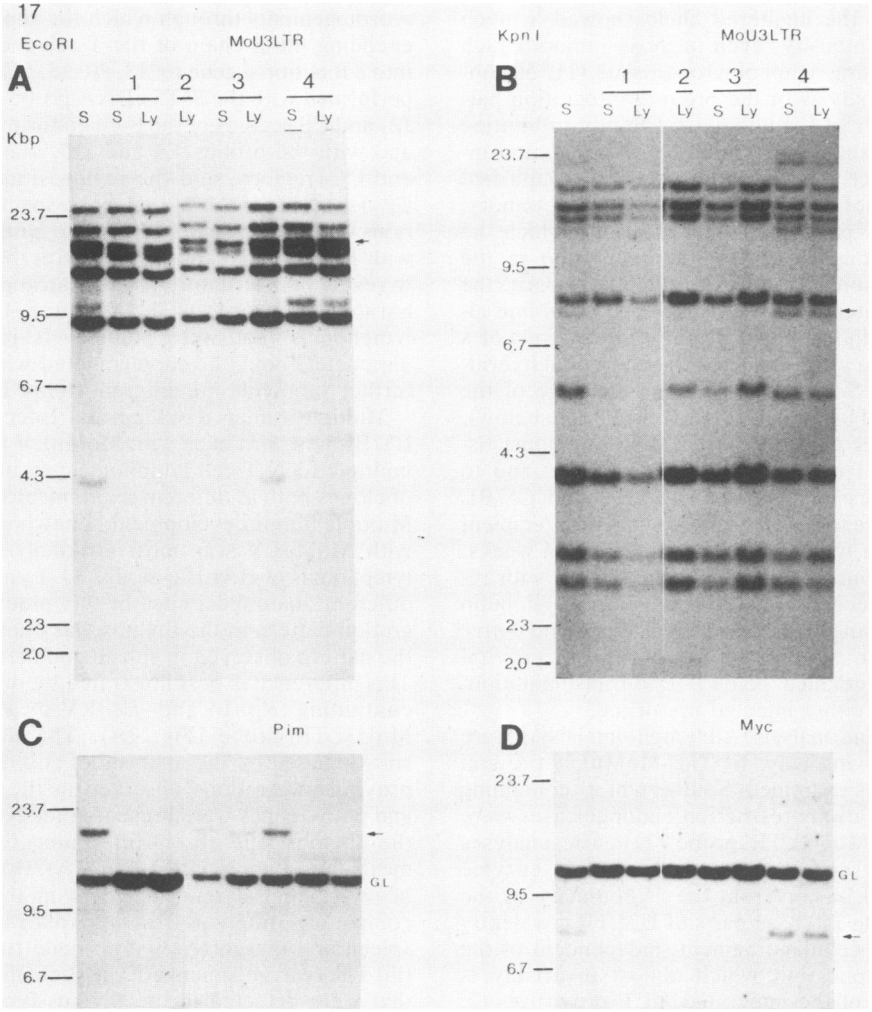


FIG. 3. DNA analyses of lymphoma from C57BL mouse 17 induced by newborn infection with MoMuLV and of lymphomas caused by transplantation of the primary spleen tumor into recipient mice. Ten-microgram samples of lymphoma DNA obtained from spleen (S) of mouse 17 and from spleen (S) and mesenteric lymph nodes (Ly) of the i.p. transplanted recipient mice 1 through 4 were digested with restriction endonucleases *Eco*RI and *Kpn*I, separated on 0.6% agarose gels, transferred to nitrocellulose filters, and annealed to probes representative for MoU3LTR (A and B), *pim-1* (C), and c-*myc* (D). The upper and lower autoradiographs were obtained from the same filters. The number of the recipient mouse is depicted above the autoradiographs. Unnumbered lanes, Primary spleen tumor; arrow, fragments diagnostic for the proviral integrations in the *pim-1* or c-*myc* region. Abbreviations are as in the legend to Fig. 1.

tissue was composed of a mixture of the two clones. One cell type, present in tumor tissues of both spleen and mesenteric lymph nodes, had a provirus integrated just inside exon 1 in the same transcriptional orientation as c-*myc*. This integration gave rise to the hybridization of fragment a (Fig. 2B). The other clone, predominantly found in the mesenteric lymph node tissue, had a proviral integration 2.0 kbp upstream from exon 1 with the transcriptional orientation directed away from c-*myc* (for a more detailed description of the analysis, see Selten et al. [50]). (iii) The biclonal character of the mesenteric lymph node tumor tissue in mice 1 through 4 was further confirmed by the pattern of the D-J rearrangements of the immunoglobulin heavy-chain gene and by the β-chain T-cell receptor rearrangements. The cell clone present in every tumor of mice 1 through 5 had D-J rearrangements of the immunoglobulin heavy chain on both alleles (Fig. 2D, a). The cell clone which was present in the lymphomatous mesenteric lymph nodes of mice 1 through 4 but absent from the splenic tumors and also from the lymph node tumor of mouse 5 had a D-J rearrangement of the heavy-chain immunoglobulin on one of the two alleles (Fig. 2D, b). The biclonal composition of the lymph node tumor tissue of mice 1 through 4 was also indicated by the somatic rearrangements of the β-chain of the T-cell receptor (Fig. 2C, a and b). Fragment a was diagnostic for the cell clones present in every tumor tissue of mice 1 through 5 and most likely represented a $VDJ_2C\beta_2$ rearrangement with deletion of the $DJ_1C\beta_1$ region on one allele and a $DJ_2C\beta_2$ rearrangement on the other. The other cell clone was characterized by the β-chain T-cell receptor rearrangement (Fig. 2C, b). At least 10 primary lymphomas out of a series of 15 induced by inoculating newborn BALB/c and C57BL mice with MoMuLV and containing proviruses integrated near both c-*myc* and *pim-1* (50) appeared to be oligoclonal. In all of these 10 primary tumors, c-*myc* and *pim-1* activation was found in independent cell clones, as became apparent after transplantation of these tumors in syngeneic hosts. The transplantation experiment with the lymphomatous cells obtained from the spleen of mouse 17 was a representative example for this group of lymphomas (Fig. 3). The primary

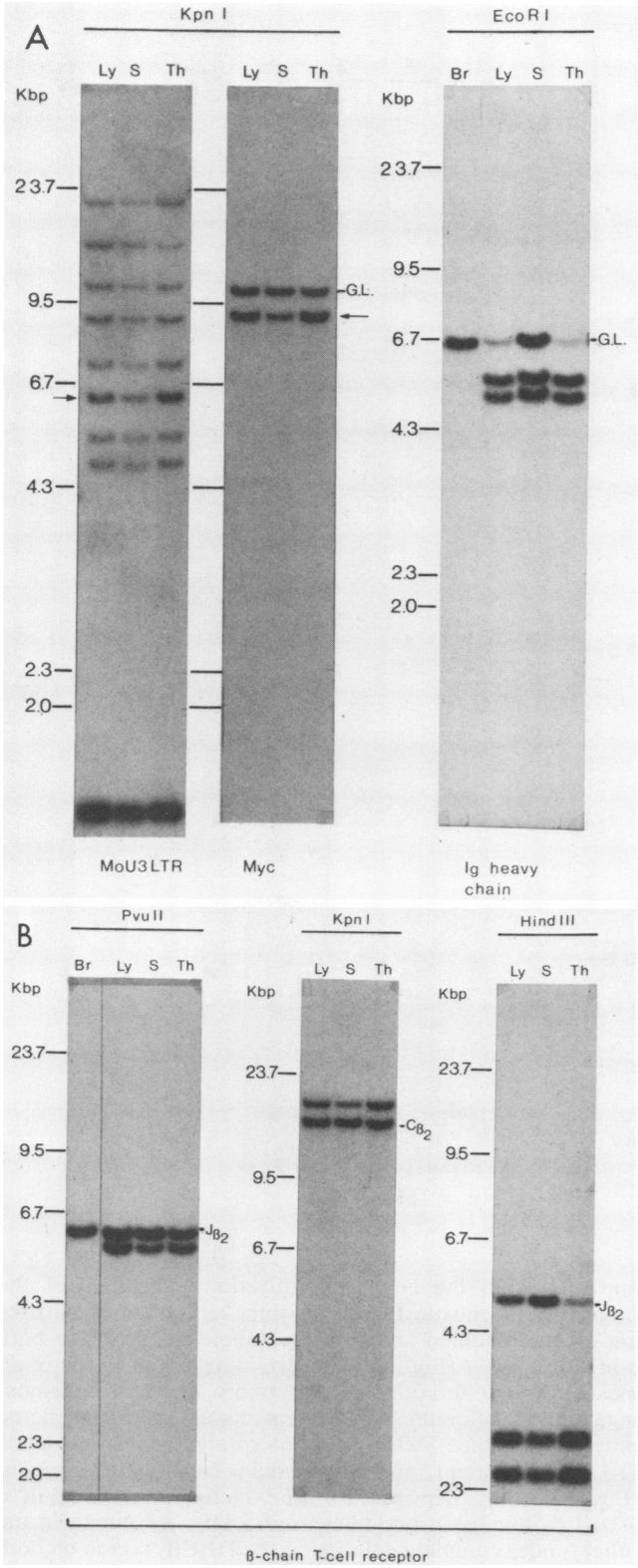
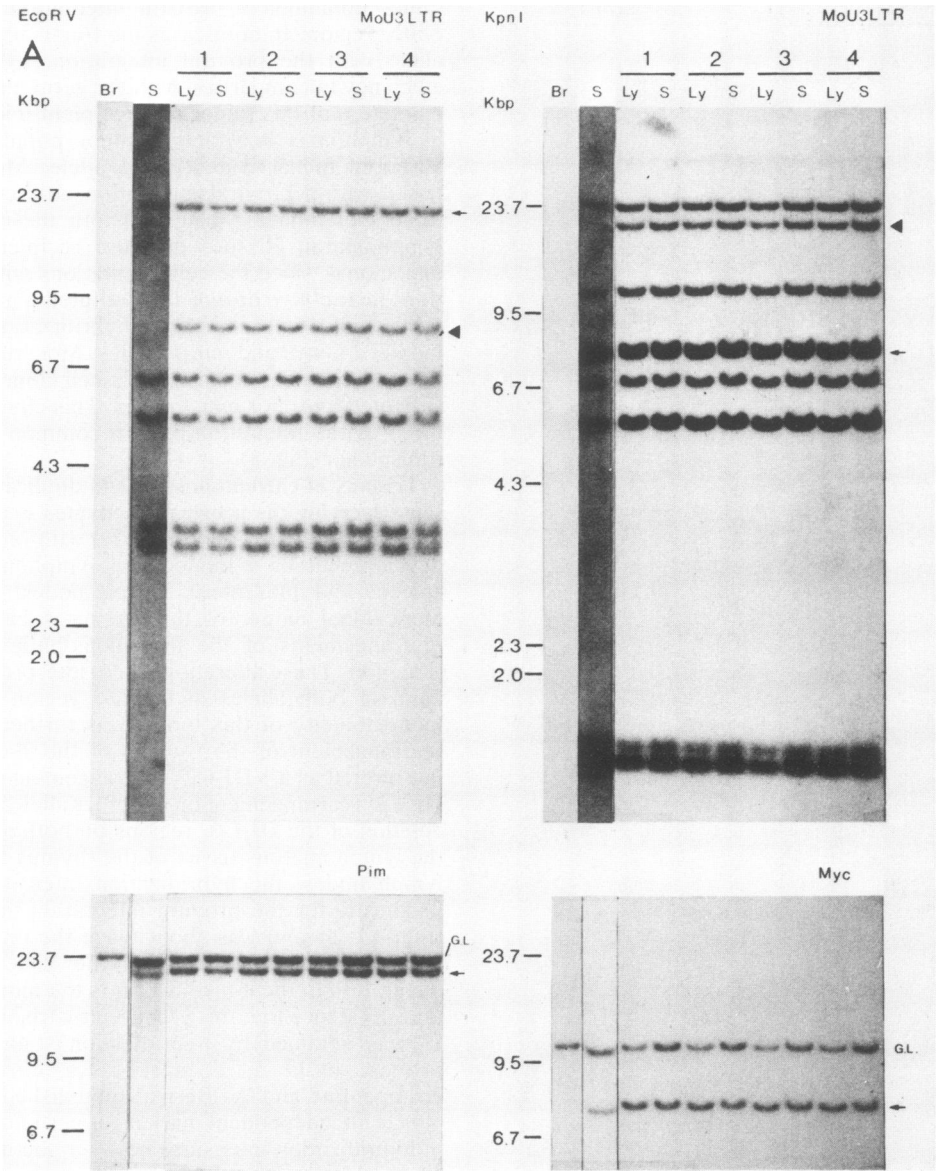


FIG. 4. DNA analyses of lymphoma from strain 129 mouse 55 induced by newborn infection with MoMuLV. Ten-microgram samples of lymphoma DNA obtained from spleen (S), thymus (Th), mesenteric lymph nodes (Ly), and brain (Br) of mouse 55 were digested with restriction endonucleases *Kpn*I and *Eco*RI (A) and with *Pvu*II, *Kpn*I, and *Hin*dIII (B), separated on 0.6% agarose gels, transferred to nitrocellulose filters, and annealed to probes representative for MoU3LTR (A, left autoradiograph), c-*myc* (A, middle autoradiograph), the immunoglobulin heavy-chain J region (pJ11) (A, right autoradiograph), and the β-chain of the T-cell receptor (J15 probe) (B). The left and middle autoradiographs of panel A were obtained from the same filter. Arrow, Fragments diagnostic for the proviral integration in the c-*myc* region. Abbreviations are as in the legend to Fig. 1.

tumor contained a proviral integration in the *pim-1* and c-*myc* region. In both cases the fragment, diagnostic for the allele with the proviral integration, hybridized with low efficiency in comparison with the germ line fragment. Transplantation of this tumor into recipient mice 2 and 3 resulted in lymphomas in which a minor population of cells still harbored the activated *pim-1* allele. Mouse 4 had tumor tissue with a proviral integration near c-*myc*, whereas the tumors of mouse 1 had lost both these populations. The lymphomatous tissues of these recipient mice were still oligoclonal, since the cell populations with proviral integrations near c-*myc* or *pim-1* constituted a minor portion of the tumor mass, as based on the hybridization intensities of the modified c-*myc* and *pim-1* alleles. Most of the tumor mass of these lymphomas consisted of a cell clone without provirally activated c-*myc* or *pim-1* genes. This clone was identified by the proviral integration pattern common to all the different transplants (Fig. 3).

Trisomy of chromosome 15 and duplication of the chromosome carrying the provirally activated c-*myc* gene. Newborn infection of mouse 55 (strain 129) with MoMuLV caused the development of a monoclonal lymphoma in the thymus, spleen, and mesenteric lymph nodes. Evidence for the monoclonal nature of this tumor is based upon the D-J rearrangements of the immunoglobulin heavy-chain gene (Fig. 4A). These alterations were interpreted as a rearrangement of both alleles of the D-J region. Evidence for the monoclonality of this tumor was further supported by the rearrangement of the β-chain of the T-cell receptor gene, interpreted as a $VDJ_2C\beta_2$ rearrangement on one allele and a $DJ_2C\beta_2$ rearrangement on the other allele under the complete deletion of the $DJ_1C\beta_1$ regions on both alleles (Fig. 4B). In the lymphomatous tissue of the thymus and the mesenteric lymph nodes, the hybridization intensity of the fragment diagnostic for the proviral integration in the c-*myc* region (Fig. 4A, arrow) was about twice the intensity of the germ line fragment. Duplication of the allele with the proviral integration in the c-*myc* region is the most likely interpretation. Evidence that this is caused by trisomy of chromosome 15 was obtained by hybridization studies with additional probes: pC17 as a marker for chromosome 18 (P. Krimpenfort and A. Berns, unpublished results) and *int-1* (40) as an independent marker for chromosome 15. The ratio of hybridization intensities between the marker for chromosomes 15 (*int-1*) and 18 (PC17) in the tumor tissues was greater than that in normal tissues by a factor 1.6, which is expected if chromosome 15 is present three times (results not shown). Unfortunately, no viable cells were obtained from this tumor to confirm the trisomy of chromosome 15 by karyotyping. However, the higher hybridization intensities of both the *int-1* and the c-*myc* probes in tumor tissues were highly suggestive of trisomy of chromosome 15 in this tumor. Hybridizations with viral probes sustained this conclusion: the proviral integration site detected by the MoU3LTR probe as a 6.5-kbp *Kpn*I fragment represents the provirus integrated with transcriptional orientation opposite to that of c-*myc*. As expected, the hybridization intensity of the pro-



viral fragment derived from the duplicated chromosome was roughly twice as high. This showed that the activation of c-*myc* by proviral integration preceded the duplication of chromosome 15. Similar results, indicating the duplication of the chromosome carrying the altered c-*myc* allele, were observed in three other mice (37, 48, and 51 [results not shown]). However, little evidence for the reverse phenomenon was obtained; i.e., duplication of the unaltered chromosome 15 next to a single copy of the modified chromosome, although this conclusion is hard to draw for lymphomas which also contain nontumor tissue.

Tumor progression in a neonatally MoMuLV-infected BALB/c mouse—subsequent proviral activation of c-*myc* and *pim-1* within the same cell lineage. The transplantation of primary lymphomatous spleen cells from BALB/c mouse 9 gave rise to the development of a monoclonal tumor in the mesenteric lymph nodes and spleen of recipient mice 1 through 4. The monoclonality of these lymphomas was apparent from the equal hybridization intensities of the fragments diagnostic for the distinct integrated proviruses and of the modified and germ line allele fragments of both c-*myc* and *pim-1* (Fig. 5A) in nearly pure tumor tissue (e.g., mesenteric lymph nodes). Furthermore, the rearrangement in the immunoglobulin heavy-chain region (Fig. 5B, depicted only for two mice), which was interpreted as a single-allele D-J rearrangement, and the β-chain T-cell rearrangement (Fig. 5C, only depicted for one mouse), resulting in a $VDJ_2C\beta_2$ joining in one allele and a $DJ_2C\beta_2$ joining in the other (under complete deletion of the $DJ_1C\beta_1$ region on both alleles), clearly established the monoclonality of the tumor at the different anatomical sites. Integration near c-*myc* and *pim-1* within the same cell lineage resulted in the enhanced transcription of both genes (49, 50). This result suggested that the subsequent activation of these genes each contributed to the proliferative capacity of the tumor.

Furthermore, this transplantation series showed an addi-

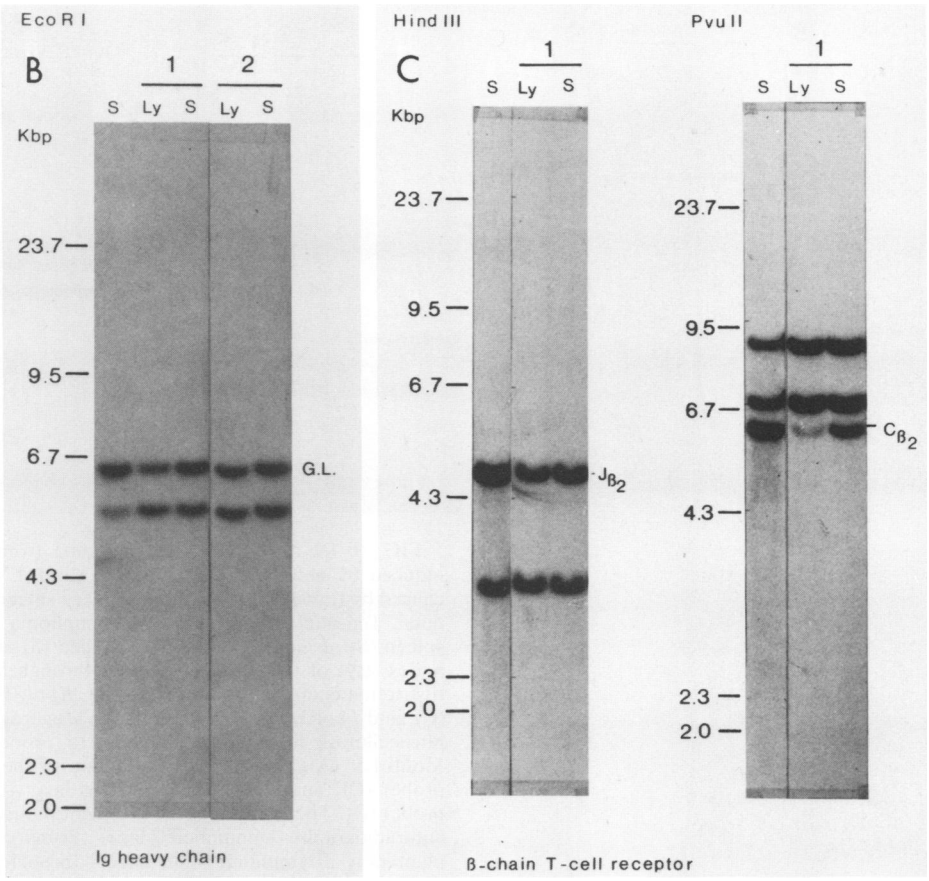


FIG. 5. DNA analyses of lymphoma from BALB/c mouse 9 by newborn infection with MoMuLV and of lymphomas caused by transplantation of the primary spleen tumor into recipient mice. Ten-microgram samples of lymphoma DNA obtained from spleen (S) and brain (Br) of mouse 9 and from spleen (S) and mesenteric lymph nodes (Ly) of the i.p. transplanted recipient mice 1 through 4 were digested with restriction endonucleases *Eco*RV and *Kpn*I (A), *Eco*RI (B), and *Hin*dIII and *Pvu*II (C), separated on 0.6% agarose gels, transferred to nitrocellulose filters, and annealed to probes representative for MoU3LTR (A, upper autoradiographs), *pim-1* (A, left lower autoradiograph), c-*myc* (A, right lower autoradiograph), immunoglobulin heavy-chain region (pJ11 probe) (B), and β-chain of the T-cell receptor (J15 probe) (C). The upper and lower autoradiographs of panel A were obtained from the same filter. The number of the recipient mouse is depicted above. Unnumbered lanes, Primary spleen tumor or control tissue; arrow, fragment diagnostic for the proviral integrations in the c-*myc* and *pim-1* region; ◀, additional integrated provirus in the recipient mice. Abbreviations are as in the legend to Fig. 1.

tional selection: all the transplanted tumors contained the same additional integrated provirus (Fig. 5A, triangle). This provirus was not detected in the DNA obtained from the primary tumor. The proviral integration pattern in the lymphomas which grew in recipient mice remained identical during further transplantations (data not shown). Most likely, the additional provirus became integrated later in the leukemogenic process, marking a cell clone with increased malignancy but unable to overgrow the large tumor mass already present in the primary tumor.

Individual cell lineages present in MoMuLV-induced T-cell lymphomas exhibited a homing preference for distinct tissues. Homing of lymphoma cell lineages was observed in different tumor tissues of mouse 54. One cell type populated predominantly spleen and lymph nodes, whereas another clone was found exclusively in the thymus (Fig. 1A, B and C; see above). The predominant clone present in the thymus of mouse 54 was not found in any of the other tissues. In contrast, the tumor cell clone present in the spleen was capable of spreading to other organs. The confinement of the thymus-specific clone could either be explained by the lack of invasive potential or reflect the requirement for factors present only in the thymus. Similar selections were seen upon transplantation of oligoclonal tumor cells into syngeneic hosts. Transplantation by i.p. injection of tumor cells from mouse 36 resulted in a characteristic and reproducible homing of one of the subclones: the subclone characterized by a specific proviral insertion near c-*myc* (Fig. 2B, b), and the distinct heavy-chain immunoglobulin and β-chain T-cell receptor rearrangements (Fig. 2C and D, b) settled almost exclusively in the lymph nodes. In the inoculating cell mass, which was derived from the splenic tumor, this cell population was present in only minute amounts (Fig. 2A and B). s.c. transplantation of the primary splenic cells of mouse 36 prevented the outgrowth of this cell clone in the lymph node tissue of recipient mouse 5 (Fig. 2), indicating that the route of inoculation can pose a barrier for distinct cell populations.

MuLV-induced lymphomagenesis were described as a continuous process of selection of cell populations which are marked by distinct proviral integrations. The transplantation of primary lymphomatous spleen cells of BALB/c mouse 9

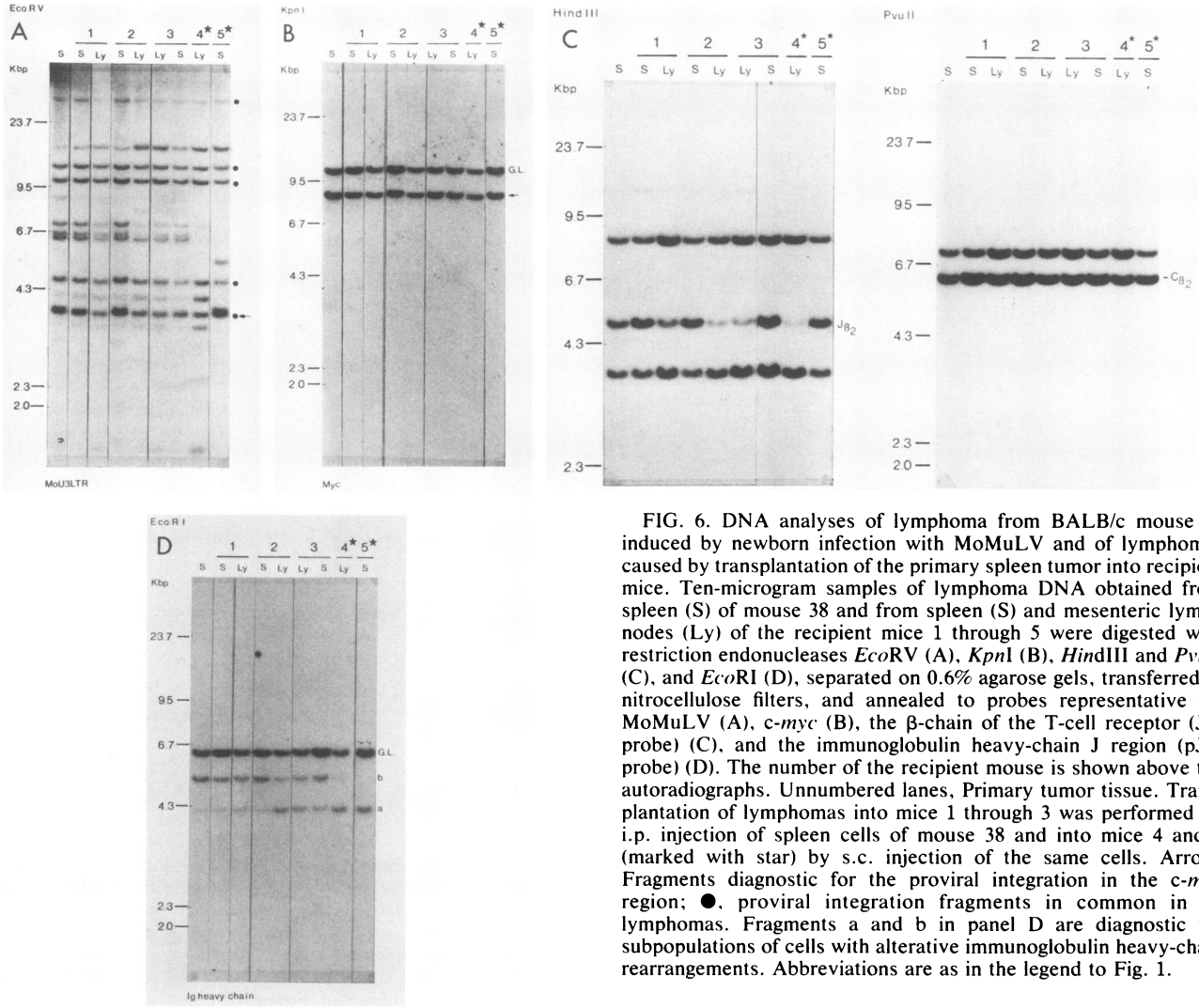


FIG. 6. DNA analyses of lymphoma from BALB/c mouse 38 induced by newborn infection with MoMuLV and of lymphomas caused by transplantation of the primary spleen tumor into recipient mice. Ten-microgram samples of lymphoma DNA obtained from spleen (S) of mouse 38 and from spleen (S) and mesenteric lymph nodes (Ly) of the recipient mice 1 through 5 were digested with restriction endonucleases *Eco*RV (A), *Kpn*I (B), *Hin*dIII and *Pvu*II (C), and *Eco*RI (D), separated on 0.6% agarose gels, transferred to nitrocellulose filters, and annealed to probes representative for MoMuLV (A), c-*myc* (B), the β-chain of the T-cell receptor (J15 probe) (C), and the immunoglobulin heavy-chain J region (pJ11 probe) (D). The number of the recipient mouse is shown above the autoradiographs. Unnumbered lanes, Primary tumor tissue. Transplantation of lymphomas into mice 1 through 3 was performed by i.p. injection of spleen cells of mouse 38 and into mice 4 and 5 (marked with star) by s.c. injection of the same cells. Arrow, Fragments diagnostic for the proviral integration in the c-*myc* region; ●, proviral integration fragments in common in all lymphomas. Fragments a and b in panel D are diagnostic for subpopulations of cells with alterative immunoglobulin heavy-chain rearrangements. Abbreviations are as in the legend to Fig. 1.

resulted in the development of clonal tumors in the spleen and mesenteric lymph nodes of all recipient mice, each with the same additional proviral integration (Fig. 4A, triangle). However, the cell population with this additional proviral integration was hardly detectable in the primary spleen tumor of mouse 9. The cell type marked by this additional proviral integration either grew far more aggressively or survived transplantation much better than did the parental cell clone, which almost exclusively populated the primary tumor.

An even more complex proviral integration pattern was seen in other transplantation studies. In one instance, mouse 38, the outgrown tumor was composed of various subclones which had five proviral integration sites in common (Fig. 6A), one of which corresponded with a provirus near c-*myc*. Although the equal intensity of the five common proviral junction fragments, the equal intensity of the altered and unchanged c-*myc* allele (Fig. 6B), and the rearranged T-cell receptor β-chain (Fig. 6C) all substantiated the monoclonal origin of this tumor, significant differences were found with respect to the immunoglobulin heavy-chain rearrangements (Fig. 6D, a and b) and the additional proviruses in the individual tumor transplants resulting from i.p. transplantation of tumor cells (Fig. 6A). s.c. transplantation of tumor cells gave rise to lymphomas with a proviral integration pattern which could be interpreted as clonal but which was different among the recipients. This established the order of events with respect to the proviral integrations and rearrangements of T-cell receptor and immunoglobulin genes. Apparently the rearrangement of the β-chain of the T-cell receptor and the five proviral integrations common to all tumors occurred before the immunoglobulin heavy-chain rearrangement. Some of the additional proviruses, present in submolar amounts, integrated in the same time span as the immunoglobulin heavy-chain rearrangement, whereas other proviruses integrated later, giving rise to additional hybridizing fragments.

DISCUSSION

The reproducible outgrowth upon transplantation of distinct tumor cell subsets in individual recipient mice indicates that this experimental model can be used to analyze progression during tumor development. We showed that a significant portion of the primary T-cell lymphomas, induced by MoMuLV in BALB/c and C57BL mice, represent heterogeneous oligoclonal cell populations. The results of the analyses of mice 54 (Fig. 1), 36 (Fig. 2), and 17 (Fig. 3) indicate

that outgrowth of independently transformed primary cells occurs frequently. During lymphoma development in mouse 54, independently transformed cells grew at different anatomical sites, whereas in animals 36 and 17 the tumor cell mass consisted of a mixture of clones, originating from separate transformation events. Oligoclonality of T-cell lymphomas was also found in stage II and III lymphomas induced by MCF69L1 virus in AKR/J mice (41).

Comparison of the different experimental models of virally induced lymphomas suggests that the occurrence of oligoclonality as the result of independent transformation events is related to the latency of the disease; e.g., BALB/c and C57BL mice inoculated as newborns with MoMuLV and AKR mice inoculated with MCF247 or MCF69L1 virus, together classified in group I, developed lymphomas within 13 to 24 weeks (11, 34, 41, 42, 50). Analyses of the DNA from these lymphomas revealed that a substantial proportion of these tumors were oligoclonal. In contrast, the spontaneous lymphomas of BALB/Mo and AKR mice and the lymphomas induced by neonatal infection of BALB/c and C57BL mice with MCF1233 or MCF1130 virus (classified in group II and III; latency longer than 35 weeks), appeared to be predominantly monoclonal (11, 22, 46, 50, 63, 69). Similarly, B-cell lymphomas in (BALB/c × strain A)F_1 mice, induced by a graft-versus-host reaction after intravenous injection of BALB/c lymphoid cells, were also found to be monoclonal in origin (43). The occurrence of oligoclonal lymphomas seemed to be restricted to the experimental models with a short latency for tumor outgrowth, probably because MoMuLV, MCF247, and MCF69L1 viruses act as very efficient (insertional) mutagens, thereby frequently causing multiple independent transformation events.

In a previous study, evidence was provided that tumorigenesis is a multistep process (28). Our experiments also indicate that MuLV-induced leukemogenesis can be described as a process of sequential selection events which can be monitored with a variety of molecular markers. This analysis reveals the following interesting features of a continuously adapting tumor cell population. (i) Tumor progression can be characterized by the sequential proviral activation of different (putative) oncogenes within the same cell lineage, as illustrated in the lymphomatous tissues of mouse 9 (Fig. 5). The proviral insertions near c-*myc* and *pim-1* in this tumor and in several other tumors (data not shown) provide evidence strongly supporting a multistep model in which a transformed cell progresses step by step to a more malignant phenotype and in which each step requires the activation of distinct cellular genes. Such synergism between oncogenes has also been observed in studies in which primary cells were transformed in vitro (19, 26, 32, 39, 47) and in the permanent cell line HL-60 (38). The development of mammary adenocarcinomas in transgenic mice that carry and express the mouse mammary tumor virus-*myc* fusion gene appears to be dependent on further transforming events (57). The concomitant appearance of proviral insertions in the MLvi-1 and MLvi-2 domains in the same populations of cells in rat thymic lymphomas induced by MoMuLV (59), the proviral insertion near both *int-1* and *int-2* in some mouse mammary tumors (44), and the cooperativity of v-*erbA* and v-*src* in both acute erythroblastosis and sarcoma development (26) also support this theory. The integration of retroviruses appears to be sufficiently random to assume that after the integration of a provirus near one of these genes, massive proliferation of cells with this particular integration must occur to make integration near the other gene statistically feasible. Integration near c-*myc* and *pim-1* within the same cell lineage resulted in the enhanced transcription of both genes. The activation of c-*myc* and *pim-1* probably contributes to different steps in the progression of the disease. (ii) Sequential selections in already expanded cell clones, which mark the various steps in tumor progression, were observed in mouse 9 (Fig. 5) and in mouse 38 (Fig. 6). In mouse 9 the clone identified by an additional proviral integration appeared to grow far more aggressively upon transplantation than the predominant constituent cell type of the primary tumor. It is possible that the additional proviruses observed after transplantation were physically linked to genes directly involved in tumor progression. Alternatively, they might represent an unlinked marker for a cell type with different oncogenic potential. Recent studies in which DNA from a human metastatic tumor was shown to increase the metastatic potential of cells indicate that this property might be encoded in a single gene and therefore might also be potentiated by a single proviral integration (3).

It is evident from the transplantation studies of the lymphomatous cells of mouse 38 that the tumor tissue in this mouse consisted of subpopulations of cells all descending from one ancestral lineage. All subpopulations had five identical proviral integrations (one near c-*myc*), but differed with respect to immunoglobulin heavy-chain D-J region rearrangements. The occurrence of D-J immunoglobulin rearrangements after clonal expansion of (pre)leukemic cells has also been observed in thymic leukemias of AKR mice. These thymomas were monoclonal with respect to the proviral integration pattern but contained subpopulations of leukemic thymocytes with unique rearrangements of the immunoglobulin heavy-chain D-J region (23). (iii) Trisomy of chromosome 15 is the most common chromosome aberration reported for murine T-cell lymphomas (12, 54, 65–67). Duplication of chromosome 15, carrying a provirally activated c-*myc* allele, was observed in four instances. We did not find indications of tumors with a provirally activated c-*myc* in which the unaltered chromosome 15 was duplicated. The increased hybridizing intensity of a rearranged c-*myc* gene in a lymphoma, which arose in an AKR mouse infected as a newborn with MCF247, can also be explained by a trisomy of chromosome 15 (34). Taken together, these results suggest that the chromosome carrying the activated c-*myc* gene is preferentially duplicated during the progression of the disease. Since proviral activation of c-*myc* seems to occur with equal efficiency in lymphomas both with and without trisomy of chromosome 15, it is possible that the duplication of chromosome 15 is unrelated to the activation of c-*myc* itself in these tumors. Instead, the duplication of chromosome 15 might promote the activation of another function, which is less abundantly expressed and which is in one way or the other physically linked to the provirally activated c-*myc* gene. Until now, five candidate oncogenes have been assigned to chromosome 15: *int-1* (40), MLvi-1 (29), MLvi-2 (58), Pvt-1 (9), and *sis* (31). However, to our knowledge, no evidence has been presented which would relate any of these (putative) oncogenes with this phenomenon.

The homing preference of individual cell lineages for distinct tissues, observed in some of the MoMuLV-induced T-cell lymphomas, could be caused by malignant transformation of lymphocytes with distinct affinities for these tissues. In this case, the pathology can be understood in terms of the normal circulation pattern of distinct classes of transformed lymphocytes (37). Evidence has been provided for the existence of two antigenically distinct receptors on lymphocytes, one interacting with endothelial cells lining the high-endothelial venules in the peripheral lymph nodes and

the other interacting with the same cells of the gut-associated lymphoid tissue. In contrast to this system, the lymphocyte recirculation through the spleen is not regulated by high-endothelial venules (17, 48, 56). The cells of the subclone characterized by fragment b (Fig. 2), which settle almost exclusively in the lymph node tissue upon transplantation, might only possess receptors enabling settlement in the lymph nodes and not in other tissues.

Comparison of the results obtained after s.c.-versus-i.p. transplantation of the lymphomatous cells of mouse 36 (Fig. 2) and those of mouse 38 (Fig. 6) showed that the tumor cells were under a different selection pressure in each of these procedures.

In summary, we believe that the system described is unique because it offers the opportunity to discern sequential steps in malignant transformation. In addition, since the most important molecular marker used in this system also represents a highly efficient insertional mutagen, it can be expected that cloning of the genes adjacent to the subclone-specific proviral integrations will yield a new class of genes likely involved in the later stages (e.g., metastasis) of tumor progression.

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