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Distinct Helper Virus Requirements for Abelson Murine Leukemia Virus-Induced Pre-B- and T-Cell Lymphomas

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Abelson murine leukemia virus (A-MuLV) can induce pre-B- or T-cell lymphomas (thymomas) in mice depending on the route and time of injection. Previous studies have shown that the choice of the helper virus used to rescue A-MuLV greatly influences its ability to induce pre-B-cell lymphomas. In this study, we investigated the role of the helper virus in A-MuLV-induced thymomas. A-MuLV rescued with the helper Moloney MuLV, BALB/c endogenous N-tropic MuLV, and two chimeric MuLVs derived from these two parents were injected intrathymically in young adult NIH Swiss mice. All four A-MuLV pseudotypes were found to be equally efficient in the induction of thymomas, whereas drastic differences were observed in their pre-B-cell lymphomagenic potential. Thymoma induction by A-MuLV was independent of the replication potential of the helper virus in the thymus, and no helper proviral sequences could be detected in the majority of thymomas induced by A-MuLV rescued with parental BALB/c endogenous or chimeric MuLVs. In the thymomas in which helper proviruses were present, none of them were found integrated in the *Ahi-1* region, a common proviral integration site found in A-MuLV-induced pre-B-cell lymphomas (Y. Poirer, C. Kozak, and P. Jolicoeur, J. Virol. 62:3985–3992, 1988). In addition, helper-free stocks of A-MuLV were found to be as lymphomagenic as other pseudotypes in inducing thymomas after intrathymic inoculation, in contrast to their inability to induce pre-B-cell lymphomas when injected intraperitoneally in newborn mice. Restriction enzyme analysis revealed one to three A-MuLV proviruses in each thymoma, indicating the oligoclonality of these tumors. Analysis of the immunoglobulin and T-cell receptor loci confirmed that the major population of cells of these primary thymomas belongs to the T-cell lineage. Together, these results indicate that the helper virus has no effect in the induction of A-MuLV-induced T-cell lymphomas, in contrast to its important role in the induction of A-MuLV-induced pre-B-cell lymphomas. Our data also revealed distinct biological requirements for transformation of these two target cells by v-*abl*.

Abelson murine leukemia virus (A-MuLV) is a highly lymphomagenic, replication-defective retrovirus. Its genome harbors the oncogene v-*abl*, responsible for its oncogenic potential (for a review, see references 32, 36, and 48). Being defective, A-MuLV needs a replication-competent helper virus to replicate in vitro and in vivo (41). When pseudotyped with competent helper virus, such as Moloney MuLV, and injected intraperitoneally (i.p.) in newborn mice of most strains, A-MuLV induces nonthymic lymphomas of the pre-B-cell phenotype after a short latent period (20 to 40 days) (1–3, 43). Previous studies have shown that the choice of helper virus used to rescue A-MuLV greatly influences its ability to induce pre-B-cell lymphomas (37, 39, 40). We found that the U_3 long terminal repeat (LTR) region of the helper Moloney MuLV harbors an important determinant involved in the development of pre-B-cell lymphoma by A-MuLV (39). These results led us to speculate that the helper virus may induce secondary genetic events important for pre-B-cell transformation by acting as an insertional mutagen (39). In support of this hypothesis, we recently identified a common proviral integration site, designated *Ahi-1*, in A-MuLV-induced pre-B-cell lymphomas. *Ahi-1* was found to be occupied by Moloney helper proviruses in 16% of tumors analyzed (30).

Interestingly, when A-MuLV pseudotypes are injected intrathymically (i.t.) into young adult mice (5 to 7 weeks old), they induce a different disease, namely, a rapid thymoma developing within 6 to 11 weeks (5). Analysis of these primary A-MuLV-induced thymomas indicated that the transformed cells belonged to the T-cell lineage (5, 42). Like the A-MuLV-induced pre-B-cell lymphomas, the A-MuLV-induced thymomas appear oligoclonal (42). The role of the helper virus, previously found to be so important for the induction of A-MuLV-induced pre-B-cell lymphomas, has not yet been studied in thymoma development with helpers having a poor replication ability in the thymus.

We therefore assessed the role of various helper MuLVs as well as the effect of helper-free stocks of A-MuLV in the induction of A-MuLV-induced thymomas. Our results showed that the choice of the helper MuLV has little effect on the incidence of thymoma induced by A-MuLV, indicating that the biological requirements for the induction of A-MuLV-induced thymoma and pre-B-cell lymphoma are drastically different.

MATERIALS AND METHODS

Mice and viruses. Outbred NIH Swiss mice were from our breeding colony. For pre-B-cell lymphoma induction, newborn (<48-h-old) mice were inoculated i.p. with 0.15 ml of filtered (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.) A-MuLV suspension. For thymoma induction, 5- to 7-week-old mice were anesthetized and injected i.t. with 0.05 ml of filtered A-MuLV suspension. A-MuLV (p160) was rescued by infecting nonproducer N54 cells with helper MuLV, and titers were determined on rat-1 cells scoring for foci of transformation (focus-forming units [FFU] per milli-

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liter). The structure and biological characteristics of the N-tropic BALB/c N-Cl-35 (pN20-7) MuLV and of the chimeric p7M1 and p7M2 MuLVs have been described previously (12, 31, 39).

Assay for thymotropism. Mice (5 to 7 weeks old) were injected i.t. with 0.05 ml of filtered MuLV suspension. Mice were sacrificed 16 to 20 days after inoculation, and the thymus was explanted. Thymic cell suspensions were seeded (2×10^2 to 2×10^6 cells) on 5×10^4 NIH 3T3 cells in Dulbecco modified Eagle medium containing 10% calf serum (GIBCO Laboratories, Grand Island, N.Y.) and 2 μg of Polybrene per ml for 18 h. After this time, the nonadherent lymphoid cells were aspirated and the adherent cells were washed with phosphate-buffered saline. These remaining adherent cells were grown until confluent in Dulbecco modified Eagle medium supplemented with 10% calf serum. Infectious centers, observable as macroscopic plaques, were identified by the XC syncytial plaque assay (38).

DNA extraction and endonuclease digestion. High-molecular-weight cellular DNA was prepared from primary A-MuLV-induced lymphomas or from kidneys by phenol extraction and was ethanol precipitated and digested with various restriction endonucleases (New England BioLabs, Inc., Beverly, Mass.; Boehringer Mannheim Biochemicals, Montreal, Quebec, Canada; and P-L Pharmacia, Montreal, Quebec) under the conditions recommended by the manufacturers as previously described (25, 27, 39).

Agarose gel electrophoresis and hybridization procedure. DNA analysis by agarose gel electrophoresis and transfer to nitrocellulose or nylon membranes were done by the technique of Southern (44). Specific DNA fragments were detected by hybridization with ^{32}P-labeled cloned DNA fragments. These cloned DNA fragments were excised from the vector with appropriate restriction endonucleases, and the inserts were separated from the vector by agarose gel electrophoresis. The DNA fragments were recovered by electroelution and ethanol precipitation and used directly as hybridization probes. Probes were labeled by incubating the denatured fragments in the presence of hexamers and the Klenow fragment of polymerase I (14). Nitrocellulose filters were hybridized in 50% formamide–3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–Denhardt solution at 42°C. Nylon filters were hybridized in 4× SET (1× SET is 0.15 M NaCl, 0.03 M Tris hydrochloride, 2 mM EDTA [pH 7.4])–0.2% sodium dodecyl sulfate–0.1% sodium PP_i–10% dextran sulfate at 65°C. After hybridization, the filters were washed sequentially in 2× SSC for 15 min at room temperature, in 0.1× SSC–0.1% sodium dodecyl sulfate for 1 h at 55°C, and in 0.1× SSC for 2 min at room temperature. Membranes were air dried and exposed at −70°C to RP-Royal X-Omat film (Eastman Kodak Co., Rochester, N.Y.) with a Cronex Lightning-Plus intensifying screen (Du Pont Co., Wilmington, Del.).

Probes. The A-MuLV-specific probe consisted of a 1.9-kilobase-pair (kbp) *Sac*I-*Hin*dIII fragment from the 5.5-kbp P120-encoding A-MuLV genome (17). The Moloney U_3 LTR probe consisted of a 0.3-kbp *Pst*I-*Sac*I fragment from a mutated Moloney MuLV LTR (39). The gp70-specific probe encompassed the *env* domain of endogenous ecotropic MuLV (21). For the detection of *myc* sequences, the *Xba*I-*Bam*HI fragment covering exons 2 and 3 of the murine c-*myc* was used (8). *Pim-1* sequences were detected by using the previously described probe A (10), whereas the *Mis-1* region was analyzed by using the murine *Eco*RI-1.4 and -1.5 probes (47). The J_H region was analyzed with a 6.2-kbp *Eco*RI germ line J_H DNA fragment (2), while the C_k probe was a 500-base-pair *Hpa*I-*Bgl*II fragment from the coding region and 3′ untranslated sequences of the C_k region (26, 28). The murine β T-cell receptor (TcR) gene was analyzed with a 700-base-pair RBL5 DNA fragment containing most of the murine C region and 3′ untranslated sequences of $C_{\beta 1}$ (4). The TcR γ probe was a 1.5-kbp *Eco*RI cDNA fragment encoding the V-J-C regions of γ_1 (22).

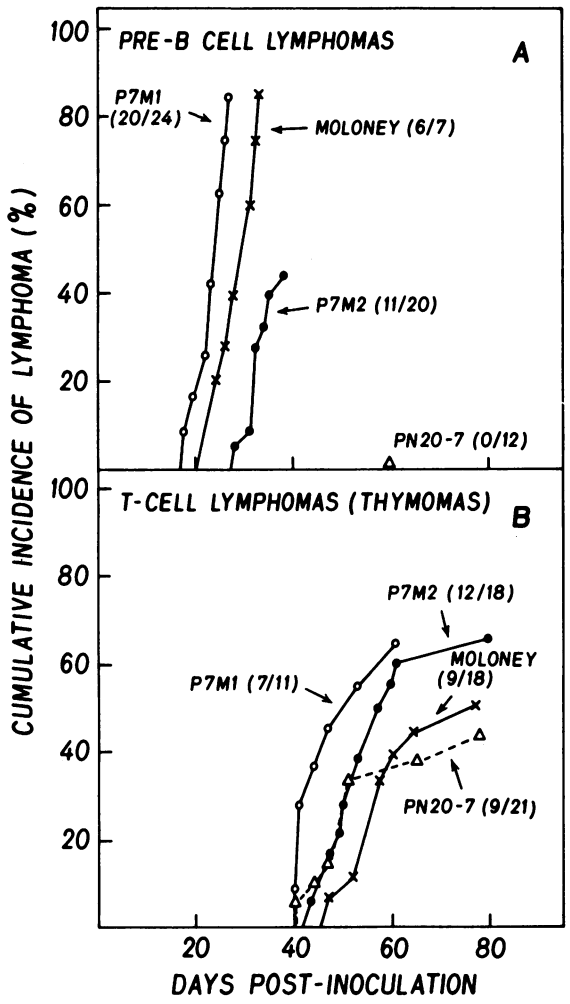
RESULTS

Oncogenic potential of A-MuLV pseudotyped with parental and chimeric helper MuLVs. The structure and biological characteristics of the parental and chimeric MuLVs used in this study have been reported previously (12, 39). Briefly, the recombinant MuLV p7M1 contains the 3′ end of *env* and the U_3 LTR region of Moloney MuLV, while p7M2 MuLV contains only the U_3 LTR region of Moloney MuLV, the remaining region of the viral genome being derived from the endogenous BALB/c ecotropic N-Cl-35 (pN20-7) MuLV (Fig. 1). In newborn NIH Swiss mice injected i.p., we previously reported that A-MuLV pseudotyped with Moloney MuLV induced pre-B-cell lymphomas in nearly 100% of inoculated mice, whereas A-MuLV pseudotyped with the helper endogenous BALB/c N-Cl-35 (pN20-7) MuLV was completely nonlymphomagenic. The recombinant p7M1 MuLV was found to be as effective as Moloney MuLV in inducing A-MuLV pre-B-cell lymphomas, whereas A-MuLV pseudotyped with p7M2 MuLV had intermediate lymphomagenic potential (39) (Fig. 1A).

To study the effect of the helper virus on A-MuLV-induced T-cell lymphoma, we injected 5- to 7-week-old NIH Swiss mice i.t. with equal amounts of A-MuLV pseudotyped with pN20-7, Moloney, p7M1, and p7M2 MuLVs (2×10^4 FFU/ml). A-MuLV pseudotyped with Moloney MuLV induced rapid thymomas in 9 of 18 injected NIH Swiss mice after a latency period of 44 to 77 days (Fig. 1B). Comparable results were previously reported in BALB/c and C57BL/6 mice (5, 11, 33, 42). As a helper, N-Cl-35 (pN20-7) MuLV was as effective as Moloney MuLV in inducing A-MuLV thymomas in 9 of 21 injected mice after 40 to 78 days, in contrast with its total inefficiency in inducing pre-B-cell lymphomas following i.p. injection in newborn mice (39). The two chimeric helper p7M1 and p7M2 MuLVs were found to be equally efficient in inducing A-MuLV thymomas, with 60 to 65% of mice developing tumors within 40 to 80 days (Fig. 1B).

These results indicate that the helper virus requirements between A-MuLV-induced pre-B- and T-cell lymphomas are different. While the type of helper MuLV used to rescue A-MuLV greatly influences the incidence of pre-B-cell lymphoma in newborn mice injected i.p., T-cell lymphoma induction following i.t. injection of A-MuLV in young adult mice is not affected by the choice of helper virus used in this study.

A-MuLV T-cell lymphoma induction is independent of helper virus replication potential in the thymus. In newborn mice injected i.p. or i.t., Moloney MuLV replicates very efficiently in the thymus, while pN20-7 MuLV replication is severely restricted (12). The equal incidence of A-MuLV-induced thymomas with Moloney or pN20-7 MuLV as the helper virus may be explained by a different replication potential of helper MuLVs in the thymus when injected i.t. in young adult mice. To test this possibility, we studied the thymotropism of the different helper MuLVs in young adult mice. NIH Swiss mice (5 to 7 weeks old) were injected i.t. with equal amounts of helper Moloney, pN20-7, p7M1, or



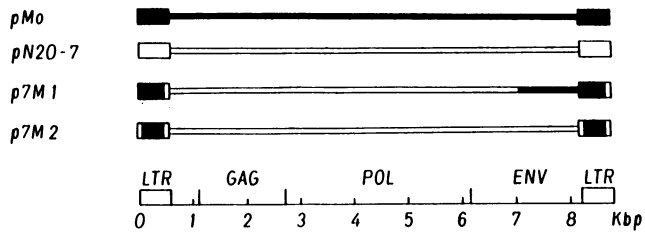


FIG. 1. Incidence of pre-B-cell lymphoma and thymoma induced by A-MuLV pseudotyped with parental and chimeric helper MuLVs in NIH Swiss mice. (A) Pre-B-cell lymphomas were induced by inoculating newborn mice (<48 h old) i.p. with 0.15 ml of filtered A-MuLV suspension (5×10^3 FFU/ml for each pseudotype). This experiment is presented for comparison and has been published previously (39). (B) Thymomas were induced in 5- to 7-week-old mice after i.t. inoculation with 0.05 ml of filtered A-MuLV suspension (2×10^4 FFU/ml for each pseudotype). The helper virus used was Moloney (×), pN20-7 (△), p7M1 (○), or p7M2 (●) MuLV. In parentheses is indicated the number of diseased animals in each group of inoculated mice/the number of mice in each group. At the bottom is shown a diagram of linear MuLV genomes identifying the origin of sequences present in the chimeric MuLVs.

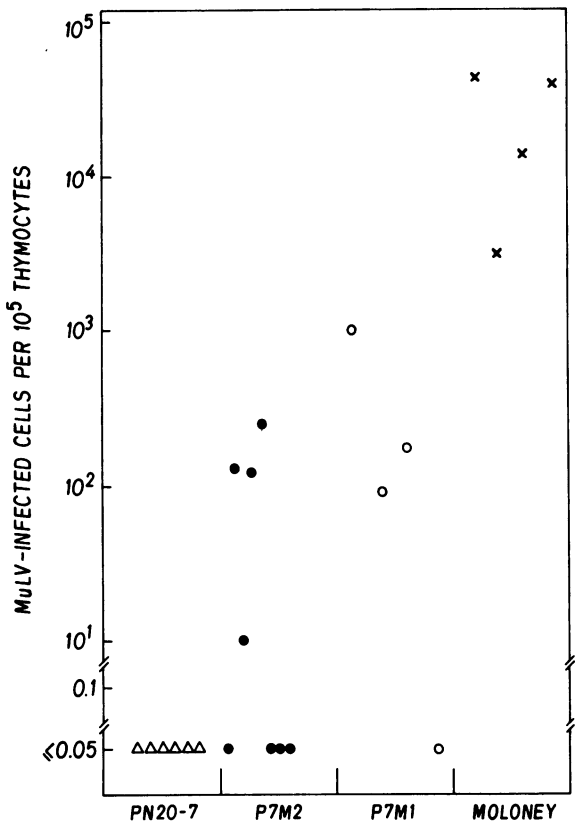


FIG. 2. Replication potential of parental and chimeric MuLVs in the mouse thymus. NIH Swiss mice (5 to 7 weeks old) were injected i.t. with 0.05 ml of filtered MuLV virus suspension (5×10^5 PFU/ml). At 16 to 20 days postinoculation, thymocytes were prepared and the number of MuLV-infected thymocytes was measured by an infectious center assay. Each symbol represents the value obtained from a single mouse. Symbols are defined in the legend to Fig. 1.

p7M2 MuLV in absence of A-MuLV. After 16 to 20 days, the thymus was explanted and the level of viral replication in thymocytes was assessed by an infectious center assay. pN20-7 MuLV was undetectable in the thymus, while Moloney MuLV replicated very efficiently in 100% of injected mice (Fig. 2). Replication of p7M1 and p7M2 chimeric MuLVs in the thymus was relatively high in 50 and 75% of injected mice, respectively, although it remained 20- to 100-fold lower than replication of Moloney MuLV (Fig. 2).

These data show that the LTR region of Moloney MuLV plays an important role in influencing the replication of MuLVs in the thymuses of young adult mice, thus confirming and extending previous data showing that the LTR region of MuLVs harbors a major determinant of thymotropism (12, 13, 35). More importantly, these results show that the ability of various A-MuLV pseudotypes to induce T-cell lymphomas is independent of the replication potential of the helper virus in the thymus. These results also suggest that A-MuLV amplification in the thymus is not required for thymoma induction, since A-MuLV rescued with a nonreplicating, nonthymotropic helper MuLV, such as pN20-7, was as efficient in inducing thymomas as if rescued with a highly thymotropic MuLV (Moloney).

The absence of replicating virus in thymomas induced by A-MuLV (pN20-7) was tested for two tumors. Thymocytes from thymomas YS6-33 and YS6-34 were cocultivated with

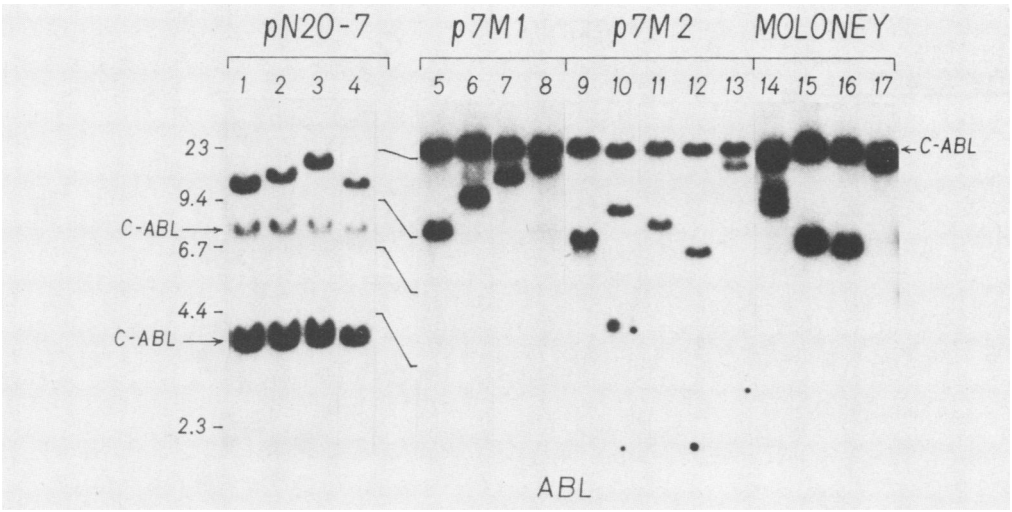


FIG. 3. Southern blot analysis of A-MuLV proviruses in A-MuLV-induced thymomas. Tumor DNAs were digested with *Hin*dIII (lanes 1 to 4) or *Eco*RI (lanes 5 to 17) and hybridized with a ^{32}P-labeled *abl*-specific probe. Tumor DNAs for lanes 1 to 17 were YS6-4, YS6-6, YS6-15, YS6-34, YS6-3, YS6-5, YS6-10, YS6-12, YS6-16, YS6-17, YS6-20, YS6-26, YS6-27, YS6-19, YS6-21, YS6-22, and YS6-23, respectively. The helper virus used to rescue A-MuLV for each group of tumors was pN20-7 (lanes 1 to 4), p7M1 (lanes 5 to 8), p7M2 (lanes 9 to 13), or Moloney (lanes 14 to 17) MuLV. The positions of c-*abl* fragments are indicated by the arrows. Numbers are lengths in kilobase pairs.

NIH 3T3 and mink cells, and supernatants were tested for the presence of retroviruses 1 month later. No replicating virus could be detected by the reverse transcriptase assay or by the XC syncytium assay (data not shown), indicating that no detectable helper virus was replicating in these tumors. This result is especially intriguing for tumor YS6-33, for which restriction enzyme analysis with the eco probe showed the presence of apparently intact pN20-7 proviral sequences (see below, Fig. 4B, lane 4).

Analysis of A-MuLV proviruses in thymomas. The proviral integration pattern of A-MuLV in thymomas was studied by restriction endonuclease analysis. Tumor DNAs were digested with *Eco*RI or *Hin*dIII and hybridized with an *abl*-specific probe. Since A-MuLV provirus harbors no *Eco*RI site and one *Hin*dIII site, the number of distinct A-MuLV proviruses present in each tumor could be assessed. For all tumors analyzed, only one to three A-MuLV proviruses could be detected, and the majority of v-*abl* fragments were of single-copy intensity, indicating that the thymomas were oligoclonal (Fig. 3). No apparent differences could be detected in tumors induced with different pseudotypes. A similar pattern of A-MuLV provirus integration has been described for A-MuLV-induced pre-B-cell lymphomas (18, 30).

Analysis of helper proviruses in A-MuLV-induced thymomas. The detection of intact helper proviruses in the induced tumors was first performed with restriction enzymes yielding internal proviral fragments of sizes unique to each helper provirus. Viral fragments were detected with a gp70-specific probe homologous to the *env* region of endogenous BALB/c ecotropic pN20-7 MuLV (eco probe) and a Moloney U_3 LTR-specific probe which hybridizes to the LTR of Moloney MuLV and A-MuLV but not to the LTR of pN20-7 MuLV (U_3 Mol probe).

DNAs from tumors induced by A-MuLV (pN20-7) were digested with *Pst*I and hybridized with the eco probe. In only one of seven tumors (thymoma YS6-33) could we detect the expected 8.2-kbp *Pst*I fragment (Fig. 4B, lane 4). No viral fragment hybridizing with this probe was detected in the other tumors. These results and the absence of detectable pN20-7 MuLV replication in the thymus (Fig. 2) indicate that the majority of A-MuLV (pN20-7)-induced tumors do not harbor pN20-7 helper proviruses.

DNAs from thymomas induced by A-MuLV rescued with p7M1, p7M2, and Moloney MuLVs were digested with *Xba*I and hybridized with the U_3 Mol or eco probe. All tumors analyzed contained the v-*abl* 5.6-kbp fragment hybridizing to the U_3 Mol probe (Fig. 4C, D, and E). Only three of seven A-MuLV (p7M1)-induced thymomas had the expected 8.2-kbp *Xba*I fragment detected by both U_3 Mol and eco probes (Fig. 4C). However, the intensity of the 8.2-kbp fragment in tumors YS6-1 and YS6-5 (Fig. 4C, lanes 1 and 4, respectively) suggests that only a minority of cells forming the tumor contained helper p7M1 proviruses.

Among the 12 A-MuLV (p7M2)-induced thymomas analyzed, only 5 tumors had the expected 7.4- and 0.85-kbp *Xba*I fragments detected by the U_3 Mol probe and the 7.4-kbp fragment detected by the eco probe (Fig. 4D). For thymomas YS6-11, YS6-16, and YS6-17, the intensity of the viral fragment (lanes 2, 5, and 6, respectively) suggests that a small fraction of cells forming the tumor contains p7M2 helper proviruses.

All eight A-MuLV (Moloney)-induced thymomas analyzed contained a Moloney MuLV-specific 2.4-kbp *Xba*I fragment, but the 5.6-kbp Moloney MuLV fragment could not be resolved from the 5.6-kbp v-*abl* fragment (Fig. 4E). As expected, no viral fragment could be detected with the eco probe in these tumors (data not shown). The additional bands detected by the U_3 Mol probe in the three groups of thymomas analyzed with *Xba*I represent cell-virus junction fragments (some of them underrepresented) of either A-MuLV proviruses or helper or recombinant proviruses (see below).

Detection of rearranged proviruses in A-MuLV-induced thymomas. Lymphomas induced by replication-competent MuLVs often show the presence of recombinant or deleted proviruses or both in their DNAs. The analysis described above did not allow the identification of possible recombinant helper proviruses. We therefore searched for evidence of rearranged proviruses in all A-MuLV-induced thymomas

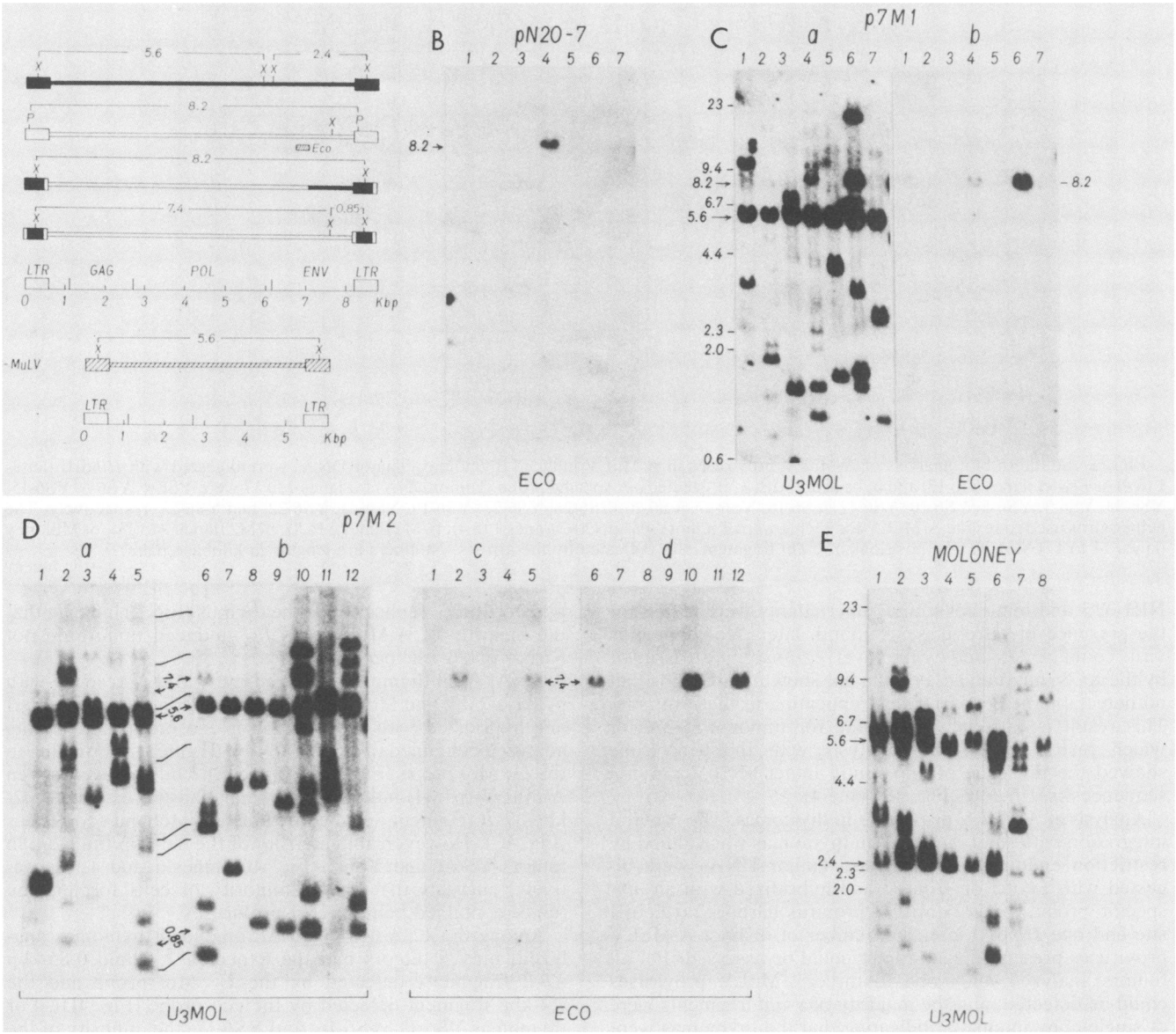


FIG. 4. Southern blot analysis of helper proviruses in A-MuLV-induced thymomas. (A) Partial restriction map of parental and chimeric p7M1 and p7M2 helper viral genomes and of the defective A-MuLV genome, showing the sizes of the expected fragments and the location of the eco probe. Restriction sites: P, *Pst*I, X, *Xba*I. Numbers are lengths in kilobase pairs. (B) DNAs from A-MuLV (pN20-7)-induced thymomas digested with *Pst*I and hybridized with the gp70-specific (eco) probe. Lanes 1 to 7 show DNAs from tumors YS6-4, YS6-6, YS6-15, YS6-33, YS6-34, YS6-35, and YS6-36, respectively. (C) DNAs from A-MuLV (p7M1)-induced thymomas digested with *Xba*I and hybridized with the Moloney U_3 LTR-specific probe (U_3 Mol) (a) or the eco probe (b). Lanes 1 to 7 show DNAs from tumors YS6-1, YS6-2, YS6-3, YS6-5, YS6-9, YS6-10, and YS6-12, respectively. (D) DNAs from A-MuLV (p7M2)-induced thymomas digested with *Xba*I and hybridized with the U_3 Mol probe (a and b) or the eco probe (c and d). Lanes 1 to 12 show DNAs from tumors YS6-8, YS6-11, YS6-13, YS6-14, YS6-16, YS6-17, YS6-18, YS6-20, YS6-26, YS6-27, YS6-29, and YS6-30, respectively. (E) DNAs from A-MuLV (Moloney)-induced thymomas digested with *Xba*I and hybridized with the U_3 Mol probe. Lanes 1 to 8 show DNAs from tumors YS6-19, YS6-21, YS6-22, YS6-23, YS6-24, YS6-25, YS-31, and YS6-32, respectively. Numbers to the sides of the gels are lengths in kilobase pairs.

by analyzing tumor DNAs with the restriction endonuclease *Eco*RI, an enzyme which allows the detection of virus-cell junction fragments. Using successively the v-*abl* U_3 Mol, and eco probes, we could confirm the presence of helper proviral sequences in A-MuLV (p7M1)-induced thymomas YS6-5 and YS6-10, in A-MuLV (p7M2)-induced thymomas YS6-17, YS6-27, and YS6-30, and in all A-MuLV (Moloney)-induced thymomas (Table 1). For A-MuLV (p7M1)- and A-MuLV (p7M2)-induced thymomas enumerated above, the fragments hybridizing with the U_3 Mol probe were comigrating with the fragments hybridizing with the eco probe (data not shown), therefore confirming the presence of intact proviruses in these thymomas. No helper virus-cell junction fragments could be detected in tumors YS6-1, YS6-11, and YS6-16, indicating that the faint signals obtained with the *Xba*I digestions (Fig. 4C and D) could be due to the presence of a small polyclonal population of infected cells in these tumors.

TABLE 1. Analysis of A-MuLV and helper proviruses in DNA of A-MuLV-induced thymomas

Helper virus	Tumor	Fragments detected				
		A-MULV (*abl* Probe)	Helper MuLV			
			Internal		Junction	
			U_3 probe	gp70 probe	U_3 probe	gp70 probe
pN20-7	YS6-4	+	ND[a]	−	−	−
	YS6-6	+	ND	−	−	−
	YS6-15	+	ND	−	−	−
	YS6-33	+	ND	+	−	ND
	YS6-34	+	ND	−	−	ND
	YS6-35	+	ND	−	−	ND
	YS6-36	+	ND	−	−	ND
p7M1	YS6-1	+	+[b]	+[b]	−	−
	YS6-2	+	−	−	+[c]	−
	YS6-3	+	−	−	−	−
	YS6-5	+	+[b]	+[b]	+[b]	+[b]
	YS6-9	+	−	−	−	−
	YS6-10	+	+	+	+	+
	YS6-12	+	−	−	−	−
p7M2	YS6-8	+	−	−	−	−
	YS6-11	+	−	+[b]	−	−
	YS6-13	+	−	−	−	−
	YS6-14	+	−	−	−	−
	YS6-16	+	+	+[b]	−	−
	YS6-17	+	+[b]	+[b]	+[b]	+[b]
	YS6-18	+	−	−	−	−
	YS6-20	+	−	−	−	−
	YS6-26	+	−	−	−	−
	YS6-27	+	+	+	+[c]	+
	YS6-29	+	−	−	−	−
	YS6-30	+	+	+	+	+
Moloney	YS6-19	+	+	−	+[c]	−
	YS6-21	+	+	−	+	−
	YS6-22	+	+	−	+	−
	YS6-23	+	+	−	+	−
	YS6-24	+	+	−	+	−
	YS6-25	+	+	−	+[c]	−
	YS6-31	+	+	−	+[c]	−
	YS6-32	+	+	−	+	−
ψ2	YS11-1	+	ND	ND	−	−
	YS11-2	+	ND	ND	−	−
	YS11-3	+	ND	ND	−	−
	YS11-4	+	ND	ND	−	−
	YS11-5	+	ND	ND	−	−
	YS11-6	+	ND	ND	−	−
	YS11-7	+	ND	ND	−	−
	YS11-8	+	ND	ND	−	−
	YS11-9	+	ND	ND	−	−
	YS11-10	+	ND	ND	−	−

[a] ND, Not done.
[b] Submolar.
[c] Rearranged provirus.

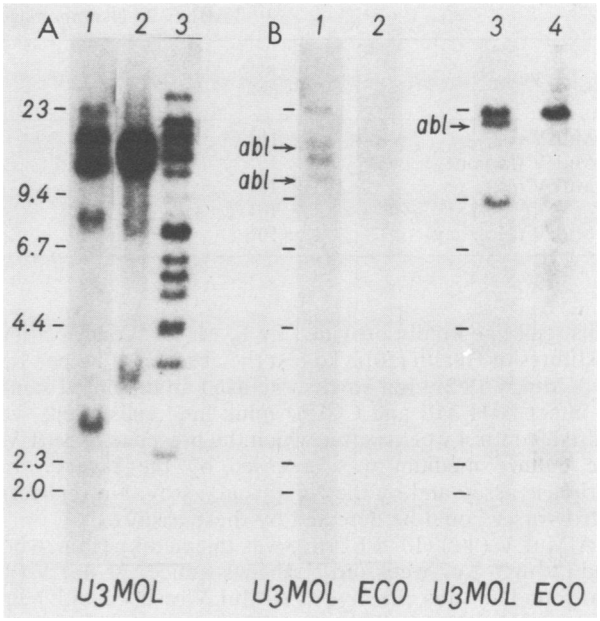


FIG. 5. Detection of rearranged proviruses in A-MuLV-induced thymomas. (A) DNAs of A-MuLV (Moloney)-induced thymoma YS6-19 (lane 1), YS6-25 (lane 2), and YS6-31 (lane 3) were digested with *Eco*RI and hybridized with the U_3 Mol probe. (B) DNAs from A-MuLV (p7M1)-induced thymoma YS6-2 (lanes 1 and 2) and A-MuLV (p7M2)-induced thymoma YS6-27 (lanes 3 and 4) were digested with *Eco*RI and hybridized with the U_3 Mol probe (lanes 1 and 3) or the eco probe (lanes 2 and 4). Arrows indicate fragments hybridizing to the *abl*-specific probe. Numbers at the left are lengths in kilobase pairs.

In A-MuLV-induced thymoma YS6-2 (p7M1 helper), four fragments were detected with the U_3 Mol probe, two of which hybridized with the v-*abl* probe, while the other two did not hybridize to either the v-*abl* or eco probe (Fig. 5B, lanes 1 and 2). Similarly, in thymoma YS6-27 (p7M2 helper), three fragments hybridized to the U_3 Mol probe. Two of them corresponded to an A-MuLV provirus and an intact helper provirus (fragments hybridizing to the v-*abl* and eco probe, respectively), while the third fragment hybridized to neither the v-*abl* nor the eco probe (Fig. 5B, lanes 3 and 4). These results could be explained by the presence of helper proviruses having sustained a deletion or recombination within their gp70 *env* region. Alternatively, the presence of A-MuLV proviruses having a deletion in the v-*abl* probe region could explain our findings. Analysis of A-MuLV (Moloney)-induced thymomas revealed that in three tumors (YS6-19, YS6-25, YS6-31), *Eco*RI fragments smaller than 6.1 kbp were detected with the U_3 Mol probe (Fig. 5A). Since Moloney MuLV or A-MuLV proviruses do not contain *Eco*RI sites, this suggests that Moloney MuLV or A-MuLV proviruses underwent rearrangements or deletions or both in these tumors.

Results from the analysis of proviruses present in A-MuLV-induced thymomas are summarized in Table 1. Although we cannot exclude the possibility that some tumors contain recombinant proviruses having deleted or rearranged both their LTR and gp70 region, our data show that more than one-half of the thymomas induced by A-MuLV pseudotyped with BALB/c ecotropic N-Cl-35 (pN20-7) MuLV or with chimeric p7M1 and p7M2 MuLVs did not contain helper proviruses. These results indicate that the presence of an intact helper provirus is not essential for tumor formation.

Oncogenic potential of helper-free A-MuLV. Since the helper virus requirement differs substantially between A-MuLV-induced pre-B- and T-cell lymphomas, we studied the lymphomagenic potential of helper virus-free A-MuLV. Stocks of helper-free A-MuLV were obtained from a cell line derived by transfection of the p160 A-MuLV DNA into Ψ2

TABLE 2. Lymphomagenesis by helper virus-free A-MuLV

Virus	Virus titer (FFU/ml)	Site of injection	Age of mice at injection	No. of diseased mice/no. of inoculated mice	Latency (days)	Tumor type (lymphoma)
A-MuLV (ψ2)	1×10^5	I.T.	5–7 wks	11/19	38–54	T cell
A-MuLV (Moloney)	2×10^4	I.T.	5–7 wks	9/18	47–77	T cell
A-MuLV (ψ2)	1×10^5	I.P.	<48 h	1/15	34	Pre-B cell
	1×10^4			0/8		
A-MuLV (Moloney)	1×10^4	I.P.	<48 h	11/12	21–36	Pre-B cell

cells (cell line kindly provided by P. M. C. Wong, National Institutes of Health [50]). To test the absence of helper virus in A-MuLV (Ψ2) viral stocks, we used an undiluted sample to infect NIH 3T3 and CCL64 mink lung cells. Cells were cultivated for 4 weeks, after which the presence of MuLV in the culture medium was assessed by the reverse transcriptase assay and by the XC plaque assay. No replicating retroviruses could be detected by these assays.

A-MuLV (Ψ2) (10^5 FFU/ml) was injected i.p. in newborn and i.t. in 5- to 7-week-old NIH Swiss mice. A-MuLV (Ψ2) was found to be as effective as A-MuLV rescued with helper Moloney MuLV in inducing T-cell lymphomas when injected i.t. (Table 2). In contrast, most newborn mice injected i.p. with A-MuLV (Ψ2) did not develop disease.

Analysis of the A-MuLV proviral integration pattern in thymomas induced by A-MuLV (Ψ2) showed the presence of one to two A-MuLV proviruses in each tumor (Table 1). No additional proviral sequences were detected with the U_3 Mol or eco probe, in agreement with the helper-free nature of the A-MuLV stock used (Table 1).

Analysis of *myc*, *Pim-1*, *Mis-1/pvt*, and *Ahi-1* loci in A-MuLV-induced thymomas. Helper MuLV proviruses have frequently been found to occupy common integration sites such as *myc*, *Pim-1*, and *Mis-1/pvt* in T-cell leukemia induced by various strains of nondefective MuLVs (8, 10, 25, 47) or such as *Ahi-1* in pre-B-cell lymphoma induced by A-MuLV (30). To determine whether these loci were involved in A-MuLV-induced thymomas in which helper proviruses were rarely found, we screened a series of DNAs from thymomas induced by A-MuLV pseudotyped with various helpers with probes for *myc*, *Pim-1*, *Mis-1/pvt*, and *Ahi-1*. A single rearrangement was detected in the *Mis-1* region of one tumor (YS6-25) induced by A-MuLV (Moloney). It was apparently caused by integration of a recombinant provirus and was clearly underrepresented, indicating that only a subpopulation of cells carried this *Mis-1/pvt* rearrangement. Only germ line fragments were detected by all the probes in the other tumors, and no rearrangement could be detected (data not shown). These results indicate that A-MuLV-induced thymomas harbor different sets of rearranged oncogenes or putative oncogenes than thymomas induced by nondefective MuLVs or than pre-B-cell lymphomas induced by A-MuLV.

Analysis of immunoglobulin and TcR loci in A-MuLV-induced thymomas. To characterize the nature of the malignant cells of the thymomas induced by A-MuLV (Ψ2) and A-MuLV rescued with different helper MuLVs in NIH Swiss mice, we examined the immunoglobulin heavy- and κ-chain genes as well as the TcR β- and γ-chain genes for evidence of rearrangements.

Analysis of the immunoglobulin heavy-chain gene was performed on *Eco*RI-digested tumor DNAs with a J_H specific probe. Nearly 50% of thymomas showed rearrangement of the J_H region, usually of only one allele. A representative example is shown in Fig. 6A. In some tumors, the underrepresentation of the rearranged allele over the germ line allele indicated that only a subpopulation of cells in these tumors have rearranged their J_H region. Analysis of *Eco*RI-digested tumor DNAs with a C_k probe showed no rearrangement at the κ locus in all 44 tumors tested except 1 (YS6-34) (Fig. 6B, lane 4).

The TcR β region was examined with a C_β probe on *Hin*dIII-digested DNAs. All thymomas showed rearrangement or deletion or both at the TcR β locus, usually involving both alleles (Fig. 6C). The apparent deletion of TcR β sequences in these thymomas could reflect continuous rearrangements occurring at random at this locus following transformation. To rule out this possibility, we digested a subset of thymoma DNAs with another restriction endonuclease, *Eco*RI, and hybridized them with the C_β probe. This analysis allowed the detection of the TcR β constant region on a 2.8-kbp fragment which does not include the J region. No TcR β sequences were revealed by this analysis (data not shown), thus confirming that deletion had occurred in these thymomas.

Analysis of the γ region with a V-J-C γ probe on *Eco*RI-digested DNAs revealed that all thymomas had this region rearranged (Fig. 6D). The majority of tumors had lost the germ line 10.5- and 5.7-kpb fragments and had gained novel 4.5- and 16-kbp fragments, while only one tumor (YS6-34, lane 4) contained a novel 22-kbp fragment. This pattern of bands is consistent with the type of rearrangements most frequently found in functional T cells as well as in thymomas, namely, rearrangements at the γ_1 (16 kbp, V plus C), γ_3 (4.5 kbp, V), and γ_4 (22 kbp, C) loci (16, 20, 23, 46).

A summary of the analysis of immunoglobulin and TcR locus rearrangements is shown in Table 3. The results indicate that the majority of cells transformed in these A-MuLV-induced thymomas belongs to the T-cell lineage. The transformed cells of thymoma YS6-34 cannot at this point be assigned to the B- or T-cell lineage, having rearrangements at the J_H, κ, and TcR β and γ loci. Finally, no significant difference in the frequency and type of immunoglobulin and TcR gene rearrangements was detected between thymomas induced by A-MuLV rescued by different helper viruses or by helper-free A-MuLV.

DISCUSSION

Role of helper MuLV in pre-B- and T-cell transformation by A-MuLV. Previous studies have shown that the choice of helper virus is critical in the induction of A-MuLV-induced pre-B-cell lymphoma (37, 39, 40). The U_3 LTR region of the helper virus was shown to harbor an important determinant involved in the development of these lymphomas (39). The present study demonstrates that thymoma induction by A-MuLV is not influenced by the choice of helper virus. Indeed, all four replicating helper MuLVs tested and the

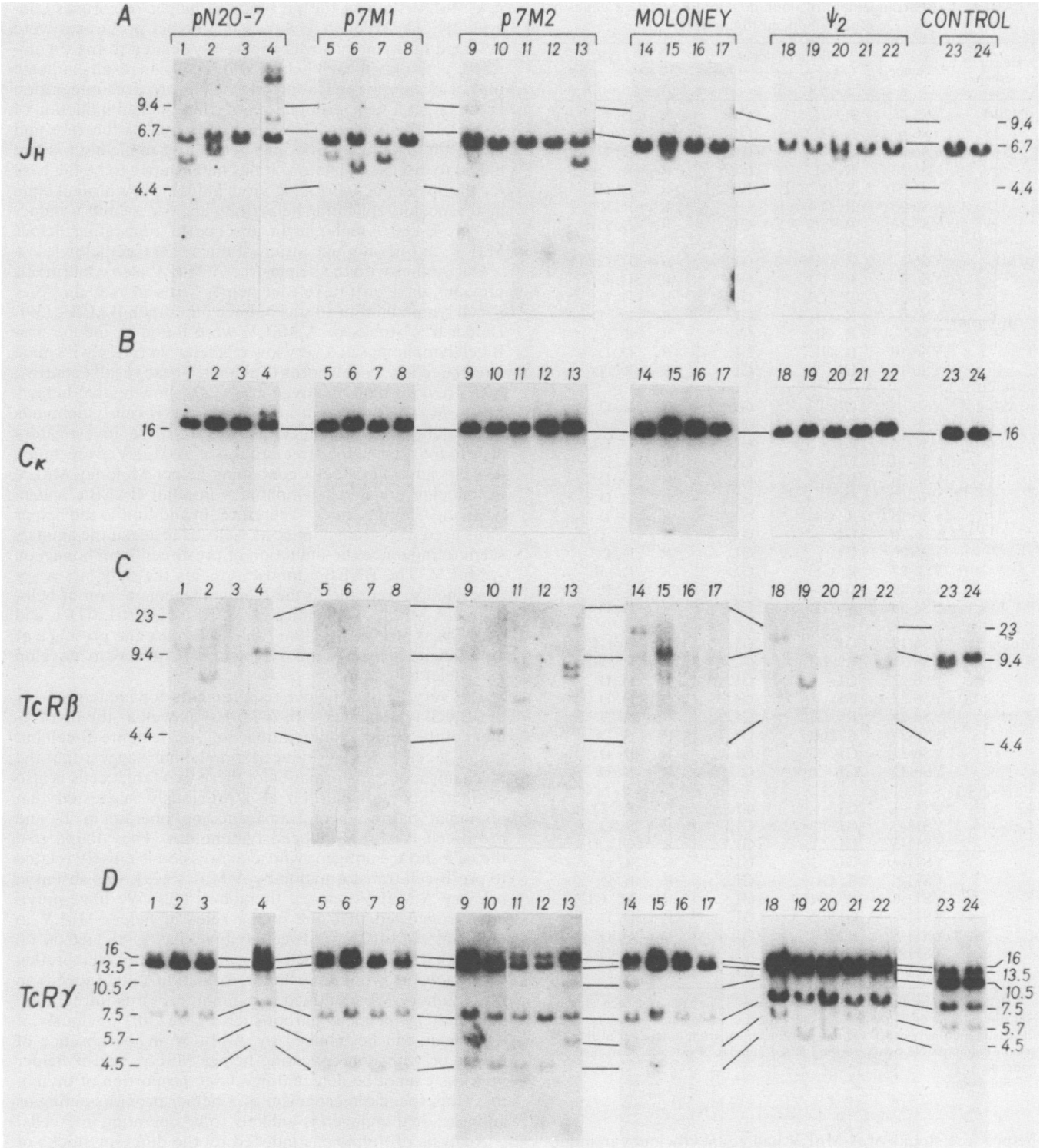


FIG. 6. Southern blot analysis of the immunoglobulin and TcR loci in A-MuLV-induced thymomas. DNAs were digested with *Eco*RI (A, B, and D) or *Hin*dIII (C) and hybridized with a J_H-specific probe (A), a κ-chain constant-region probe (B), a TcR β-chain constant-region probe (C), and a TcR γ_1-chain (V-J-C) probe (D). Lanes: 1 to 4, DNAs from A-MuLV (pN20-7)-induced thymomas YS6-4, YS6-6, YS6-15, and YS6-34, respectively; 5 to 8, DNAs from A-MuLV (p7M1)-induced thymomas YS6-3, YS6-5, YS6-10, and YS6-12, respectively; 9 to 13, DNAs from A-MuLV (p7M2)-induced thymomas YS6-16, YS6-17, YS6-20, YS6-26, and YS6-27, respectively; 14 to 17, DNAs from A-MuLV (Moloney)-induced thymomas YS6-19, YS6-21, YS6-22, and YS6-23, respectively; 18 to 22, DNAs from A-MuLV (Ψ2)-induced thymomas YS11-1, YS11-2, YS11-8, YS11-9, and YS11-10, respectively; 23 and 24, control brain DNAs from mice YS6-1 and YS6-2, respectively. Note that in NIH Swiss mice, the β-chain TcR locus is polymorphic. Owing to the lower intensity of the shorter fragments of TcR γ, overexposure was necessary. This led to an apparent loss of resolution of the 16.5- and 13.5-kpb bands, which appear in some lanes as a single thick band. These were well resolved on the original film after a shorter exposure. Numbers to the sides of the gels are lengths in kilobase pairs.

TABLE 3. Rearrangement of immunoglobulin and TcR genes in A-MuLV-induced thymomas

Helper virus	Tumor	Immunoglobulin[a]		TcR[a]	
		J_H	κ	γ	β
pN20-7	YS6-4	R, GL	GL	R	D, GL[b]
	YS6-6	R, GL	GL	R	R[b], D
	YS6-15	GL	GL	R	D, D
	YS6-33	R, GL	GL	R	R, D
	YS6-34	R, R[b], GL	R[b], GL	R	D, GL
	YS6-35	R, D	GL	R	R, R[b]
	YS6-36	R, GL	GL	R	D, D
p7M1	YS6-1	R, GL	GL	R	D, GL[b]
	YS6-2	R[b], GL	GL	R	D, D
	YS6-3	R[b], GL	GL	R	D, D
	YS6-5	R, GL	GL	R	R[b], D
	YS6-9	R, GL	GL	R	R, R
	YS6-10	R, GL	GL	R	D, D
	YS6-12	GL	GL	R	R[b], D
p7M2	YS6-8	GL	GL	R	R, D
	YS6-11	GL	GL	R	R[b], R[b], GL[b]
	YS6-13	GL	GL	R	D, GL[b]
	YS6-14	GL	GL	R	R, D
	YS6-16	R[b], GL	GL	R	D, D
	YS6-17	GL	GL	R	R, D
	YS6-18	R, GL	GL	R	D, D
	YS6-20	GL	GL	R	R[b], GL[b]
	YS6-26	GL	GL	R	D, D
	YS6-27	R, GL	GL	R	R, GL
	YS6-29	GL	GL	R	R, R
	YS6-30	R, GL	GL	R	D, D
Moloney	YS6-19	GL	GL	R	R, GL[b]
	YS6-21	R[b], GL	GL	R	R[b], GL
	YS6-22	GL	GL	R	D, D
	YS6-23	GL	GL	R	D, D
	YS6-24	R[b], GL	GL	R	R, D
	YS6-25	R[b], GL	GL	R	R, D
	YS6-31	GL	GL	R	D, D
	YS6-32	GL	GL	R	D, D
ψ2	YS11-1	GL	GL	R	R[b], D
	YS11-2	GL	GL	R	R, D
	YS11-3	GL	GL	R	GL, D
	YS11-4	GL	GL	R	R, D
	YS11-5	R, GL	GL	R	R, D
	YS11-6	R[b], GL	GL	R	R[b], GL[b]
	YS11-7	GL	GL	R	D, D
	YS11-8	R[b], GL	GL	R	D, D
	YS11-9	GL	GL	R	D, D
	YS11-10	GL	GL	R	R, D

[a] R, Rearranged; GL, germ line; D, deleted. The status of each chromosome at each locus is indicated, except for the TcR gamma (γ) locus, where the distinction between a second rearrangement from a deletion of the TcR region is impossible owing to the limited number of possible rearrangement patterns.

[b] Submolar.

helper-free stocks of A-MuLV had equal efficiency in inducing A-MuLV thymomas, despite their distinct ability to replicate in the thymus. The BALB/c endogenous N-tropic N-Cl-35 (pN20-7) helper MuLV, which by itself was unable to replicate in the thymus, was as efficient as the highly thymotropic Moloney MuLV in inducing thymomas when complexed with the A-MuLV. Moreover, the majority of tumors induced with stocks containing helper virus pN20-7, p7M1, or p7M2 showed no helper proviral sequences. Only thymomas induced by A-MuLV rescued with helper Moloney MuLV showed the presence of helper proviruses consistently. The frequency with which helper proviruses were detected in thymomas was apparently related to the thymotropic potential of each helper MuLV. These results indicate that helper virus replication and helper provirus integration in the target T cells is not necessary for efficient induction of A-MuLV thymomas. This constitutes one of the rare and interesting examples in which retrovirus replication is not linked to disease induction. It has been shown to be the case for the spleen focus-forming virus inducing erythroleukemia in absence of replicating helper (49) and for A-MuLV inducing pre-B-cell lymphoma in absence of replicating helper MuLV, but in only one strain of mice (19) (see below).

Our studies with the helper-free A-MuLV also confirm our previous data on the role of helper virus in inducing pre-B-cell lymphomas in strains of mice other than BALB/c (39). Helper-free stocks of A-MuLV were found to induce pre-B-cell lymphomas at a very low efficiency in NIH Swiss mice inoculated i.p. as newborns (Table 2). These results contrast with previous data by Green et al. (19) showing that helper-free A-MuLV is efficient in inducing pre-B-cell lymphomas in weanling BALB/c mice. However, these investigators also found that helper-free stocks of A-MuLV were much less efficient than stocks containing helper Moloney MuLV in inducing pre-B-cell lymphomas in adult BALB/c and in weanling C57BL/6 mice. Therefore, in addition to the helper virus used, the strain of mice as well as the age of the animals seem to influence the induction of pre-B-cell lymphomas by A-MuLV. The BALB/c mouse remains the only strain yet described susceptible to the lymphomagenic action of helper-free A-MuLV, while mice of C57BL/6, SWR/J, CD-1, and NIH Swiss strains (19, 39) (Table 2) require the presence of an efficient helper pseudotyping the A-MuLV to develop pre-B-cell lymphoma.

The very distinct helper requirements for inducing T- or pre-B-cell lymphomas with A-MuLV as well as the involvement of the common integration site, *Ahi-1*, in pre-B-cell but not in T-cell A-MuLV-induced lymphomas suggest that the mechanism of transformation of these two target cells is also distinct. Scott et al. (42) also previously suggested that dissimilar pathways of transformation operate in T- and pre-B-cell A-MuLV-induced lymphomas. They found that the 6C3 surface antigen, whose expression is closely related to pre-B-cell transformation by A-MuLV (29), was absent in primary A-MuLV-induced thymomas (42). We have previously suggested that one of the roles of helper MuLV in inducing A-MuLV pre-B-cell lymphomas is to act as an insertional mutagen to activate cellular genes collaborating with v-*abl* in promoting tumor formation (39). Indeed, in these tumors, we identified a common provirus intregration site (*Ahi-1*) which may harbor such a gene (30). Obviously, if thymomas can be induced by A-MuLV in the absence of helper or with nonreplicating helper MuLV, and if helper provirus cannot be detected in a large proportion of thymomas, this specific mechanism of a helper provirus acting as an insertional mutagen is unlikely to be operating in T cells.

Analysis of thymomas induced by the different stocks of A-MuLV showed that all thymomas appear oligoclonal with a number of probes (*abl*, U_3 Mol, J_H, TcR β, TcR γ) and that all contained one or a few A-MuLV proviruses, suggesting that v-*abl* was essential for tumor induction but not sufficient to fully transform these cells, assuming that a large number of target cells were initially infected. This assumption seems reasonable since some of the helper MuLVs used to pseudotype A-MuLV can infect up to 10 to 20% of thymic cells (Fig. 2) as soon as 20 days postinfection. Therefore, other

genetic events (second hit) may be required for complete transformation of these target cells or to favor outgrowth of some clones. These putative genetic events do not appear to involve *myc*, *Pim-1*, or *Mis-1/pvt*, previously found to be rearranged frequently in thymomas induced by nondefective MuLVs (8, 10, 25, 47), or *Ahi-1*, found rearranged in 16% of A-MuLV-induced pre-B-cell lymphomas (30). A higher target cell population or a higher mutation rate of target T cells or both could provide conditions for spontaneous activation of other loci yet to be identified. Alternatively, all stocks of A-MuLV, including helper-free stocks, may contain other retroviral sequences such as those of the IAP or VL30 family. These would be reverse transcribed, and their proviruses could act as insertional mutagens, presumably more efficiently in T cells than in pre-B cells. The Ψ2 helper-free cell line has been shown to produce virus particles containing VL30 RNA, which was reverse transcribed and produced a provirus (34). Whatever the mechanism of activation of these putative loci favoring outgrowth of specific clones in the presence of v-*abl*, they are likely to be distinct from those identified in thymomas induced by nondefective MuLVs (*myc*, *Pim-1*, *Mis-1/pvt*) or in A-MuLV-induced pre-B-cell lymphomas (*Ahi-1*).

Target cells in thymomas induced by A-MuLV. The analysis of the immunoglobulin and TcR loci in our primary A-MuLV-induced thymomas indicated that the majority of cells constituting the tumor mass of most thymomas belong to the T-cell lineage. All thymomas showed rearrangements at both TcR β and γ loci, while having frequent immunoglobulin heavy-chain locus rearrangements and, with one exception, no κ-chain locus rearrangement. Rearrangement of the immunoglobulin heavy-chain locus has frequently been found in T-cell lines (9, 15, 24), including T-lymphoma lines derived from A-MuLV-induced thymomas (7). In contrast, TcR β and γ rearrangements can be found only rarely in B cells (45). However, the submolar J_H rearrangements detected in some tumors may reflect the presence of a subpopulation of transformed pre-B cells. Scott et al. (42) have previously observed that in some A-MuLV-induced thymomas, a fraction of transformed cells stained positively for the surface antigen B220, a marker of early B cells, thus suggesting the presence of pre-B cells in these tumors. Together, our results confirm earlier data on the nature of primary thymomas induced in C57BL/Ka mice by A-MuLV pseudotyped with Moloney MuLV (42).

We did not detect significant differences in the presence of various markers among thymomas induced with the different A-MuLV pseudotypes or with helper-free A-MuLV, indicating that the helper virus used to pseudotype the A-MuLV did not affect markedly the choice of target cells transformed. This result appears to contrast with data of Cook (6) claiming that the helper Moloney MuLV or radiation leukemia virus influences the subtype of lymphoid cells, respectively, the medulla and the cortical cells, which can be established in vitro from A-MuLV-induced thymomas. In fact, all the Thy-1^- cell lines studied were induced by either virus, and among the small number of Thy-1^+ cell lines in each group which was examined, a significant overlap of the values of each marker was documented, raising the possibility that other variables, besides the helper, might have influenced the establishment of these cell lines in vitro. Moreover, later work with molecular probes specific for cell lineages did not document any significant difference between cell lines derived from A-MuLV-induced tumors induced with Moloney MuLV or radiation leukemia virus helper (7).

Several cell lines have previously been established from thymomas induced by A-MuLV pseudotypes (6, 7, 11, 42). The molecular and biological characterization of these cell clones indicates that they belong to a cell lineage different from that of the majority of cells present in primary thymomas (42). As suggested previously (5, 42), they probably constitute a minority of cells of the thymomas which have a selective growth ability in vitro. The cells constituting the major population of the tumor mass of these thymomas seem difficult to establish in culture. Occasionally, a unique cell population is present in a greater proportion of the tumor mass, such as in tumor YS3-34, and shows a phenotype (J_H, TcR β, TcR γ, and C_K rearrangements) resembling the phenotype of thymoma cell lines previously reported (6, 7, 42). Together, these results clearly indicate that cell lines established from A-MuLV-induced thymomas rarely reflect the major cell population of the primary tumors.

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