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EVIDENCE OF RECOMBINANT ECOTROPIC PROVIRUS INTEGRATION IN THYMIC LYMPHOMAS INDUCED BY DIRECT OR INDIRECT RADIATION EFFECTS

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Abstract—Several investigators described the occurrence of ecotropic recombinant proviruses in the DNA of *in-vivo* or *in-vitro* propagated radio-induced lymphomas, but such proviruses were never detected in primary tumors. To assess their biological significance in the tumorigenic process, we reinvestigated the presence of new proviruses chiefly in primary radio-induced tumors and in models of radioleukemogenesis which could give additional support for their role. Such models included thymic lymphomas originating after (i) graft of non-irradiated thymuses in thymectomized irradiated mice and (ii) the injection of a B-ecotropic retrovirus (T1223/B) in association with a subleukemogenic dose of irradiation. We report for the first time that new ecotropic proviral sequences are encountered in a significant number (30%) of primary lymphomas induced directly by irradiation or indirectly in non-irradiated thymuses grafted in irradiated hosts. The existence of a 3.5-kbp Kpn1 restriction fragment with ecotropic sequences in the digested DNA of these tumor cells indicates that these new sequences belong to an ecotropic provirus recombinant in the *gag-pol* region. We observed that most of the primary radio-induced tumors in which novel recombinant provirus could be detected, displayed the integration at a single or at a few sites, demonstrating their clonality with respect to viral integration. The same was observed in thymic lymphomas arising after T1223/B virus injection and irradiation and in *in-vivo* or *in-vitro* propagated tumors. Altogether, these data bring the first evidence of the integration of ecotropic recombinant proviral genomes in a significant number of primary radiation induced thymic lymphomas and of their possible role in view of their frequent occurrence in grafted thymomas.

Key words: Thymic lymphomas, radiation, ecotropic provirus.

INTRODUCTION

WHEN DELIVERED according to the protocol of Kaplan and Brown [1], fractionated doses of X-irradiation, i.e. 4 × 1.75 Gy at weekly intervals, induce a high rate (over 90%) of T-cell lymphomas in C57BL/6 or C57BL/Ka mice, whereas such tumors occur spontaneously in less than 5% of non-treated animals. Several lines of evidence suggest that retroviruses could play a role in radioleukemogenesis. Indeed, murine leukemia viruses (MuLV) were detected in cell-free extracts of radio-induced thymic lymphomas [2–5] as well as in established *in vitro* cell lines [6, 7]. Some of these retroviruses (termed "radiation leukemia virus", or RadLVs) were shown to initiate a similar type of disease upon injection into non-irradiated mice. Furthermore, non-irradiated thymuses could become neoplasic upon graft into thymectomized irradiated hosts [8], and conversely immunization of C57BL/6 mice with B-ecotropic retrovirus significantly reduced the incidence of radiogenic thymomas [9, 10]. Thus the hypothesis according to which radiation is not tumorigenic by itself but acted via the activation of endogenous retrovirus sequences was generally accepted. However, it was also shown that X-irradiation could induce lymphomas in mouse strains lacking endogenous ecotropic viruses [11], and MuLV expression was seldom detected in primary tumors [12, 13].

Abbreviations: Gy, Gray; *MuLV*, murine leukemia virus, *MCF*, mink cell focus forming virus.

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Besides, Southern blot analyses did not reveal novel ecotropic recombinant proviruses integrated in the DNA of primary lymphoma cells, even though retrovirus was frequently observed after *in-vivo* or *in-vitro* propagation of the cells [14]. These retroviruses, *gag-pol* ecotropic recombinants, exerted a weak leukemogenic activity in mice after a longer latency period than that observed in the case of radiation-induced lymphomagenesis [15, 16].

Apart from the results of Haas and Jongstra [17] which as far as we know have yet to be confirmed, all investigations aimed at the detection of mink cell focus-forming (MCF) dual tropic viruses in radiation-induced tumors of C57BL mice gave negative results. Therefore the search for the only putative etiological candidates observed so far, i.e. the *gag-pol* recombinant ecotropic retroviruses, seems perfectly logical.

One possible explanation of the contradictory data cited above is that some B-ecotropic recombinant retroviruses might transform only thymic cells of a given compartment generated or selected in irradiated animals.

On the basis of this working hypothesis, we reinvestigated the role of such a type of retroviruses in a model in which we combined the injection of a B-ecotropic recombinant retrovirus and a subleukemogenic dose of irradiation (2×1.75 Gy). The virus (T1223/B) was isolated from RadLV/Rs induced tumors [18]. It does not induce any lymphoma within the time required for radiation tumorigenesis (6 months), although 80% of the inoculated mice develop extrathymic lymphomas after a latency period exceeding 500 days [19]. Injected in association with a subleukemogenic irradiation, it resulted in a 30% incidence of thymic lymphomas [20]. It should be added that in the protocol of Kaplan *et al.* [21], the radiation induced leukemogenic process is abrogated by bone marrow restoration. The same was observed in our model associating virus and irradiation. This contrast with the lack of inhibitory effect of bone marrow cells on RadLV induced leukemogenesis. Altogether, these observations strongly support that, at least in these experimental conditions, certain retroviruses and radiation may cooperate for thymic lymphoma induction.

Following injection of T1223/B into animals in combination with irradiation, the appearance of its provirus in the DNA of the subsequently arising tumor cells is a logical expectation. However, a clonality of the tumors with respect to the viral integration site(s) would suggest that the provirus was inserted early in the cell transformation and that it played a role in the leukemogenic process. Previous experiments have shown that an ecotropic recombinant provirus was indeed present in the chromosomal DNA of every tumor induced by the combined protocol, although in an apparently random fashion [22]. This result was gained by using an ecotropic molecular probe excised from Friend MuLV [23]. Due to the distant homology between this probe and the endogenous proviral sequences of the C57BL/6 mouse, only a weak hybridization signal could be observed. We thus reinvestigated the presence of new proviral sequences by using a more homologous probe, i.e. a segment of the AKV MuLV *env* gene (pEC-B4) cloned by Chan *et al.* [24].

It seemed relevant to perform such experiments not only on thymic lymphomas induced by the combined effect of virus and irradiation, but also on tumors induced by irradiation alone. The latter model offers the additional possibility of approaching the indirect mechanism of tumor induction. Indeed it has been repeatedly demonstrated that non-irradiated thymic cells may become neoplasic after transplantation into an irradiated recipient animal, even when the latter had been surgically deprived of its thymus. This suggests that a "diffusible leukemogen" [25] is implied in radio-leukemogenesis, the targets of which are the grafted thymic cells. It could be either a retrovirus or a cellular factor. A strong support for a retroviral causality of radiation induced leukemogenesis would of course be the detection of provirus integration in the lymphomas having developed from the grafted cells.

The experiments reported below give the first evidence of the integration of ecotropic recombinant proviral genomes in a significant number of primary radiation induced thymic lymphomas and of its possible role in view of its frequent occurrence in grafted thymomas. Furthermore, tumors arising after either irradiation alone or following a combined effect of virus and irradiation were often shown to be clonal or oligoclonal with respect to viral integration.

MATERIALS AND METHODS

Animal models

Inbred C57BL/6 and C57BL/Ka mice were maintained in our own facilities. In order to be able to distinguish donor from recipient cells in grafting experiments, we used C57BL/Ka/Thy-1.1/Lb mice, congenitally derived from C57BL/Ka but bearing the Thy-1 gene (which codes for a lymphocyte cell membrane antigenic determinant) in the AKR-derived Thy-1.1 instead of the Thy-1.2 allotype form. These mice were originally obtained from Dr M. Lieberman (Department of Radiology, Stanford University School of Medicine, CA, U.S.A.).

Cell lines and viruses

Isolated from RadLV-Rs induced tumors [26], the T1223/B virus was biologically cloned and propagated in

normal C57BL/6 thymic epithelial TAC7 cells, as described previously [18]. Like most of the B-tropic viruses isolated from C57BL/6 mice [27], the T1223/B virus is a recombinant between endogenous ecotropic and xenotropic sequences of the mouse [22, 28].

Lymphoma induction, transplantation and cultivation.

Thymic lymphomas were induced in 30-day old C57BL/6 mice either by four 1.75 Gy X-irradiation at weekly intervals, or by two 1.75 Gy irradiations at one-week interval prior to or after injection of T1223/B virus [20]. *In-vivo* transplanted tumors were obtained by injecting intravenously 10^6 primary lymphoma cells into C57BL/6 mice. In these conditions, T-lymphoma cells grew preferentially in the spleen.

The indirect lymphoma induction protocol was as follows: 4–6-week old C57BL/Ka/Thy-1.1/Lb were surgically thymectomized and 2–4 weeks later, submitted to the first of the four weekly 1.75 Gy irradiations. After they had received the last irradiation, they underwent on the same day subcutaneous implantations of 2–6 thymuses, freshly collected from 1 to 48-h old C57BL/Ka newborns. Transplantations were done by implanting subcutaneously a fragment of the primary tumor (or, occasionally, of an invaded tissue) into C57BL/Ka mice. Cell cultures were established according to Lieberman *et al.* [29], with a slight modification of the culture medium: RPMI 1640 medium was supplemented with 10% fetal calf serum, 10^{-5} M 2-mercaptoethanol, 1 mM Na pyruvate and MEM-non-essential amino-acids (Gibco BRL).

DNA analysis

High molecular weight DNA was extracted from normal and from tumor tissues as described by Maniatis *et al.* [30]. Ten-microgram samples of DNA were cleaved with restriction enzymes under the conditions recommended by suppliers (Boehringer Mannheim or Gibco BRL). In part of the work, Asp718 was used instead of Kpn1. The two enzymes recognize the same sequence GGTACC but cleave it at a different site. After electrophoresis in 0.8% agarose gels, the DNA restriction fragments were blotted into either Hybond N Nylon membranes (Amersham) or Gene Screen Plus membranes (Du Pont, NEN products) and hybridized with a ^{32}P-labelled probe as described previously in the case of Hybond N [22] or according to the user's manual in the case of Gene Screen Plus. The ecotropic-specific *env* probe was a 0.4-kbp BamHI-SmaI fragment of the AKV *env* gene, obtained from the plasmid pEC-B4 [24] which was kindly supplied by Dr Malcolm Martin (NIH, Bethesda, MD, U.S.A.). The DNA probe was labelled with ^{32}P by using the Multiprime DNA Labelling System from Amersham.

RESULTS

(a) *Presence of ecotropic recombinant proviruses in radio-induced thymic lymphomas*

To search for new ecotropic recombinant provirus in tumor DNA, Kpn1 restriction fragments were analysed by the Southern procedure and hybridized with the *env* ecotropic specific probe. In 11 control spleen and thymus DNA of C57BL/6 or C57BL/Ka mice analysed, this probe detected only a 4.0-kbp restriction fragment (Fig. 1) corresponding to the single endogenous N-ecotropic MuLV provirus. All the B-ecotropic recombinant viruses isolated so far from the C57BL/6 mouse have an additional Kpn1 site in their genome [15, 31, 32]. Thus, another internal Kpn1 fragment (3.5 kbp) is recognized by the *env* ecotropic probe in cells infected with such viruses. DNAs from 35 primary or transplanted radio-induced tumors were analysed. A 3.5-kbp Kpn1 ecotropic fragment was detected in five out of 18 primary tumors and in seven out of 16 transplanted tumors (Table 1 and Fig. 1A). It is noticeable that the intensity of the band observed was weak in three out of the five primary tumors as compared with that of the endogenous virus (Table 1, tumors Nos 3647,

TABLE 1. ADDITIONAL ECOTROPIC MuLV SEQUENCES IN THYMIC LYMPHOMAS INDUCED BY LEUKEMOGENIC DOSE OF IRRADIATION (4 × 1.75 Gy)

Tumor No.	*In vivo* passage No.	Size of additional fragments		
		Kpn1	HindIII	EcoR1
3532	0	—*	—	—
3533	0	—	—	—
3534	0	—	—	—
3540	2	—	—	—
3547	0	—	—	—
3550	2	—	—	—
3555	0	—	—	—
3647	0	3.5	13.5	—
3651	3	3.5	14.5	13
3656	1	—	—	—
4065	10	3.5	14.5	13
4073	10	3.5	14.5	13
4080	0	—	—	—
4081	5	3.5	14.5	14
4088	0	3.5	—	—
5060	0	—	—	—
5062	0	—	—	—
5063	0	3.5	14.5	20;13
5063	3	3.5	17;14.5	13;6.2
5064	0	—	8.6	8.6;5;4.5
5064	2	—	18	21.5
5065	0	3.5	13	20
5065	2	3.5	22	17
5068	4	—	—	—
5069	0	—	—	—
5069	4	—	—	—
5070	0	—	—	—
5070	3	—	—	—
5072	0	—	—	—
5072	2	—	—	—
5073	0	ND†	—	—
5073	3	—	—	—
5199	0	3.5	—	—
5199	4	3.5	—	—

* "—" means no visible band.
† ND means not done.

4088, 5063 and Fig. 1A, lane 1). This suggests that the new recombinant virus is integrated in a limited number of cells per tumor. This contrasts with the transplanted tumors in which, the intensity of the 3.5-kbp band, when present, indicated that an ecotropic recombinant provirus is integrated in a high percentage of cells of the corresponding tumor. Interestingly enough, in every case ($n = 8$) in which the primary and the corresponding transplanted tumors were analysed, both were identical with regard to the presence (or absence) of the 3.5-kpb band.

These experiments show that in radio-induced tumors (whether primary or transplanted) a new ecotropic recombinant retrovirus is observed in a significant number of instances. This contrasts with the results of Jolicoeur *et al.* [14] who also observed a new ecotropic recombinant retrovirus but only after *in-vivo* or *in-vitro* propagation of radio-induced thymic lymphomas.

Because a new ecotropic recombinant retrovirus is encountered in a significant number of radio-induced thymic lymphomas, one may assume that it plays a role in the neoplastic transformation. In view of the genomic organization of the related retroviruses known so far [16], such a virus could become pathogenic via its integration in a critical domain of the cellular genome. Such a mechanism implies that the cells of a tumor are clonal and harbor a new provirus integrated at the same site. Consequently, in the tumor DNA digested with restriction enzymes which do not cleave (or cleave only once) the genome of the B-ecotropic retroviruses, one may expect to detect ecotropic sequences in new restriction fragments. DNAs from radiation-induced thymic lymphomas were digested by restriction enzymes HindIII or EcoR1. In control C57BL/6 and C57BL/Ka mice DNA digested with these enzymes, the *env* ecotropic probe hybridized with a 7.0-kbp (HindIII) and a 25-kbp (EcoR1) fragment corresponding to the endogenous N-ecotropic retrovirus (Figs 1B and C).

The results presented in Table 1 and Figs 1B and C indicate that in tumors in which a new ecotropic recombinant retrovirus is integrated, additional HindIII or EcoR1 restriction fragments with ecotropic sequences are nearly always detected. Indeed, in DNAs of the seven out of nine such tumors, one or two additional HindIII or EcoR1 restriction fragments with ecotropic sequences were detected. With respect to tumors 4088 and 5199 (Figs 1B and C, lanes 1 and 2) one may speculate that the lack of visible HindIII and EcoR1 restriction fragments may reflect a random integration of the new provirus. Besides, the negative results obtained with tumor 4088 may be due to technical limitations in view of the low percentage of tumor cells harboring a new recombinant provirus as suggested by the weakness of the 3.5-kbp Kpn1 restriction fragment described above. Apart for these two tumors for which more information is required, our results demonstrate the clonal or oligoclonal nature of the radio-induced thymic lymphomas with respect to the integration of a recombinant B-ecotropic retrovirus. It could be objected that the additional ecotropic sequences observed after HindIII or EcoR1 digestion do not belong to a recombinant ecotropic provirus. In a preliminary attempt to clarify this, the 13-kbp EcoR1 restriction fragment of tumor 4081 was purified by glycerol gradient. It was found that it contained a proviral genome with restriction sites identical to that of ecotropic recombinant retroviruses of the C57BL/6 mouse (data not shown).

It should be mentioned that in a tumor such as 5064, additional HindIII and EcoR1 fragments were detected in the absence of a 3.5-kbp Kpn1 restriction fragment. This means that this tumor contains either a reinserted N-ecotropic endogenous retrovirus or a recombinant ecotropic provirus lacking the specific Kpn1 restriction site.

(b) *Presence of ecotropic recombinant proviruses in indirectly induced thymic lymphomas*

Using the *env* ecotropic-specific probe in the Southern procedure, we searched for the presence of new ecotropic recombinant proviruses in the DNA of 19 primary lymphomas having developed in non-irradiated thymuses that had been implanted in thymectomized, irradiated recipient mice (Fig. 2 and Table 2). Four of those lymphomas (BLGM7, FG2, MG7, BLGM12) showed, in addition to the 4.0-kbp Asp718 restriction fragment of the single endogenous N-ecotropic MuLV provirus, the presence of the novel diagnostic 3.5-kbp Asp718 fragment. In three out of the four cases, the intensity of the hybridization signal was weaker than that of the endogenous provirus, strongly suggesting that the new recombinant provirus was integrated in only a limited proportion of the cells. In three of the provirus-positive tumors (BLGM7, FG2, MG7), the absence of detectable novel HindIII and EcoR1 fragments reflected the lack of a common integration site shared by the cells of these tumors. However, the absence of detectable hybridization signals could also be due to technical limitations, in view of their possible weakness. In contrast, one tumor (BLGM12) was clonal with respect to the proviral integration site, as suggested by the occurrence of a novel 8.0 kbp HindIII fragment in a non-negligible proportion of the cells. An alternative explanation could be that the novel provirus might contain two HindIII sites separated by a 8.0-kbp distance. The same reasoning can be

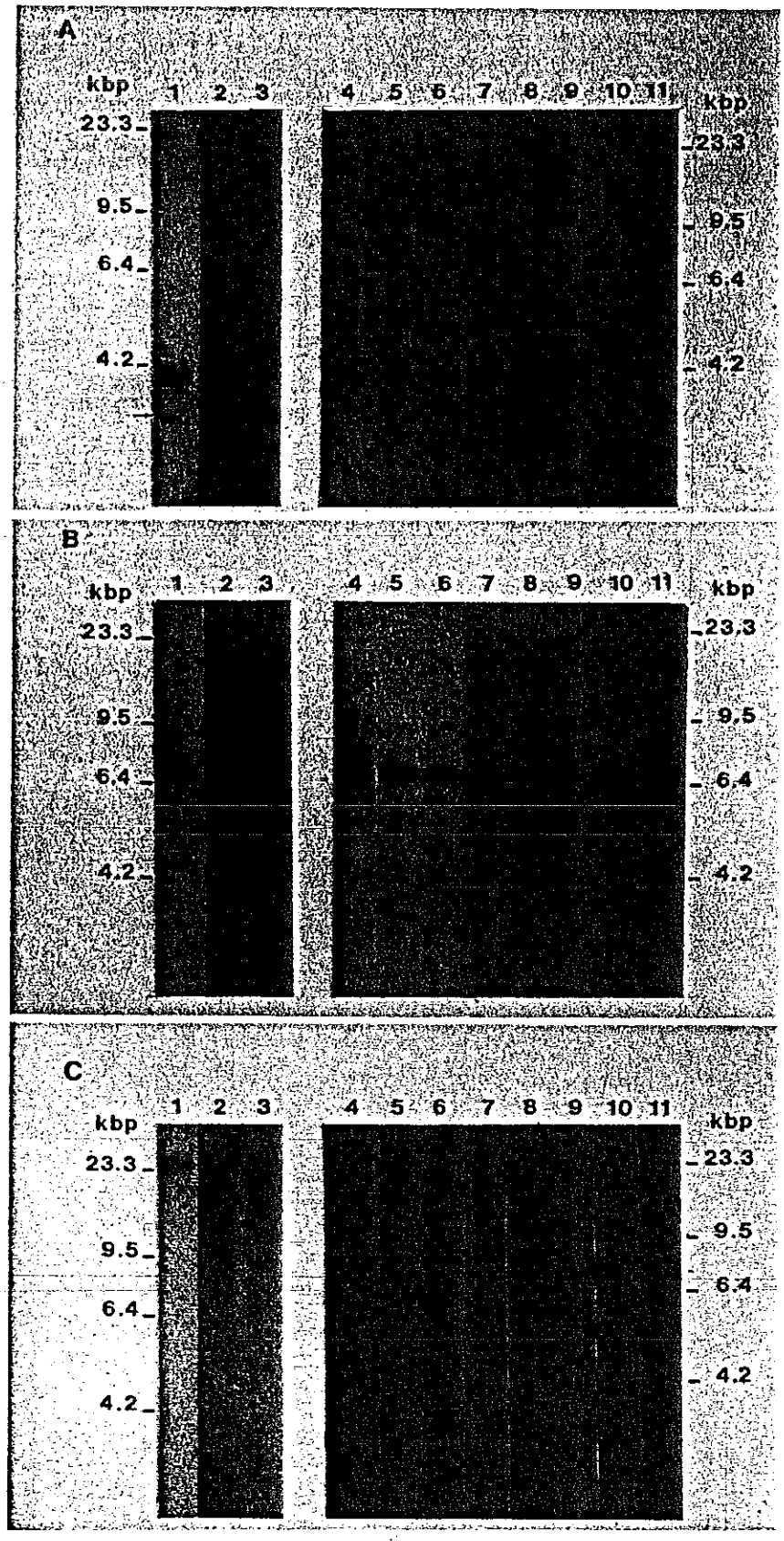

FIG. 1. Restriction pattern of ecotropic sequences in DNA from thymic lymphomas induced by irradiation (4 × 1.75 Gy); (A) after Kpn1 digestion; (B) after HindIII digestion, (C) after EcoR1 digestion. 4088 (lane 1), 5199 (lane 2), 5065 (lane 3), normal spleen (lane 4), 3547 (lane 5), 3533 (lane 6), 4073p10 (lane 7), 3651p3 (lane 8), 3656p1 (lane 9), 4065p10 (lane 10), 4081p5 (lane 11). "p #" means *in-vivo* passage number. The arrow indicates the position of the weak Kpn1 restriction fragment of tumor 4088.

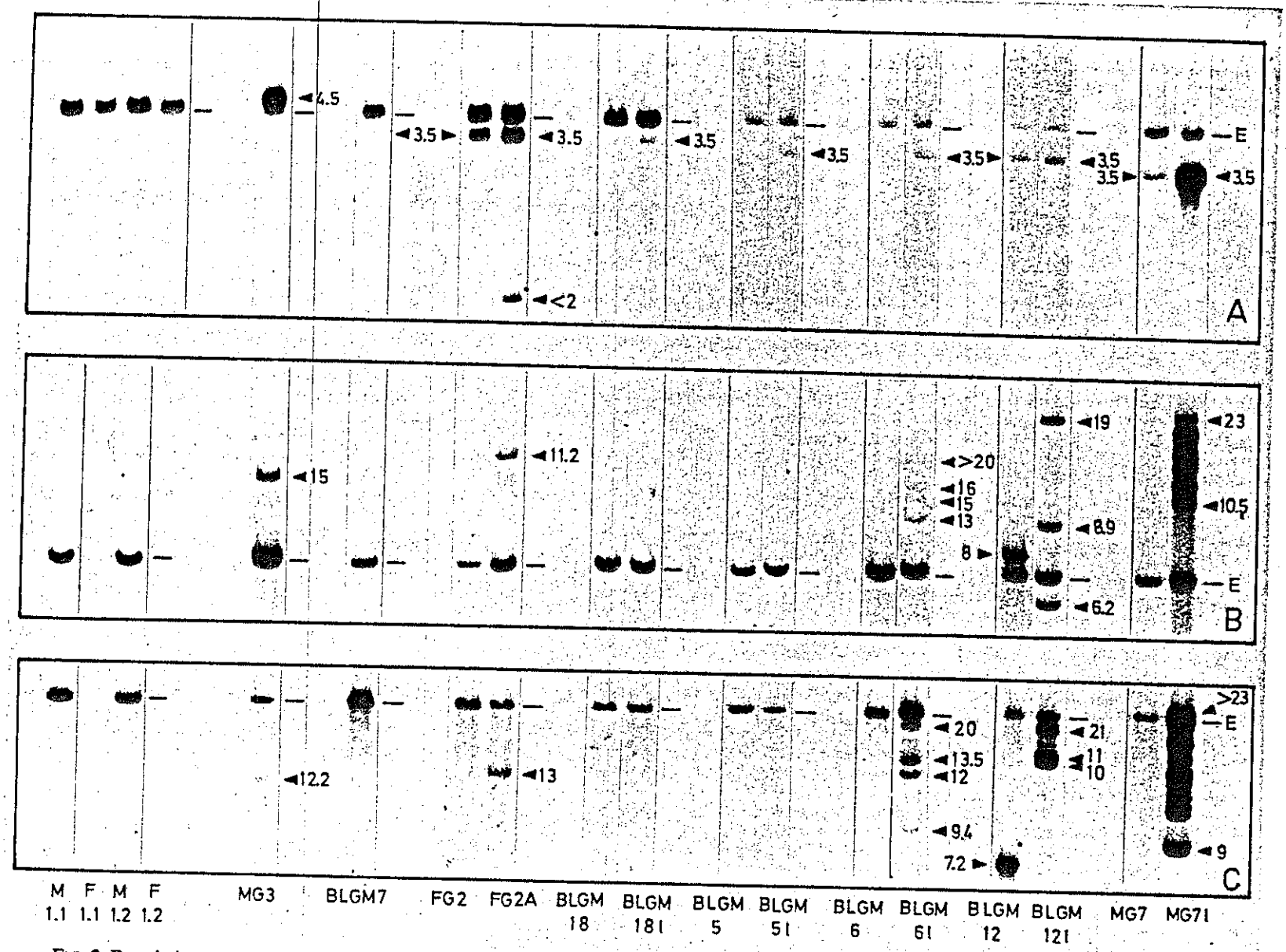

FIG. 2. Restriction pattern of ecotropic sequences in DNA from thymic lymphomas originating from thymic grafts (from C57BL/Ka newborns) implanted in thymectomized, 4 × 1.75 Gy-irradiated C57BL/Ka/Thy-1.1/Lb hosts. (A) after Asp718 digestion; (B) after HindIII digestion; (C) after EcoR1 digestion. M 1.1 and F 1.1, DNA from thymuses of respectively male and female (for which HindIII and EcoR1 patterns are not shown) control C57BL/Ka/Thy-1.1/Lb mice. M 1.2 and F 1.2, DNA from thymuses of respectively male and female (for which HindIII and EcoR1 patterns are not shown) control C57BL/Ka mice. FG2A is an *in-vivo* passage of FG2. The letter "I" indicates an *in-vitro* culture of the corresponding primary tumor.

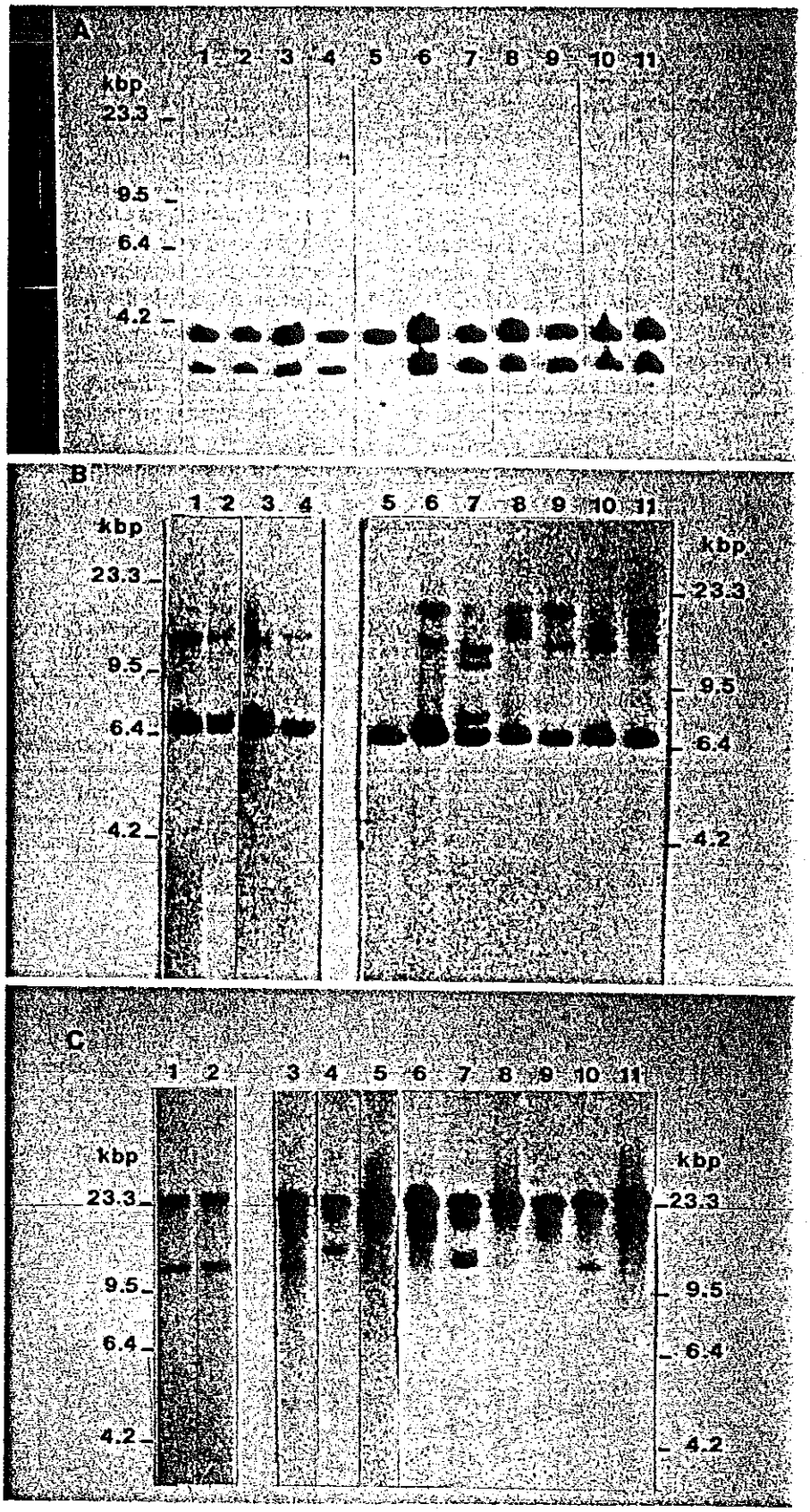

FIG. 3. Restriction pattern of ecotropic sequences in DNA from thymic lymphomas induced by T1223/B virus injection and irradiation (2 × 1.75 Gy); (A) after Kpn1 digestion; (B) after HindIII digestion; (C) after EcoR1 digestion; 3963p4 (lane 1), 3966p8 (lane 2); 3873p2 (lane 3); 3792p4 (lane 4); normal spleen (lane 5); 4750p2 (lane 6), 4891p2 (lane 7); 4749p6 (lane 8), 4750 (lane 9), 4891 (lane 10), 4749 (lane 11). "p #" means *in-vivo* passage number.

applied to the novel 7.2-kbp EcoR1 fragment of the same tumor.

One of the primary tumors (MG3), showed another Asp718 pattern than that yielding the usual 3.5-kbp diagnostic fragment. It displayed a clonal pattern for a novel provirus, as attested by a 15-kbp HindIII and a 12.2-kbp EcoR1 fragments. This result clearly demonstrates that novel proviruses may have appeared, without being necessarily characterized by the classical diagnostic Asp718 site. Such a phenomenon was also observed at one occasion in the lymphomas induced by the direct induction protocol as previously described. Anyway, novel proviruses were observed in one third of the lymphomas arising in thymuses grafted in thymectomized irradiated mice. This is in perfect agreement with the results described above for the lymphomas obtained by the direct irradiation protocol.

In order to analyse the proviral pattern after passage, nine of the investigated, indirectly induced, tumors were submitted to either *in-vivo* transplantation or *in-vitro* cell cultivation. Among the six that were originally negative for the occurrence of the novel diagnostic Asp718 fragment, three became positive after passage (BLGM5L, BLGM6L, BLGM18L). One of these three tumors (BLGM6L) displayed multiple novel HindIII and EcoR1 fragments; this may mean whether the tumor contains one cellular clone harboring multiple viral insertions or that it is composed of several cellular clones with different proviral insertions. The three Asp718-positive tumors (FG2, MG7, BLGM12) remained posi-

TABLE 2. ADDITIONAL ECOTROPIC MuLV SEQUENCES IN THYMIC LYMPHOMAS INDUCED IN NON-IRRADIATED THYMUSES UPON GRAFTING IN IRRADIATED RECIPIENTS

		Size of additional fragments (kbp)		
Tumor classification	Tumor name	Asp718	HindIII	EcoR1
Primary tumors (not available as *in-vivo* or *in-vitro* passage)	MG3	4.5	15	12.2
	BLGM3	—*	—	—
	BLGM4	—	—	—
	BLGM7	3.5	—	—
	BLGF8	—	—	—
	BLGM11a	—	—	—
	BLGM11b	—	—	—
	BLGM13	—	—	—
	BLGM14	—	—	—
	BLGM15	—	—	—
Primary tumors and their *in-vivo* progeny	FG2	3.5	—	—
	FG2A	3.5; <2	11.2	13
	FG3	—	—	—
	FG3B	—	—	—
Primary tumors and their *in-vitro* progeny	FG6	—	—	—
	FG6L	—	—	—
	MG7	3.5	—	—
	MG7L	3.5	10.5 to 23†	9 to >23†
	BLGM2	—	—	—
	BLGM2L	—	—	—
	BLGM5	—	—	—
	BLGM5L	3.5	—	—
	BLGM6	—	—	—
	BLGM6L	3.5	>20;16; 15;13	20;13.5; 12;9.4
	BLGM12	3.5	8	7.2
	BLGM12L	3.5	19;8;9;6.2	10;11;21
	BLGM18	—	—	—
	GLGM18L	3.5	—	—

* Means no visible band.
† means 7 or 8 fragments.

tive after passage, two of them (FG2, MG7) acquiring a clonal proviral pattern, as shown by new HindIII and EcoR1 fragments. The third (BLGM12) underwent a modification of the initial provirus distribution.

The results suggest that integration of new recombinant proviruses may confer, to one or a few cells of the original population, a selective growth advantage leading to a selection during subsequent *in-vivo* or *in-vitro* cultivation.

(c) *Ecotropic recombinant proviruses in thymic lymphomas arising after associating a subleukemogenic dose of irradiation and injection of T1223/B virus*

It was previously shown that infection by the ecotropic recombinant T1223/B virus in association with a subleukemogenic dose of irradiation induced a significant percentage of thymic lymphomas. This suggests that in this model retroviruses and irradiation may cooperate for thymic lymphoma induction.

The DNAs from 29 (primary or transplanted) such tumors were analysed as above. As expected the internal 3.5-kbp Kpn1 restriction fragment was detected in every instance indicating that a recombinant ecotropic virus (presumably the injected T1223/B virus) integrated in cells of all tumors (Table 3 and Fig. 3A).

All thymic lymphomas but one (5084) had additional HindIII or EcoR1 restriction fragment(s) with ecotropic sequences (Table 3, Figs 3B and C). Most of these tumors (17) harbored only one such additional band. Five tumors had two additional bands, and the remaining five tumors had three or more extrabands. It may be concluded that the majority of the thymic lymphomas obtained in our protocol are clonal or oligoclonal in nature with regard to the integration of ecotropic proviruses. Thus the frequent clonal integration of new ecotropic viral sequences appears to be a common feature of thymic lymphomas arising after either a leukemogenic irradiation or the association of a subleukemogenic irradiation and B-ecotropic recombinant retrovirus injection.

The thymic lymphomas induced by T1223/B virus injection and a subleukemogenic irradiation studied were mainly transplanted tumors. Thus it was logical to compare the pattern of ecotropic sequences in primary and transplanted tumors. In three instances, we had the opportunity to perform the same experimentation on both the primary and the corresponding transplanted tumors. A 3.5-kbp Kpn1 restriction fragment was detected in all tumors, whether primary or transplanted, indicating the integration of a recombinant ecotropic provirus.

TABLE 3. ADDITIONAL ECOTROPIC MuLV SEQUENCES IN THYMIC LYMPHOMAS INDUCED BY SUBLEUKEMOGENIC IRRADIATION ASSOCIATED WITH VIRAL INJECTION

Tumor No.	*In-vivo* passage No.	Size of additional fragments		
		Kpn1	HindIII	EcoR1
3153	4	3.5	25	18.5
3792	4	3.5	15.5	16
3840	3	3.5	15.5	ND†
3840	4	3.5	15.5	20
3848	3	3.5	16.5	15
3865	7	3.5	14	13
3873	3	3.5	14	13
3962	3	3.5	14	13
3963	4	3.5	14	13
3966	8	3.5	14	13
3971	7	3.5	14.5	14
3988	4	3.5	14;12	20;14
3993	14	3.5	14.5	ND
4002	7	3.5	15	13
4009	4	3.5	14	20
4112	9	3.5	Numerous fragments	Numerous fragments
4113	13	3.5	14.5	13
4114	6	3.5	18.5;14	11
4144	3	3.5	18.5;14	11
4749	0	3.5	20;14.5	Numerous fragments
4749	6	3.5	Numerous fragments	Numerous fragments
4750	0	3.5	19;14.5	19;17.5
4750	2	3.5	19;14.5	19;17.5
4751	0	3.5	19;9;3.6	14.5;12;5.7
4891	0	3.5	14.5	13
4891	2	3.5	13.5;11.5;8	19;13.5
4989	0	3.5	14.5	ND
5084	0	3.5	—*	—
5085	0	3.5	17	19;23

* "—" means no visible band.
† ND means not done.

After HindIII or EcoR1 digestion, the new ecotropic fragments had the same size in the DNAs of one primary (4750) and transplanted tumor (Figs 3B and C, lanes 6 and 9). In the DNAs of the two other tumors (4891 and 4749), new additional bands were detected in the transplanted tumor as compared to the primary tumor (Figs 3B and C, lanes 7, 8, 10 and 11). This finding could be explained either by viral reinsertions or by amplification of minor cell populations during transplantation.

DISCUSSION

The only results showing a frequent expression of

ecotropic retroviruses in C57BL radiation-induced lymphomas were obtained with *in-vitro* cultured lymphoma cells [7, 29, 33]. Despite numerous experiments, their origin remained unknown because the expression of such retroviruses in primary non cultured radio-induced thymic lymphomas was seldom observed [13]. Besides, new ecotropic proviral integrations were found in radio-induced thymic lymphomas after *in-vivo* or *in-vitro* propagation, but never in primary tumors [14, 22]. Two mechanisms could explain those observations. One may speculate that the new ecotropic recombinant retroviruses are generated during *in-vivo* or *in-vitro* propagation of the lymphoma cells. Alternatively, a minor cell population harboring the virus, and undetectable in the primary tumor, could be selected by *in-vivo* or *in-vitro* passage.

In contrast to these previous observations, we report here the detection of new ecotropic sequences in both directly and indirectly radio-induced primary lymphomas in a significant number (30%) of cases. This new finding may best be explained by the use of a different probe having a high homology with the C57BL ecotropic endogenous sequences. The existence of a 3.5-kbp Kpn1 (or Asp718) restriction fragment in the digested DNA of primary tumor cells indicates that these sequences belong to an ecotropic provirus recombinant in the *gag-pol* region. This virus is thus similar to the B-ecotropic recombinants isolated by Rassart *et al.* [15] from radio-induced thymoma cell lines.

The frequent occurrence of ecotropic retrovirus expression in cultured tumor cells and proviral integration in primary radio-induced tumors allow to speculate in favor of their implication in the tumorigenic process. If so, this would confirm the hypothesis of Kaplan and Brown [8], by which they postulated that irradiation of a mouse (even if thymectomized) results in the appearance of a diffusible factor—may be a retrovirus—capable of provoking the malignant transformation of thymic cells (even if non-irradiated and transplanted into the irradiated, thymectomized host). Assuming this hypothesis to be correct, one would expect the appearance of novel proviruses in all the indirectly induced, primary tumors. In fact, although only 25% (4 out of 19) displayed a novel Asp718 diagnostic restriction fragment, many more might contain new recombinant proviruses. Indeed, we found that, in both directly and indirectly radiation-induced tumors, novel proviruses could be present, lacking the diagnostic Kpn1 (or Asp718) site. However, one should not underestimate that the absence of detection of recombinant ecotropic provirus in some radio-induced lymphomas could mean that retroviruses are not involved in development of such tumors or that virus of other types are implicated.

If B-ecotropic retroviruses, when present, are implicated in the neoplastic process, one may expect the tumors to be clonal, or at least oligoclonal with respect to provirus integration as described in many other systems, particularly in retrovirus-induced thymic lymphomas [34–41]. In the present study, we observed that most of the primary radio-induced tumors in which novel recombinant provirus could be detected displayed the integration at a single or at a few sites. The same observation was made on *in-vivo* or *in-vitro* propagated tumors. However, primary tumors that were devoid of a Kpn1 (or Asp718) diagnostic fragment could acquire it after *in-vitro* propagation, showing sometimes clonality or oligoclonality with respect to proviral integration. The appearance of a clonality with respect to proviral integration, as well as modifications of a preexisting clonal pattern, can best be explained in terms of either proviral reinsertions or selection of minor cell populations during *in-vitro* or *in-vivo* propagation due to a selective growth advantage confered by the new provirus.

Although our experiments give new arguments for a role of retroviruses in the genesis of radio-induced thymic lymphomas, it remains to be explained why recombinant ecotropic proviruses were not encountered in all tumors. It is well accepted that X-irradiation leads to the emergence of a radio-resistant T-cell compartment from which tumor cells originate [42–44]. Our finding that proviral integration was not observed in every tumor may mean that the virus is not integrated in a sufficient number of cells to be detected. The faintness of some diagnostic bands argues for such an hypothesis. Altogether this suggests that the genesis and the integration of the new recombinant provirus can be relatively late events. A possible role of the new provirus could be to induce lymphoproliferation of the infected cells as well as the non-infected cells via a diffusible factor. Our observation also indicates that other mechanisms can be implicated in thymic lymphoma development, particularly at early stages. One such additional event could be a *ras* gene activation [45].

Another striking observation is that B-ecotropic recombinant retroviruses isolated from radio-induced thymic lymphoma cell lines seldom induce thymic lymphomas and if so with a long latency period. This latter observation was gathered in experiments performed without an accompanying irradiation. However, if the radio-resistant thymic cell compartment generated by irradiation is the target for the new recombinant provirus, then such a

virus may exhibit its transforming potential only after irradiation. This would explain why B-ecotropic recombinant retroviruses isolated from cultured radio-induced lymphoma cells are not leukemogenic when injected without an accompanying irradiation.

So, we examined the pathogenic potential of the T1223/B recombinant ecotropic retrovirus when injected in association with a subleukemogenic dose of irradiation. As mentioned above this led to the occurrence of thymic lymphomas in a high percentage of treated mice and with a latency comparable to that observed with a leukemogenic dose of radiation. As expected an ecotropic recombinant virus (presumably the injected one) was integrated in cells of all primary or transplanted tumors. The majority of these tumors were clonal or oligoclonal with respect to viral integrations.

Altogether, our data demonstrate the frequent clonal integration of new ecotropic viral sequences in thymic lymphomas induced whether by a leukemogenic dose of radiation or by B-ecotropic recombinant retrovirus injection accompanied by a subleukemogenic irradiation. Assuming that the new ecotropic viral sequences encountered in radio-induced thymic lymphomas play a role in tumorogenesis and in view of their clonal integration, one may speculate that the virus acts via its integration in a specific chromosomal region by acting on the expression of adjacent cellular gene(s). The molecular cloning of some proviruses together with flanking sequences could give support to such an hypothesis and allow to examine if the genomic organization of the provirus itself could be related to its tropism and pathogenic potential.

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