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Murine Thymomas Induced by Fractionated-X-Irradiation Have Specific T-Cell Receptor Rearrangements and Characteristics Associated with Day-15 to -16 Fetal Thymocytes

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We report here that specific T-cell receptor rearrangements were observed in fractionated-X-irradiation-induced murine leukemias. Consistent γ-chain rearrangements, limited β-chain rearrangements, and no detectable α-chain rearrangements were observed. Gene expression studies revealed that, in comparison with normal thymus tissue, expression of α T-cell receptor genes was lower in the thymomas, β expression was much higher but approximately equal to that of normal thymocytes, and γ expression was significantly increased. After coupling these data with those from analyses using reagents against other surface markers, such as Lyt-2, L3T4, H-2, IL-2R and MEL-14, we concluded that the target T cells for fractionated-X-irradiation-induced transformation resemble fetal thymocytes from days 15 and 16 of gestation.

Whole-body fractionated-X-irradiation (FXI; 4 × 1.75 Gy at weekly intervals) results in thymic lymphomas in most animals of certain mouse strains (19). It has been suggested that the cellular process leading to development of thymic tumors may reflect interactions of the oncogenic radiation treatment with the T-cell differentiation pathway (7, 16). Radiation is presumed to affect one or several sets of cells in the lymphohematopoietic system. These target cells then transform into preleukemic cells, which then proceed to a neoplastic state during a latency period of 3 months or longer. The autonomous lymphoma cells proliferate and then give rise to thymic tumors (3). The evidence indicates that target cells for FXI are immature T cells; i.e., marrow prothymocytes or intrathymic precursor cells (18).

The study of T-cell receptors provides an excellent tool to study whether particular cells in the T-cell maturation pathway are in fact affected as has been proposed. It has been suggested that specific T-cell receptor rearrangements, because they mark various maturational stages of T-cell development, may help identify particular T subsets involved in certain neoplasias. This indeed appears to be the case for at least some human leukemias (11) and for thymomas arising spontaneously in AKR mice (27). In addition, studies of the effects of FXI on T-cell receptor genes may prove of interest for additional reasons. Involvement of T-cell receptor genes in leukemogenesis has been suggested by numerous investigators. Rearrangements of the immunoglobulin heavy-chain locus are thought, in some instances, to deregulate cellular oncogenes like c-*myc* (28). A similar involvement of T-cell receptor rearrangements has been proposed for certain T-cell neoplasms (26, 42).

Here we report that, indeed, specific T-cell receptor rearrangements were observed in FXI-induced murine leukemias, suggesting interaction of FXI with a specific maturational stage of T-cell development. Rearrangements were detected by using β and γ probes. No α rearrangements were detected. The expression of these genes and those encoding other cell surface antigens during the course of leukemogenesis suggests that target cells of FXI resemble fetal thymocytes at the day-15 to -16 fetal stage. The characterization and isolation of these target cells would provide an important tool for studying the mechanisms of leukemogenesis.

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MATERIALS AND METHODS

Leukemogenic FXI. Mice (4 to 5 weeks old) were irradiated unanesthetized with a ^{137}Cs source (model A Gammator; Radiation Machinery Corp., Parsippany, N.J.) at 175 rads weekly for 4 weeks.

Mice. All of the mice used in our studies were bred in our colony at New York University Medical Center.

Cell culture. In vitro-adapted tumors of C57BL/6 X-irradiated tumors were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin solution, and 0.1% Fungizone.

Hybridization probes. The DNA probes used for all Northern (RNA blot) and Southern hybridizations were as follows: (i) 8/10-2γ1.1 (γ), kindly provided by Tak W. Mak and Aikichi Iwamoto (17), and (ii) pHDS203 (γ), (iii) pHDS4 (C_γ), (iv) pHDS11 (β), and (v) pHDS58 (α), all kindly provided by Haruo Saito (32, 33) (see Fig. 1). The T-cell surface marker probes used were as follows: MEL-14/5, kindly provided by Irving Weissman; IL-2R, kindly provided by Ethan M. Shevach, T3d (murine), kindly provided by Cox Terhorst; Lyt-2 and L3T4, kindly provided by Dan Littman. The H-2 probe (pH-2II), a cDNA probe which cross-hybridizes with virtually all mouse class I genes and has been previously described (36), was kindly provided by Leroy Hood.

DNA sample preparation. The DNAs used in our studies were isolated by the procedure of Blin and Stafford (2). All DNA concentrations were determined spectrophotometrically by the diphenylamine method.

Southern blots. Southern blot analyses of mouse liver DNA (T-cell receptor genes are not rearranged in liver) and mouse thymus DNA were done as follows. Restriction enzyme-digested DNA (10 μg) was separated by electrophoresis on 0.8% agarose gels and transferred to nitrocellulose (Schleicher & Schuell, Inc., or Biotrace, Gelman Sciences, Inc.) as described by Southern (35). Hybridization was for 18 h at 65°C with a probe having a specific activity of 1×10^8 to 3×10^8 cpm/μg. The filters were then washed successively in

2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% sodium dodecyl sulfate (SDS), 20 mM sodium phosphate, 0.06% disodium pyrophosphate at 65°C, 1× SSC, 0.4× SSC, and 0.1× SSC when Schleicher & Schuell filters were used. The Biotrace filters were washed in 2× SSC–0.1% SDS at room temperature, followed by 2× SSC–0.1% SDS at room temperature and 6× SSC–0.1% SDS and 2× SSC–0.1% SDS at 65°C.

Total RNA preparation. Total RNA samples were prepared by a modified guanidinium-CsCl method (24). Tissue samples were homogenized in 10 ml of 4 M guanidine thiocyanate–140 mM β-mercaptoethanol–2% sarcosyl–50 mM Tris hydrochloride (pH 7.5)–10 mM EDTA. The homogenate was then layered onto a cushion of 2.5 ml of 5.7 M CsCl–0.1 M EDTA (pH 5.0) and centrifuged for 18 h at 35,000 rpm (Beckman L7-55). The resultant pellet was reconstituted with sterile 1 mM Tris hydrochloride (pH 7.5)–0.2 mM EDTA, after which a sample was taken for an optical density reading. The remaining sample was stored as an ethanol suspension at −70°C.

Northern blots. Total RNA (10 μg) was suspended in 4.5 μl of H_2O–10 μl of 99% formamide–3.5 μl of 37% formaldehyde–2 μl of 200 mM morpholinepropanesulfonic acid–50 mM sodium acetate–10 mM EDTA. Samples were then heated at 65°C for 10 min and placed on ice for 5 min, and then 5 μl of bromophenol blue loading buffer was added. The samples were loaded into a 1% agarose gel containing formaldehyde and transferred to Nytran filters (Schleicher & Schuell). Hybridization was for 18 h at 42°C with a probe having a specific activity of 1×10^8 to 3×10^8 cpm/μg. The filters were washed at room temperature with 2× SSC–0.1% SDS, followed by 1×, 0.4×, and 0.1× SSC at 65°C.

Completeness of digestion. To ensure that the DNA samples were completely digested during the initial phases of our studies, we ran a completeness-of-digestion control as follows. At 20 min after the DNA digestion samples had been incubated at 37°C, a small sample was removed and added to a tube containing lambda DNA, after which sample and control tubes were incubated overnight at 37°C. On the following day, a minigel was run to assure that the correct lambda digestion was present. Only if this control was completely satisfactory were the test tubes processed for hybridization studies.

RESULTS

Thymomas induced in C57BL/6 mice by FXI display similar γ rearrangements. DNA digests from 25 FXI-induced tumors and three cell lines derived from FXI-induced tumors were analyzed with the three γ-chain probes diagrammed in Fig. 1. These probes were derived from cytotoxic T lymphocytes (CTL) clones (8/10-2, a K^b-[*N*-iodoacetyl-*N*(5-sulfonic-1-napthylyl)ethylene diamine]-specific clone, and 2C, an alloreactive [anti-L^d] CTL clone). Probe 8/10-2γ1.1 was derived from the former clone, and probes pHDS203 and pHSD4 were from the latter clone (17, 21, 32, 33). pHDS203 contains the 5′ untranslated region, *V*, *J*, and portions of the *C* region represented by the pHDS4/203 protein; pHDS4 encodes only a small portion of the V region, the *J* the *C* regions, and the 3′ untranslated region.

All of the thymomas tested, including those tested directly after removal from leukemic mice and those adapted to in vitro growth and maintained as cell lines, contained similar rearranged γ-chain genes (a representative set of these results is shown in Fig. 2A to G). The rearrangement present in the cell lines was representative of the findings in tumors. There were no consistent differences in T-cell receptor rearrangements in tumors compared with the cell lines. Such rearrangements were never discerned in splenocytes derived from these animals, even though in some cases tumor cells had already metastasized to the spleen. However, we were unable to detect rearrangements in spleen cells, probably because, in most cases, the ratio of tumor cells to normal cells in the spleens was low.

Rearrangements in the γ-chain genes in thymus-derived cells was observable after digestion of the thymoma DNAs with all of the three restriction enzymes used: *Hin*dIII, *Eco*RI, and *Pvu*II. These enzymes were selected because they have been used to detect rearrangements. *Eco*RI is the most commonly used, but *Pvu*II and *Hin*dIII have also been used by other investigators (10, 13, 32). The restriction maps for these enzymes are in papers by Saito et al. (32) and Hayday et al. (14). The germ line arrangement of the α, β, and γ genes is in Fig. 1E. Figures 2A to C show the Southern blot analysis of DNAs derived from normal and leukemic C57BL/6 mice digested with these enzymes. The 8/10-2γ1.1 (*C* and *V*) probe detected eight bands in *Hin*dIII digests of normal liver DNA (considered to be an unrearranged control for T-cell receptor probes) and seven bands in normal

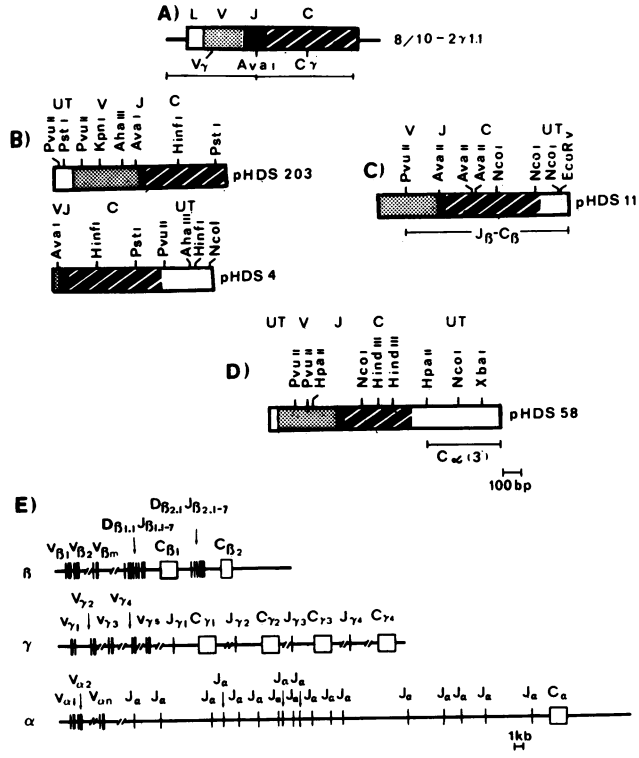

FIG. 1. Restriction maps of the T-cell receptor probes used in these studies. (A) 8/10-2γ1.1 is a cDNA probe of the γ-gene family of the CTL clone from B10. The transcript is composed of the *V-J-C* (*Vγ10.8A* and *JCγ10.5*) region found in several CTLs (14). (B) pHDS203 and pHDS4 were derived from CTL clone 2C of BALB.B origin and code for the γ-chain gene. (C) pHDS11 encodes the β subunit of CTL 2C. (D) pHDS58 corresponds to the *V* and *C* regions of the α-chain gene. The probes derived from each clone are indicated. 8/10-2γ1.1 was kindly provided by Tak W. Mak and Aikichi Iwamoto. PHDS203, pHDS4, pHDS11, and pHDS58 were kindly provided by Haruo Saito. (E) Germ line drawing of the α, β, and γ genes described elsewhere (15, 37, 40). Distances between gene segments are indicated. bp, Base pairs.

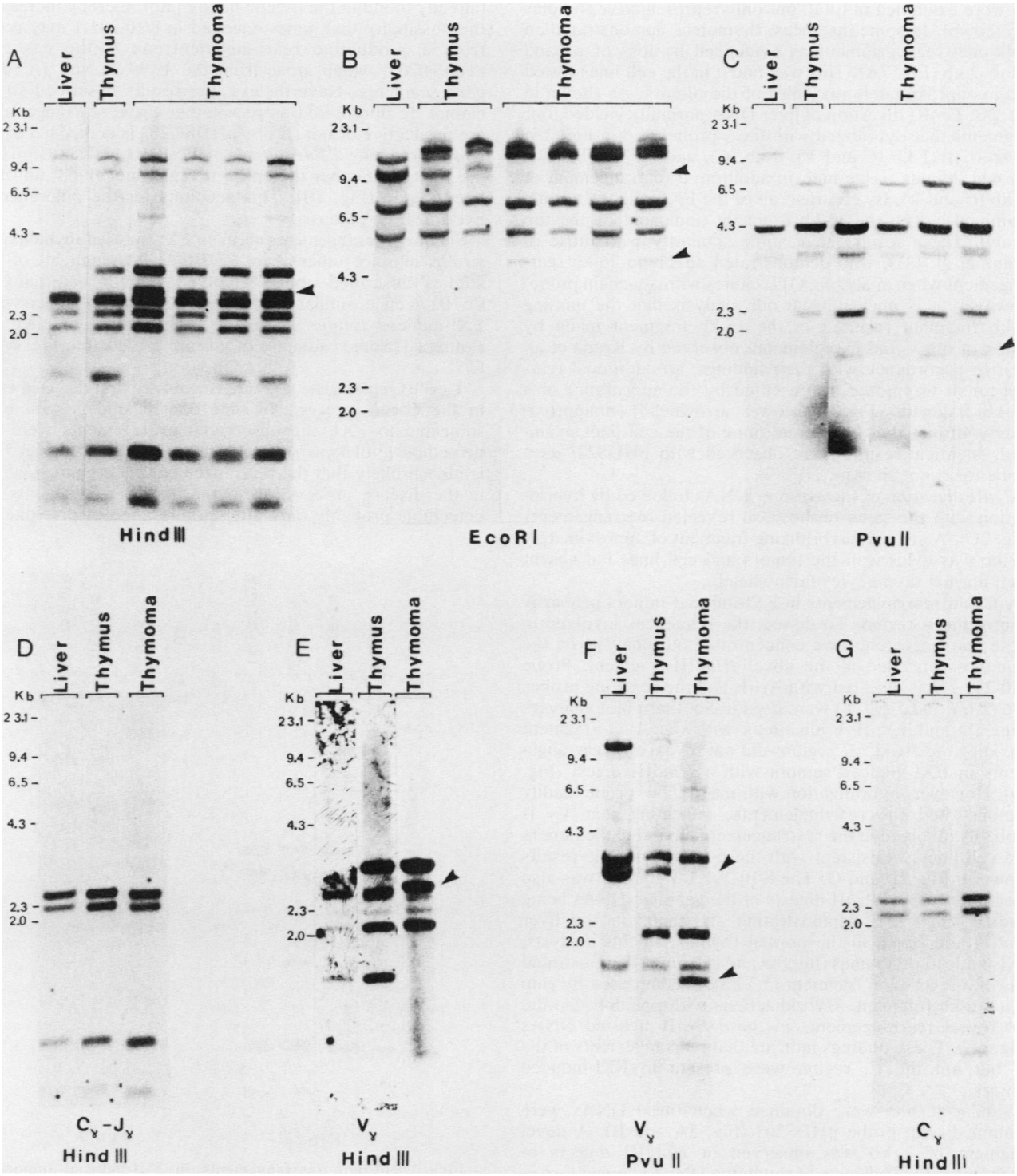

FIG. 2. Thymomas induced in C57BL/6 mice by FXI displayed similar γ rearrangements. Panels A to C demonstrate representative Southern blot analyses of normal liver, thymus, and thymoma DNAs from C57BL/6 mice. Ten micrograms of DNA per lane were digested with (A) *Hin*dIII, (B) *Eco*RI, or (C) *Pvu*II and hybridized with a ^{32}P-labeled, nick-translated total insert from 8/10-2γ1.1. Panels D to F represent hybridization with *C*γ-*J*γ and *V*γ probes from 8/10-2γ1.1 with an *Ava*I digest of the insert (Fig. 1) and *Hin*dIII and *Pvu*II digests as indicated. Panel G represents hybridization with a total insert from probe pHDS4 corresponding to the *C* region of the γ-chain gene. The migration distances of *Hin*dIII-cut lamda DNA molecular size markers are indicated. The arrowheads indicate the rearrangements noted in the text.

thymocyte DNA below 4.3 kilobases (kb) (Fig. 2A). A novel fragment of 3 kb was observed in all FXI-induced thymomas (25 were examined in total, but only representative samples are shown). In some instances, thymomas demonstrated an additional rearrangement as evidenced by loss of a band below 2 kb (Fig. 2A). This was noted in the cell lines tested and in approximately one-third of the tumors. As shown in Fig. 2B, *Eco*RI digestion of liver DNA normally yielded four fragments that hybridized with the γ probe used in Fig. 2A: 14 kb (*C*), 11 kb (*C* and *V*), 8 kb (*C*), and 5.2 kb (*V*) (41). Normal thymus tissue had, in addition, a rearrangement of 16 kb (*C* and *V*). By contrast, all of the FXI-induced tumors examined lacked the 11-kb fragment (indicated by the top arrow). These results have some similarity with those of Kranz et al. (21), who demonstrated an 11- to 16-kb rearrangement when analyzing CTL clones with a γ-chain probe. However, it is unclear from our analysis that the missing 11-kb fragment resulted in the 16-kb fragment made by fusion of the *V* and *C* regions are observed by Kranz et al. (21). In accordance with their findings, an additional rearrangement was noted, represented by the appearance of a 4.5-kb fragment (Fig. 2B, lower arrowhead) in approximately 40% of the tumors and none of the cell lines examined. Identical results were observed with pHDS203 as a probe (data not shown).

*Pvu*II digestion of these tumor DNAs followed by hybridization with the same probe, also revealed rearrangements (Fig. 2C). A strongly hybridizing fragment of approximately 1.7 kb was evident in the tumors and cell lines but absent from normal thymocytes (arrowhead).

γ-Chain rearrangements in FXI-induced tumors primarily involve the *V* region. To dissect the sequences involved in these rearrangements, we concentrated our analysis on the sequences encoded in the novel *Hin*dIII fragment. Probe 8/10-2γ1.1 was digested with *Ava*I, and the resulting probes (*J-C-3'UT* and *5'UT-V*) were used in Southern blot analyses (Fig. 2D and E). Hybridizations with the *Ava*I fragment encoding the *J-C-3'UT* regions did not reveal any rearrangements in FXI-induced tumors with a *Hin*dIII digest (Fig. 2D). However, hybridization with the *5'UT-V* probe readily demonstrated the rearrangements, indicating that *V*γ is probably involved in the rearrangements found in the tumors and cell lines. Consistent with these results are the results shown in Fig. 2F and G. The 8/10-2γ1.1 *V*γ probe was also used to hybridize *Pvu*II digests of the genomic DNAs being studied (Fig. 2F), demonstrating six bands in the liver control and seven in the normal thymus. In this analysis, FXI-induced thymomas (tumors and cell lines) demonstrated loss of a restriction fragment (3.3 kb), accompanied by gain of a 1.6-kb fragment. Hybridizations with pHDS4 (*C*γ) did not reveal rearrangements in these *Pvu*II-digested DNAs (Fig. 2G). These findings indicate that rearrangements of the *V*, but not the *C*, region were present in FXI-induced tumors.

Similar results were obtained when these DNAs were examined with probe pHDS203 (Fig. 3A and B). A novel fragment of 3 kb was observed in *Hin*dIII digests of thymomas. *Pvu*II digests of thymoma DNAs showed loss of a fragment of 3.2 kb and enhanced intensity of the 1.2-kb fragment observed in normal thymus or liver controls.

The published sequences of the genes coding for 8/10-2γ1.1 and pHDS4/203 are identical except for two extra nucleotides (AT) in pHDS4/203 at the *V-J* junction. This difference results in a translational frameshift in 8/10-2γ1.1, thereby creating a termination codon at position 441 not present in pHDS4/203 (17). Extra nucleotides at *V-J* junctions of immunoglobulin genes are common (37). It has been proposed that *D* segments may exist in the γ-gene family (already found in the β-gene family [20]), thereby increasing the possibility that genes encoded in 8/10-2γ1.1 may result from a productive rearrangement, as is the case for pHDS4/203, which arose from the *Vγ10.8A* and *J-Cγ10.5* gene segments. Nevertheless, the results described so far cannot be interpreted as to whether these rearrangements are productive or not. Probe pHDS4/203 is composed of the two overlapping cDNA clones pHDS4 and pHDS203. pHDS 203 contains neither the entire *C* region nor the 3' untranslated region (Fig. 1B). This accounts for the difference in hybridization patterns.

Similar rearrangements occur in FXI-induced thymomas in strains of mice other than C57BL/6. Although all of the studies described above involved thymomas arising in C57BL/6 mice, similar rearrangements can be obtained when FXI-induced tumors arising in other strains of mice are examined. Some examples of this are provided in Fig. 4A to C.

T-cell receptor gene rearrangements are first detected early in the disease process. In time course studies with mice subjected to FXI, the observed rearrangements were not detectable until approximately 12 weeks post-FXI (Fig. 5). It is indeed likely that the rearrangement occurs much earlier in the disease process. However, the rearrangements are detectable probably only after proliferation of preleukemic

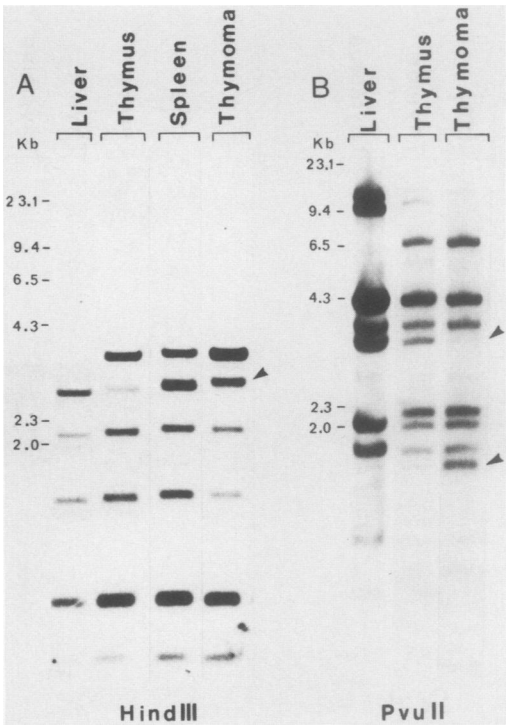

FIG. 3. γ-Chain rearrangements in FXI-induced tumors of C57BL/6 mice involve primarily the *V* region. A ^{32}P-labeled, nick-translated total insert from pHDS203 (Cγ and Vγ) was used. Panel A is a representative gel of *Hin*dIII digest of normal liver, thymus, thymoma, and spleen from leukemic mice. Panel B is a representative gel of *Pvu*II digests of normal liver, thymus, and thymomas. Ten micrograms of DNA was used per lane. The migration distances of *Hin*dIII-cut lambda DNA molecular size markers are indicated. The arrowheads indicate the rearrangements noted in the text.

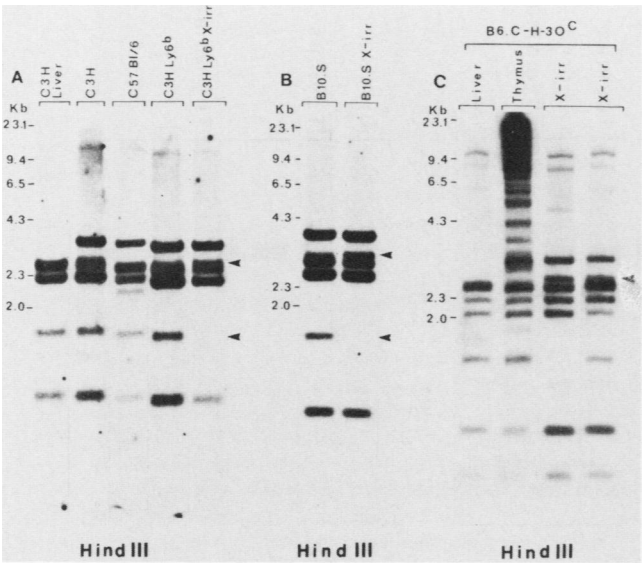

FIG. 4. Similar rearrangements occur in FXI-induced thymomas in strains of mice other than C57BL/6. Evidence for γ-gene rearrangement in C3H.B-*Ly6*^{b}, B10.S, and B6.C-*H-30*^{c} thymomas. Ten micrograms of *Hin*dIII-digested normal liver, thymus, and thymoma DNAs per lane was used. A ^{32}P-labeled, nick-translated total insert from 8/10-2γ1.1 was used as a probe. Migration of lambda *Hin*dIII markers on the gels is indicated. The arrowheads indicate the observed rearrangements.

or leukemic cells has been sufficiently extensive to raise their representation in the population above a certain level.

Expression of γ-chain genes increases as the FXI-induced leukemogenic process advances. Northern blot analyses of total RNA from thymocytes at the indicated times post-FXI demonstrated that the γ-chain genes rose from their initial basal level to levels significantly higher than those in normal thymocytes (Fig. 6). Some of the cells examined in this way (those from 32 and 52 weeks post-FXI) were also examined by Southern blot analysis and shown to contain the γ-chain gene rearrangement.

Radiation-induced tumors in mice contain primarily two forms of β-chain rearrangements. The known β-gene family contains numerous *V*β, *D*β, and *J*β gene segments (6). Evidence dictates that β-chain rearrangements occur in the thymus and that *D-J* joining occurs before *V-D* joining (4). This gene family is considerably more extensive than the γ-gene family. It should, therefore be less likely to detect unique-type rearrangements of the β-gene family than of the γ-gene family. Nevertheless, in Southern blots of *Pvu*II digests of radiation-induced thymomas (tumors and cell lines), only two prevalent rearrangements were observed (Fig. 7A). In addition, the intensity of the germ line fragment of approximately 3 kb seemed to be diminished even in normal thymocytes. Genomic DNAs of the liver and thymus controls gave one major band which corresponds to the $C\beta_1$ 6.2-kb fragment reported by Born et al. (4). The hybridization pattern that they observed for BW5147, an AKR tumor-derived cell line, is the same as one of the rearrangements observed in our tumors (e.g., Fig. 7A, lane 4). Given the predicted restriction enzyme fragment length resulting from *D*β-to-*J*β rearrangements as reported by Born et al. (4), it appears that the rearrangement observed in approximately half of the tumors (approximately 8- and 5-kb fragments) involve $J\beta_2$.

This observation was also noted by Kronenberg et al. (22), who used a $J\beta_2$ probe on blots of *Pvu*II-digested DNA derived from T-cell hybridomas. This similarity is further supported by the fact that the β rearrangements we noted primarily involve the *J*β-to-*C*β regions (Fig. 7A).

*Eco*RI digests of the control samples (normal liver and thymus) gave the same pattern as that reported by Saito et al. (32). In addition, the same rearrangement was observed in tumors and cell lines (loss of the 2.3- and 2.0-kb frag-

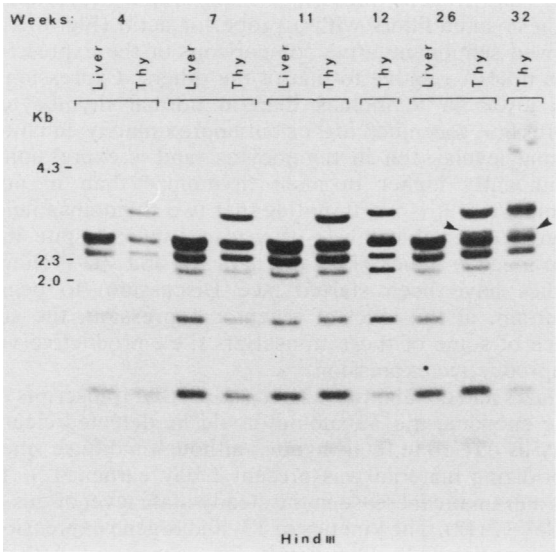

FIG. 5. T-cell receptor gene rearrangements are first detectable early in the disease process. Time course studies using mice subjected to FXI demonstrated that the observed γ rearrangements (arrowheads) were not detectable until approximately 12 weeks post-FXI. The periods noted signify times after the last radiation dose. *Hin*dIII digests of 10-μg of DNA per lane were used. Southern blot analysis was performed with ^{32}P-labeled 8/10-2γ1.1 insert as a probe. The sizes of *Hin*dIII-cut lambda DNA fragments are indicated.

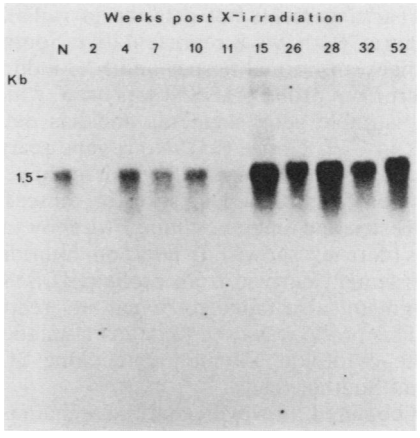

FIG. 6. Expression of γ-chain genes increases as the FXI-induced leukemogenic process advances. Northern blot analysis of total cellular RNA of thymus tissue from normal (N) and previously X-irradiated C57BL/6 mice. The periods noted signify times after the last irradiation. Ten micrograms of RNA was used per lane. The blot was hybridized with a ^{32}P-labeled 8/10-2γ1.1 insert. The approximate size of the hybridizing RNA is indicated. This was determined by coelectrophoresing *Escherichia coli* (1.6- and 2.9-kb) and mouse (1.9- and 4.8-kb) rRNAs as molecular size markers.

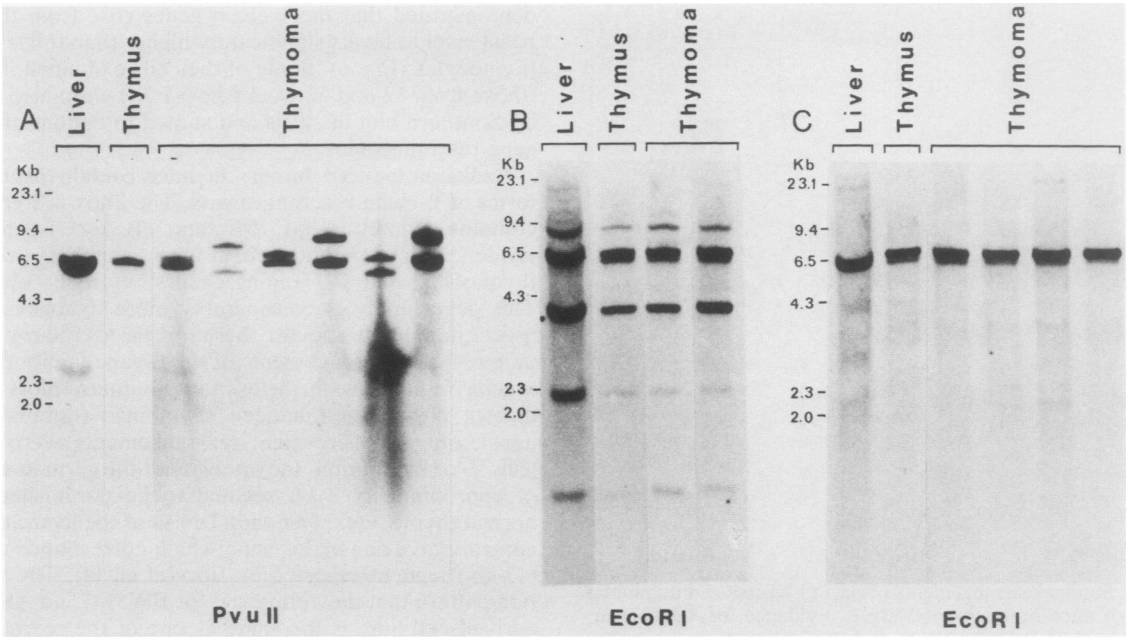

FIG. 7. α-Gene rearrangements were not detected in radiation-induced tumors from C57BL/6 mice. DNAs were digested as indicated and analyzed by the Southern blot technique. (A) Hybridization with a *J*β-*C*β probe (*Pvu*II fragment from pHDS11 insert [Fig. 1]) from pHDS11 demonstrated that, in *Pvu*II digests of radiation-induced thymomas, only two prevalent rearrangements occur. In addition, the intensity of the germ line fragment of approximately 3 kb seemed to be diminished even in normal thymocytes. (B) Hybridization with a total insert from pHDS58 of an *Eco*RI digest of normal tissues and FXI-induced tumors. No α-gene rearrangements were apparent in the tumors shown or in any other FXI-induced tumor examined. (C) Hybridization with an *Hpa*II fragment derived from probe pHDS58 (Fig. 1), a Cα (3′) fragment, also failed to reveal any rearrangements. Ten micrograms of DNA was used per sample. The migration of lambda *Hin*dIII markers is indicated to the left of the gels.

ments) that occurs in the cytotoxic T-cell clone 2C (data not shown).

This limited rearrangement of T-cell receptors is consistent with the notion that a specific maturational stage of T-cell development appears to be targeted by neoplasia-inducing FXI (see Discussion). No *V*β rearrangement was observed by us in any FXI-induced tumor (data not shown).

α-Gene rearrangements not detected in radiation-induced tumors. Figure 7B shows a representative Southern blot of an *Eco*RI digest of normal tissues and FXI-induced tumors. A total insert from probe pHDS58 was used, which contains five or six variable gene segments and has detected rearrangements in CTL clones (33). No α-gene rearrangements were apparent in the tumors shown or in any other FXI-induced tumor examined. Digestion of these DNAs with additional restriction enzymes failed to show any α rearrangements (data not shown). In addition, hybridization with an *Hpa*II fragment derived from probe pHDS58 (Fig. 1), a *C*α (3′) fragment, also failed to reveal any rearrangements (Fig. 7C). This probe was used to detect a unique rearrangement in the cytotoxic T-lymphocyte clone 2C (13) with conventional Southern gels.

It should be noted, however, that the α chain is composed of many *V*α-gene segments, a large number of *J*α genes, and a single *C*α gene. The *J*α genes are dispersed over a 60-kb segment of DNA 5′ to *C*α, and of those analyzed most are different (40). Therefore, the unique genomic organization of the α chain makes detection of specific rearrangements difficult in heterogeneous populations. Because of this, Haars and collaborators (12) were not able to analyze the timing of rearrangements in this gene family.

α-, β-, and γ-gene expression in FXI-induced tumors and other characteristics of these cells. Total RNAs prepared from numerous thymomas were analyzed for the presence of α-, β-, γ-, and T3-gene transcripts (Fig. 8 A to D). Hybridization of these same filters with a probe for actin (Fig. 8E to G) allowed semiquantitative comparisons of the expression of each mRNA relative to that of the others. Expression of α was lower in thymomas than in normal thymocytes; β expression was much higher but approximately equal to the normal levels seen in thymocytes, and γ expression was significantly higher in most thymomas than in normal thymocytes. It is worth noting that two thymomas failed to express detectable levels of γ transcripts, despite having rearranged γ genes (Fig. 8C, lanes 4 and 7). Preliminary studies have been started (see Discussion) to begin to ascertain, at the level of receptor expression, the significance of some of these transcripts (i.e., productive versus nonproductive expression).

Haars and collaborators have shown that transcripts of the gene encoding the T3 subunit could be detected clearly as early as day 16 in fetal thymus, although a diffuse smear of hybridizing material was present 1 day earlier (12). There was a dramatic increase in the steady-state level of this RNA on day 17 (12). The kinetics of T3- and α-gene expression are quite similar (12). Our results are consistent with these kinetics. As with α T-receptor chains, T3 expression was low in most FXI-induced thymomas (Fig. 8D).

These results suggested that the thymomas represented an early stage of T-cell development. RNA samples from these thymomas were therefore probed with T-cell maturation markers. Coupled with the low-to-normal levels of Thy-1 mRNA observed in these cells (Fig. 9A), very low levels of L3T4 and Lyt-2 mRNAs (Fig. 9B and C), normal levels of

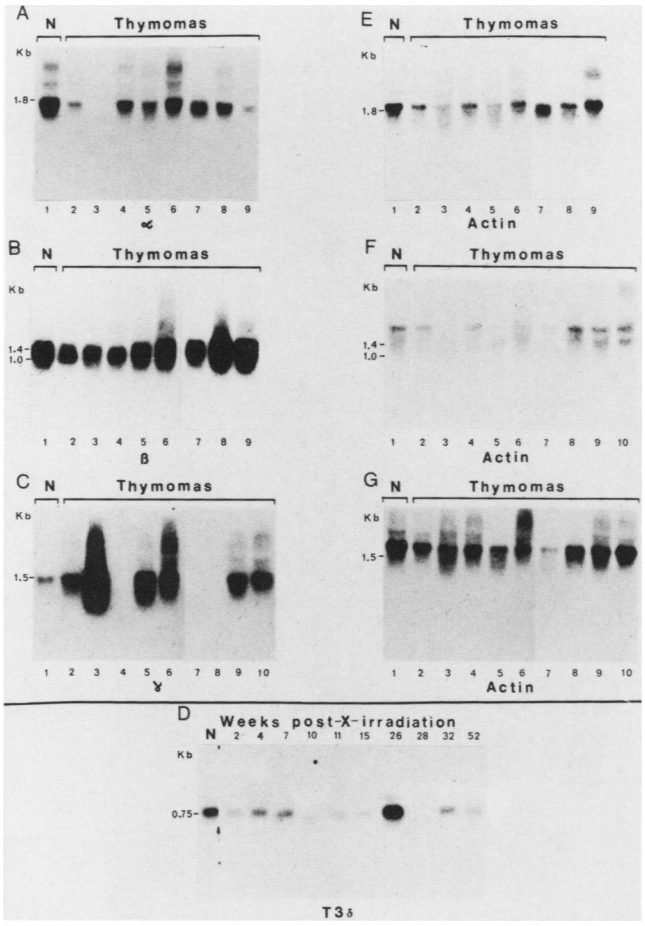

FIG. 8. α-, β-, and γ-gene expression in FXI-induced tumors and other characteristics of these cells. Total RNAs prepared from numerous thymomas were analyzed for the presence of (A) α (pHDS58 insert)-, (B) β (pHDS11 insert)-, and (C) γ (8/10-2γ1.1 insert)-gene transcripts. Hybridization of these same filters with a probe for actin (E to G) allowed semiquantitative comparisons of the rate of each mRNA relative to those of the others. Northern blot analysis of normal thymus (N) and thymoma RNAs. Ten micrograms of total RNA was used per sample. (D) Time course study using mice previously subjected to FXI. The number of weeks noted for the T3δ receptor was used. The sizes of the RNA transcripts were determined by coelectrophoresis of *E. coli* (1.6- and 2.9-kb) and mouse (1.9- and 4.8-kb) rRNAs as molecular size markers.

H-2 mRNA (Fig. 9D), and high levels of interleukin 2 (IL-2) receptors (Fig. 9E) and MEL-14 antigens (Fig. 9F), FXI-induced tumor cells resemble fetal thymocytes from days 15 to 16 of gestation. Day-15 to -16 thymocytes are low in Lyt-2 and L3T4 expression (5, 12, 39), express low-to-normal levels of Thy-1 (12), have significant amounts of IL-2 receptors (30), express peak amounts of MEL-14 (12), and express high levels of T-cell receptor β and γ mRNAs but low amounts of TCRα mRNA (12, 30).

These findings have prompted us to take advantage of the elevated expression of MEL-14 antigens on these cells to attempt to isolate them earlier in the leukemic process than when γ rearrangements are normally first seen (12 weeks post-FXI) in experimentally unselected cells (work in progress). MEL-14 is an antigen defined by a monoclonal antibody directed against the mouse lymph node homing receptor. Most (80%) cells emigrating from the thymus are MEL-14^{hi}. These cells originated from a MEL-14^{hi} population in the thymus that contains most immunocompetent thymic cytotoxic T-lymphocyte precursors (9). Time course analysis of MEL-14 expression after FXI treatment demonstrated that, by 13 to 14 weeks post-FXI, approximately 25% of the total thymocyte population expressed high levels of MEL-14, whereas in unirradiated animals the level was about 4%. This is approximately the same time period in which the γ rearrangement was first detected.

DISCUSSION

Significance of the consistency of γ rearrangements associated with FXI-induced tumors. It has been reported (21) that the repertoire of T-cell-specific γ genes is limited and that different T-cell clones use identical *Vγ* (*V10.8A*), *Jγ* (*V10.8A*), and *Cγ* (*JCγ10.5*) gene segments. However, analysis of organization of the γ-chain family in BALB/c mice revealed the existence of three cross-hybridizing constant regions, each associated with its own *Jγ* gene segments (13). In addition, as discussed earlier, Iwamoto et al. (17) have analyzed cDNA sequences from cytotoxic T-cell lines derived from mouse strain C57BL/10 and defined yet other constant- and variable-region genes of the γ-chain family. More recently, Heilig and Tonegawa (15) have defined another *Vγ* segment (*V5*). None of these germ line gene segments, with the possible exception of *C3*, have obvious structural defects (15).

Thus, it is possible to argue that the appearance of a constant rearrangement of these genes in every FX-induced tumor examined may result from the limited pool of γ genes available. However, as just stated, others have detected more than one type of γ gene rearrangement (14, 15, 17). Furthermore, within each type of rearrangement there exists sequence variability in the *V-J* junction, indicating that the structural diversity of the TCRγ gene products is not as limited as previously thought (15). Additional TCRγ segments may exist which do not hybridize with the available γ probes. In humans, at least two *Cγ* and six *Vγ* gene segments have been defined (23, 39). These facts suggest that the invariant detection of one γ rearrangement in this type of tumor may have significant implications.

Furthermore, when examined by *Eco*RI digestion, 22- and 16-kb rearrangements (γ4 and γ1, respectively) are by far the most frequent γ-gene rearrangements noted, and they have been found in all functional T-cell clones analyzed (38). By contrast, in our hands, all of the FXI-induced tumors examined by *Eco*RI digestion lacked the 11-kb fragment. While these results have some similarity with those of Kranz et al. (21), who demonstrated an 11- to 16-kb rearrangement when analyzing CTL clones with a γ-chain probe, our analysis provides no evidence that the 11-kb fragment that is missing has resulted in a 16-kb fragment made by fusion of *V* and *C* regions from the V_A in the 10.8-kb fragment joining to the *J-C* γ1 cluster located in the 10.5-kb fragment (47). In addition, some FXI-induced thymomas have a novel 4.5-kb fragment. The latter type of rearrangement is similar to those observed by others for the γ3 genes (38). γ3 and γ2 rearrangements are said to be rare in functional T-cell clones but to be common in thymomas, which generally show more γ-gene rearrangements (38).

Expression of T-cell receptor chains and other markers on FXI-induced thymomas suggests that these cells resemble day-15 to -16 fetal thymocytes. Work from many laboratories (1, 4, 8, 10, 12, 15, 30, 31, 34) has generated an approximate picture of T-cell differentiation based on T-cell receptor and

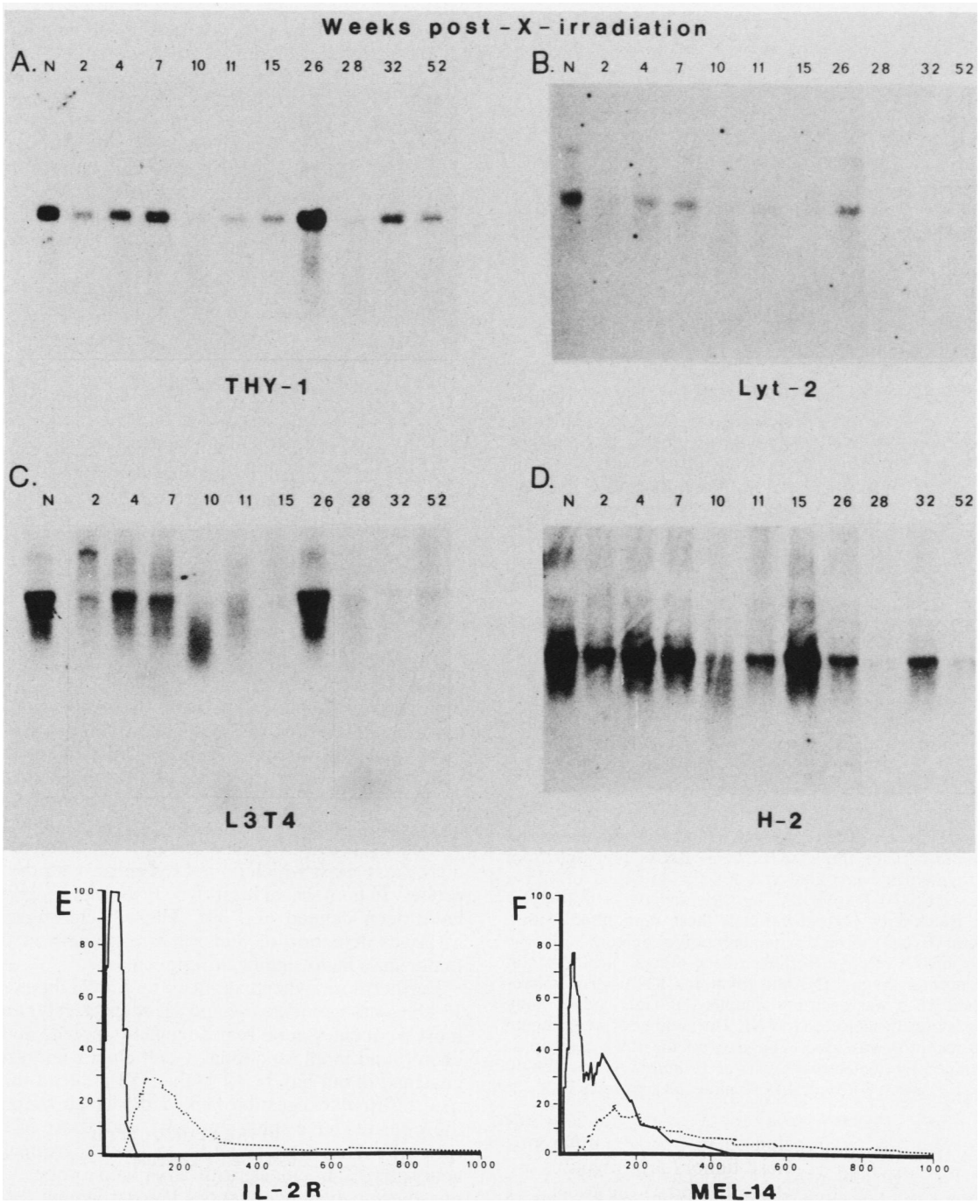

FIG. 9. FXI-induced tumor cells resemble fetal thymocytes from days 15 and 16 of gestation. Northern blot analysis of total cellular RNAs of thymus from normal (N) and previously X-irradiated C57BL/6 mice. The periods noted signify times after the last irradiation. FXI-induced tumor cells expressed low-to-normal levels of Thy-1 mRNA (A), very low levels of L3T4 and Lyt-2 mRNAs (B and C), normal levels of H-2 mRNA (D), and high levels of IL-2 receptors (E) and MEL-14 antigens (F). The probes used in panels A to D are as noted below each panel (10 μg was used per sample). Panels E and F represent Fluorescence-Activated Cell Sorter one-color fluorescence of C57BL/6 thymocytes stained for IL-2R and MEL-14. ——, Normal thymus; ---, thymoma.

cell surface markers which may provide a framework in which the current findings may be discussed. For example, it is known that γ-chain genes demonstrate two unique characteristics, i.e., increased expression at the RNA level in immature thymocytes (33) and expression on the surface of 16-day-old fetal thymocytes (25). During ontogeny, α-gene transcripts first appear on day 16 of gestation in the fetal thymus. At this stage, most of the transcripts are 1.4 kb long. The 1.7-kb transcripts which appear later are similar in size to the transcripts found in mature T cell and are presumed to encode a functional α polypeptide. The structure of the 1.4-kb transcript has not been determined, although, by analogy with the β-gene locus, it may be derived from a hypothetical *Dα-Jα* rearrangement, or it may originate from

a promoter located in front of a germ line $J\alpha$ gene segment. There is a large increase in the amount of 1.7-kb α transcript on day 17 of gestation; at this stage it is present at approximately 50% of the level detected in the adult thymus. Expression of this RNA increases steadily after day 17 until adult levels are reached. After day 16, the shorter, 1.4-kb $C\alpha$ is much less abundant than the 1.7-kb $C\alpha$ transcript in total thymus.

From this perspective, it appears that the thymocytes in FXI-induced thymomas, which, in general, express levels of α transcripts of 1.7 kb at lower levels than those found in normal adult thymocytes, are less differentiated than mature T cells. The 1.4-kb transcript was not detected.

A similar picture emerged from β-gene expression. Expression of small amounts of a 1.0-kb $C\beta_2$ RNA could be detected on days 14 and 15. All or most of this 1.0-kb transcript was derived from a partial $D\beta$-$J\beta$ gene rearrangement (12). Very low levels of the 1.3-kb $C\beta_2$ RNA, presumably derived from fully assembled $V\beta$-$D\beta$-$J\beta$ gene segments, became detectable at day 15 of gestation. There was a larger increase in the level of the 1.3-kb RNA on day 16 of gestation, 1 day earlier than the similar increase observed for the α transcript. On day 16, the 1.3-kb $C\beta_2$ RNA was present at approximately 50% of the adult level. Analogous to the kinetics of α-gene expression, β-gene RNA levels increased steadily from this day until the adult level was reached. In contrast to α-gene expression, nearly equal amounts of both the shorter 1.0-kb and the full-length, functional 1.3-kb species could be detected in thymus RNA on day 17 of gestation and thereafter, including adult thymus RNA.

From these data, it appears that FXI-induced thymomas, which, in general, express levels of β transcripts of molecular weights comparable to those of transcripts found in normal adult thymocytes, are more differentiated with respect to β genes than they are with regard to α genes and resemble day-15 to -16 fetal thymocytes.

Relatively high levels of γ gene expression can be detected in the fetal thymus on day 14, the earliest day tested (12). The γ transcripts on this day could be derived from a few cells (<5%) that are rearranged and contain abundant γ-gene transcript, or they could be derived from germ line γ genes (12). Maximum expression of γ-gene RNA is found on day 15 of gestation, followed by a slow decline until day 18 (12). Finally, there is a significant decrease in the RNA level at birth (12). γ-Gene expression at day 15 is at least 50-fold higher than in adult thymus tissues (12).

Given the high expression of γ transcripts in most FXI-induced thymomas and the finding that, during the period of leukemogenesis, transcription of these genes gradually increases, one might again conclude that, from the differentiation point of view, cells of these tumors are frozen at the day-15-to-16 fetal thymocyte stage defined as the pro-T-cell stage by Haars et al. (12).

Further evidence for this staging of FXI-induced thymoma cells is provided by the very low levels of Lyt-2 and L3T4 mRNAs observed in most of these cells, the normal levels of H-2 and Thy-1 mRNA, and the high levels of IL-2 receptors and MEL-14 antigens. Fluorescence-Activated Cell Sorter (Ortho Diagnostics, Inc., Raritan, N.J.) analysis of some of these markers (Thy-1.2, Lyt-2, and L3T4) reflected similar results, although differences between normal and FXI-induced thymocytes were less pronounced at some time points. Drastically lower levels of Lyt-2 and L3T4 were observed in tumors versus normal thymocytes (data not shown). Day-15 to -16 fetal thymocytes are low in Lyt-2 and L3T4 expression (5, 12, 39), have significant amounts of IL-2 receptors (12, 30), express peak amounts of MEL-14 (12), and express high levels of TCRβ and TCRγ mRNAs but low amounts of TCRα mRNA (12, 30).

Owen et al. (27) have published evidence for a similar T-cell subpopulation involvement in AKR murine leukemias. These investigators have suggested that AKR murine thymic leukemias derive from a distinct thymic cell lineage that does not express the β chain of the T-cell antigen receptor. The α, β, and γ DNA contents of all 10 AKR thymic leukemias examined showed that all tumors rearranged their β-chain genes and 9 of 10 rearranged their γ-chain genes. However, while 9 of 10 tumors showed a normal α-chain mRNA, none of these thymomas contained a normal-length β-chain mRNA, and almost all lacked γ-chain mRNA. From what has been described here, it is clear that the population involved in FXI-induced leukemias is different from that involved in AKR neoplasias.

An alternative explanation to the proposed subpopulation targeting by FXI is, of course, that the increased transcription of γ genes during leukemogenesis reflects activation of a functional T-cell population. This argument could also explain the high levels of expression of γ genes in the thymomas. However, if so, one would expect to observe functional γ-chain rearrangements. Three of four FXI-induced cell lines examined by J. Coligan for us have shown no evidence of γ T-cell receptor chain expression (J. Coligan, personal communication). Thus, this explanation appears less palatable than the subpopulation-targeting concept. The proposed interruption of T-cell maturation by FXI according to the model of T-cell development proposed by Haars et al. (12) would not require that any of the observed γ rearrangements be functional.

The studies reported here begin to define some of the characteristics associated with cells susceptible to transformation of FXI and strongly suggest that tumorigenesis by FXI involves interruption of T-cell maturation at a specific stage. The mechanism by which FXI accomplishes this interruption is the subject of current studies in our laboratory. We might be aided in this goal by the fact that isolation and study of such target cells now appears possible because of their elevated MEL-14 expression.

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