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Genetics

Viral integration near c-*myc* in 10–20% of MCF 247-induced AKR lymphomas

(leukemia/*myc* rearrangements/MCF virus)

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ABSTRACT **We induced AKR thymomas by injecting retrovirus MCF 247 that we had tagged with a fragment of phage λ in its U3 region. About 10–20% of the 26 primary tumors studied showed both rearranged and germline c-*myc* bands in Southern blots. The rearranged c-*myc* genes were cloned from two of the tumors and studied by Southern analysis in two others. In all four cases, rearrangement was due to integration of MCF 247 proviruses about 2 kilobases (kb) 5′ of the three c-*myc* exons and in the opposite transcriptional orientation. Thus, viral integrations were clustered within a 1-kb range in the tumors that revealed c-*myc* rearrangements. Three of the four proviruses appeared to be intact, whereas the fourth had an internal deletion of about 3 kb.**

High leukemic AKR mice have lifelong retrovirus infections and, as a result, develop T-cell lymphomas between 6 and 12 months of age. Considerable evidence indicates that mink cell focus-forming (MCF)-type retroviruses are the proximal leukemogenic agents in these mice (1). MCF viruses arise during the lifetime of the mouse by recombination between distinct classes of inherited viruses (2–4). They are nondefective and they lack cell-derived oncogenes. The mechanism of tumor induction by MCF viruses has remained unclear, and several models have been suggested. According to one, the envelope glycoproteins of MCF viruses are mitogenic for T cells (3, 5), a possibility bolstered by analogy to the acute defective component of Friend virus, spleen focus-forming virus (SFFV), whose aberrant, MCF-like envelope glycoprotein appears to be a mitogen for erythroid cells (6). Another model is that MCF-induced leukemias arise in a manner analogous to avian leukosis virus (ALV)-induced B-cell and erythroid cell tumors—namely, by integration adjacent to a cellular proto-oncogene resulting in its activation (7–10). Evidence for this mechanism has also been obtained for mammary tumors induced by the mouse mammary tumor virus (11) and for T-cell lymphomas induced in rats by the Moloney murine leukemia virus (12).

To look for viral integrations near preferred sites, possibly known oncogenes, in MCF-induced tumors, we injected AKR mice with the prototype MCF virus, MCF 247, whose genome we had tagged with a fragment of phage λ genetic information. We examined DNA from the resulting T-cell lymphomas by Southern transfer, both for MCF 247 viral integrations by using the λ DNA probe and for rearrangements of known oncogenes. The large number of proviral integrations precluded a simple search for preferred integration sites with the λ probe. However, using known oncogene probes, we found rearranged c-*myc* genes in about 10–20% of the tumors. These rearrangements, which were caused by MCF viral integrations, are the subject of this report.

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MATERIALS AND METHODS

The construction of MCF 247λPst virus will be described elsewhere. Recombinant phage and plasmids were constructed and DNAs were prepared according to published procedures (13). Preparation of genomic DNA from the thymic tumors was as published (14), except that the whole thymus tissue was ground to a fine powder in liquid nitrogen. Enzymes were used according to commercial suppliers' recommendations.

Probes were obtained from the following sources: pBS-9 and pHiHi-3 were obtained from E. Scolnick (Merck Sharp & Dohme), pAB3sub3 was from S. Goff (Columbia University), pAE-Pvu 11 and pVM-2 were from J. M. Bishop (University of California, San Francisco), and pMS 1 was from G. Vande Woude (National Institutes of Health). Two *myc* probes were used. One, cDNA *myc*, contains the second and third c-*myc* exons from a human Burkitt lymphoma (C. Wood, Massachusetts Institute of Technology). The second, untranslated (UT) *myc*, is a 1.3-kilobase (kb) *Bam*HI subclone containing the first exon of mouse germline *myc*. It was cloned from one of the AKR tumors (S35893) used in this study. The 220-nucleotide λPst probe was ligated to form concatamers before labeling. All probes were labeled by nick-translation (15) of the gel-purified insert.

Southern analyses were as described (16), except that in some cases GeneScreen Plus (New England Nuclear) or Zetabind (AMF, Meriden, CT) was used to immobilize DNA. Stringent and relaxed hybridization conditions were as described (17). Filters were washed, blotted dry, and exposed to Kodak XAR-5 film with intensifying screen at −70°C (18).

RESULTS

Induction of T-Cell Lymphomas in AKR Mice by MCF 247λPst. The retrovirus MCF 247 was isolated from an AKR mouse and is the prototype of a class of leukemogenic MCF viruses that can induce T-cell lymphomas, primarily thymomas, in AKR mice within 3–6 months (1). To trace exogenous MCF 247 genomes in tumor DNAs against a background of cross-hybridizing endogenous viruses, we introduced a 0.22-kb fragment of phage λ DNA into the *Pst* I site in the U3 region of the long terminal repeat of a molecular clone of MCF 247 (Fig. 1). The resulting recombinant yielded infectious virus following transfection, and this virus, called MCF 247λPst, induced lymphomas in AKR mice. We obtained 26 such tumors for further study. As we shall describe elsewhere, the λPst sequences remained stably associated with the MCF 247λPst viral genome during biological cloning of the virus and tumor induction. The resulting tumors contained multiple integrated copies of MCF 247λPst proviruses.

Abbreviations: kb, kilobase(s); UT, untranslated; ALV, avian leukosis virus; MCF, mink cell focus-forming; SFFV, spleen focus-forming virus.

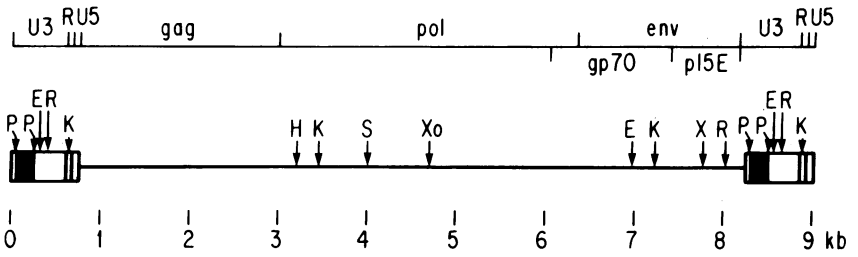

FIG. 1. Location of restriction enzyme sites in MCF 247λPst viral DNA. The top line depicts the viral genes. The middle line shows the restriction enzyme sites for *Pst* I (P), *Eco*RI (E), *Eco*RV (R), *Kpn* I (K), *Hin*dIII (H), *Sac* I (S), *Xho* I (Xo), *Xba* I (X). The inserted λ sequences are drawn as a solid bar in the U3 region of the long terminal repeat.

Rearrangement of c-*myc* Is Detected in Some MCF 247λPst-Induced Lymphomas. Because of the stunning precedent for retroviral integrations near known oncogenes in ALV-induced tumors, we analyzed Southern blots of AKR DNA and of DNA from the 26 MCF 247λPst-induced tumors with probes for the Ha-*ras*, Ki-*ras*, *abl*, *myb*, *myc*, *erb*A and *erb*B, and *mos* oncogenes. We failed to observe gene rearrangements in tumor DNAs using the following probes against DNAs digested with the restriction enzymes indicated: pBS-9 (Ha-*ras*, *Eco*RI), pHiHi-3 (Ki-*ras*, *Eco*RI), pAB-3sub3 (*abl*, *Eco*RI), pVM2 (*myb*, *Eco*RI, *Hin*dIII, *Kpn* I, *Bam*HI), pAE-Pvu II (*erb*A and *erb*B, *Hin*dIII), pMS 1 (*mos*, *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn* I). However, the cDNA *myc* probe revealed a new band in addition to the germline band(s) in each of 6 of the 26 tumors when DNAs were digested with *Eco*RI, *Eco*RV, or *Xba* I. A typical result for *Eco*RI-digested DNAs is shown in Fig. 2. In AKR DNA, germline c-*myc* resides on a 20-kb *Eco*RI fragment. *Eco*RI digests of 6 tumors have *myc* hybridizing bands of about 14 kb in addition to the 20-kb band (Fig. 2).

A striking feature of Fig. 2 is that the relative intensities of the germline and rearranged *myc* bands are different in different tumors. Additional experiments confirmed this finding. For example, Fig. 3 shows a Southern blot of *Kpn* I-digested tumor DNAs from 4 tumors with abnormal *myc* bands. Here germline c-*myc* is on an ≈10 kb band, whereas the rearranged genes are on bands of 8–9 kb. In 2 tumors (S35893 and S36005) the germline and rearranged *myc* bands are of equal intensity (Fig. 3, lanes 3 and 5; Fig. 2, lane 9), whereas in 1 tumor (S35845: Fig. 3, lane 2; Fig. 2, lane 4) the abnormal band is heavier than the germline band. The fact that c-*myc* is on chromosome 15 (19), whereas AKR leukemias are associated with trisomy of chromosome 15 (20), might explain the increased intensity of the abnormal *myc* band in this tumor. Most curiously, in 3 tumors the rearranged *myc* band is less intense than the germline band. One such case (S35894) is shown in Figs. 2 (lane 10) and 3 (lane 4). In the other two cases (tumors S35351, Fig. 2, lane 3, and S36059) the rearranged band is extremely faint. These 2 tumors were not analyzed further in this study. The weak intensity of the rearranged *myc* bands is presumably not due to a deletion in the *myc* gene since the relative intensity of the normal and rearranged genes was the same when either a cDNA *myc* probe homologous to the second and third c-*myc* exons or the UT *myc* probe encompassing the first c-*myc* exon was used.

One tumor (S36005), whose germline and rearranged *myc* bands were of equal intensity, was cultured *in vitro* and a cell line was established. In the cultured cells, the rearranged c-*myc* band is more intense than the germline band (Fig. 3, lanes 5 and 6).

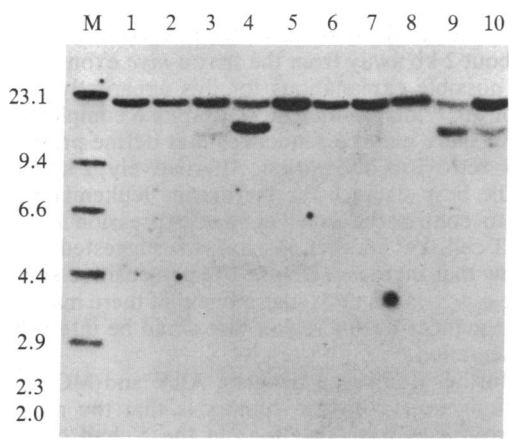

FIG. 2. Autoradiogram of a Southern blot of *Eco*RI-digested tumor DNAs hybridized with UT *myc*. DNAs from MCF 247λPst virus-induced AKR thymomas (lanes 2–10) and a spontaneous AKR thymoma (lane 1) were digested with the enzyme *Eco*RI. The blot was hybridized with the UT *myc* probe that contains the first c-*myc* exon. Lane M shows the electrophoretic mobility of fragments of *Hin*dIII-digested λ DNA and *Taq* I-digested φX174 replicative form DNA with the sizes in kb shown.

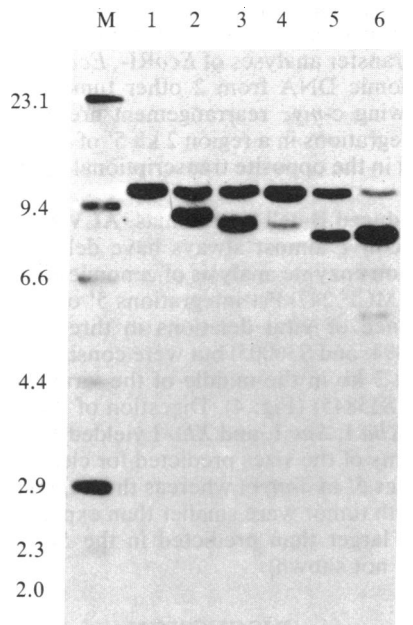

FIG. 3. Autoradiogram of a Southern blot of *Kpn* I-digested tumor DNAs hybridized with cDNA *myc*. DNA was isolated from tumors with rearranged c-*myc* (lanes 2–5) or without c-*myc* rearrangement (lane 1) and from cultured cells (lane 6) derived from a tumor (lane 5) and digested with *Kpn* I. The blot was hybridized with the cDNA *myc* probe that consists of the second and third c-*myc* exons. Lane M is the same as shown in Fig. 2. The identity of some faint bands in lane 6 is not known and they were not observed in other Southern analyses. Sizes are shown in kb.

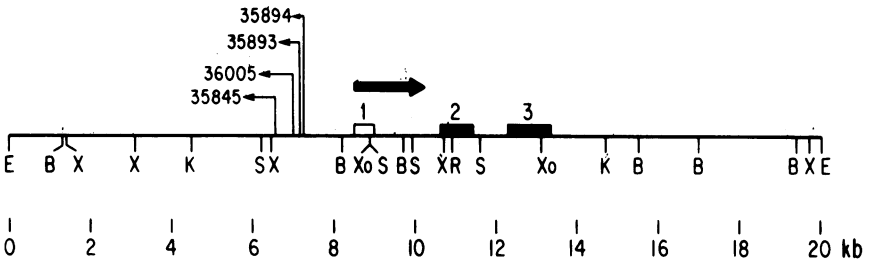

FIG. 4. MCF 247 integrations near AKR c-*myc*. Location of restriction enzyme sites in a 20-kb *Eco*RI fragment containing the germline c-*myc*. The thick arrow indicates the c-*myc* transcriptional orientation. Numbers 1, 2, and 3 show the positions of the first, second, and third exons of AKR c-*myc*. The open box shows the position of the UT first exon. Each vertical line represents the site of integration of an MCF 247 provirus in separate tumors. The small arrows on top of each line depict the orientation of transcription of each provirus. Restriction enzyme abbreviations are the same as shown in Fig. 1, except *Bam*HI (B).

MCF 247λPst Integration 5′ of c-*myc* in the Opposite Transcriptional Orientation. Southern blots of *Eco*RI-digested tumor DNAs probed with the λPst sequence revealed bands at the same positions as the four aberrant *myc*-containing bands in the 4 tumors we studied further (data not shown). This result suggests that the c-*myc* rearrangements might be due to nearby integrations of MCF 247λPst viruses. To confirm the integration of MCF 247λPst viruses adjacent to c-*myc* and to determine their orientation and precise location relative to c-*myc*, we cloned the 14-kb *Eco*RI *myc*-containing fragments from 2 of the tumors, S35845 and S35893. A recombinant phage library was prepared from enriched 14- to 20-kb *Eco*RI-digested DNA and screened with the cDNA *myc* probe. Clones of both rearranged (clone 113) and germline c-*myc* were obtained from tumor S35893. A clone of rearranged c-*myc* (clone 91) was obtained from tumor S35845. The c-*myc* gene inserts were subcloned in pBR322. Restriction enzyme analysis of the germline gene yielded a map virtually identical to that obtained by Adams *et al.* (21). Analysis of the two rearranged genes showed that both contained the λPst tag at the 5′ end of c-*myc*, about 2 kb from the first exon (Fig. 4) and that the proviruses are pointed in the opposite transcriptional orientation from c-*myc*.

Southern transfer analyses of *Eco*RI-, *Eco*RV-, and *Xba* I-digested genomic DNA from 2 other tumors (S36005 and S35894) showing c-*myc* rearrangement are also consistent with viral integrations in a region 2 kb 5′ of c-*myc*, as shown in Fig. 4, and in the opposite transcriptional orientation from c-*myc*.

In ALV-induced B-cell lymphomas, ALV proviruses integrated near c-*myc* almost always have deletions (7, 9, 22, 23). Restriction enzyme analysis of genomic DNA from the 4 tumors with MCF 247λPst integrations 5′ of c-*myc* failed to reveal evidence of viral deletions in three cases (tumors S35893, S35894, and S36005) but were consistent with a deletion of about 3 kb in the middle of the viral genome in the fourth case (S35845) (Fig. 4). Digestion of the first 3 tumor DNAs with *Xba* I, *Sac* I, and *Xho* I yielded UT *myc* hybridizing fragments of the sizes predicted for cleavage within intact proviruses 5′ of c-*myc*, whereas the fragment sizes seen with the fourth tumor were smaller than expected in the *Xba* I digest and larger than predicted in the *Sac* I and *Xho* I digests (data not shown).

DISCUSSION

We found integrated MCF 247 proviral DNA 5′ of the c-*myc* gene in some AKR thymomas induced by this virus. All four of the proviruses analyzed were in the opposite transcriptional orientation from c-*myc*. Although we have no direct evidence that these integrations near c-*myc* played a role in tumor formation, analogy to ALV-induced bursal lymphomas (7–9) makes this seem likely. Also consistent with these proviral integrations playing a causal role in tumor development is the failure to obtain evidence for preferred proviral integration near c-*myc* during routine viral infection. No c-*myc* rearrangements were detected in 20 cell lines cloned from a culture of fibroblastic Sc-1 cells infected *in vitro* with MCF 247λPst, even though these lines had on average twice as many integrated proviruses as the tumors we analyzed (data not shown). Assuming then that the c-*myc* rearrangements we studied were important in tumorigenesis, it is interesting to note that there are significant differences between the mechanism of MCF-induced AKR thymomas and ALV-induced bursal lymphomas. In the majority of bursal tumors the ALV promoter plays an important role in activating c-*myc* (7–9, 23, 24), whereas in the AKR thymomas we studied c-*myc* activation is more likely due to the enhancer element in the U3 region of the long terminal repeat because the proviruses and c-*myc* are in opposite transcriptional orientations. Enhancer activation of c-*myc* or other oncogenes is not without precedent. Some ALV-induced bursal lymphomas probably involve this type of activation (25); as yet ill-defined oncogenes may be activated this way in mouse mammary tumor virus-induced tumors (11); and in human Burkitt lymphomas chromosome translocations sometimes bring c-*myc* adjacent to an immunoglobulin gene locus, where it may utilize an immunoglobulin gene enhancer (26).

In mouse plasmacytomas the majority of translocations involving c-*myc* break the gene within the first exon (21). Similarly, ALV proviruses integrated near c-*myc* in chicken bursal lymphomas usually interrupt the first c-*myc* exon or intron (27). In contrast, the MCF 247λPst proviruses we found near c-*myc* in AKR thymomas were integrated within a 1-kb range about 2 kb away from the first c-*myc* exon. There are several possible explanations for this apparently preferred region of MCF integration. First, despite a complete lack of evidence, there may be sequences that define preferred regions for retrovirus integration. Alternatively, 2 kb may represent the best distance for the murine leukemia virus enhancer to control the level of *myc* expression in a transformed T cell. Yet another possibility is suggested by studies that show that there is a DNase I hypersensitive site 2–3 kb 5′ of the c-*myc* locus (28), suggesting that there may be regulatory sequences in this region that could be interrupted by viral integration.

One further difference between ALV and MCF proviral integrations near c-*myc* in tumors is that the majority of ALV proviruses have deletions in the 5′ half of their genomes (7–9, 22), whereas three of four MCF proviruses analyzed here appeared to be intact. This difference might reflect the use of the promoter by ALV versus only the enhancer by MCF to activate c-*myc*.

A curious finding in our study was 3 tumors in which an aberrant-sized c-*myc* band was present but hybridized less intensely than the germline band. It is possible that much of the DNA in these preparations was from normal rather than

tumor cells, although this seems unlikely since the DNA was obtained from grossly enlarged thymic tissue. As noted above, it is extremely unlikely that deletion of the rearranged c-*myc* gene explains our result. Possibly MCF 247 integration near c-*myc* in these tumors occurred as a late event after the tumor was already fairly large. Alternatively, it is possible that these tumors are not clonal.

We noted that the increased intensity of a rearranged c-*myc* gene in one of the tumors we studied might be explained by a trisomy of chromosome 15 (20) carrying the rearranged gene. Interestingly, cultured tumor cells derived from a tumor that had germline and rearranged c-*myc* bands of equal intensity retained mainly the rearranged c-*myc* band. Possibly the rearranged c-*myc* gene confers a growth advantage on cells *in vitro* and even greater amounts of this gene may be needed for growth *in vitro* than for growth as a tumor *in vivo*.

The tumors containing rearranged c-*myc* represent only about 12% of the total we analyzed. What about the other 80–90% of the tumors? If we consider that viral integration near proto-oncogenes plays a major role in AKR tumorgenesis, then there must be other specific sites of integration that are important. We failed to find evidence of viral integration near Ha-*ras*, Ki-*ras*, *abl*, *myb*, *erb*A and *erb*B, and the *mos* proto-oncogenes, but it is possible that there are other oncogenes that may be activated by viral integration in AKR leukemogenesis. Alternatively, viral integrations near c-*myc* may be present in other MCF-induced tumors but outside the ≈20 kb 5′ and ≈15 kb 3′ of the gene that we scanned in these studies.

While this manuscript was in preparation, two reports of similar observations appeared, one demonstrating proviral integrations near c-*myc* in 25% of spontaneously arising AKR thymomas (29) and the other describing occasional integration of Moloney leukemia virus sequences near c-*myc* in rat lymphomas (30). The similarity between c-*myc* alterations in spontaneous (29) and MCF 247-induced AKR thymomas is particularly striking.

It was our privilege during the early stages of this work to have had Dr. Wallace P. Rowe as a collaborator. His tragic death in July 1983 deprived us of an extraordinary mind and a much loved friend. We are grateful to have had his input and shall continue to regret his loss. We thank Adrian Hayday for many helpful discussions, Drs. E. Scolnick, S. Goff, J. M. Bishop, G. Vande Woude, and C. Wood, who provided us with probes, Lucinia Pilapil for excellent technical assistance, and Concepta Siembab for preparation of this manuscript. This work was supported by Grant POI-CA19308 and Program Project Grant CA26717 from the National Institutes of Health to N.H. and partially by Grant CA19308 (Core) to S. E. Luria. Y.L. acknowledges the support of the Damon Runyon–Walter Winchell Cancer Fund (DRG 705). C.A.H. acknowledges the support of the Muscular Dystrophy Association. A portion of this work was done on Contract NOI-AI-22673 at Microbiological Associates, Bethesda, MD.

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