Restriction enzyme analysis of mouse cellular type C viral DNA: Emergence of new viral sequences in spontaneous AKR/J lymphomas

(Southern gel blotting/inbred mouse strains/monoclonal tumors)

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ABSTRACT   The topography of endogenous type C viral sequences in mouse cellular DNA was investigated by EcoRI nuclease restriction and application of the Southern blotting technique. The DNAs from one outbred and five inbred strains were resolved into 20–35 fragments containing viral sequences, distributed in unique, though related, patterns for each mouse strain. Different normal tissues from the same animal were indistinguishable in their DNA patterns, suggesting that tissue differentiation is not associated with gross alteration in the topography of endogenous type C virus sequences. Tumor tissues from spontaneous lymphomas of AKR/J mice were similarly analyzed. In four out of seven individual tumors we detected the emergence of one or two new virus-containing DNA fragments. The mass of these fragments varied, indicating different insertion sites of the new viral sequences. The detection of these new viral sequences suggests that each tumor was composed of descendents of only one or a few cells.

In recent years it has been established that type C viruses are transmitted genetically within the mouse cellular genome. Various inbred mice strains show different patterns of expression of endogenous type C virus information. This is correlated with the frequency and time of leukemia development. Thus, strains such as AKR/J and C58/J yield high titers of virus from early age and develop leukemia with high incidence at 6–12 months of age, whereas strains such as BALB/c and C57BL/6J express low titers of virus only after the age of 4–6 months and have a moderate incidence of leukemia later in life. A third class of strains such as NIH Swiss and 129 do not produce infectious viruses and very rarely develop leukemia. The application of molecular hybridization techniques provided evidence that multiple copies of viral sequences are present in the chromosomal DNA of all mouse strains (1–3). Some of these copies represent the genomes of ecotropic viruses, whose structural information is present only in some inbred strains (2). Other viral copies presumably represent the genomes of xenotropic viruses (4), while others, structural genes of as yet uncharacterized viruses or partial virus genomes.

Recent advances in the technology of separation of DNA fragments and their detection by hybridization in situ have made it possible to study the arrangement of endogenous viral sequences within mouse cellular DNA. We applied the Southern blotting technique (5) to study the size and topographical distribution of these sequences in several mouse strains and in a variety of normal and malignant tissues. In particular, we have asked whether normal tissue differentiation is accompanied by rearrangements of endogeneous type C viral sequences and whether naturally occurring lymphomas of inbred mice are associated with alteration or specific addition of type C virus-containing DNA fragments.

MATERIALS AND METHODS

Mice. AKR/J and C57BL/6J mice were generously supplied by M. Cloyd (National Institutes of Health) and M. Dinowitz (Microbiological Associates, Bethesda, MD), respectively. Inbred C57L/J and BALB/c strains were obtained from Jackson Laboratory, and Charles River Breeding Laboratories, respectively. Inbred NIH Swiss (NFS) and NZB/N mice were raised at the National Institutes of Health colony. Mice from an outbred colony of wild mice were generously provided by M. Collins (Microbiological Associates).

Extraction and Purification of Tissue DNA. DNA was prepared from individual fresh organs by mincing (thymus) or homogenization (all other organs) in 5 vol of 150 mM NaCl/70 mM EDTA at pH 8.0. Sodium dodecyl sulfate was added to a concentration of 1.0%, and the homogenate was incubated with 1 mg of autodigested Pronase per ml at 37°C overnight. The DNA was extracted three times with phenol and CHCl$_3$/isoamyl alcohol. After extensive dialysis against 1 M NaCl, and then against 10 mM Tris·HCl at pH 7.5/1 mM EDTA, the nucleic acids were incubated with 100 µg of pancreatic ribonuclease per ml at 37°C for 1 hr and then deproteinized and dialyzed as described. DNA was fractionated on preparative sucrose gradients; DNA sedimenting at 25–50 S was concentrated by ethanol precipitation.

Restriction Endonuclease Digestion and Gel Electrophoresis. High molecular weight DNA was digested to completion with EcoRI (New England BioLabs). DNA at a concentration of 40 µg/ml was incubated in a reaction mixture containing 100 mM Tris·HCl at pH 7.5, 50 mM NaCl, 5 mM Mg(OAc)$_2$, 100 µg of bovine serum albumin per ml, and 2 mM dithiothreitol. The extent of digestion of cellular DNA was monitored by inclusion of radiolabeled λ phage DNA in the reaction mixtures. Incubation for 2 hr at 37°C was followed by phenol extraction and ethanol precipitation. The resolution by agarose gel electrophoresis of multiple fragments containing viral sequences was found to require the use of small amounts of DNA, long gels, low agarose concentrations, and low voltage. Thus, about equal amounts of the different DNAs (10–15 g) were redissolved in 50 µl of 10 mM Tris·HCl at pH 7.5/1 mM EDTA and loaded into 6 × 2 × 8 mm deep slots in a 22 × 13.5 × 1.0 cm horizontal slab gel. The gels (0.5% or 0.7% agarose) were run at room temperature for 40 hr at 25 V and 30 mA. One hour after the beginning of the run, the gels were overlaid with electrophoresis buffer (40 mM Tris·HCl at pH 7.7/5 mM NaOAc/1 mM EDTA).

Blotting and Hybridization. Gels were blotted and the DNA was fixed on nitrocellulose sheets as described (6). The filters were incubated for 2 hr at 65°C in a solution containing 0.45 M NaCl, 0.045 M Na citrate, 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone. This solution was replaced with a similar solution supplemented with 0.1%

sodium dodecyl sulfate, 1 mM EDTA, and 50 μg each of sheared salmon sperm DNA and denatured sheared rat DNA per ml. After 4–10 hr it was replaced with a similar solution containing 1–2 × 10⁶ cpm/ml of ³²P-labeled cDNA probe. [This probe was obtained by reverse transcription of a 1:1 mixture of 35S RNA from ecotropic AKR and xenotropic NZB viruses. The RNA in this reaction was primed by calf thymus DNA fragments (7); ³²P-labeled dCTP (Amersham/Searle) had a specific activity of 2000–3000 Ci/mmol (1 Ci = 3.7 × 10¹⁰ becquerels).] Hybridization was conducted in a volume of 10 ml for 22 × 13 cm strips for a period of 2 days at 65°C. The filters were then washed at 65°C for 1 hr with five changes of complete hybridization solution (omitting ³²P-labeled cDNA), followed by 30-min wash at 65°C in 15 mM NaCl/1.5 mM citrate supplemented with 0.1% sodium dodecyl sulfate and 50 μg of denatured sheared salmon sperm DNA per ml. The strips were dipped in a solution of 3 mM Tris·HCl at pH 9.3 at 4°C, transferred into a solution of 1 M NH₄OAc, dried, and autoradiographed by exposure for 2–6 days to Kodak X-Omat R film.

RESULTS

Specific Patterns of Endogenous Viral Sequences in the DNAs of Different Mouse Strains. High molecular weight DNAs from livers of six inbred mouse strains—NFS (NIH Swiss), AKR/J, BALB/c, NZB/N, C57BL/6J, C57L/J—and from one wild mouse were digested with the site-specific endonuclease EcoRI (8). Unless viral sequences were integrated in tandem, most of the fragments should contain viral sequences flanked on 1 or 2 ends with cellular sequences. In addition, some fragments might be composed entirely of viral sequences.

The DNA digest was fractionated by agarose gel electrophoresis and then directly transferred to nitrocellulose sheets by the Southern blotting technique (5). The location of the fragments containing viral sequences was determined by hybridizing the DNA on the filters to ³²P-labeled viral cDNA (Fig. 1). DNA from all the mice yielded complex patterns of hybridization. This reflects the presence of viral sequences in DNA fragments of many different sizes; 20–35 bands were resolved on each gel lane. The high signal intensity associated with some bands reflects either a closer relationship between the viral sequences in these fragments and the cDNA probe or superimposition of several bands. The molecular weights of the fragments were determined by comparing their electrophoretic mobilities with those of a reference set of fragments from a HindIII digest of λ phage DNA (9). Many of the bands had a mobility slower than that of a 6 × 10⁶ dalton marker and, thus, were larger than unit length viral DNA.

The distribution of DNA fragments was unique for each mouse strain. However, a substantial number of bands appeared to be shared by DNAs of most of the strains analyzed (Fig. 1). Individuals of a given inbred mouse line showed identical distribution of viral sequences. However, the pattern of each outbred wild mouse was unique and different from one individual to another (data not shown). There was a remarkable similarity between the patterns of C57BL/6J and C57L/J cellular DNAs, a result that may be due to the fact that these two inbred strains were derived relatively recently from a common progenitor (10). The C57BL/6J strain is known to possess the genome of an inducible ecotropic virus, identifiable by molecular hybridization (ref. 2; K. Robbins and S. Aaronson, unpublished), whereas the C57L/J mouse lacks this virus. Thus, it is conceivable that one of the few extra bands detectable in the C57BL/6J DNA pattern represents the genome of this ecotropic virus.
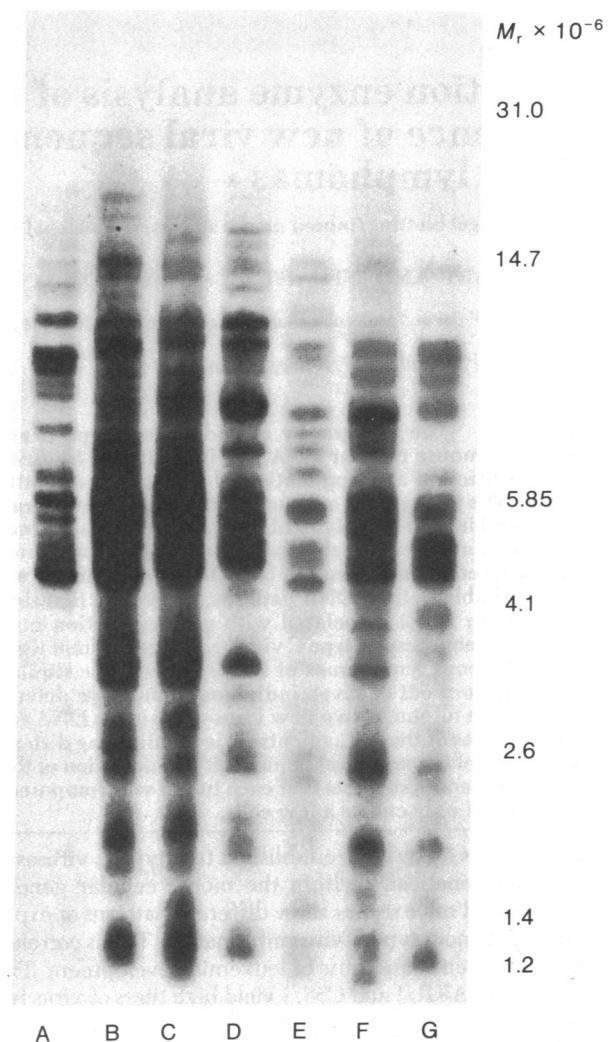

FIG. 1.   EcoRI digests of DNA from different mouse strains. Liver DNAs from (A) wild, (B) C57BL/6J, (C) C57L/J, (D) NZB/N, (E) BALB/c, (F) AKR/J, and (G) NIH Swiss (NFS) mouse strains were digested with EcoRI endonuclease, fractionated on a 0.5% agarose gel, blotted into nitrocellulose sheets, and hybridized to a ³²P-labeled viral cDNA probe. The strips were then autoradiographed by exposure for 2 days at room temperature to Kodak X-Omat R film. Molecular weight markers of intact λ phage DNA (31 × 10⁶) and HindIII λ DNA fragments were electrophoresed in parallel.

Patterns of Viral Sequences in the EcoRI DNA Fragments of Differentiated Tissues. Recently it has been reported that some rearrangement of mouse DNA takes place during differentiation. Specifically, it has been shown that the genes for κ and λ immunoglobulin chains are found to be arranged differently in the DNAs of antibody-producing lymphocytes as compared to embryonic DNA (11, 12). We investigated the possibility that rearrangements of DNA segments containing viral sequences might occur during differentiation.

High molecular weight DNA was extracted from the brain, spleen, testis, liver, and kidney of an NFS mouse. Similarly, DNA was obtained from the liver, brain, spleen, and kidney of a wild mouse. The DNA was digested with EcoRI endonuclease, fractionated on agarose gels, blotted into nitrocellulose filters, hybridized to ³²P-labeled cDNA, and autoradiographed. As shown in Fig. 2, the band patterns of DNA from the five organs from the NFS mouse were indistinguishable from one another, as were those of four tissues from the wild mouse. Because most of the bands are composites of viral and cellular sequences, we

can conclude that there is no gross rearrangement of endogenous viral sequences and their flanking DNA in the various differentiated organs analyzed.

Appearance of New Virus-Related Sequences in the DNA of AKR Thymic Lymphomas. Because normal mouse tissues appeared to have identical patterns of viral DNA sequences, we asked whether tumor tissues showed the same distribution as normal tissues. We analyzed the DNA from AKR/J thymic lymphomas, which appear at high incidence in AKR mice at the age of 6–12 months (13). High molecular weight DNA was extracted from normal thymuses of 1- to 2-month-old AKR/J mice and from the grossly enlarged thymuses of leukemic animals. The DNAs were digested with EcoRI endonuclease and analyzed by the blotting technique.

Four individual thymomas out of the seven tested appeared to contain extra bands lacking either in the DNA of normal AKR thymus (Fig. 3, lane B) or in normal tissues of the leukemic animal (data not shown). The pattern of AKR thymoma 2 DNA showed one extra band of mass 3.5×10^6 daltons (Fig. 3, lane C). AKR thymoma 3 DNA revealed the presence of new 12.5×10^6 and 7.3×10^6 dalton fragments (Fig. 3, lane D), whereas DNAs from AKR thymomas 6 and 7 contained one additional band each of masses 16×10^6 and 17×10^6 daltons, respectively (Fig. 3, lanes E and F). Due to the large number of bands on the blot, it proved difficult to resolve some of the new bands from those present in the blot of normal AKR/J thymus DNA (Fig. 3, lanes A and B). Thus, the band of mass 12.5×10^6 daltons (Fig. 3, lane D) was superimposed on a faint band apparent in normal thymic tissue DNA, and the band of mass 7.3×10^6 daltons (Fig. 3, lane D) did not resolve well from an adjacent preexisting band. It has been our experience that for a particular DNA preparation both relative intensities and relative width of virus-containing bands are reproducible from one experiment to another. The data presented in Fig. 2 demonstrate this point. Therefore, a reproducible enhancement of signal intensity or of band width was considered significant. As shown here, each thymoma possessed new DNA fragment(s) with
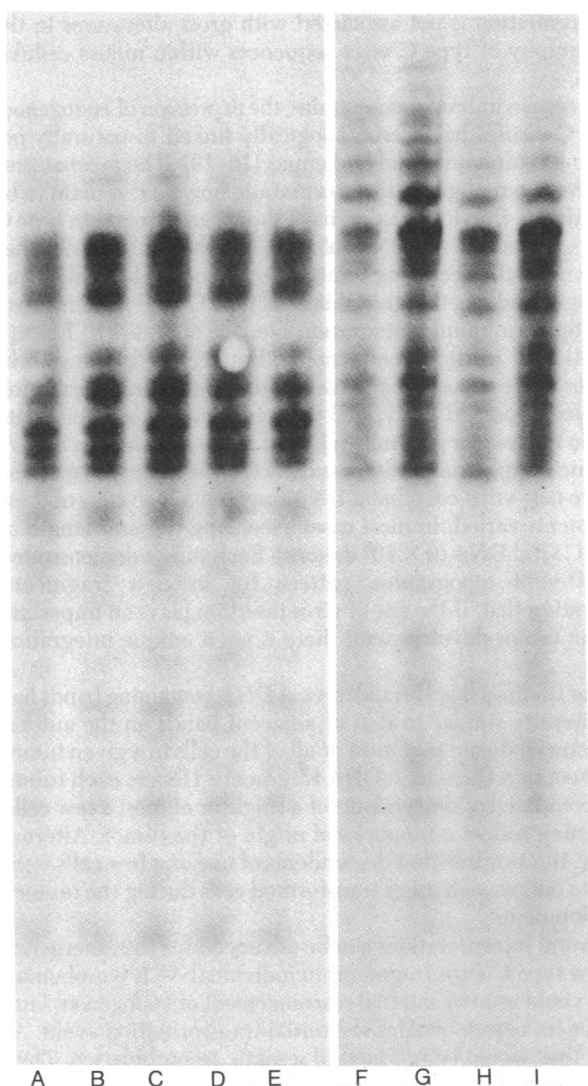

FIG. 2. Detection of fragments containing type C viral sequences in the DNA from differentiated tissues of NIH Swiss and wild mouse strains. EcoRI digests of DNA from (A) brain, (B) spleen, (C) testis, (D) liver, and (E) kidney of one NIH Swiss mouse were fractionated on a 0.7% agarose gel and analyzed for the presence of viral sequences. The digests of DNA from (F) liver, (G) kidney, (H) brain, and (I) spleen of one wild mouse were fractionated on a 0.5% agarose gel and similarly analyzed.
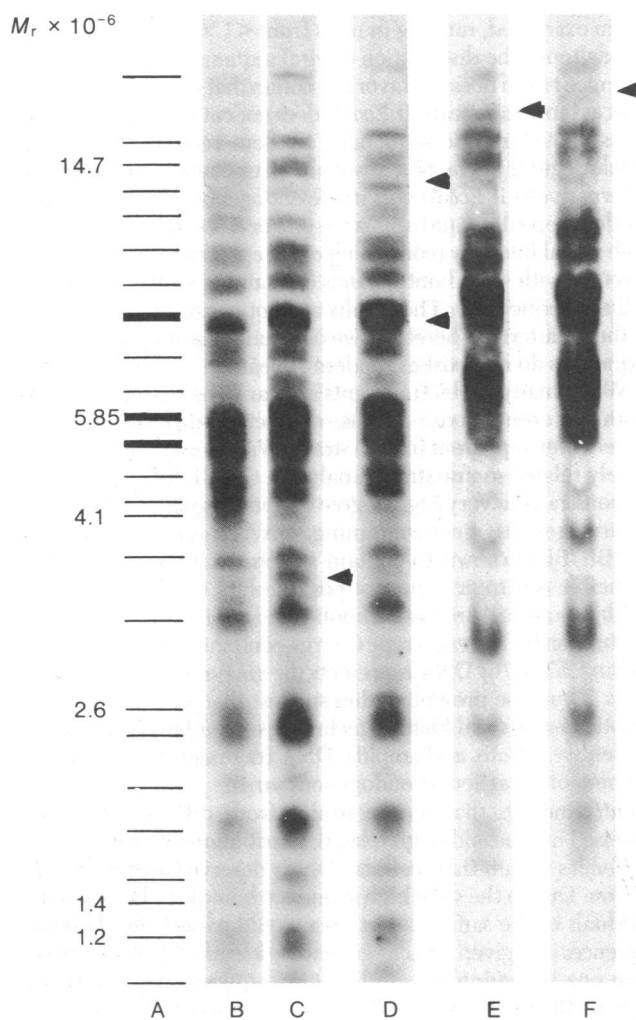

FIG. 3. Detection of new type C virus-containing fragments in the DNAs of spontaneous AKR/J thymic lymphomas. The DNAs from one AKR/J normal thymus (B) and four AKR/J thymoma (C, thymoma 2; D, thymoma 3; E, thymoma 6; F, thymoma 7) were analyzed for the presence of viral sequences as described in the legend to Fig. 1. A schematic representation of the band pattern for normal AKR/J thymus DNA is shown in lane A. The DNA preparations shown in lanes B, C, and D were analyzed in one gel, and those of lanes E and F were analyzed in a separate gel. Arrows point to the new virus-positive fragments.

different size. Most of the new fragments were larger than 6 × 10^6 daltons, the mass of a complete type C viral genome. Therefore, they presumably contain both viral and cellular sequences.

DISCUSSION

The application of molecular hybridization techniques has established the existence of multiple copies of type C viral sequences within mouse cellular DNA (1–3). Recently, it has become possible to study the arrangement of endogenous type C viral sequences within mouse cellular DNA, due to advances in restriction endonuclease technology, in resolution of DNA fragments by gel electrophoresis, and the development of techniques for direct transfer of DNA fragments from gels to nitrocellulose filters for molecular hybridization.

In the present report, refinement of these techniques made it possible to achieve a high degree of resolution of cellular DNA fragments containing endogenous type C viral sequences. DNAs of several mouse strains were demonstrated to possess unique patterns of EcoRI fragments containing type C viral sequences. At least 20–35 such fragments were resolved for DNAs of each strain examined, ranging in mass from <1 × 10^6 to about 20 × 10^6 daltons. The distribution of viral sequences in the DNAs of the mice tested does not favor the notion that the viral sequences exist as tandem repeats. If EcoRI does not cut inside endogenous virus-related mouse sequences, tandem repeated sequences would register as one or a few giant virus-containing molecules. Alternatively, if EcoRI cuts inside endogenous viral sequences, tandem repeats would appear as one or a few bands with very high signal intensity (containing only repeated viral sequences) together with some fainter bands (containing viral and flanking cellular sequences). The results are not consistent with either of these patterns. Therefore, we conclude that most of the viral sequences do not exist as tandem repeats.

While many of the fragments appear to be shared by DNAs from different mouse strains, distinct modifications of the pattern were apparent in each strain. We observed that the most closely related mouse strains analyzed, C57BL/6J and C57L/J, demonstrated a very high degree of homology in their patterns of endogenous virus-containing DNA fragments. Because C57BL/6J is known to contain structural information of an inducible ecotropic virus not possessed by C57L/J (ref. 2; K. Robbins and S. Aaronson, unpublished), it is conceivable that one or more of the extra type C virus-containing bands observed within C57BL/6J DNA represents the genome of this ecotropic virus. Thus, the present studies suggest that a combination of genetic analysis and blotting techniques should make it possible to identify, isolate, and amplify DNA fragments containing the genome of a particular endogenous virus.

Our findings that mouse strains from different lineages possessed unique, although related, distributions of virus-related sequences within their cellular DNAs deserve further consideration. Due to the very high sequence homology between individuals of the same species, one would expect the flanking sequences of a given cellular gene to be identical or very similar from one individual to another. Consequently, the Southern blots of the same gene in different individuals of the same species would be expected to be very similar. In fact, it recently has been demonstrated that the localization of α-globin sequences in human DNA was identical in specimens obtained from five normal and four β-thalassemic subjects (14). The heterogeneity observed here in the distribution of viral sequences in the DNAs of different mouse strains suggests that endogenous viral genes possess some unusual properties. One possible interpretation is that the viral genes are unstable elements, capable of dissociating from the host chromosome and

reinserting into different sites. In this respect, they would be the eukaryotic equivalents of certain bacterial plasmids (15). Alternatively, after the time of speciation of Mus musculus, continued exogenous infections into the germ line may have been accompanied by insertions into different sites within the cell genome. Consequently, individual animals may have acquired different patterns of viral genes and transmitted them to their progeny.

Recent studies have demonstrated that differentiation of an antibody producing lymphoid cell can be accompanied by rearrangement of genes associated with this differentiated function (11, 12). The high degree of resolution of viral DNA-containing cellular DNA fragments made it possible to study whether normal tissue differentiation might be associated with somatic rearrangement of type C virus genetic information. There were no detectable differences in the chromosome topography of endogenous viral sequences and their adjacent cellular DNA in a variety of normal tissues. While studies with other restriction endonucleases may be useful, it is possible to conclude on the basis of the present results that normal tissue differentiation is not associated with gross alterations in the topography of type C virus sequences within mouse cellular DNA.

In certain imbred mouse strains, the expression of endogenous type C viruses has been etiologically linked to naturally occurring lymphomas and leukemias (16–19). The mechanisms involved in malignant transformation, however, remain to be elucidated. Previous studies have shown an increase in type C viral sequences in the DNAs of AKR lymphomas (20). Whether these naturally occurring tumors are monoclonal or polyclonal in origin, and whether the additional type C viral sequences are inserted at a unique integration site, are not known. The application of restriction enzyme and molecular blotting techniques made it possible to investigate whether transformation was accompanied by type C virus insertion at a specific site within the cellular genome. In four of seven spontaneous AKR/J thymic lymphomas tested, we detected the emergence of one or two new virus-containing DNA fragments. The mass of these fragments varied, in most cases exceeding the unit length of type C viral DNA (6 × 10^6 daltons). Each tumor demonstrated a different topographic pattern for its new fragments, suggesting that, if the site of virus insertion plays an important role in tumor development, there is not a unique integration site.

Our findings that the added viral DNA-containing bands had an intensity similar to that of adjacent bands on the autoradiograms indicate that most or all of the cells in a given tumor must contain these new DNA fragments. Hence, each tumor was composed of descendents of a single or at most a few cells. This may reflect a monoclonal origin of the tumor. Alternatively, it is possible that descendents of one or a few cells were able to outgrow all other transformed cells during the tumor's development.

Several interpretations can be advanced for the emergence of new type C virus fragments in individual AKR lymphomas. They could arise by internal rearrangement of endogenous viral sequences before or after the initial transformation event. As such, they would be products of somatic recombination. They could also reflect the initiating event in the development of the tumor, namely the emergence and reintegration into mouse cellular DNA of an additional copy of type C virus information. Finally, the new fragments might be observed as the result rather than as the cause of the tumor. There is a high level of ecotropic virus release and reinfection in the AKR mouse during the course of its development. Thus, transformation due to any mechanism might lead to amplification during tumor devel-

Biochemistry: Canaani and Aaronson

Proc. Natl. Acad. Sci. USA 76 (1979)   1681

opment of a virus-infected cell. The identity of new virus-containing DNA fragments in spontaneous AKR lymphomas may be determined by the isolation of these fragments, their amplification by transfection, and further biochemical characterization after molecular cloning (21). By such approaches, it may be possible to determine whether the added viral sequences possess properties that might be expected of a more malignant variant or recombinant virus (22).

Note Added in Proof. Using similar techniques, Steffen and Weinberg (23) have recently demonstrated the appearance of unique viral bands in individual thymomas induced in NIH/Swiss mice by inoculation with Moloney leukemia virus.

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