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The Presence of Unique DNA Sequences after Viral Induction of Leukemia in Mice

(RNA tumor virus/nucleic acid hybridization/insertion of viral DNA)

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ABSTRACT **From previous studies, lymphocyte DNA from human leukemias and DNA from involved tissues of patients with Hodgkin's disease or Burkitt's lymphoma contain sequences that are absent from their normal counterparts. These sequences are related to those found in particulate elements associated with these neoplasias and possessing biochemical properties characteristic of RNA tumor viruses. Similar observations have been made of unique sequences related to those of the feline virus RD-114 and found in spontaneous mastocytomas in cats. Here we extend these results to the classical murine model of virus-induced leukemias.**

Splenic DNA from BALB/c mice with leukemia induced by Rauscher leukemia virus (RLV) possess some RLV-related sequences that do not exist in normal BALB/c DNA. Furthermore, these leukemia-specific sequences were absent in all other mouse strains examined, including AKR, a strain with a high incidence of spontaneous leukemia.

The DNA of all noninfected mouse strains possesses considerable homology with the RLV genome. Temperature denaturation studies indicate, however, that although the RLV-related sequences found in all normal mice are similar to each other, they are not exactly homologous with RLV sequences.

We conclude that RLV-induced leukemia in BALB/c results in the insertion of RLV sequences into cellular DNA that itself possesses only partial homology with the RLV genome.

One of the key questions of viral oncology may be usefully put in the following terms: "Does the DNA of a malignant cell contain viral-related sequences that are *not* found in the DNA of its normal counterpart?" Phrasing the issue in this manner leads to the design of experiments that avoid the uncertainties generated by the demonstrated fact that many indigenous tumor viruses share *some* sequences with the normal DNA of their natural hosts (1–5, 19, 23). The crucial point is of course whether *all* of the viral sequences are to be found in normal DNA. The strategy adopted requires removal of those viral sequences that are shared with normal DNA by exhaustive hybridization of the viral probe to normal DNA in vast excess. If any unhybridized residue is left, it can be used to determine whether malignant DNA contains viral-related sequences not detectable in normal tissue.

We first reported experiments along these lines with human leukemias (6). Leukemic cells contain viral-related 70S RNA and an RNA-directed DNA polymerase encapsulated in particles possessing a density of about 1.16 g/cm^3 (7, 8). These particles were used to generate [^{3}H]DNA probes from which normal sequences were removed by hybridization to normal leukocyte DNA in vast excess. The resulting residue of unpaired [^{3}H]DNA was then annealed alternatively to the white blood cell DNA from leukemic patients and to normal DNA. The data obtained with 10 patients, including leukemic members of two sets of identical twins (9), revealed in every instance that the DNA of leukemic cells contained particle-related sequences that could not be found in the leukocyte DNA of normal individuals. The sensitivity of these experiments was such that 1/50th of the hybridizable DNA observed in leukemic cells would have been readily detected. Since the leukemia-specific sequences are present at a level of about one per genome equivalent, these findings suggest that the vast majority of normal cells do not contain a single copy of the leukemia-specific sequences.

A parallel series of investigations was made in human lymphomas with [^{3}H]DNA probes generated by the particles isolated from Burkitt's lymphomas and Hodgkin's disease spleens (10). The nuclear DNAs of both of these human lymphomas contain particle-related sequences that could not be detected in the DNA of normal cells (11).

One could argue that these findings are not relevant to animal systems that involve viral agents of established oncogenic ability. The present investigation seeks to provide the necessary data using DNA from BALB/c mice before and after infection with the Rauscher leukemia virus (RLV), a murine virus originally isolated from an induced leukemia in BALB/c (12).

The results indicate that leukemic spleen DNA contains RLV-related sequences that are absent in normal BALB/c DNA. The data from the murine system and the conclusions derivable from them are therefore similar to those obtained with the human disease. Leukemic cells contain particle-related sequences not found in normal DNA.

MATERIALS AND METHODS

Virus. Rauscher leukemia virus (RLV) was purified from RLV-infected BALB/c plasma (University Laboratories, Highland Park, N.J.) by isopycnic sucrose centrifugation (15). This virus stock was passaged only in BALB/c or Swiss Webster mice since its isolation by Rauscher (12).

Isolation of Nucleic Acids. Mouse tissues were minced, suspended in 5% sucrose TNE [0.01 M Tris·HCl, pH 8.2, 0.15 M NaCl, 3 mM ethylenediamine tetraacetate (EDTA)] and

Abbreviations: RLV, Rauscher leukemia virus; AMV, avian myeloblastosis virus; EDTA, ethylenediaminetetraacetate; PB, sodium phosphate buffer; C_0t, product of initial single-stranded nucleic acid concentration and time; $T_{e^1/_2}$, temperature at which half of the hybrid was eluted from hydroxyapatite.

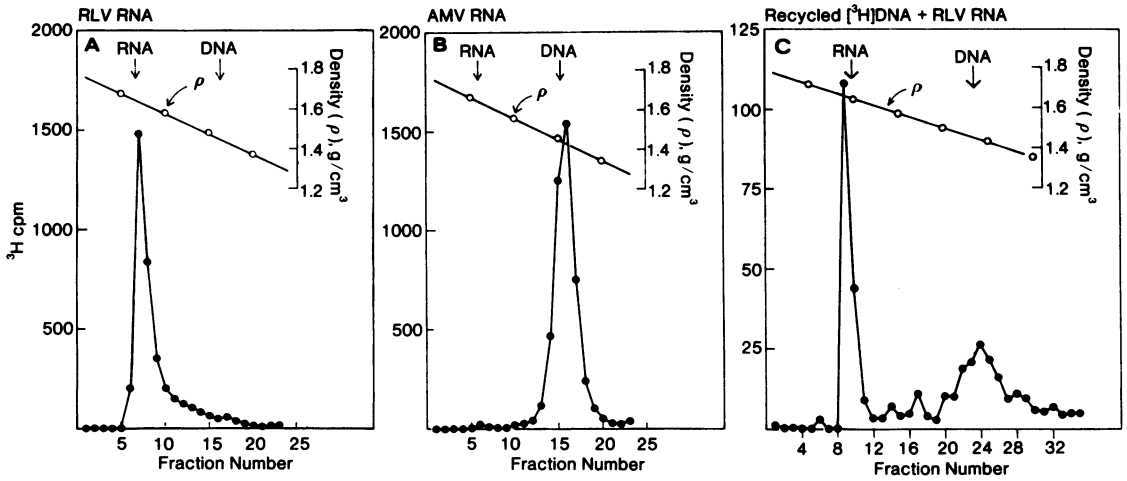

FIG. 1. Hybridization of total and recycled RLV [^{3}H]DNA with viral RNAs. The hybridizations with total RLV [^{3}H]DNA (*A*, *B*) contained (in 20 μl): 0.4 M NaCl, 0.05 M EDTA, pH 7.5, 0.1% sodium dodecyl sulfate, 50% formamide, 0.5 μg of RLV RNA or 2.5 μg of AMV RNA, and 0.44 pmole of RLV [^{3}H]DNA (10,000 cpm/pmole). The samples were heated at 80° for 1 min and then incubated at 37° for 14 hr. One-fourth of the sample was diluted into 0.12 M PB for hydroxyapatite chromatography and the remainder was analyzed by Cs_2SO_4 gradient centrifugation. In *C*, 0.08 pmole of recycled [^{3}H]DNA (see *legend* to Fig. 3) was annealed with 1 μg of RLV RNA under the same conditions (50 μl reaction). The reaction was incubated at 37° for 13 hr and then analyzed by Cs_2SO_4 gradient centrifugation.

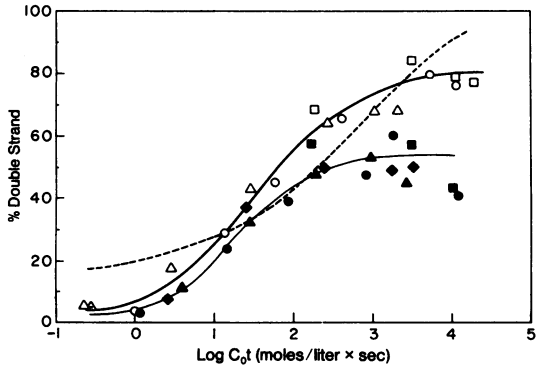

FIG. 2. Kinetics of annealing of RLV [^{3}H]DNA to DNA from normal and RLV-infected BALB/c mice. (C_0t is the product of initial single-stranded nucleic acid concentration and hybridization time.) Annealing of RLV product to normal BALB/c DNA is shown by closed symbols (–●–, –▲–, –■–, –◆–); annealing to infected BALB/c DNA is shown by open symbols (–○–, –△–, –□–). The different symbols represent separate experiments performed with RLV product 1 (*circles* and *triangles*) and product 2 (*squares* and *diamonds*) and different DNA preparations. The *dashed line* (– – –) describes the renaturation of the cellular DNA in the same experiments; the renaturation kinetics of normal and infected BALB/c DNA were indistinguishable. The hybridizations contained: 0.4 M NaCl, 0.05 M EDTA 0.18 M [Na^+], 0.1% sodium dodecyl sulfate, 0.2–12 mg/ml of DNA, and 2–140 pmole/ml of RLV [^{3}H]DNA (10,000 cpm/pmole). The ratio of cellular DNA to RLV [^{3}H]DNA varied from 3×10^5 to 3×10^7 in different experiments. Hybridization mixtures were heated to about 105° and then incubated at 66°. Samples from the hybridization reactions were analyzed by hydroxyapatite chromatography; each aliquot contained at least 800 cpm. DNA from BALB/c mice (Life Sciences, Inc., St. Petersburg, Fla.) was isolated from adult pooled tissue (spleen, kidney, and liver). DNA from RLV-infected BALB/c was prepared from the enlarged spleens (University Laboratories, Highland Park, N.J.) of these leukemic mice. DNA from spleen, kidney, or liver of normal BALB/c does not differ in its content of RLV-related sequences, although differences do exist in the leukemic mice (J. -R. Cho and S. Spiegelman, manuscript in preparation).

disrupted with a Silverson homogenizer. Centrifugation at 5000 × *g* for 10 min yielded a crude nuclear pellet, which was repeatedly homogenized and pelleted until the supernatant was clear. DNA was extracted from the pellet as previously described (16).

RLV 70S RNA was extracted from purified virus according to previous procedures (15).

RLV [^{3}H]DNA. Two preparations of [^{3}H]DNA complementary to RLV RNA were synthesized from detergent-disrupted virions with tritiated TTP (50 Ci/mmole—New England Nuclear Corp.) as the radioactive label. All reactions contained 100 μg/ml of actinomycin D (Sigma Corp.) and 50 μg/ml of distamycin A (Farmitalia, Milan, Italy) (17). The specificity of the [^{3}H]DNA products for RLV RNA was shown by hybridization to 70S RNA from RLV and avian myeloblastosis virus (AMV). The cesium sulfate density gradient profiles obtained with product 2 are shown in Fig. 1. Approximately 90% hybridization (1.52–1.68 g/cm^3) was observed with RLV RNA,whereas only 2.5% hybrid was obtained with AMV RNA. By hydroxyapatite chromatography, the percent [^{3}H]DNA eluting above 60° was 87% for the hybrid with RLV RNA (mean elution temperature, $T_{e1/2}$ = 87°) and 2% for that with AMV RNA. Virtually identical results were obtained with product 1.

Analysis of DNA·DNA and DNA·RNA Hybridization Reactions. The extent of hybridization of the viral [^{3}H]DNA products with cellular DNA was measured by temperature-elution hydroxyapatite chromatography (18). The percent hybridization was calculated from the fraction of [^{3}H]DNA eluted from hydroxyapatite (Bio-Gel-HTP, Bio·Rad) with 0.12 M sodium phosphate buffer (PB), pH 6.8, at 80° and 98° after extensive elution at 60° with the same buffer. Column fractions were counted directly in Aquasol (New England Nuclear Corp.). Renaturation of the cellular DNA was determined from the A_{260} of the fractions. In the temperature-elution experiments (Fig. 4), elution was at 4° intervals with 0.12 M PB. DNA was precipitated with trichloroacetic acid,

TABLE 1. *Annealing of total and recycled RLV [³H]DNA to DNA of different mouse strains*

DNA	Total [³H]DNA cpm (>80°)	Total [³H]DNA % (>80)	[³H]DNA recycled against: BALB/c DNA cpm (>80°)	BALB/c DNA % (>80°)	NIH DNA cpm (>80°)	NIH DNA % (>80°)
NIH	116	21	1	0.4	13	2.5
C57BL	96	15	6	2.0	—	—
XGF	159	27	2	0.7	—	—
AKR	119	19	5	1.9	—	—
BALB/c	172	28	10	4.6	5	1.0
RIII	—	—	9	2.8	—	—
BALB/c (RLV-infected)	347	62	71	31.0	191	36.0

Hybridizations with the total RLV [³H]DNA (product 1) were performed as described in Fig. 2. Each sample contained approximately 0.06 pmole of [³H]DNA (600 cpm). Hybridization reactions with the recycled [³H]DNA were as described in Fig. 3; the results for the BALB/c-recycled [³H]DNA with normal and infected BALB/c DNAs are also shown in Fig. 3 (*circles*). The NIH-recycled [³H]DNA was prepared as described in Fig. 3 except that NIH rather than BALB/c DNA was used. All samples were incubated to a C_0t of 1.0×10^4. The cpm and percent [³H]DNA eluting *above* 80° are tabulated. DNAs of NIH (Life Sciences, Inc., St. Petersburg, Fla.), C57BL/6, and XGF (14) mice were isolated from pooled adult tissue (spleen, kidney, and liver). DNA of AKR mice (Jackson Lab., Bar Harbor, Me.), was extracted from embryos and DNA of the Paris RIII strain (13) was prepared from breast tumor tissue.

collected on membrane filters, and counted in Omnifluor–toluene (New England Nuclear Corp.).

RNA·DNA hybridization products were analyzed by cesium sulfate (E. M. Laboratories) density centrifugation as previously described (15) and, in some instances, also by hydroxyapatite chromatography.

RESULTS

Hybridization of total RLV [³H]DNA

We first measured the total amount of RLV-related information in the splenic DNA of normal and RLV-induced leukemic BALB/c mice by annealing single-stranded RLV [³H]DNA product with a vast excess of the two kinds of cellular DNAs (Fig. 2). In agreement with previous studies of other murine RNA tumor viruses (4, 5, 19), extensive homology is observed with the DNA from normal as well as infected spleens. However, as may be seen from Fig. 2, even in such experiments with total RLV [³H]DNA it would appear that the infected BALB/c DNA contains RLV-related sequences that are absent from normal DNA. Thus, when more than 90% of the cellular DNA (*dashed line* of Fig. 2) was renatured (at a C_0t of 1×10^4) 82% hybridization was observed with the infected DNA and only about 55% with normal DNA. For comparison it may be noted that, using the same hydroxyapatite procedure and RLV [³H]DNA, 85–87% hybridization was obtained with RLV 70S RNA. That the lower level of annealing to normal BALB/c DNA was not due to inadequate amounts of DNA was demonstrated by showing that the extent of annealing to normal DNA was invariant over a 100-fold range in the ratio of [³H]DNA to cellular DNA.

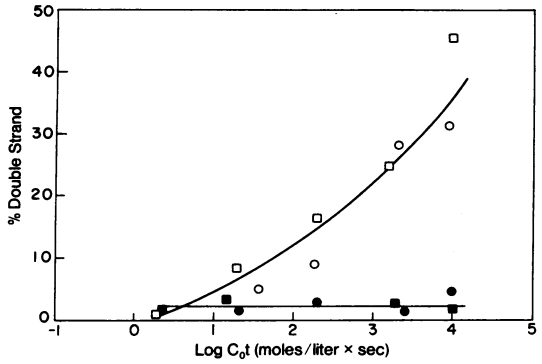

FIG. 3. Kinetics of annealing of recycled RLV [³H]DNA to normal and infected BALB/c DNA. RLV product, recycled against normal BALB/c DNA, was annealed to DNA from normal (-●-, -■-) or infected (-○-, -□-) BALB/c. The different symbols represent two separate preparations of recycled product from RLV product 1. The hybridization reactions contained 8–12 mg/ml of cellular DNA and 0.5–2.5 pmole/ml of recycled product, but were otherwise as described in Fig. 2. The per cent of product eluting above 80° is plotted versus C_0t; each sample was at least 200 cpm. To prepare the recycled probe, RLV [³H]DNA was first annealed with an excess of normal BALB/c DNA to a C_0t of 2×10^3. The unhybridized [³H]DNA was recovered by passage through hydroxyapatite at 60° in 0.12 M PB and was again annealed with BALB/c DNA ($C_0t = 1.1 \times 10^4$). The unhybridized probe recovered from this reaction was used in the above experiments.

Hybridization of "recycled" RLV DNA

The small but detectable difference in the annealing of RLV [³H]DNA to normal as compared with leukemic DNA (Fig. 2) can be greatly amplified and its significance revealed by recourse to the type of "recycling" experiment (6) used with human leukemic material. In this approach sequences in the [³H]DNA shared with normal DNA are first removed by exhaustive annealing with normal DNA in vast excess. The residue of unreactive [³H]DNA can then be isolated by passage through a hydroxyapatite column at 60° in 0.12 M PB as described in *Methods*. These recovered strands should no longer hybridize to normal DNA, since all normal sequences have been eliminated. To further accentuate the specificity of the search for RLV-related sequences in normal and infected DNA, attention is focused on hybrids of comparatively high thermal stability (80° and above). Exclusive hybridizability of such recycled [³H]DNA to leukemic DNA would establish that leukemic cells contain specific sequences not found in normal DNA. Fig. 3 shows the outcome of such a test with a recycled RLV [³H]DNA. About 35% hybridization (65% total hybrid) is observed at a C_0t of 10,000 with DNA from RLV-infected spleen and few, if any, such complexes are observed with normal DNA (20% hybrid eluting below 80°). Again for comparison, 52% of the recycled [³H]DNA hybridized with RLV 70S RNA by Cs_2SO_4 gradient analysis (Fig. 1*C*). The results of Figs. 2 and 3 indicate that the recycled [³H]DNA is annealing with nonrepeated sequences in the RLV-infected DNA. It is estimated that less than one in 50 normal BALB/c cells can contain leukemia-specific viral-related sequences.

Hybridizations to DNAs of other mouse strains

Table 1 compares the abilities of total and recycled RLV [³H]DNA to hybridize to a variety of murine DNAs. The

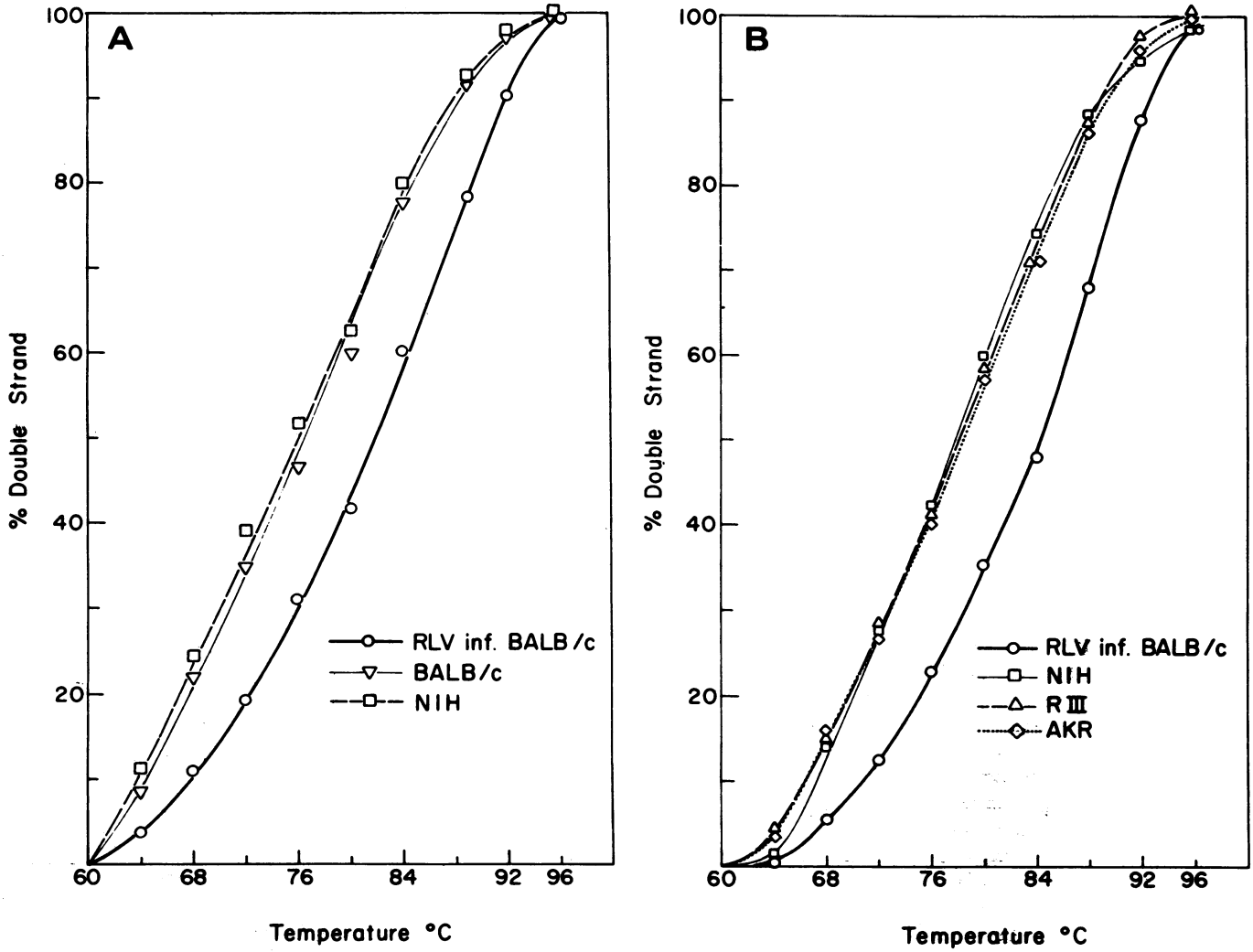

FIG. 4. Melting curves of RLV [^{3}H]DNA–cellular DNA hybrids. RLV [^{3}H]DNA was annealed with the indicated cellular DNAs to $C_0t = 1.3 \times 10^4$ and the hybrids were melted on hydroxyapatite as described in *Methods*. The hybridization reactions (50 μl) contained: 0.12 M PB, pH 6.8, 20 mM EDTA, 0.1% sodium dodecyl sulfate, 350 A_{260} units (per ml) cellular DNA and 0.1 pmole of [^{3}H]DNA product 2 (10,000 cpm/pmole). The cumulative percent of the total hybrid eluting at each temperature (corrected for background) is plotted versus the temperature. The [^{3}H]DNA alone had less than a 2% background on hydroxyapatite. (*A*) Comparison of RLV-infected (inf.) BALB/c (-O-), normal BALB/c (-▽-), and NIH (-□-) DNAs. The actual extents of hybridization were 94%, 79%, and 78%, respectively. (*B*) Comparison of AKR (-◇-) and RIII (-△-) with NIH (-□-) and RLV-infected BALB/c (-O-). The extents of hybridization were 69%, 60%, 67%, and 89%, respectively.

degree of homology with the total RLV [^{3}H]DNA is about the same in all the strains examined including AKR, the strain having a high frequency of spontaneous leukemia. Again, as in Fig. 2, DNA from RLV-infected BALB/c tissue hybridizes to a significantly greater extent than does any normal DNA. The data shown in Table 1 with the *recycled* RLV [^{3}H]DNA emphasize once again that significant levels of the leukemia-specific sequences are found only in the DNA of leukemic tissues and not in the normal DNA of any of the strains examined, whether they be of high or low leukemic frequency.

The fact that all the normal DNAs tested showed the same level of hybridization with the total RLV [^{3}H]DNA suggests that the RLV-related sequence found in normal DNA also occurs in the DNAs of the other strains examined. This conclusion was tested more directly by an experiment summarized in Table 1. Here total RLV [^{3}H]DNA was recycled on NIH rather than on BALB/c DNA and the residue rechallenged with normal and infected BALB/c as well as with NIH DNA. It is evident from Table 1 that prior annealing to NIH DNA is as effective in removing the normal sequences from the RLV [^{3}H]DNA as is recycling to normal BALB/c DNA. The RLV [^{3}H]DNA recycled on NIH DNA did not anneal significantly to either normal DNA but still detected the same levels of leukemia-specific sequences in the DNA of infected mice.

Thermal stability of RLV DNA complexes with cellular DNAs

Further information on the nature of the complexes between the RLV [^{3}H]DNA and the various cellular DNAs can be obtained by examining the thermal stability of the hybrid structures. In such experiments the total RLV [^{3}H]DNA is annealed to the cellular DNA of the different mouse strains and the hybrids melted on hydroxyapatite as described in Fig. 4. It will be noted that the hybrids formed with the DNA from

the low leukemia incidence strains (BALB/c and NIH) did not differ from one another, and, perhaps of greater interest, were indistinguishable in their melting profiles from the hybrid formed with the DNA from the high leukemia frequency AKR strain. Further, in keeping with the results shown in the table, the DNA from the RIII mammary tumor behaved as normal DNA with respect to RLV-related sequences.

It may be concluded that the RLV-related sequences found in nonleukemic DNAs from different sources are very similar if not identical. On the other hand, it is evident from Fig. 4 that the duplexes formed with the leukemic DNA are different and possess a higher (4–6°) thermal stability as measured by the mean elution temperature ($T_{e^1/_2}$). This distinction was consistently observed with several leukemic and normal cellular DNA preparations and with various samples of RLV [^{3}H]DNA and agrees with a previous report (5) comparing lymphomatous DNA and normal DNAs from the same mouse strains employed here.

It is unlikely that the higher melting temperature with leukemic DNA can be explained in terms of its additional 20–30% annealing with the viral probe. A more plausible explanation is that the sequences inserted as a result of the infection represent a more faithful copy of viral information than those detected in the normal DNA of the different strains. From the difference in $T_{e^1/_2}$, one would estimate (20, 21) that there is 7–8% mismatching of bases in the complexes formed with the normal as compared with the leukemic DNA. This adds further weight to the conclusion that the leukemia-specific sequences are different from those detected in normal DNAs. These results concur with the observed addition of viral sequences to cellular DNA in *in vitro* transformation of rodent (23, 24), avian (25), and simian (16) cells by nonindigenous RNA tumor viruses.

DISCUSSION

The present investigation reverses the usual order of biomedical research, since its principal purpose is to extend to an animal model information gained from a study of human disease. To this end we chose to study BALB/c mice, a strain with a low incidence of leukemia and the indigenous murine virus RLV, an established leukemogenic agent. In complete parallelism to the human disease, we found RLV sequences in the DNA of leukemic spleens of infected mice that could not be detected in normal DNA. In the case of the human leukemias and lymphomas, the viral and etiologic relation of the particles detected was, and still remains, a moot point. The results described here with RLV establish with a known viral etiologic agent that the same pattern of unique leukemia-specific sequences exists in the mouse as has been observed in man. It will be of great interest to extend such experiments to a spontaneous neoplasia in an animal model.

It has been suggested (22) that RLV exists in some latent form in the genome of BALB/c mice, the strain from which the virus was originally isolated (12). The data reported here do not support this contention, since we have shown that some RLV sequences are missing from normal BALB/c DNA and that even those RLV-related sequences that are present in normal DNA appear not to be entirely homologous to RLV. Such sequences appear to be present only in leukemic DNA.

In summary, recycling experiments in lymphoid neoplasias of humans (6, 11), cats (1), and mice all lead to the conclusion that malignancy is associated with the addition of new particle-related sequences to the normal DNA complement. In a global biological sense no collection of individual experiments can disprove hypotheses (26, 27) that invoke the germ line transmission of a complete copy of some unspecified viral information required for malignancy and the production of an RNA tumor virus. The experiments described here and those previously reported make this hypothesis unlikely only for those particular neoplasias and associated particulate elements subjected to examination.

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