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Nuclear DNA Sequences Present in Human Leukemic Cells and Absent in Normal Leukocytes

(70S RNA/hybridization/RNA tumor viruses/oncogene/protovirus)

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ABSTRACT **The central purpose of the present study was to test the proposition that the nuclear DNA of every human cell contains whatever information is necessary and sufficient for transformation to malignancy. The experiments were made possible by our earlier identification in human leukemic cells of particulate elements encapsulating 70S RNA and RNA-directed DNA polymerase. The [^{3}H]DNA synthesized by these particles was used as a probe, through molecular hybridization, to normal and leukemic DNA. The results obtained establish that leukemic nuclear DNA contains particle-related sequences that cannot be detected in normal leukocytes. This outcome does not support the virogene–oncogene theory, which postulates the inclusion of at least one complete copy of oncogenic information in the genome of every normal cell.**

The data suggest that we may not be forced to cope with an omnipresent DNA segment coding for malignancy. Under the circumstances, we can perhaps entertain more hopeful pathways leading to the control and cure of cancer.

We have over the past few years focused our efforts on an attempt to determine whether the knowledge gained from animal viral oncology is relevant to the etiology of human cancer. We first searched in human tumors for RNA molecules related in sequence to those found in the RNA tumor viruses known to cause corresponding cancers in experimental animals. Radioactive DNAs synthesized on the appropriate viral RNA templates with RNA-directed DNA polymerase were used as probes. The data obtained revealed a pattern of specificities that agreed remarkably with what was known from the animal experimental systems. Human breast carcinomas were found (1) to contain RNA homologous to that of the mouse mammary tumor virus. As expected, the human mammary tumor RNA showed no homology to that of the unrelated Rauscher leukemia virus. This observation gained in interest and significance when we turned our attention to human leukemias (2), sarcomas (3), and lymphomas (4). Here we found RNA homologous to that of the Rauscher leukemia virus, an agent related to a group that causes similar neoplasias in mice.

Finding the pertinent RNA molecules related to oncogenic viruses in the different human tumors did not, of course, establish a viral etiology. We next had to design and perform experiments that could answer the following questions with respect to the RNA molecules we had identified: (*a*) Were they as large as the 70S-RNA molecules characteristic of the RNA tumor viruses?, (*b*) Were they physically associated in a particle containing RNA-directed DNA polymerase, also characteristic of RNA tumor viruses?, and (*c*) Did they possess the density characteristic of tumor viruses?

Answering these questions became feasible with the development of an assay for the simultaneous detection of a 70S-RNA template associated with an RNA-directed DNA polymerase (5). Mouse mammary tumor was used to resolve the technical problems encountered in applying this technique to malignant tissue (6). The methodology thus developed permitted us to establish that the viral-related RNA encountered in human mammary carcinomas (7) and leukemic cells (8) contained molecules 70S in size, and physically encapsulated with a RNA-directed DNA polymerase in a particle possessing a density between 1.16 and 1.19 g/ml (Hehlmann, R. and Spiegelman, S., in preparation). Furthermore, the DNA synthesized endogenously by these complexes hybridized uniquely to the RNAs of the viral agents that caused the corresponding neoplastic diseases in mice.

These experiments provided evidence for the existence in human neoplasias of particulate elements possessing four features diagnostic of the homologous oncogenic murine viruses. Though compelling, the data did not establish that the particles thus identified are causative, or even contributory. Final proof requires the demonstration that the human particles are in fact infectious and transforming agents. Nevertheless, the results were sufficiently impressive to warrant the initiation of the next stage of our investigation into the applicability of animal viral oncology to human cancer.

It is true that virologists can satisfy Koch's postulates with purified animal tumor viruses. The question, however, is being raised with increasing frequency of whether this fact is relevant to the natural history of cancer. Indeed, the "virogene–oncogene" hypothesis (9, 10) implicitly uses this argument, since it suggests that normally the disease is caused not by an external virus, but by the activation of an oncogene DNA segment already present, and one that is vertically transmitted (inherited) in the germ lines of all organisms prone to cancer. An alternative is the provirus theory (11, 12), which postulates the exogenous introduction of new DNA sequences through viral infection with an RNA tumor virus. The insertion of this viral-related information into the genome is presumed to involve the synthesis of the requisite DNA by a DNA polymerase directed by the viral RNA.

It is obvious that an experimental decision between the provirus and virogene hypotheses is a necessity for the development of a rationale for controlling the disease. The two hypotheses can be distinguished by their logical consequences. The virogene hypothesis predicts that normal DNA includes at least one complete copy of the information required for transformation and virus production. The provirus hypothesis denies this, and stipulates that if some of this infor-

mation previously exists in normal cells, it is incomplete, and that infection with a virus is a necessary requisite to provide the information needed for tumorigenesis. The issue can be settled by experiments designed to answer the following question: *Does the DNA of transformed cells contain viral-related sequences that are not found in the DNA of normal cells?*

We explored the technical feasibility of performing the necessary experiments with an animal system by use of DNA·DNA hybridization because of its superior sensitivity. The data obtained showed (Goodman, N., Ruprecht, R. M., Sweet, R., Deinhardt, F., Massey, R., and Spiegelman, S., in preparation) that it is readily possible to demonstrate with nonindigenous RNA oncogenic viruses that the DNA of transformed cells contains sequences that are not present before transformation.

These results encouraged us to apply this technology to human leukemia, and the purpose of this paper is to describe the outcome. We have shown (8) that human leukemic cells contain viral-related 70S RNA encapsulated with an RNA-directed DNA polymerase. These particulate elements can be used to generate radioactive DNA probes, which in turn can be used to detect viral-specific information in human leukocyte genomes. The experiments to be described show that leukemic nuclear DNA contains sequences that cannot be detected in the DNA of normal leukocytes.

METHODS

Preparation of [^{3}H]DNA. Leukemic leukocytes were gently opened with a Dounce homogenizer, and the extract was centrifuged at 16,000 × *g* for 15 min at 2° to remove nuclei and mitochondria. The resultant supernatant was layered on 25% sucrose–TNE buffer [0.01 M Tris·HCl (pH 8.3)–0.15 M NaCl–0.01 M EDTA] and centrifuged in a Beckman SW-41 rotor at 40,000 rpm for 1 hr at 2°. The resulting pellet was resuspended in 1 ml of 0.01 M Tris·HCl (pH 8.3) and used in a typical endogenous RNA-directed DNA polymerase reaction to generate [^{3}H]DNA (8). The product was brought to 0.4 M NaOH and incubated at 37° for 2 hr to destroy RNA. To provide acceptable backgrounds for such probes, it is imperative that all self-complementary material be removed before use. To this end, the alkali-treated material is neutralized with 0.4 M HCl and 1 M Tris·HCl (pH 7.4) and then self-annealed at 60° for 48 hr, corresponding to a C_0t value of 0.01–0.25, as defined by Britten and Kohne (18). The annealed material is then passed over a 2-ml bed volume of hydroxyapatite (Biorad) at 60°. The column was washed with 100 ml of 0.01 M $NaHPO_4$ (pH 6.8), and the single-stranded [^{3}H]DNA was eluted with 4 ml of 0.15 M $NaHPO_4$ (pH 6.8), a technique that recovers 90% of the input [^{3}H]DNA. The DNA was then layered upon a Sephadex G-50 column (coarse) of 100-ml bed volume. The DNA region of the column was pooled, and the [^{3}H]DNA was precipitated with 2 μg/ml of carrier yeast RNA and two volumes of 95% ethanol. The pellet was dissolved in 3 mM EDTA and stored at 2°. The [^{3}H]DNA banded in Cs_2SO_4 gradients at densities between 1.42 and 1.45.

Nuclear DNA was prepared as detailed elsewhere (Goodman, N., Sweet, R., Ruprecht, R. M., and Spiegelman, S., manuscript in preparation). The ratios of A_{260} to A_{280} of the preparations were all close to 2; on denaturation, these preparations reannealed to better than 80%.

Annealing Conditions. Annealing reaction mixtures contained 6 A_{260} units of cellular DNA, 0.2–1.0 pmol of [^{3}H]DNA, and 15 μmol $NaHPO_4$ (pH 7.2), in a final volume of 0.1 ml. The reaction was brought to 98° for 60 sec and 0.04 mmol of NaCl was added. The reaction mixture was then incubated at 60°, and aliquots were removed at the indicated intervals. The reaction was stopped by the addition of 2 ml of 0.01 M $NaHPO_4$ (pH 6.8). The sample was then passed over a column of hydroxyapatite of 3-ml bed volume (15, 16) at 60°. The column was washed with 20 ml of 0.15 M $NaHPO_4$ (pH 6.8) at 60°, 80°, 88°, and 95°. 4-ml Fractions were collected, the A_{260} of each fraction was read, and the DNA was precipitated with 2 μg/ml of carrier yeast RNA and 10% trichloroacetic acid. The precipitate was collected on Millipore filters, which were dried and counted (8).

The method identifies unpaired strands that elute at 60° and poorly paired duplexes that disassociate at 80°. *Only the duplexes dissociating and eluting at 88°–95° are counted here as properly hybridized.*

Recycling of Probe on Normal DNA to Separate Leukemic from Normal Sequences. The reactions were performed with normal DNA as above and the eluates were collected in 1-ml fractions at 60°, 80°, 88°, and 95°. The peak fractions were combined and the resulting pools were passed over a Sephadex G-50 column of 10-ml bed volume. The DNA regions were collected, and the DNA was precipitated with 2 μg/ml of carrier yeast RNA and 2 volumes of 95% ethanol. The material eluted at 88°–95° was pooled and used for the determination of sequences common to normal and leukemic DNA. The material eluted at 60° was used as the recycled product unable to hybridize to normal DNA.

RESULTS

The strategy of the present investigation may be outlined in the following steps: (*a*) Isolate from leukemic cells the fraction containing the particles encapsulating the 70S RNA and RNA-directed DNA polymerase (8); (*b*) Use this fraction to generate [^{3}H]DNA endogenously synthesized in the presence of high concentrations of actinomycin D to inhibit host and viral DNA-directed DNA synthesis (9, 15, 17); (*c*) Purify the [^{3}H]DNA by hydroxyapatite and Sephadex chromatography, with care being exercised to remove all self-complementary material; (*d*) Use the [^{3}H]DNA as a probe to detect complementary sequences in normal- and leukemic-leukocyte DNA; (*e*) If sequences are detected in both types of leukocyte, remove those found in normal DNA by exhaustive hybridization; and (*f*) Test the residue for specific hybridizability to leukemic DNA.

Fig. 1 shows typical C_0t reannealing curves (18) determined by hydroxyapatite chromatography (13, 14). The purpose here is to compare annealing of [^{3}H]DNA probe to normal (Fig. 1*A*) and leukemic DNA (Fig. 1*B*). Since each reaction mixture contains large amounts of nuclear DNA, the behavior of this component can be readily followed by absorbance readings at 260 nm, and can be used as an internal control for the adequacy of the annealing reaction. As seen from the A_{260} readings, the rates and extents of annealing are the same for both normal and leukemic nuclear DNA. However, comparison of Fig. 1*A* and *B* shows that annealing of the [^{3}H]DNA probe to leukemic DNA appears to be faster and more extensive than to normal DNA.

The observation that the [^{3}H]DNA synthesized by the

leukemic particles hybridized to nuclear DNA of normal leukocytes was not surprising in view of previous experience with murine and avian systems (19–26). Whenever indigenous RNA tumor viruses were used, DNA from normal cells were found to contain viral-related sequences. We have already noted that such findings cannot decide between the provirus and virogene mechanisms. What is noteworthy here is the quantitative difference revealed in Fig. 1 between the responses of normal and leukemic DNA. The greater extent and rate of annealing of the leukemic [^{3}H]DNA with leukemic nuclear DNA suggests either one, or a combination, of the following two possibilities; (*a*) Leukemic DNA contains more of the same sequences detected by the [^{3}H]DNA in normal DNA, or (*b*) Leukemic DNA contains additional unique sequences not found in normal DNA and detectable by the [^{3}H]DNA.

A decision between the two possibilities is readily made by the last two steps of the experimental plan outlined above. Sequences in [^{3}H]DNA common to both normal and leukemic nuclear DNA are first removed by exhaustive annealing with normal DNA in vast excess. The residue of unreactive [^{3}H]DNA can then be isolated and purified by hydroxyapatite chromatography, as described in *Methods*. These recovered strands should no longer contain sequences complementary to those found in normal DNA. Exclusive hybridizability of such recycled [^{3}H]DNA to leukemic DNA would establish that the genome of leukemic cells contains specific sequences not detectable in normal DNA.

In such experiments, strands complementary to normal-leukocyte DNA are removed by annealing to a C_0t of greater than 10,000. Fig. 2 shows a typical outcome of hybridizing such recycled [^{3}H]DNA to normal and leukemic DNA. It is evident that no complexes stable at temperatures above 88° are formed with normal DNA. On the other hand, 57% of the recycled [^{3}H]DNA added forms well-paired duplexes with the leukemic DNA.

Table 1 summarizes the data obtained in a series of experiments with [^{3}H]DNA and nuclear DNA obtained from eight untreated patients with either acute myelogenous leukemia or chronic myelogenous leukemia. In all instances the leukemic [^{3}H]DNA probe hybridized to normal nuclear DNA as in Fig. 1*A*. However, in every case, after it was recycled by exhaustive annealing to normal DNA, the residual [^{3}H]DNA formed stable duplexes only with leukemic DNA, in agreement with the experiment of Fig. 1*B*.

To provide further information on the quantitative aspects of the annealing reactions, we performed experiments to see whether the hybridization with leukemic nuclear DNA removed the leukemia-specific strands from the [^{3}H]DNA probe. To accomplish this, initial hybridizations were performed between the [^{3}H]DNA probes and their homologous nuclear leukemic DNA. The residual strands were then isolated and rechallenged against the same leukemic DNAs. It is clear from the results summarized in Table 2 that no further hybridizations occur, a result that implies that the first annealing to leukemic DNA had indeed removed virtually all of the [^{3}H]DNA strands complementary to leukemic DNA.

The data of Table 2, combined with the information on the same five patients in Table 1, permit another quantitative comparison of independently performed hybridizations on the same set of materials. The percent hybridization to leukemic DNA (column 2 of Table 2) should approximate the sums estimated from the serial hybridizations to normal and leukemic DNA (columns 2 and 4 of Table 1). The resulting sums are given in column 5 of Table 2; comparisons with column 2 of the same table indicate reasonable agreement.

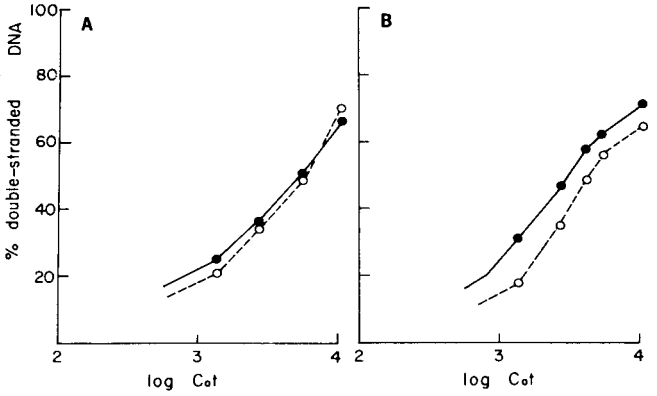

FIG. 1. Annealing curves of [^{3}H]DNA synthesized by leukemic particles to leukocyte nuclear DNA from normal pooled blood (*A*) and from a leukemic patient (*B*). The eluates from the hydroxyapatite columns at 88° and 95° were summed and counted as hybrids. The *abscissas* represent log C_0t, where C_0 is mol/liter of nucleotides and t is time in seconds (18). O– – –O, A_{260}; ●——●, ^{3}H.

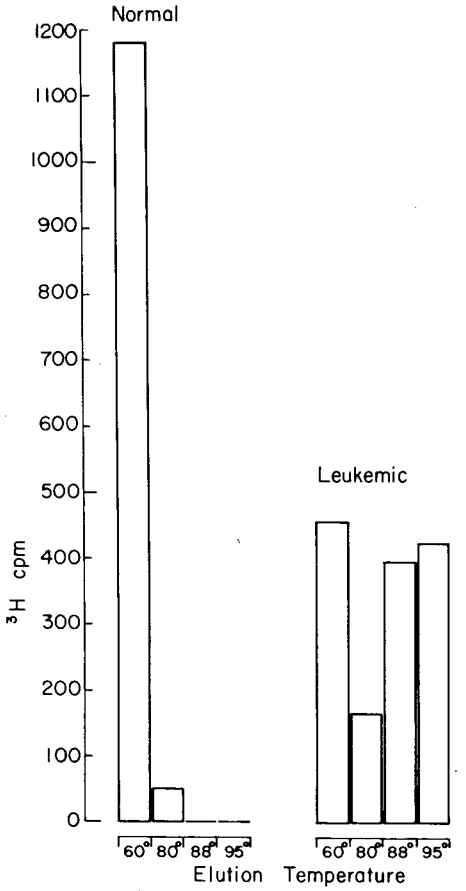

FIG. 2. Hydroxyapatite elution profile of a hybridization reaction of the recycled leukemic [^{3}H]DNA probe to nuclear DNA from normal leukocytes and leukocytes from the same leukemic patient. The background was 30 cpm.

TABLE 1. *Exhaustive hybridization of [^{3}H]DNA probe synthesized by leukemic particles with normal-leukocyte nuclear DNA, followed by hybridization of the nonhybridizing recycled leukemic [^{3}H]DNA probe to normal DNA and to leukocyte nuclear DNA from the same leukemic patient*

	Leukemic [^{3}H]DNA hybridized to normal-leukocyte DNA		Recycled leukemic [^{3}H]DNA hybridized to leukocyte DNA			
			Leukemic		Normal	
	cpm	% Hybridization	cpm	% Hybridization	cpm	% Hybridization
Ne (AML)	3020	61	523	56	0	0
Ha (CML)	1350	40	1020	51	0	0
Co (CML)	2580	51	431	35	3	0
Mi (CML)	510	45	101	36	0	0
Go (AML)	1100	43	303	48	4	0
Si (AML)	4520	49	1130	46	1	0
El (AML)	390	42	45	69	0	0
Mac (AML)	1450	49	510	52	0	0

Background was 30 cpm and all counts recorded represent cpm above background. *CML* = Chronic myelogenous leukemia. *AML* = Acute myelogenous leukemia. First column indicates initials of patients.

The concordance we have just noted includes the sequences in the [^{3}H]DNA probes that are common to both normal and leukemic DNA, and implies that the proportion of these shared sequences in the two types of DNA will not be very different. This conclusion can be checked more directly by the technique of Gelb, Kohne, and Martin (27). Exhaustive hybridization of [^{3}H]DNA to normal nuclear DNA was used to isolate the duplexes stable above 80°. This material was then annealed in the presence of either normal or leukemic nuclear DNA. If either nuclear DNA contained a significantly higher content of these shared sequences, its addition would result in a significantly greater acceleration of the annealing reaction. As can be seen from the two experiments described in Fig. 3, the addition of normal and leukemic DNA made no obvious difference on the rate of reassociation of the [^{3}H]DNA probe containing sequences common to both. This result suggests that the [^{3}H]DNA sequences shared by normal and leukemic DNA are present in equal numbers in the genomes of leukemic and normal cells.

DISCUSSION

It is informative to consider the sensitivity of the hybridization experiments summarized in Tables 1 and 2. The specific activity (50.1 Ci/mmol) of the [^{3}H]TTP used, when corrected for counting efficiency and composition of the product, results in a specific activity of 3×10^7 cpm/μg of [^{3}H]DNA synthesized. If we assume that only 10% of the 70S RNA has been effectively transcribed, the ratio of the molecular weight of the [^{3}H]DNA to that of a human genome is 5×10^{-7}. If there were one probe equivalent per genome, the 250 μg of nuclear DNA used in the hybridizations would contain 120 pg of probe sequences, and would correspond to about 3.7×10^3 cpm, if all were complexed with [^{3}H]DNA. Since hybridizations were carried to a C_0t of 10,000, well beyond the point where the nuclear DNA has reannealed, one would expect to see at least 50% of the reaction completed, corresponding to 1.8×10^3 cpm. It should, however, be noted that in many instances the amount of [^{3}H]DNA probe available was not sufficient to saturate cellular DNA containing one equivalent per genome. Comparison of the cpm observed with recycled [^{3}H]-DNA on normal and leukemic nuclear DNA (Table 1), and assuming we can easily detect 30 cpm over a background of the same value, one can conclude from the two instances where over 1000 cpm were complexed to leukemic DNA that normal DNA contains considerably less than 2.5% of a probe equivalent per genome.

We would like to explicitly emphasize the biological difference between our earlier studies and the present one. We previously focused (1–8) attention on the RNA content of tumors and, therefore, only on active gene transcripts or on autonomous extrachromosomal RNA entities. The experiments reported here center on the nuclear DNA and, as such, include all genes—active or silent. It is evident from the results described that at least a portion of the viral-related se-

TABLE 2. *Exhaustive hybridization of leukemic [^{3}H]DNA probe to leukocyte nuclear DNA, from the same patient, followed by hybridization of the residue to nuclear DNA*

	Leukemic [^{3}H]DNA hybridized to leukemic-leukocyte DNA		Recycled leukemic [^{3}H]DNA hybridized to leukemic-leukocyte DNA		Estimated total % hybridization from serial hybridizations*
	cpm	% Hybridization	cpm	% Hybridization	% Hybridization
Ne (AML)	1310	79	0	0	82
Ha (CML)	4450	63	2	0	70
Co (CML)	4040	63	9	0	68
Mi (CML)	372	59	0	0	65
Go (AML)	1250	75	0	0	70

Background was 30 cpm and all counts recorded represent cpm above background.

* See columns 2 and 4 of Table 1.

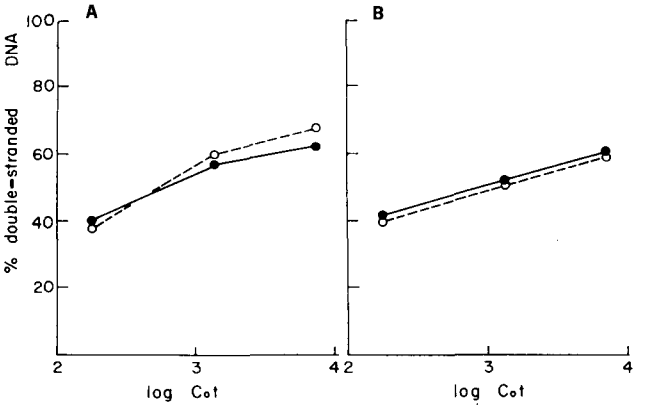

FIG. 3. C_0t curves of [^{3}H]DNA probe recovered from normal DNA at 88° and 95° and hybridized to normal DNA and the same leukemic patient's DNA. *A* and *B* represent two different patients. O– – –O, acute myelogenous leukemia DNA; ●——●, Normal leukocyte DNA.

quences we detected in the RNA are also to be found in the nuclear DNA of the tumor cells. This finding establishes a generic relation between RNA and DNA viral-related sequences, and makes it difficult to argue that the viral-specific RNA we detected (1–8) is associated with an irrelevant passenger in the cancer cell.

The fact that we cannot detect these sequences in the DNA of normal leukocytes argues against the virogene–oncogene theory as an explanation of human leukemia. Our data are more consistent with either the provirus hypothesis, or the more recent protovirus concept (28), and—at the very least—imply a requirement to add new genetic information for conversion of a normal cell to a cancer cell, in agreement with our studies of simian cells transformed by nonindigenous tumor viruses (Goodman, N., Sweet, R., Ruprecht, R. M., and Spiegelman, S., manuscript in preparation).

One striking feature exhibited by the experiments described is the necessity of first removing shared sequences before attempting to use such probes to search for sequences unique to a neoplastic cell. Many of the previous attempts (19–26) to detect significant differences between normal and transformed cells probably failed because they did not include such a recycling step.

The experiments reported here generate the possibility for various potentially illuminating investigations. Thus, one would like to know whether [^{3}H]DNA probes made with one type of leukemia can be used to detect sequences in the DNA of other leukemias, or related neoplastic disorders. In addition, it is of key importance to determine whether the DNA in the peripheral cells of a leukemic patient in remission still contain the leukemia-specific sequences. Further, one would like to understand the significance of the indigenous viral-related sequences shared between normal and cancer cells. The fact that they do not appear to be amplified (Fig. 3) in the leukemic cells and that are not necessary for transportation (Goodman, N., Sweet, R., Ruprecht, R. M., and Spiegelman, S., manuscript in preparation) suggests that these sequences may ultimately tell us more about the origin and evolutionary history of oncogenic viral elements than about the mechanism of their pathogenesis.

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Correction. In the article "Nuclear DNA Sequences Present in Human Leukemic Cells and Absent in Normal Leukocytes," by Baxt, W. G. & Spiegelman, S., which appeared in the December 1972 issue of the *Proc. Nat. Acad. Sci. USA* **69,** 3737–3741, the second line, under *Annealing Conditions*, top right-hand column, p. 3738, should read: "tained 60 A_{260} units of cellular DNA,. . .," and the last sentence of the text, p. 3741, bottom, left-hand column, should read: "The fact that they (shared sequences) do not appear to be amplified (Fig. 3) in the leukemic cells and that they are not necessary for transformation (Goodman, N., Sweet, R., Ruprecht, R. M. & Spiegelman, S., manuscript in preparation) suggests that these sequences may ultimately tell us more about the origin and evolutionary history of oncogenic viral elements than about the mechanism of their pathogenesis."