Heterogeneity of Murine Leukemia Virus in vitro DNA; Detection of Viral DNA in Mammalian Cells

Abstract. *Kinetic analysis of the reassociation of DNA synthesized in vitro by a murine leukemia virus DNA polymerase revealed two classes of double-stranded product representative of 25 and 100 percent of viral genetic information. The DNA product representing the smaller portion of the viral genome comprised 85 percent of the double-stranded DNA generated in vitro and was extensively duplicated in the genomes of both normal cells and cells containing RNA tumor virus.*

RNA-dependent DNA polymerizing activity has been detected in many tumor viruses (*1*). The products of this reaction appear to be single-stranded DNA, DNA-RNA hybrids, and double-stranded DNA (*2*). The in vitro DNA sediments slowly in neutral and alkaline sucrose gradients, indicating that it has a molecular weight of 1 to 2×10^5 (*3*). Because the RNA tumor virus genomes have sedimentation coefficients between 60*S* and 70*S* (*4*), the corresponding double-stranded DNA with a similar genetic constitution would have a molecular weight of about 2×10^7. Duesberg and Canaani have reported that in vitro DNA made with Rous sarcoma virus renders most of the 70*S* viral RNA resistant to ribonuclease digestion (*5*). This experiment suggests that the in vitro DNA is complementary to the entire viral genome.

Using a different experimental approach, we have examined the informational content of in vitro *double-stranded* DNA made from murine leukemia virus to determine whether it represents the entire RNA genome or is a homogeneous population of molecules transcribed from a very limited portion of the viral RNA. Our studies are based on the work of Britten and Kohne (*6*) who showed that the rate of DNA reassociation can be directly correlated with the number of base pairs in a given nucleic acid. Lambda phage DNA, for example, reassociates about 143 times more rapidly than an equal concentration of *Escherichia coli* DNA, and these rates reflect the difference in their molecular weights. In addition, we have used the double-stranded DNA as a probe to detect the presence of viral DNA sequences in normal and transformed animal cells.

The cells used included the mouse BALB/3T3 and NIH/3T3 (*7*) and the normal rat kidney (NRK) lines (*8*). The Rauscher strain of murine leukemia virus (R-MuLV) was obtained from the plasma of infected mice (*9*). The Kirsten strain of murine leukemia virus (Ki-MuLV) (*10*) was propagated in NIH/3T3 cells and purified from tissue culture fluids by two cycles of sedimentation in sucrose gradients (20 to 50 percent). Both R-MuLV and Ki-MuLV in vitro DNA's were made in reaction mixtures containing 0.05*M* tris-HCl, *p*H 7.8, 0.002*M* dithiothreitol, 0.06*M* NaCl, $5 \times 10^{-4}M$ deoxynucleoside triphosphates (specific activity of each deoxynucleoside triphosphate was 161 mc/mmole), 0.006*M* magnesium acetate, 0.014 percent Triton X-100, and 100 μg of virus protein per milliliter. Samples were incubated at 37°C for 18 hours. The specific activity of the in vitro DNA was calculated from the specific activities of the precursor nucleoside triphosphates. The values for both ^{3}H-labeled Ki-MuLV and R-MuLV DNA's were 2.51×10^5 count min^{-1} μg^{-1}.

Double-stranded DNA was prepared by first extracting the reaction mixture with phenol at room temperature in the presence of 0.05*M* ethylenediaminetetraacetate (EDTA) and 1 percent sodium dodecyl sulfate. The aqueous phase was then precipitated with two volumes of ethanol after the addition of yeast RNA (50 μg/ml). The precipitate was suspended in 0.007*M* phosphate buffer (PB; consists of equimolar parts of Na_2HPO_4 and NaH_2PO_4, *p*H 6.8) and digested with pancreatic ribonuclease (Worthington) (10 μg/ml) for 2 hours at room temperature. The PB concentration was adjusted to 0.14*M*, and the DNA preparation was applied to a hydroxyapatite column equilibrated with 0.14*M* PB at 60°C. The column was washed with 50 ml of 0.14*M* PB at 60°C to remove the single-stranded DNA; the double-stranded DNA was then collected by eluting the hydroxyapatite with a small volume of 0.14*M* PB at 100°C. Double-stranded DNA comprised 35 to 50 percent of each reaction mixture.

The preparation of normal and transformed cellular DNA's has been

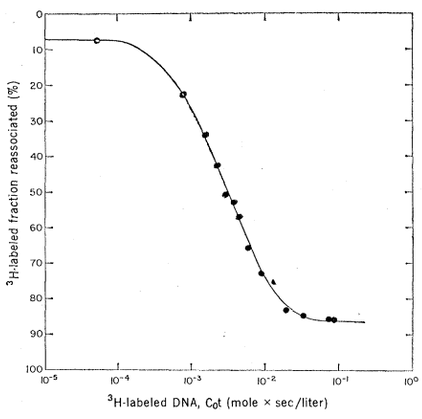

Fig. 1. Reassociation of ^{3}H-labeled in vitro double-stranded DNA of Ki-MuLV plotted as a function of C_0t. At a concentration of 0.152 μg/ml Kl-MuLV double-stranded DNA was denatured at 100°C in 0.14*M* PB and 0.0025*M* EDTA; the denatured virus DNA was kept at 60°C, and periodically monitored for reassociation by passage through a hydroxyapatite column; C_0 concentration; *t*, time.

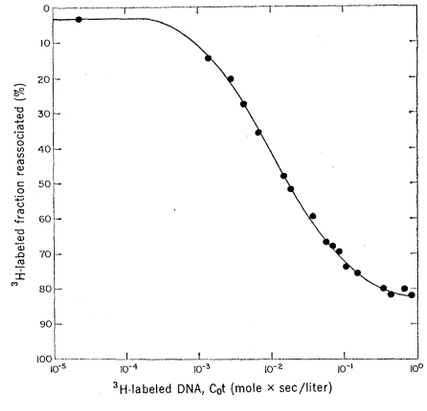

Fig. 2. Reassociation of the slowly reassociating fraction of ^{3}H-labeled in vitro double-stranded DNA from Ki-MuLV plotted as a function of C_0t. Ki-MuLV double-stranded DNA was denatured by heat and incubated at a concentration of 1.10 μg/ml to a C_0t of 2.0×10^{-2} at 60°C in 0.14*M* PB and 0.0025*M* EDTA. The reannealed DNA was removed by passage through a hydroxyapatite column. The single-stranded DNA (0.14 μg/ml) recovered was again denatured at 100°C (10 minutes); the product was held at 60°C in 0.14*M* PB and periodically monitored for reassociation on hydroxyapatite columns. A portion of the single-stranded DNA was concentrated to 2.75 μg/ml so that reassociation at high C_0t values could also be monitored.

Table 1. Kirsten strain of murine leukemia virus in vitro DNA equivalents in mammalian cells.

Cell line	Increased rate of reassociation	Viral DNA equivalents* per diploid cell
AGM	1.50	8.7
NRK	3.14	37.4
Ki-MuLV–infected NRK	2.75	30.6
Ki-MSV–infected NRK	2.13	19.8
Ki-MSV–infected BALB/3T3	3.88	50.4

* The number of viral in vitro DNA equivalents in each of these cells can be calculated by multiplying ^{3}H Ki-MuLV double-stranded DNA copies per mammalian diploid cell added to the control reaction mixture by (factor of increased rate − 1) (*11*). The number of copies of in vitro double-stranded DNA per diploid cell in the control reaction is

$$\frac{0.018\ \mu g/ml\ ^3H\ Ki\text{-}MuLV\ double\text{-}stranded\ DNA}{818\ \mu g/ml\ animal\ DNA} \times \frac{4 \times 10^{12}}{5 \times 10^{6}} = 17.49\ copies\ per\ cell.$$

described (*11*). Mammalian DNA was sheared at 50,000 psi (1 psi = 6.9 kN/m^2) in a Ribi cell fractionator in 0.001*M* EDTA and 0.01*M* PB, passed through cellulose acetate filters, and dialyzed against 0.1*M* NaCl before use.

The selective binding of double-stranded DNA to hydroxyapatite was used to follow the percentage of ^{3}H-labeled DNA which had reannealed with time and which was then plotted as a function of the C_0t, where C_0 is the concentration of nucleotides in moles per liter and t is the time of incubation in seconds (*6*, *12*). Tritiated viral, double-stranded DNA was denatured by heating in a boiling water bath for 10 minutes and then incubated at 60°C for various periods of time. Portions of the reaction mixture were applied to hydroxyapatite columns and analyzed for reassociated DNA (*11*). Figure 1 shows the results of such an experiment when the percentage of ^{3}H-labeled Ki-MuLV DNA that is reassociated is plotted as a function of the C_0t. The reaction was half completed at a C_0t of about 2.8×10^{-3}. In another experiment, the $C_0t_{1/2}$ value obtained with ^{3}H-labeled R-MuLV double-stranded DNA was 3.4×10^{-3}. Under identical experimental conditions, SV40 DNA (molecular weight of 3×10^6) exhibited a $C_0t_{1/2}$ of 1.67×10^{-3}. These results indicate that, although the physical size of the in vitro DNA is quite small, the double-stranded DNA's prepared from Ki-MuLV and R-MuLV exhibit reassociation kinetics compatible with a total sequence length equivalent to a molecular weight of 5.0 and 6.1×10^6, respectively. These values, while much larger than the small molecular weight product of the polymerase reaction in vitro, were still lower than one would expect for a double-stranded DNA product representing the entire viral genome.

The experiment illustrated in Fig. 1 indicated that 85 percent of double-stranded Ki-MuLV DNA reassociated with the C_0t employed. Similarly, about 80 percent of the R-MuLV double-stranded DNA reannealed under the same conditions. We therefore examined more closely the nature of the double-stranded DNA that failed to reassociate. Ki-MuLV double-stranded DNA (3.29 μg) was denatured by heat and incubated to a C_0t of 2.0×10^{-2}. This C_0t was sufficient to permit the dominant species of DNA to reanneal (Fig. 1). The remaining unannealed DNA from this reaction was collected in the 0.14*M* PB washings from the hydroxyapatite column. A portion (3 ml containing 0.14 μg/ml) was used directly after the passage through the hydroxyapatite column, and the remainder was dialyzed against 0.001*M* EDTA, concentrated to a volume of 0.09 ml (2.75 μg of double-stranded DNA per milliliter), and adjusted to 0.14*M* PB. The solutions were denatured by heat and incubated at 60°C for various periods of time. Figure 2 shows the reassociation kinetics of this slowly reannealing fraction of Ki-MuLV double-stranded DNA. In contrast with the results of Fig. 1, this DNA reassociated with a $C_0t_{1/2}$ of about 1.10×10^{-2}. This value suggests a genome size approximately four times larger (molecular weight of 19×10^6) than the most abundant in vitro product and is equivalent in informational content to the 70*S* viral RNA.

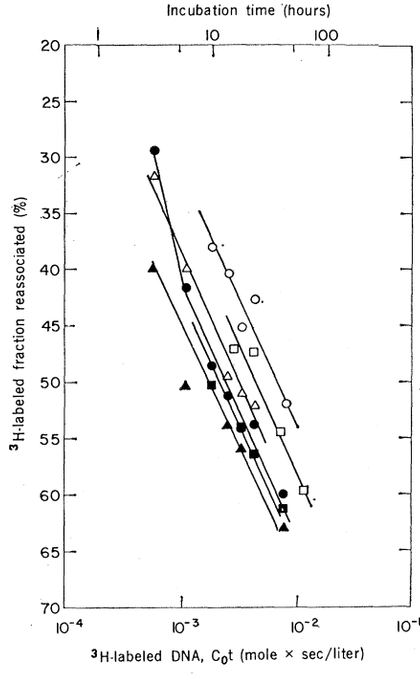

Fig. 3. Reassociation of ^{3}H-labeled Ki-MuLV in vitro double-stranded DNA with DNA's from normal and RNA tumor virus containing mammalian cells. Each reaction mixture contained 0.018 μg/ml of ^{3}H-labeled Ki-MuLV in vitro double-stranded DNA, 0.13*M* PB, 0.0025*M* EDTA, and 818 μg/ml of sheared DNA from either salmon sperm (open circles), AGM (open squares), NRK (solid squares), Ki-MuL–infected NRK (solid circles), Ki-MSV–tranformed NRK (open triangles), or Ki-MSV–transformed BALB/3T3 (solid triangles). The DNA mixture was denatured at 100°C, incubated at 60°C, and monitored for reassociation on hydroxyapatite columns.

Since the rate of DNA reassociation is proportional to the concentration of the DNA in the solution measured, the concentration of viral sequences present in animal DNA can be measured from the effect of unlabeled normal DNA and transformed cell DNA on the reassociation of labeled viral DNA. We have shown that virus-specific polynucleotide sequences in the cellular DNA preparations will increase the rate of reassociation of the labeled indicator double-stranded DNA (*11*). We have used a similar method of analysis here to quantitate the amount of leukemia virus DNA in a series of normal and transformed cell genomes. Figure 3 shows that the rate of Ki-MuLV double-stranded DNA reassociation increased over threefold when NRK DNA was present in the reaction mixture. The DNA from NRK cells infected with Ki-MuLV increased the reassociation rate by a factor of 2.75. NRK and BALB/3T3 cells transformed by Kirsten murine sarcoma virus (Ki-MSV), which contain the sarcoma genome in a nonreplicating state (*13*), increased the reaction rate to a similar extent. African green monkey (AGM) cells, in which Ki-MuLV has not been propagated or known to infect, were also examined. Figure 3 shows

that DNA from AGM cells also speeded up the reassociation of ^{3}H-labeled Ki-MuLV DNA, although the effect was much less than that of any of the rodent DNA's tested. The number of viral equivalents per diploid cell can be calculated from these data and is listed in Table 1.

Our results indicate that at least two classes of double-stranded DNA are synthesized by the RNA-dependent DNA polymerase of Ki-MuLV. The major class of DNA represents the selective transcription of a relatively small portion of the viral RNA. It is not clear whether the dominant double-stranded DNA product is an artifact of the in vitro reaction or whether it represents a functionally important gene product. It has been shown that 70*S* viral RNA can be dissociated by heat or treatment with dimethyl sulfoxide into several major subunits each with a molecular weight of about 3×10^6 (*14*). Such a subunit or portion of it may function as a template and generate double-stranded DNA having the reassociation characteristics shown in Fig. 1. Although it was a minor fraction, there was a class of double-stranded DNA that approximated the size of the entire viral genome.

It is possible that the in vitro viral DNA also plays a role in viral replication in vivo and may represent a form of the viral genome that can be integrated into host DNA during the process of transformation. However, it is not yet clear whether these in vitro DNA products are related in any way to the replicative or oncogenic potential of RNA tumor viruses. The process of transformation with RNA tumor viruses may be similar to that reported with oncogenic DNA viruses; the latter are thought to be integrated into the genome of transformed cells (*15*). With the DNA tumor virus SV40, normal uninfected cells appear to contain only a partial copy of the DNA viral genome (*11*). When labeled Ki-MuLV double-stranded DNA with an informational content equivalent to that of a molecule having a molecular weight of 5×10^6 was used as a probe to detect "integrated" viral DNA sequences, normal and transformed cell DNA's could not be differentiated (Fig. 3). In addition, the number of "integrated" genome equivalents detected in these cells exceeded that for comparable DNA tumor viruses by as much as 50-fold (*11*). Our results imply the presence of mammalian polynucleotide sequences within the viral RNA genome. Perhaps a more valid probe would be the in vitro DNA product with the informational content of the entire viral genome. The relation between the polynucleotide sequences shared by the major double-stranded DNA product and the normal cell genome is not yet known.

LAWRENCE D. GELB
Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014
STUART A. AARONSON
Viral Leukemia and Lymphoma Branch, National Cancer Institute, Bethesda, Maryland 20014
MALCOLM A. MARTIN
Laboratory of Biology of Viruses

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Rubidium and Lithium: Opposite Effects on Amine-Mediated Excitement

Abstract. *In mice the activation caused by morphine was antagonized by previous treatment with lithium and was potentiated by previous treatment with rubidium. Other antimanic drugs antagonized the morphine activation as well. The effect of rubidium was similar to that of the antidepressant drugs imipramine and pargyline. Rubidium may merit clinical evaluation as an antidepressant agent in man.*

The alkali metal lithium is a useful agent for the treatment of acute mania (*1*) and may also be of value in the prevention of recurrent manic and depressive illnesses (*2*). The lithium ion is thought to act through an effect on monoamine metabolism in the brain (*3*); the neuropharmacology of lithium indirectly supports the monoamine theory of depression (*4*). Rubidium is an alkaline earth metal of the same series as lithium, but it possesses contrasting properties. Its effects on the binding of sodium and potassium to a preparation of neuronal membrane ganglioside in vitro (*5*) are opposite to those of lithium (*6*). Rubidium causes increased activity, aggression, and electroencephalogram (EEG) activation in monkeys; whereas lithium produces EEG slowing and reduces hyperactivity (*6*). Recently these two ions were shown to have opposite effects on brain norepinephrine (NE) metabolism in rats (*7*). Rubidium produces a marked shift in the metabolism of NE toward normetanephrine formation; this action of rubidium is similar to the effect of imipramine on NE metabolism (*8*). Lithium, on the other hand, alters NE catabolism toward deamination and decreases normetanephrine formation (*9*). Both ions cause an increase in the rate of disappearance of NE from brain; the increased turnover seen with lithium may reflect intraneuronal NE degradation, whereas the pattern of catabolite production after treatment with rubidium indicates increased release of physiologically active NE (*7*). Rats treated with rubidium became aggressive and irritable when handled. These effects on behavior and NE metabolism have suggested a possible use for rubidium in the treatment of depression (*6*, *7*). However, the behavioral actions of rubidium have not previously been compared directly with those of known antidepressant drugs.

Our experiments were designed to evaluate the effects of the two ions on