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Endogenous Guinea Pig Virus: Equability of Virus-Specific DNA in Normal, Leukemic, and Virus-Producing Cells

(DNA·RNA hybridization/BrdU activation/oncornavirus/reiteration)

D. P. NAYAK

Department of Microbiology and Immunology, School of Medicine, University of California, Los Angeles, Calif. 90024

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ABSTRACT **The kinetics of hybrid formation between the RNA of BrdU-activated endogenous guinea pig virus and the DNA of leukemic, normal, or BrdU-activated guinea pig cells were measured by the technique of RNA·DNA hybridization in DNA excess. The results suggest that virus-specific sequences representing some 60–70% of the viral genome are unique (2–3 copies per haploid cell genome), while the remainder (30–40%) are reiterated (147 copies), and that the reiterated virus-specific DNA may be composed of more than one species, each having a different reiteration frequency. No difference was found in the quantity of viral DNA sequences contained in normal, leukemic, or bromodeoxyuridine-activated guinea pig cells. These data are considerably different from those reported for exogenous (infectious) oncornaviruses, where cells infected or transformed by exogenous RNA tumor viruses have been shown to contain increased amounts of virus-specific DNA. The data reported here are consistent with the contention that preexisting viral genes are activated by bromodeoxyuridine treatment. Results of hybridization experiments done at different DNA/RNA ratios suggest that although the virus-specific DNA is partly unique and partly reiterated, the viral RNA does not contain any detectable internal reiteration. Total mass of the viral RNA sequences is around 0.7 to 1 × 10^7 daltons.**

Since we reported that leukemic chicken myeloblasts contained increased amounts of oncornavirus-specific DNA (v-DNA) when compared to normal cells (1, 2), a number of investigators have confirmed and extended these observations to other systems (3–5, 9). In our initial studies, an excess of viral RNA from avian myeloblastosis virus (AMV) was hybridized with the DNA of myeloblastosis virus-producing leukemic chicken myeloblasts and with the DNA of normal chicken embryos immobilized on filters (1, 2). Since then, however, a more refined methodology of quantitating virus-specific DNA has developed. Such procedures usually investigate the kinetics by which radioactively labelled viral probes (RNA or DNA) reassociate with excess cellular DNA. Except in a few cases (6–8), increases in v-DNA have been reported in cells infected with exogenous RNA tumor viruses. This increase has been observed in both virus-producing cells, whether or not they are transformed, and in transformed non-virus-producing cells (3, 11). Baxt and Spiegelman, using the techniques of molecular hybridization, have demonstrated DNA sequences in human leukemic cells which are absent in nonleukemic cells (10). Such a finding is consistent with the idea that an exogenous virus may bring new information into leukemic cells, even though these cells are not producing viruses.

During these studies, it became obvious that even DNA from normal cells is homologous to some oncornavirus-specific sequences (1–9, 11). Although normally repressed, these viral genes can be activated to produce virus in a number of ways (12–17). These activated viruses, known as endogenous viruses, are mostly noninfectious for the host in which they are induced. They possess the same morphological, physical, and biochemical properties as exogenous (infectious) viruses. Their role in malignancy is uncertain at present.

We have recently activated and characterized an endogenous oncornavirus (GPV) from cultured guinea pig cells (17) which do not have any known infectious (exogenous) oncornaviruses. In this report, evidence is presented which suggests that unlike exogenous RNA tumor viruses, the amount of virus-specific DNA in cells induced to produce an endogenous virus does not increase. In fact, the same amount of viral DNA was found in normal, leukemic and bromodeoxyuridine (BrdU)-induced (virus producing) guinea pig cells.

MATERIALS AND METHODS

Animals and Cells. Random bred and inbred Wright's strain 2 guinea pigs were obtained from Curd's Caviary (Los Angeles). Leukemia is maintained in strain 2 guinea pigs by transplanting leukemic cells into healthy animals. Animals inoculated in this way develop leukemia within 2–4 weeks. Leukemic animals with a white cell count of 2 to 3 × 10^5/mm^3 were bled by cardiac puncture and only the leukemic blood cells were used in these experiments. Over 95% of these cells were leukemic lymphoblasts. The methodology involved in establishing cultures from guinea pig embryos or adult leukemic animals has been described previously (17).

Activation of Endogenous Guinea Pig Oncornavirus by BrdU. As reported previously (17), nonconfluent cultures were treated with BrdU (10^{-4} M) for 24 hr at which time the BrdU medium was replaced by fresh medium without BrdU and allowed to incubate an additional 24 hr. BrdU-treated cells were subsequently exposed to the medium containing [^{3}H]-uridine and [^{3}H]cytidine (34 μCi/ml, each) at a time when maximum virus synthesis was observed (17). For 2 days the labeled medium was changed every 4–6 hr.

Isolation of Labeled GPV and GPV-RNA. GPV was concentrated and purified in sucrose gradients as described previously (17). Viral RNA was purified from the peak virus fractions by the phenol–sodium dodecyl sulfate method (18). Viral RNA was analyzed in sucrose velocity gradients con-

Abbreviations: BrdU, bromodeoxyuridine; v-DNA, oncornavirus-specific DNA; GPV, endogenous oncornavirus from cultured guinea pig cells; cRNA, RNA complementary to DNA; GLV, Gross leukemic virus.

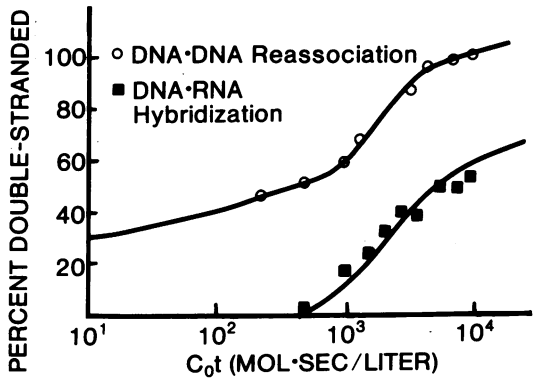

FIG. 1. Kinetics of reassociation of guinea pig DNA (O) and hybridization of complementary guinea pig RNA (■) with guinea pig DNA. The reassociation reaction was carried out with the denatured guinea pig DNA (7 S) at 70° in 0.5 M sodium phosphate buffer. The DNA concentration was 4 mg/ml. At appropriate intervals 50-μl DNA aliquots (200 μg) were diluted into 5 ml of ice-cold sodium phosphate buffer (0.01 M) and stored at 4°. Single- and double-stranded DNA were separated by step-wise elution with 0.17 M and 0.40 M phosphate, respectively, from a hydroxyapatite column at 60°. The reaction was considered complete at a C_0t of 10,000, at which 85% of input DNA reassociated into double-stranded form and 15% remained single-stranded. All values of double-stranded DNA are normalized using 85% reassociation as 100%. DNA·RNA hybridization with complementary guinea pig RNA (cRNA) was carried out in a separate experiment. The conditions of hybridization were the same as the conditions of reassociation. cRNA had a specific activity of 10^7 cpm/μg and DNA/RNA ratio was 2×10^6. Hybrid formation was monitored by determining ribonuclease-resistant counts as described in *text* (20, 21).

taining NaCl, 0.1 M, Tris·HCl, pH 7.4, 0.01 M, EDTA, 0.001 M (17, 18). Only the 65–70S GPV-RNA which was free from smaller molecular weight RNA components was used; one batch of viral RNA with a specific activity of 10^6 cpm/μg was used throughout.

Liquid DNA·RNA Hybridization in DNA Excess. Cellular DNA was isolated by procedures previously reported (1, 2). DNA was fragmented by sonication to a size of 7 S (about 180,000 daltons), treated with alkali to remove RNA, neutralized and precipitated with ethanol. Hybridization conditions are essentially the same as described by Melli *et al.* (19). DNA and RNA are mixed at a DNA/RNA ratio of 8×10^5 in the appropriate salt concentration (usually 0.5 M sodium phosphate buffer). DNA-RNA mixture was boiled for 8 min and cooled quickly. Final DNA concentration was 6–8 mg/ml. For determining the kinetics of hybridization at lower C_0t, reaction was carried out at lower DNA concentration (1 mg/ml) and in 0.1 M phosphate buffer. The reaction mixture was incubated at 70°. Hybridization was followed by determining the acid precipitable counts after RNase treatment. Aliquots (usually 50 μl) were taken out at intervals, diluted with 5 ml of 0.12 M sodium phosphate and chilled. RNase A (20 μg/ml) and RNase T_1 (10 units/ml) were added, mixed in a vortex mixer and incubated along with the untreated controls, in a 37° shaking waterbath for one-half hour. Trichloroacetic acid precipitable radioactivity was determined. Background RNase resistance of single-stranded RNA after boiling was 0.8–1.5% and was subtracted from all results. C_0t (mol·sec/liter) was calculated and converted to 0.12 M phosphate according to Britten and Kohne (20, 21). Conversion factors for 0.1 and 0.5 M phosphate were 0.6867 and 5.8157, respectively (21).

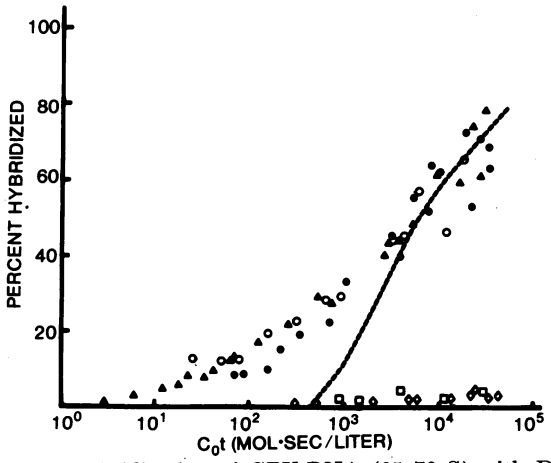

FIG. 2. Hybridization of GPV-RNA (65–70 S) with DNAs from *E. coli*, chickens, guinea pig embryos, and leukemic guinea pig lymphoblasts. 65–70S GPV-RNA with a specific activity of 1×10^6 cpm/μg was isolated and separated from small-molecular-weight RNA as described in the *text*. The reaction was carried out as in Fig. 1 except that the salt (0.1 or 0.5 M sodium phosphate) and DNA (1 or 8 mg/ml) concentration varied depending on whether hybrid formation was measured at high or low C_0t. The DNA/RNA ratio was 8×10^5. The results of two independent experiments are plotted (each using DNA from normal and leukemic cells run simultaneously), leukemic cells (△,O), and normal guinea pig embryo (▲,●). Absence of complementarity of GPV-RNA with *E. coli* (□) and chicken (◇) DNA is also shown in the figure. The reference curve of cRNA hybridization is plotted from Fig. 1 (– –).

RESULTS

Calibration of the Technique. The technique of liquid hybridization in DNA excess was standardized by determining the reassociation rate of denatured DNA and the hybridization rate of complementary guinea pig RNA (cRNA). cRNA was synthesized *in vitro* (5, 22) on a guinea pig DNA template using *Escherichia coli* RNA polymerase (Miles Laboratories). Purified cRNA was prepared by DNase treatment and phenol extraction. *In vitro*-synthesized cRNA has a sedimentation value of 3–4 S.

The kinetics of reassociation of denatured guinea pig DNA show that about 40% of the DNA renatured quickly (below a C_0t of 200) while the remainder reassociated with a $C_0t_{1/2}$ of 1800 (Fig. 1), indicating complexity of 2×10^{12} daltons for haploid genome. This approximates the value of 1.86×10^{12} daltons for haploid guinea pig DNA calculated from published data (23, 24). The kinetics of hybridization of cRNA with guinea pig DNA are also shown in Fig. 1. The maximum hybridization of cRNA was 58% of the input. There was no hybrid formation below a C_0t of 500, indicating that rapidly reassociating DNA was not transcribed *in vitro*. Self-annealing of cRNA was less than 1%. As expected, the rate of hybridization of cRNA to DNA (K^h) was slower than the reassociation rate (K^d). The $C_0t^h{}_{1/2}$ of guinea pig cRNA was 6000 [extrapolating the hybridization curve (Fig. 1) to 100%]. $C_0t^h{}_{1/2}$ of *E. coli* cRNA under similar condition is 22 (Shoyab, Baluda, and Evans, personal communication). A reiteration frequency of 2.5 for guinea pig cRNA per haploid guinea pig DNA was estimated using the equation described by Melli *et al.* (19).

Hybridization of Endogenous Viral RNA to Cellular DNA. Next, GPV-[^{3}H]RNA was hybridized to DNAs from guinea pig embryos and leukemic lymphoblasts as well as to chicken

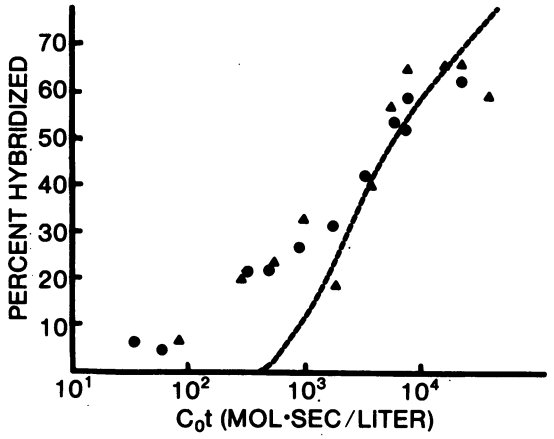

FIG. 3. Hybridization of GPV-RNA with DNA from normal (▲) and BrdU-treated (●) guinea pig cells. BrdU-treated guinea pig cells were examined for GPV production. DNA was isolated from guinea pig cells at 72 hr after BrdU treatment when GPV production is maximum in activated cells. DNA/RNA ratio was 8×10^5 and hybridization was carried out as in Fig. 2. The curve shows the position of reference graph of cRNA plotted from Fig. 1 (- - -).

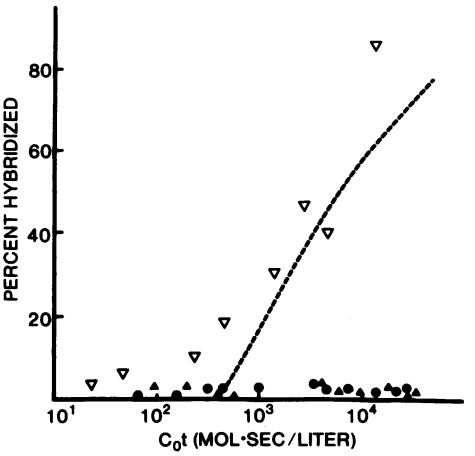

FIG. 4. Hybridization of Gross leukemia virus (GLV) RNA with DNA of Gross virus-producing mouse cells (H-111) (▽) and leukemic (●) and normal (▲) guinea pig embryo DNA. Reference curve of cRNA hybridization is drawn from Fig. 1 (- - -). 70S GLV-RNA with a specific activity of 875,000 cpm/μg was used.

and *E. coli* DNA. High-molecular-weight viral RNA (65–70 S) alone, or in the presence of *E. coli* or chicken DNA, formed only 2–4% RNase resistance (Fig. 2). The kinetics of hybrid formation between GPV-[^{3}H]RNA and normal or leukemic guinea pig DNA suggest two points of consideration (Fig. 2): (*1*) No difference exists between the kinetics of hybridization of leukemic and normal DNA; (*2*) The kinetics of viral RNA hybridization, when compared to that of cRNA, is complex and spreads over five decades (C_0ts 10^0–10^5). Approximately 72–78% of the viral RNA hybridizes to normal or leukemic DNA suggesting that this technique detects almost the entire viral genome. When compared to the reference curve of cRNA hybridization, the curve for viral RNA appears to be multiphasic. Around 30–40% of the viral RNA hybridizes at a faster rate then cRNA and has a $C_0t_{1/2}$ of 100. The rest (60–70%) hybridizes at a rate similar to that of cRNA and has a $C_0t_{1/2}$ of 6000. The reiteration frequencies of fast (reiterated) and slow (unique) reacting DNA sequences are 147 and 2.4, respectively. These values were obtained by extrapolating the experimental curve to 100%.

Although no clear plateau can be seen in Fig. 2, we have divided the curve into unique and reiterated areas using cRNA curve as a reference for unique segment. The technical limitation restricting complete hybridization to a plateau value for unique sequence have been pointed out by others (19, 27, 29). The plateau of the reiterated portion may be masked either by the trailing effect of the unique portion or because of multiple DNA segments within the reiterated DNA, each with a different reiterated frequency. In either case, it is almost impossible to estimate the frequency of reiterated v-DNA sequences precisely. Thus, the value of reiteration frequency of 147 is merely an average estimate rather than the actual number of DNA copies.

In spite of these limitations, it is obvious that there is no difference in the RNA·DNA hybridization kinetics of leukemic lymphoblasts and normal embryos (Fig. 2). These data indicate that v-DNA representing the entire viral genome is found in both normal and leukemic cells, and that it is composed of species with identical reiteration frequency (Fig. 2). This conclusion was derived from two independent experiments (each using DNA from both normal and leukemic cells) (Fig. 2). In addition, no difference was found between the virus-specific DNA content of the cultured guinea pig cells and guinea pig embryo (not shown here).

The next experiments consider if the activation of virus by BrdU requires amplification of viral DNA. First, we ensured that the majority of cells after BrdU treatment are producing viruses. In our conditions, the entire population became characteristically altered by the fourth day of BrdU treatment (guinea pig embryo fibroblasts became flattened and enlarged with increased cytoplasm). In the electron microscope, over 50% of cells contained characteristic vacuoles and virus particles. Similar effects of BrdU treatment in guinea pig cells have been reported by others (16). Hybridization results of GPV-RNA and DNA from normal and BrdU-induced guinea pig cells are shown in Fig. 3. The reference graph of cRNA hybridization is plotted for comparison. The hybridization rate again shows that the same amount of viral DNA was present in both uninduced and BrdU-activated virus-producing cells. Thus, it appears that GPV-DNA is not amplified in cells actively producing GPV particles.

Next, Gross leukemic virus (GLV) RNA was hybridized with mouse and guinea pig cell DNA (Fig. 4). The reason was 2-fold: (*i*) to determine if there is homology between guinea pig and mouse oncornaviruses; and (*ii*) to compare virus-specific DNA sequences, complementary to an exogenous mammalian RNA tumor virus, in leukemic and normal guinea pig cells. The results show that GLV-RNA hybridized to murine DNA as expected, but did not hybridize to the DNA of either leukemic or nonleukemic guinea pig cells (Fig. 4). This lack of homology between the GLV-RNA and guinea pig DNA also indicates that guinea pig virus is not related to murine viruses. Indeed, the common group specific mammalian viral antigen (gs-3) has not been detected in normal, leukemic or BrdU-activated guinea pig cells (ref. 25, 26 and unpublished data) nor in guinea pig virus.

Competitive Hybridization. To obtain more information regarding the nature of intracellular v-DNA and of the viral RNA, a constant amount of labeled viral RNA and cellular DNA was mixed with increasing amounts of unlabeled 65–70S

viral RNA. The mixture was hybridized to a C_0t of 24,500. A similar dilution experiment has been used by Bishop *et al.* (27) to determine the mass and the frequency of hemoglobin genes.

In Fig. 5, one first observes a drop in hybrid formation (*I*) at a high DNA/RNA ratio. This drop is followed by a plateau and a second drop (*II*) of hybrid formation at a relatively low DNA/RNA ratio. Several conclusions can be drawn from these results: (*1*) Assuming that 100% of viral RNA will hybridize to guinea pig DNA at a very high DNA/RNA ratio, the initial drop (*I*) represents about 60% of the viral genome (Fig. 5). Since this rapid drop of hybrid formation occurs at a relatively high DNA/RNA ratio, DNA sequences complementary to this portion of viral RNA are unique in guinea pig cells. Indeed, this part of the viral DNA (60%) corresponds fairly close to the value (65%) obtained from Fig. 2 for the unique region. (*2*) A long plateau after the first drop in hybridization indicates that the remaining sequences have a higher reiteration frequency. (*3*) A second drop of hydridization (*II*) reflects the dilution of these reiterated sequences at lower DNA/RNA ratios. The second drop represents about 40% of viral genome which is close to the value (35–40%) obtained from Fig. 2 for reiterated sequences. (*4*) The ratio between D/R (50) values of the first and second drop is 140. This gives an ndependent estimate of the frequency of the reiterated DNA (*II*) relative to the unique DNA (*I*). (*5*) The mass of RNA sequences can be calculated using a predetermined reiteration frequency according to the following equation of Bishop *et al.* (27, 28):

$$M = (N_d/N_r) \times (C/F)(D/R) \quad [1]$$

where M = mass of a single-stranded RNA sequence in daltons; mass N_d/N_r is the ratio of viral DNA to viral RNA; C is the analytical complexity of the haploid cellular genome; F is the frequency of reiteration; D/R is an overall DNA/RNA ratio (as seen on the abscissa) (Fig. 5). Assuming that 100% of RNA will hybridize at a high N_d/N_r (i.e., >20), the value of N_d/N_r at 50% hybrid formation is estimated to be around 2.0 (27, 28). Therefore, at 50% hybrid formation Eq. 1 becomes:

$$M = 2 \times (C/F)/(D/R)(50) \quad [2]$$

where D/R (50) is the overall DNA/RNA ratio at 50% hybridization. Eq. 2 can be used to estimate the mass of RNA corresponding to the unique and the reiterated portion of v-DNA from Fig. 5. For the portion of v-RNA which hybridizes with the unique DNA, D/R (50) = 3.5×10^5 (Fig. 5) extrapolating the curve to 100%, the mass of the RNA will be either 3.5×10^6 or 5.3×10^6 daltons for F = 3 or 2, respectively.

As for the portion of the viral genome which hybridizes with the reiterated v-DNA sequences, there is difficulty in estimating the mass because the reiteration frequency 147 could not be determined accurately (Fig. 2). However, an independent frequency estimate of 140 for the reiterated DNA relative to that of the unique DNA was derived from Fig. 5. Thus, the mass of the viral RNA corresponding to the reiterated v-DNA is either 5.3 or 3.5×10^6 daltons when F = 2 or 3 $\times$ 140 and D/R (50) = 2500. If the reiteration frequency of 147 (Fig. 2) is used the estimated mass of the RNA sequences corresponding to the reiterated v-DNA will be much higher (10^7 daltons). In either case, this precludes the possibility of detecting any internal reiteration within the viral RNA because it will require that the calculated mass of RNA sequence must be much smaller than the expected mass of the RNA. The total mass of the RNA sequences hybridizing to the unique and reiterated DNA sequences was 7 to 10 $\times$ 10^6 daltons. In conclusion, the data presented in Fig. 5 show that (*a*) v-DNA is partly unique and partly reiterated; (*b*) there is no detectable internal reiteration within the viral RNA; and (*c*) the mass of the viral RNA sequence is 7 to 10 $\times$ 10^6 daltons (36).

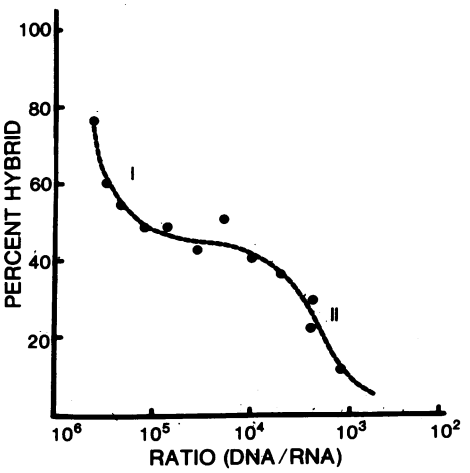

FIG. 5. Hybridization of GPV-RNA and guinea pig embryo DNA at different DNA/RNA ratios. Unlabeled 65–70S GPV-RNA was isolated from BrdU-activated GPV. Varied amounts of unlabeled GPV-RNA were added to the mixture of a constant amount of labeled GPV-RNA and guinea pig embryo DNA. All hybridization was carried out as described in Fig. 2 to a C_0t of 24,500. Hybrid formation was determined by RNase resistance and plotted as a function of DNA/RNA ratio.

Thermal Stability of RNA · DNA Hybrids. Hybridization was carried out to a C_0t of 900 and 15,000 to obtain hybrids formed at low and high C_0t's. Salt concentration was 0.12 M sodium phosphate, pH 7.2. Aliquots were exposed to various temperatures for 8 min in a waterbath equilibrated to a given temperature and chilled. One-half of the sample was treated with RNase. The RNase resistance of untreated samples was taken as 100% and the RNase resistance of treated samples is determined relative to that of untreated sample. Both high (15,000) and low C_0t (900) hybrids had an identical thermal transition profile, T_m, at 90°.

DISCUSSION

The results presented here show that essentially the entire copy of GPV genome (70–80%), as expected, could be detected in normal guinea pig cells by these experiments. The kinetics of hybridization between GPV-RNA and cellular DNA are complex. DNA sequences representing 65% and 35% of viral RNA are present with an average reiteration frequency of 2–3 and 147 copies per haploid guinea pig DNA, respectively. These results also suggest that more than one species of DNA may be present within the reiterated sequences. Similar results have been reported in other systems (5, 29). Data in Fig. 5 further show that there is little if any internal reiteration within the viral RNA.

Technical factors which limit the precise frequency estimation of a DNA sequence have been described by others (27–29). However, these limitations do not have a significant bearing on our overall conclusion that there is no quantitative difference in the amount of virus-specific DNA in normal, leukemic and BrdU-treated guinea pig cells. The kinetics of hybrid forma-

tion between GPV-RNA and cellular DNA were the same for either leukemic, normal or BrdU-treated cells over a C_0t of 5 decades. Our results are then considerably different from the ones reported in the case of cells infected or transformed by exogenous oncornaviruses (1–5).

There are three obvious explanations to account for the absence of any increase virus-specific DNA sequences in normal, leukemic and BrdU-treated cells. (*1*) Endogenous virus genes are not expressed in leukemic or BrdU-treated cells. (*2*) The expression of GPV-genome does not require any amplification or additional synthesis of new DNA. (*3*) The rates of synthesis and decay of virus-specific DNA sequences are the same; and, therefore, there will not be any net increase in the amount of DNA. It is obvious that the entire virus genome is expressed in BrdU-treated cells since BrdU-activated viral RNA is used as the probe. Evidence which will be presented elsewhere indicates that the GPV genome is also expressed in leukemic cells. This evidence is based on: (*a*) non-infectious particles morphologically resembling GPV are consistently present in leukemic cells (33–35); (*b*) leukemic plasma pellets contain both virus particles and a DNA polymerase (33, 37); (*c*) fresh leukemic cells placed in culture release particles which have a density of 1.16 g/ml in a sucrose gradient and possess an oncornavirus-specific reverse transcriptase; and (*d*) GPV-RNA, undetectable in normal cells, is present in leukemic cells. The third possibility of a high turnover rate of DNA is unlikely and without any known precedent.

Absence of increased v-DNA sequence in BrdU-activated cells also supports that the virus production after BrdU treatment is due to the activation of repressed v-DNA sequences and not to the facilitation of infection. It is conceivable that a few cells are chronically infected and release virus at an undetectable level. The majority of the cells which are normally resistant to virus infection become susceptible after BrdU treatment. If this was the case, more v-DNA copies per cell would be found in BrdU-induced cells because oncornaviruses which contain an RNA-dependent DNA polymerase (reverse transcriptase) (31, 32) replicate via a DNA intermediate (30).

If oncornaviral genes are expressed in a specific cancer as detected by the presence of viral RNA or viral proteins, it should then be technically possible to determine if the virus in question is an endogenous or an exogenous virus. Using a reference endogenous and exogenous virus RNA (or DNA) as probes, one would determine if an increased amount of virus-specific DNA, over its normal counterpart, is present in these cancer cells. An increased amount of virus DNA in a cancer tissue (or cell) would indicate an exogenous infection; its absence would suggest an endogenous virus expression. Spiegelman and his colleagues (10) have shown new information is present in some human cancer cells which would suggest an exogenous infection. Whether or not an endogenous virus is also expressed in some human cancerous cells, remains to be seen.

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