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Detection of Avian Tumor Virus-Specific Nucleotide Sequences in Avian Cell DNAs

(reassociation kinetics/RNA tumor viruses/*gs* antigen/Rous sarcoma virus/chick cells)

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ABSTRACT **The effect of unlabeled cellular DNA upon the reassociation kinetics of labeled double-stranded DNA made by DNA polymerase from avian tumor viruses has been used to measure virus-specific nucleotide sequences in cells. Multiple copies of these sequences were found equally in normal chick cells, in chick cells transformed in culture, and in Rous tumor cells. Copies were also present equally in cells harboring chick helper factor and group-specific antigen, and in cells lacking those characteristics. Quail DNA also contains multiple copies of these sequences, but no copies were detected in HeLa-cell DNA or salmon-sperm DNA.**

Several lines of evidence suggest that transformation by both DNA and RNA oncogenic viruses is accompanied by integration of DNA copies of viral genes into cell DNA, but direct evidence for this phenomenon has been obtained only with the DNA tumor viruses (1). The most sensitive method developed thus far for detection of virus-specific sequences in cells was described by Gelb, Kohne, and Martin, who measured the accelerating effect of unlabeled cellular DNA upon the reassociation of labeled DNA from simian virus 40(SV40) (2). In this report, we apply this technique to a search for sequences from avian RNA tumor viruses in various avian cell types, using double-stranded DNA synthesized *in vitro* by RNA-dependent DNA polymerases (3). We find that multiple copies of nucleotide sequences from avian tumor viruses are present equally in normal and transformed chick cell DNAs; they are also present in quail DNA, but not in HeLa DNA or salmon-sperm DNA.

Apparently normal chick cells can be classified according to phenotypic evidence of viral genetic information. These cells may or may not carry a viral group-specific (*gs*) antigen, detected by the Complement Fixation Avian Leukosis (COFAL) test, and harbor a latent avian virus (also called chick helper factor) (4–7). Occasional embryos spontaneously produce a complete virus, called Rous associated virus (RAV-0), which carries the helper-factor genome (7). Using double-stranded product synthesized by polymerase from RAV-0 virus, we can detect viral sequences in equal numbers in cells with a negative COFAL test and without helper factor and in cells with a positive COFAL test and with helper factor.

Abbreviations: RSV, Rous sarcoma virus; RAV, Rous associated virus; *gs*, group specific; SSC, standard saline-citrate (0.15 M NaCl–0.015 M Na citrate); chf, chick helper factor; RR-DNA, rapidly-reassociating DNA; SR-DNA, slowly-reassociating DNA; COFAL, Complement Fixation Avian Leukosis (test).

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MATERIALS AND METHODS

Virus Purification. Schmidt–Ruppin strain of Rous sarcoma virus (RSV) was propagated in tissue culture of transformed chick cells, assayed, and purified as described (8). RAV-0 was harvested from a spontaneously producing line of Line 7 (C/A) chick cells and similarly purified.

Sources of Cell DNA. 11-day chick embryos were obtained from Kimber Farms (Berkeley, Calif.) and from Heisdorf and Nelson Farms (Kirkland, Wash.). 11-day quail embryos were provided from the University of Washington, Department of Zoology flock. Rous sarcomas were induced by the infection of about 5×10^5 focus-forming units (FFU) of Schmidt–Ruppin strain of RSV into the wing webs of 1- to 3-week-old chicks (Kimber Farms). Tumors generally developed within 2.5 weeks, were dissected away from normal tissues, and were minced and homogenized before DNA extraction. DNA from RSV-transformed cells was obtained from cultures of chronically transformed cells producing infectious SR-RSV. HeLa-cell DNA was purified (9) from cultures grown in our laboratory. Sheared salmon-sperm DNA was supplied by Drs. L. Gelb and M. Martin (NIH).

Characterization of "Positive" and "Negative" Embryos. The ability of chick cells to produce infectious RSV(0) of the Bryan high-titer strain, now called RSV(RAV-0) (10), was shown to depend on helper factor endogenous to the chick cells (4–7). The presence of *gs* antigen and helper factor is genetically controlled by the gs^+ allele of an autosomal locus (11, 12). However, occasional embryos that carry the helper factor do not synthesize detectable *gs* antigen. Therefore, all chick embryos were examined both for *gs* antigen by the COFAL test, and for helper factor by the ability of cells to complement defective Bryan RSV, leading to the release of infectious progeny. Table 1 indicates the satisfactory correlation between the two types of tests. The COFAL test was performed as described by Vogt and Friis (7), and the latent virus complementation test as described by Weiss *et al.* (10).

Preparation of Cellular DNA. DNA was extracted according to a modification (2) of the method of Berns and Thomas (13).

The final concentration was adjusted to 200–300 μg/ml in 0.015 M NaCl–1.5 mM Na citrate (0.1 × SSC), and the DNA was sheared at 45,000–50,000 pounds/inch² in a pressure cell designed by the American Instrument Co. This procedure produces DNA fragments that migrate at 5.5 S after denaturation relative to a 4S RNA marker in a 5–20% neutral sucrose gradient. Thus, the length of these double-stranded fragments is about 150–200 nucleotide pairs.

Preparation of Double-Stranded Polymerase Products. RNA-dependent DNA polymerase activity was elicited by disruption of purified virus particles with the nonionic detergent Nonidet-P-40 (Shell) and DNA was synthesized for 18 hr at 37°C with ^{3}H- or α-^{32}P-labeled deoxynucleoside triphosphate precursors (3). The single- and double-stranded DNA products were extracted and separated by batch elution from hydroxyapatite powder (Bio-Gel, DNA grade) (3, 14). The concentration of the double-stranded DNA was computed from trichloroacetic acid-precipitable cpm on the basis of the specific activity of the labeled precursor (3). (This material does not require mechanical shearing, since it cosediments with sheared DNA in 5–20% sucrose gradients.) The rapidly-reassociating (RR) fraction of double-stranded product was separated from the slowly-reassociating (SR) fraction according to their reassociation kinetics (3).

Reannealing Techniques. Labeled DNA synthesized by viral polymerase, with or without sheared unlabeled cell DNA, was denatured in a boiling-water bath in 3 mM EDTA. The solution was permitted to cool for 1 min at room temperature and 4.0 M phosphate buffer was added, to a final concentration of 0.40 M. The mixture was then incubated for up to 100 hr in a water bath at 68°C, and samples were removed periodically for analysis on hydroxyapatite. The reassociation of labeled DNA was followed by precipitation of eluates with cold 5% trichloroacetic acid with calf-thymus DNA carrier. The reassociation of cell DNA was monitored by recording the absorbance of each eluate from hydroxyapatite before precipitation. [The latter results are not presented, but were consistent with data reported by Britten and Kohne (15).]

Computations. Extent of DNA reassociation was plotted against the product of initial DNA concentration (Co) and time (t), according to the convention established by Britten and Kohne (15). Points of 50% reassociation (half-Cots) were used to determine the molecular weight of unique sequences of each polymerase product by comparison with the half-Cots of DNA standards (3). The number of viral gene copies found in each cell was calculated by the method of Gelb, Kohne, and Martin (2); acceleration of annealing of polymerase product by cell DNA reflects the presence of viral nucleotide sequences in the cell DNA. The number of copies of labeled unique sequences in each reaction is determined from the specific activity and the complexity of the DNA, and the number of unlabeled cell genomes is determined from the concentration of DNA, based upon absorbance and the published values for cell content of DNA (16). Typical computations are illustrated in the description of the results and in ref. 2.

RESULTS

The nature of the viral probes

We have described the purification and characterization of two populations of double-stranded molecules synthesized by the Schmidt–Ruppin strain of RSV (3). The rapidly-reassociating fraction (RR-DNA) has a half-Cot of 1.7×10^{-3} mole-sec/liter and an estimated complexity of about 10^6 daltons, whereas the slowly-reassociating fraction (SR-DNA) has a half-Cot of $7–8 \times 10^{-3}$ mole-sec/liter and a complexity of about $4–5 \times 10^6$ daltons. According to current estimates of the molecular weight of the 70S viral RNA genome (10^7) these results indicate that RR-DNA (which comprises about 85% of double-stranded product) is copied from about 5% of the genome, and SR-DNA (which comprises 5–10% of the double-stranded product) is transcribed from about 25%.

In addition to the RR- and SR-DNA probes synthesized by the viral polymerase, we made a double-stranded probe with the polymerase activity associated with Rous associated virus-0 (RAV-0) spontaneously produced by Line 7 chick cells (7). Most of the double-stranded DNA synthesized by this polymerase reassociates slowly, with a half-Cot of 8×10^{-3} mole-sec/liter, consistent with a molecular weight of unique sequences of about 5×10^6. Other avian tumor virus polymerases we have examined—those associated with Bryan (RAV-1) strain and Prague strain of RSV, as well as with Schmidt–Ruppin RSV—produce principally rapidly-reassociating double-stranded DNA.

There appears to be a considerable amount of homology among the sequences copied by each of these polymerases. If small amounts of ^{3}H-labeled double-stranded product of the Bryan (RAV-1) or Prague strain enzymes are incubated with large amounts of ^{32}P-labeled double-stranded DNA made with the Schmidt–Ruppin strain enzyme, 70% of the [^{3}H]-DNA will anneal with the [^{32}P]DNA sequences. Similarly, as much as 70% of the tritiated product of the RAV-0 polymerase appears to be homologous to slowly-reassociated [^{32}P]DNA made with Schmidt–Ruppin strain polymerase. These results suggest that our probes detect genetic sequences that may be present in any or all of the avian tumor viruses, regardless of the source of enzyme and template used to synthesize these probes.

We have shown that the double-stranded products of the RSV polymerase reaction hybridize to viral 70S RNA (17).

TABLE 1. *Characterization of embryos used for DNA extraction*

Number of embryos	CF Titer of *gs* antigen 0	1:2	1:4	1:8	Average number of foci* No x-rays	X-rays + quail cells	X-rays + C/BE cells	Type
6	6	0	0	0	315	14	15	−
8	0	1	2	5	309	276	14	+

* Three 50-mm plates of each embryo, each containing 1×10^6 cells, were infected with 300 FFU of RSV(RAV-1). After 24 hr, one plate was overlaid with agar for direct focus assay and the other two were irradiated with 5000 R. One irradiated plate was then seeded with unirradiated quail cells [susceptible to RSV(RAV-1) and RSV(RAV-0)] and the other was seeded with unirradiated C/BE cells [susceptible to RSV(RAV-1) only]. The difference in focus formation due to infective centers between plates seeded with quail cells and those seeded with C/BE cells is a measure of the capacity of the embryo cells to produce RSV(RAV-0) (10).

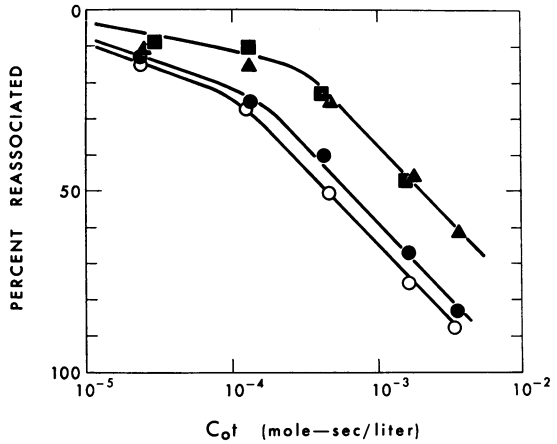

FIG. 1. Detection of rapidly-reassociating sequences. RR-DNA (1.5 ng/ml) labeled (3) with [^{3}H]dCTP (Schwarz), [^{3}H]-dGTP (Amersham-Searle), [^{3}H]dATP (New England Nuclear), [^{3}H]TTP (Schwarz), was incubated with salmon-sperm DNA (420 μg/ml, ▲), HeLa-cell DNA (2.1 mg/ml, ■), normal chick-embryo DNA (660 μg/ml, O) or Rous tumor cell DNA (550 μg/ml, ●). Reassociation was studied as outlined in the *text* and percent of labeled DNA reassociated was plotted logarithmically against Cot values, corrected for Na^+ concentration (32).

Therefore, they do not represent sequences copied from the several species of cellular RNA or cellular DNA also contained in the virus particles (8, 18).

Detection of sequences homologous to RR-DNA

As shown in Fig. 1, when RR-[^{3}H]DNA made with the Schmidt–Ruppin RSV polymerase is incubated with salmon-sperm or HeLa-cell DNA, it reassociates with a half-Cot of 2×10^{-3} mole-sec/liter. This represents a slight slowing of the reassociation reaction, attributable to increased viscosity of the annealing solution (19). If DNA extracted from 11-day chick embryos is incubated with RR-[^{3}H]DNA, there is a marked acceleration of annealing. In the experiment shown in Fig. 1, 660 μg/ml of chick DNA and 1.5 ng/ml of RR-[^{3}H]-DNA are used, representing four copies of labeled viral sequences for each complement of diploid cell DNA. The half-Cot of the RR-[^{3}H]DNA in the presence of chick DNA is 5×10^{-4} mole-sec/liter, representing a 4-fold increase in the rate of reannealing and, therefore, a 4-fold increase in the concentration of viral sequences. Thus, each cell complement of DNA contains 12 copies of rapidly-reassociating sequences (e.g., $4 \times 4 = 16$ total copies, $16 - 4$ labeled copies $= 12$ copies).

Fig. 1 also shows acceleration of annealing by DNA from sarcomas induced in chicken wing webs with Schmidt–Ruppin RSV. The concentration of tumor DNA is slightly lower than that of normal chick DNA, and the acceleration is proportionately less; the half-Cot is 6×10^{-4} mole-sec/liter, consistent with the presence of about 11 copies of rapidly-reassociating sequences per cell. If a similar experiment is performed with higher concentrations of cell DNA (2.1 mg/ml for both normal chick embryo and Rous tumor DNA), proportionately greater acceleration of reannealing is observed, with the half-Cot reduced to 1.5×10^{-4} mole-sec/liter. Again, the number of viral gene copies per diploid cell is about 13, with no significant differences between normal and tumor cells.

To rule out the possibility that an increased number of copies of rapidly-reassociating genes in tumor cells was masked by a high proportion of normal cells in the tumor tissue, we also examined DNA from cells transformed in tissue culture by the Schmidt–Ruppin strain of RSV. 75% of these cells were capable of forming foci when cloned onto chick feeder layers. 790 μg/ml of DNA from these cells lowered the half-Cot of RR-[^{3}H]DNA to 4×10^{-4} mole-sec/liter, indicating 14 copies of viral sequences per diploid cell, a result not significantly different from that obtained with normal chick embryo or Rous tumor DNA.

Detection of sequences homologous to SR-DNA

Because we detected no differences between normal and transformed cell DNA using the limited number of viral sequences present in RR-DNA, we also used SR-DNA, which is generally 4- to 5-fold more complex, as a probe for viral sequences in cells. The preparation of SR-[^{32}P]DNA used in the experiments shown in Fig. 2 reannealed in the absence of cell DNA with a half-Cot of 1.2×10^{-2} mole-sec/liter; this value is about 50% higher than that of the several other SR-DNA preparations we have made. The discrepancy is due either to an error in determination of the specific activity of the DNA or to more extensive copying by the polymerase during the reaction in which this SR-DNA was made. Again, salmon-sperm and HeLa-cell DNAs retard reassociation, the half-Cot rising to 3.2×10^{-2} mole-sec/liter at high concentrations of cell DNA. When normal chick or Rous tumor cell DNA is used, however, the reassociation is hastened. Chick DNA at 3.4 mg/ml accelerates the reaction 12.5-fold, lowering the half-Cot to 2.6×10^{-3} mole-sec/liter; tumor DNA (2.5 mg/ml) reduces the half-Cot to 3.8×10^{-3} mole-sec/liter. The results are consistent with the presence of 17.5 copies of slowly-reassociating sequences in normal diploid cells and 15 copies in RSV-transformed cells. In similar experiments, lower concentrations of chick DNA produce proportionately less acceleration.

Detection of viral sequences in *gs*$^-$ chick cells

To investigate further the finding of multiple copies of viral sequences in normal chick cells, we asked whether such se-

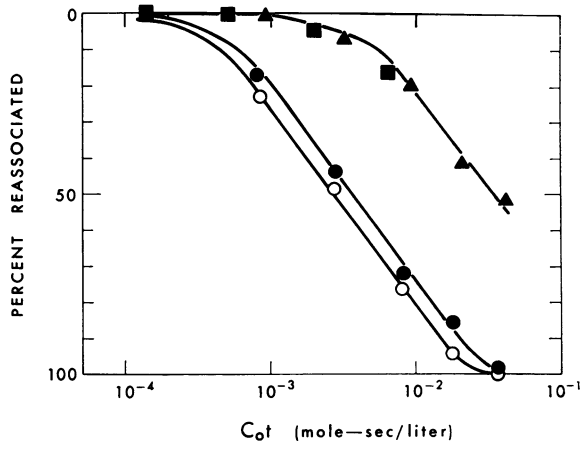

FIG. 2. Detection of slowly-reassociating sequences. SR-DNA (14 ng/ml) labeled with [α-^{32}P]dGTP (International Chemical and Nuclear Corp.), was prepared (3) and reannealed in the presence of salmon-sperm DNA (3.9 mg/ml, ▲), HeLa DNA (2.1 mg/ml, ■), normal chick-embryo DNA (3.4 mg/ml, O), or Rous tumor DNA (2.5 mg/ml, ●). Cot curves are plotted as in Fig. 1.

quences could be identified in cells that had no evidence of avian tumor virus genes, as judged by absence of the group-specific antigen of avian tumor virus and of active helper factor.

Double-stranded product labeled with ^3H of the RAV-0 polymerase reanneals with a half-Cot of 1×10^{-2} mole-sec/liter in the presence of salmon-sperm (or HeLa-cell) DNA, as shown in Fig. 3. 1.7 mg/ml of DNA extracted from either gs^+ or gs^- embryos (Table 1) accelerates this reaction, yielding a half-Cot of 2×10^{-3} mole-sec/liter, consistent with the presence of about six copies per diploid cell of those RAV-0 genes transcribed *in vitro* into double-stranded DNA. Both types of cell DNA have also been shown to contain about equal numbers of RR-DNA sequences. The results suggest that the gs^+ and gs^- cells differ in the extent of expression of viral genetic information, rather than in the inheritance of structural genes, although they could possess genetic differences not detected with the limited probe we have used.

Detection of avian tumor virus sequences in quail cells

Quail embryo cells in our hands are invariably negative in COFAL testing, and they do not have active helper factor. Since some strains of RSV can cause tumors in quails, we tested quail cell DNA for the presence of avian tumor virus genes. A stringent test of the provirus theory would be afforded by a species susceptible to RSV whose normal DNA did not contain viral sequences. However, as Fig. 4 illustrates, DNA from normal quail embryos contains sequences present in all three viral probes used in these experiments—RR- and SR-DNA synthesized by RSV polymerase and double-stranded DNA synthesized by the RAV-0 polymerase. About four copies of rapidly-reassociating and RAV-0 sequences and two copies of slowly-reassociating sequences are present per diploid cell.

DISCUSSION

As summarized in Table 2, we find multiple copies of avian virus genes in several types of chick cells and in quail cells, but not in HeLa cells or salmon sperm. Moreover, we observe a similar number of viral genes in chick cells regardless of the degree of expression of viral information. Thus, Rous tumor cells, cells transformed in culture with RSV, random chick embryos, or selected gs^+ or gs^- embryos all appear to contain about 15 copies of rapidly-reassociating sequences. This result suggests that all chick cells contain a considerable amount of avian tumor virus genetic information and that such information may be expressed in response to various factors, including inheritance of regulatory genes (11, 12) or stimulation by physical, chemical, or biological agents (6, 10). These results support the several hypotheses that postulate the presence of viral genes in normal cells (11, 20–23).

The observation of tumor virus genes in gs^- cells suggests that these cells might be induced to produce viral antigen or complete virus under appropriate conditions. Since completion of these experiments, it has been discovered that gs^- chicken

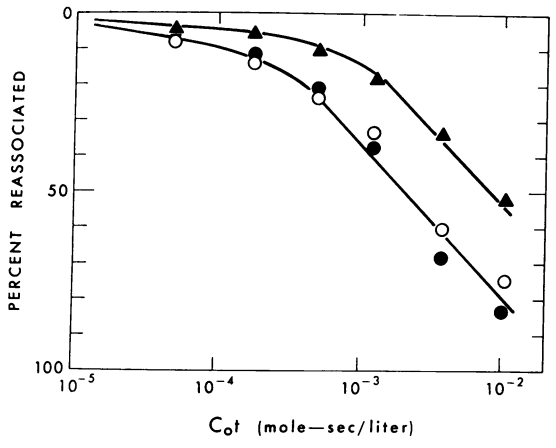

FIG. 3. Detection of RAV-0 sequences in gs^+ and gs^- cells. Double-stranded product of the RAV-O polymerase was labeled with [^3H]TTP and renatured in the presence of salmon-sperm DNA (1.7 mg/ml, ▲) or DNA (1.7 mg/ml) extracted from COFAL (+), RAV-0 (+) embryos (●) or COFAL (−), RAV-0 (−) embryos (○).

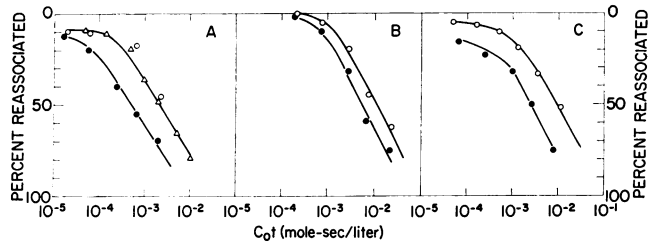

FIG. 4. Detection of viral sequences in quail cells. Viral DNA probes synthesized *in vitro* were reassociated with salmon-sperm DNA (△, ○; two experiments) and with DNA, extracted from quail embryos (●). (*A*) RR-[^3H]DNA (1.5 ng/ml) annealed with 1.55 mg/ml of salmon-sperm or quail DNA. (*B*) SR-[^{32}P]-DNA (9 ng/ml) annealed with 1.2 mg/ml of salmon-sperm or quail DNA; and (*C*) [^3H]RAV-0 DNA (6 ng/ml) annealed with 1.45 mg/ml of cell DNAs. Quail DNA reduces the half-Cot for RR-DNA from 2.0×10^{-3} to 5.0×10^{4}, the half-Cot for SR-[^{32}P]DNA from 1.2×10^{-2} to 6.0×10^{-3}, and the half-Cot for [^3H]RAV-O DNA from 1.0×10^{-2} to 3×10^{-3} mole-sec/liter.

TABLE 2. *Number of viral gene copies per diploid cell DNA*

Source of cell DNA	Probe*		
	Rapidly-reasso-ciating	Slowly-reasso-ciating	RAV-0
Random chick embryos	12–15	17.5	4
RSV-induced sarcomas†	11–13	15.0	4
RSV-transformed cell cultures	14	N.T.	N.T.
COFAL (+), RAV-0 (+) chick embryos	16	N.T.	6
COFAL (−), RAV-0 (−) chick embryos	16	N.T.	6
Quail embryos	4	2	4
HeLa cells	0	0	0
Salmon sperm	0	0	0

* Labeled double-stranded DNAs synthesized by crude RNA-dependent DNA polymerase preparations were used as probes for the detection of viral nucleotide sequences in the listed cell types. Rapidly-reassociating DNA and slowly-reassociating DNA were made by Schmidt–Ruppin RSV polymerase; RAV-0 = double-stranded DNA made by RAV-0 polymerase; N.T. = not tested.

† Schmidt–Ruppin strain.

cells, which appear to possess no helper activity by routine testing, can be induced to reveal endogenous viruses by treatment with physical or chemical carcinogens (10). Further, the results reported here raise the possibility that phenotypic mixing or exchange of RNA subunits between exogenous and endogenous viruses may occur in gs^-, as well as gs^+, cells.

These studies do not provide a definitive answer to the question of whether viral DNA synthesis is required for cell transformation by the RNA tumor viruses, as proposed by Temin (24, 25). Investigators using RNA–DNA hybridization techniques have detected homology between avian tumor virus RNA and normal chick DNA (26–28), and some have reported greater homology with DNA from cells transformed by these viruses (29, 30). Measuring reassociation kinetics of the viral genes transcribed into double-stranded DNA *in vitro* by crude preparations of polymerase, we do not find an increased number of copies of those genes in transformed, as opposed to normal, chick cells. Similar results with labeled DNA synthesized by murine leukemia virus polymerase and unlabeled DNA from normal and transformed rat and mouse cells have recently been reported by Gelb, Aronson, and Martin (31).

Although our method of detection permits a degree of quantitation of gene copies not possible with RNA–DNA hybridization techniques, it is not sufficiently sensitive to measure a small increase in a relatively large number of viral gene copies. Moreover, the probes we use in these experiments probably do not represent the complete viral genome and, therefore, might not detect selected viral genes transcribed into DNA during transformation.

The use of reassociation kinetics, however, does allow detection of less than one copy of a viral genome per cell (2). Therefore, a more stringent test of Temin's provirus hypothesis will be provided by study of the effect of Rous transformation upon DNA from cells that normally do not contain avian tumor virus sequences. Mammalian cells may meet these specifications.

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