Use of DNA-DNA Annealing to Detect New Virus-Specific DNA Sequences in Chicken Embryo Fibroblasts After Infection by Avian Sarcoma Virus

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Labeled, virus-specific DNA synthesized in vitro by the virion-associated polymerase of avian sarcoma virus (ASV) was used to measure virus-specific sequences in cell DNA in three ways: (i) by determining the effect of cell DNA upon the reassociation rate of double-stranded polymerase products; (ii) by measuring the kinetics of annealing of single-stranded polymerase product (cDNA) to cell DNA; or (iii) by measuring the amount of cDNA which anneals to a large excess of cell DNA. With these three assays and modifications of them, we show that fewer than five copies of ASV-specific DNA sequences are present per diploid cell in uninfected chicken embryos; that a two- to several-fold increase in copy number of viral DNA follows infection by ASV; that infection introduces to the cell viral sequences not present before infection; and that DNAs from uninfected Pekin duck and Japanese quail embryos show no homology with DNA synthesized by the ASV polymerase. Some of these results differ from data in a previous report from this laboratory (H. E. Varmus, R. A. Weiss, R. R. Friis, W. Levinson, and J. M. Bishop, 1972) and, in general, reconcile our observations with those from other laboratories.

Several lines of experimental evidence now support the notion that infection by RNA tumor viruses is followed by synthesis of virus-specific DNA and its insertion into the genome of the infected cell (23). Direct measurement of newly acquired viral DNA by nucleic acid hybridization is unequivocal when the virus infects a heterologous host which does not normally contain nucleic acid sequences homologous to those in the viral genome (1, 28-30). However, there exist discrepancies among the several reports addressed to the question of whether new viral DNA is detectable after infection of the natural host. In the case of avian sarcoma virus (ASV), it is generally agreed that all normal chicken cells contain DNA at least partially homologous to the genome of ASV, regardless of whether the cells express endogenous viral information (1, 15, 19, 26). However, the evidence is less consistent regarding the appearance of additional viral sequences in chick cells infected by ASV. In general, hybridization experiments using labeled ASV 70S RNA as a hybridization reagent have documented differences between normal and infected cells in several laboratories, including our own (1, 2, 14, 19, 21, 22, 27). On the other hand, we previously reported that the double-stranded virus-specific DNA synthesized in vitro by virion-associated DNA polym-

erase was represented equally in normal and infected chick cells (26). We speculated that the known incomplete representation of the viral genome in the polymerase products might have been responsible for the discrepant results (27). This possibility was strengthened by the recent report of Schincariol and Joklik (20), who found a threefold increase in the number of ASV DNA sequences after infection, using single-stranded DNA synthesized in vitro as a hybridization reagent.

In this manuscript we describe three varieties of DNA-DNA hybridization experiments, the results of which conflict with our earlier report by demonstrating a substantial increment in virus-specific DNA in ASV-infected chick cells. Moreover, we show that viral nucleotide sequences unrelated to viral sequences present in normal chick cells are added to the cell during infection. The differences between these results and those reported previously, and the merits of the various DNA-DNA annealing techniques used, are presented in the Discussion.

MATERIALS AND METHODS

Cell and viruses. B77 strain (subgroup C) of ASV was obtained from R. Friis, Schmidt-Ruppin strain (subgroup A) (SR) from H. Rubin, and Prague strain (subgroups B and C) from P. Vogt; viruses were

propagated in chicken embryo fibroblasts and purified as described (4). Embryonated chicken eggs (specific pathogen-free) were obtained from Kimber Farms, Fremont, Calif.; Pekin duck embryos from Reichardt Duck Farm, Petaluma, Calif.; and Japanese quail embryos from Life Science, Inc., St. Petersburg, Fla., through the courtesy of P. Vogt. The chicken embryos were not tested for ASV antigens, chick helper factor, or susceptibility to viral subgroups. Chicken embryo cells were transformed by infection with ASV (multiplicity of infection about 1 focus-forming unit per cell), and propagated for at least a week before harvesting of cells for DNA extraction. Induction of tumors in chicken wing webs by ASV has been described (26).

Preparation of cell DNA. Our procedures for DNA extraction have been published (26, 28, 29). In general, DNA from embryos and from transformed cells was collected after Pronase-sodium dodecyl sulfate treatment and phenol extraction by spooling the ethanol precipitate on a plastic pipette. After pancreatic RNase treatment and phenol extraction, the DNA was extensively dialyzed against $1 \times \text{SSC}$ and $0.1 \times \text{SSC}$ ($\text{SSC} = 0.15 \text{ M NaCl}$ plus 0.015 M sodium citrate). Before hybridization, all DNA preparations were sheared to a length of 200 to 300 nucleotides at $50,000 \text{ lb/in}^2$ (6, 26). Chick DNA labeled for 3 days with $10 \mu\text{Ci}$ of [^{14}C]thymidine per ml was kindly provided by L. Hallick. The unique-sequence [^{14}C]DNA was prepared by reannealing the labeled DNA to a C_0t value of 240 mol-s/liter in a reaction driven by an excess of unlabeled chicken embryo DNA; the reassociated DNA was removed by fractionation on a hydroxyapatite column. The 60% of the DNA which failed to reanneal at this C_0t value was assumed to represent unique sequences (6) and had a final specific activity of approximately 2,500 counts per minute per μg .

Preparation of labeled virus-specific DNA and RNA. RNA-directed DNA polymerase activity was elicited by treatment of purified ASV with 0.02% Nonidet P-40 (Shell Oil Co.). For synthesis of single-stranded DNA complementary to the viral RNA genome (cDNA), reactions were performed in the presence of $100 \mu\text{g}$ of actinomycin D (Calbiochem) per ml. High specific activities (about 2×10^7 counts per min per μg) were achieved by labeling with [^3H]dATP (7.8 Ci/mmol; ICN), [^3H]dTTP (17 Ci/mmol, Schwarz/Mann), and [^3H]dCTP (20 Ci/mmol; ICN). We find that double-stranded DNA may constitute as much as 40 to 60% of the product despite the presence of actinomycin D. Consequently, all preparations were fractionated by batch elution on hydroxyapatite. When ready for use as a hybridization reagent, the cDNA was over 97% digestible by S1 nuclease; and it hybridized completely (as assayed by S1 nuclease) to 70S RNA prepared from ASV. Although we have demonstrated that ASV cDNA synthesized in the presence of actinomycin D is copied from the entire genome (9), the copying is nonuniform; 10- to 50-fold excesses of cDNA to 70S RNA are required to anneal labeled 70S RNA completely. (For unknown reasons, copying is more uniform by SR-ASV or Prague subgroup C ASV than by B77

virus polymerase [E. Stavnezer, N. Quintrell, and authors, unpublished data].) Consequently, the extent of cDNA annealing to cell DNA can only provide a qualitative estimate of the percentage of the viral genome present in cell DNA.

Double-stranded ASV polymerase product was prepared in the absence of actinomycin D by using high specific activity [^3H]dTTP (57 Ci/mmol, Schwarz/Mann) as the only labeled precursor. Two populations of double-stranded molecules can be identified and separated by their reassociation kinetics (25). The slowly reassociating fraction (srDNA) has a $C_0t_{0.5}$ of 8×10^{-3} to $10 \times 10^{-3} \text{ mol-s/liter}$ and, according to our current estimates (5), represents approximately 30% of the ASV genome, assuming the genome is haploid (32).

[^{32}P]70S RNA was prepared by labeling cultures of virus-producing chick cells with 2 mCi of carrier-free $^{32}\text{PO}_4$ per ml (17). Preparation of 70S RNA has been described (3, 17). The specific activity was estimated from the specific activity of RNA extracted from the virus-producing cells. Values so obtained were in the vicinity of 10^7 counts per min per μg and presumably represent an underestimate of specific activity of 70S RNA.

Hybridization conditions and assays. Annealing was generally performed at 68°C in either 0.6 M NaCl or 0.4 M phosphate buffer (equimolar Na_2HPO_4 and NaH_2PO_4) with 0.002 M EDTA. Volumes smaller than $30 \mu\text{l}$ were incubated in sealed capillary pipettes, and larger volumes were incubated in conical tubes under a layer of mineral oil. Samples were diluted into 0.01 M phosphate buffer and assayed by batch or column fractionation on hydroxyapatite (BioRad); diluted into 0.3 M NaCl , 0.003 M ZnCl_2 , and 0.1 M sodium acetate, pH 4.5, containing $10 \mu\text{g}$ of sheared calf thymus DNA per ml, and assayed by digestion at 50°C for 2 h with single strand-specific S1 nuclease from *Aspergillus oryzae* (12); or diluted into $2 \times \text{SSC}$ and digested with $50 \mu\text{g}$ of pancreatic ribonuclease per ml at 37°C for 45 min.

Computation of copy number. cDNA can be used to determine the amount of virus-specific DNA per cell by assessing the kinetics of annealing in relation to the kinetics of annealing of differentially labeled unique-sequence DNA. The ratio of the $C_0t_{0.5}$ for cDNA to the $C_0t_{0.5}$ for unique-sequence DNA equals the copy number per haploid genome. As noted above, since the cDNA is not uniformly copied from its template, the extent of its reaction with cell DNA does not provide a quantitative estimate of the percentage of the viral genome present in the cell.

Double-stranded DNA (srDNA) can be used to compute copy numbers for viral sequences by determining the effect of a defined amount of unlabeled cell DNA upon the reassociation kinetics of the labeled viral DNA (11). Reassociation is accelerated (and the $C_0t_{0.5}$ reduced) in proportion to the amount of virus-specific DNA present in the cell DNA, since the effective concentration of viral sequences is augmented. This method for estimating viral DNA copies has several inherent limitations, however, introduced by the heterogeneity of the labeled DNA, its incomplete representation of the genome, and the uncertainties

involved in determining its concentration and complexity (28; H. E. Varmus et al., *Comparative Leukemia Research*, in press).

RESULTS

Hybridization of cDNA to excess cell DNA.

To reinvestigate the question of whether differences between normal and ASV-infected chick cells can be detected by hybridization of cell DNA with labeled viral polymerase products, we first asked whether single-stranded DNA (cDNA) synthesized by the B77 virus polymerase anneals to a greater extent with DNA from infected cells than with DNA from normal chicken embryos. Figure 1 demonstrates the effect of incubating a constant amount of labeled B77 cDNA to increasing amounts of cell DNA from various normal avian cells and from ASV-infected chicken cells to C_0t values of 2×10^4 mol-s/liter. DNA from a variety of ASV-infected chick cells in culture and from ASV-induced chicken wing-web tumors hybridized

with a larger fraction of cDNA than did equal amounts of DNA from uninfected chicken embryo cells, and the percentage of cDNA annealed increased to over 70% with increasing amounts of infected-cell DNA. This indicates that the infected cells contain more ASV-specific DNA than the uninfected embryo cells, a result consistent with several reports of RNA-DNA hybridization experiments (1, 2, 14, 19, 21, 22, 27) and one recent report of DNA-DNA annealing experiments (20).

B77 cDNA annealed to a maximum of 30 to 40% with large excesses of DNA from three pools of uninfected chicken embryos (Fig. 1 and 4A; Table 1). The limited extent of the reaction suggested that not all of the sequences present in the cDNA were present in the chicken embryo DNA; this is supported by the "exhaustive" hybridization experiments discussed below (Table 1) and by the limited (about 35%) hybridization of B77 70S RNA to chicken embryo DNA (Table 2). The RNA-DNA hybridiza-

FIG. 1. Hybridization of ASV cDNA to avian cell DNA. One thousand counts per minute of B77 cDNA was incubated for 66 h at 68 C in 0.6 M NaCl with increasing amounts of DNA from various sources; in each case, the reaction volume was increased in proportion to the amount of DNA to maintain a DNA concentration of 5 mg/ml and a final C_0t value of 2×10^4 mol-s/liter. Hybridization was assessed by S1 nuclease digestion. Normal duck embryos (two experiments), (Δ); pool of normal Japanese quail embryos, ($\blacktriangledown$); three pools of normal chicken embryos, ($\diamond$, $\times$, $\square$); tumor produced in chickens with SR-ASV, ($\bullet$); B77-transformed chicken embryo cells, ($\bullet$); SR-ASV-transformed chicken embryo cells, ($\circ$). The results have not been corrected for the intrinsic resistance of the cDNA to S1 nuclease (3%).

TABLE 1. *Effect of prior annealing to chicken embryo DNA upon the annealing of ASV cDNA to embryo and B77-transformed chick cell DNA^a*

No. of previous annealings with CE DNA	DNA newly added	Amount added (mg)	S1 resistance (%)	Increment in S1 resistance (%)
0	CE	6.0	43.5	
0	B77/CE	0.6	68	
1	CE	4.0	47	7
1	B77/CE	1.0	73	58
2	CE	3.0	50	6
3	CE	1.0	48.5	0
3	B77/CE	1.0	69	38

^a Hybridization mixtures, containing approximately 1,000 counts/min of ASV cDNA per mg of cell DNA in 0.6 M NaCl, were incubated to a C_{ot} value of 7×10^3 mol-s/liter. The extent of annealing was determined by S1 digestion of a portion (in the case of annealing with chicken embryo [CE] DNA) or all of the reaction mixture (in the case of B77-ASV transformed chick cell [B77/CE] DNA). Additions of CE or B77/CE DNA were made to undigested portions of reaction mixtures which contained CE DNA, the Na^+ concentration was adjusted to 0.6 M, and the mixtures were incubated to a C_{ot} of 7×10^3 for the newly added DNA. This process was repeated to allow as many as three exposures to normal chick DNA before annealing to transformed cell DNA. The cumulative DNA concentration was maintained at 5 mg/ml throughout the experiment. The "increment in S1 resistance" represents the difference in S1 resistant fractions before and after each incubation, divided by the S1 susceptible fraction before that incubation.

TABLE 2. *Hybridization of RSV 70S RNA to uninfected cell DNA^a*

Source of cellular DNA	Hybridization (%)
Normal cat liver (20)	3
Duck embryo	2
Quail embryo	2.5
Chicken embryo	33

^a ³²P-labeled B77 70S RNA (1,000 counts/min, 0.1 ng) was incubated at 68 C in a solution of 0.6 M NaCl with vast excesses (1 mg) of sheared, denatured cellular DNA to C_{ot} values of 3×10^4 to 7×10^4 mol-s/liter. Hybridization was determined by resistance to 50 μ g of pancreatic RNase A per ml in $2 \times$ SSC at 37 C for 45 min, after correction for the intrinsic RNase resistance of the RNA (12%).

tion results are consistent with reports from other laboratories (14, 15, 21).

No homology was detected between B77 cDNA and DNA from normal Pekin duck embryos, as expected from previous work (29, 30); in addition, no homology was found with DNA from a pool of 15 Japanese quail embryos, even

when as much as 1.2 mg of DNA was used. (Use of this quantity of DNA permits detection of as little as 0.01 copy per cell [unpublished data].) These negative findings were supported by hybridization with labeled B77 70S RNA (Table 1) and by reassociation kinetics assays under stringent and nonstringent conditions (Fig. 2 and 3). However, the results with quail embryos differ from our earlier report of low numbers of ASV sequences in quail DNA (26) and from the reports of Neiman (15) and Rosenthal et al. (19) in which low levels of annealing of 70S RNA to quail embryo DNA were observed.

Test for new virus-specific nucleotide sequences in chicken cells after infection by ASV. An increased amount of ASV-specific DNA in infected chick cells could be due to addition of sequences not present in the uninfected cells, to the addition of more copies of sequences present in the uninfected cells, or to both. We approached these possibilities by asking (i) whether we could identify sequences in the labeled cDNA which hybridized to DNA from infected cells after exhaustive hybridization to DNA from uninfected cells; (ii) whether repetition of our earlier attempts to enumerate copies of a defined population of ASV sequences (srDNA) in normal and transformed cells would now reveal differences between them; and (iii) whether the kinetics of annealing of cDNA to DNA from normal and transformed chick cells would indicate a difference in copy numbers.

(i) Exhaustive hybridization with cDNA. When B77 cDNA was incubated with large amounts of DNA from normal chicken embryos, about 30 to 40% became resistant to S1 nuclease (Fig. 1 and 4A; Table 1). When samples from this reaction were subjected to repeated incubation with additional quantities of chicken embryo DNA before nuclease assay, as much as 45 to 50% of the cDNA was rendered nuclease resistant. After repeated additions of chicken embryo DNA, however, no further annealing occurred, but a considerable portion of the remaining, unannealed B77 cDNA was capable of interacting with DNA from B77-transformed chick cells (Table 1). This result demonstrates that infection by ASV introduces at least some new viral sequences into chicken cells. Similar results obtained by using DNA from SR-ASV transformed chicken cells or from SR-ASV induced tumors and B77 cDNA indicate that at least some of the new sequences found in the cells infected by SR-ASV are shared by the B77 strain of ASV (data not shown).

(ii) Acceleration of DNA reassociation by cell DNA. In Fig. 2, we illustrate attempts to measure the number of copies of B77 srDNA in

FIG. 2. Effect of avian DNAs upon the reassociation of B77 srDNA. The slowly reassociating double-stranded DNA (specific activity, 15×10^6 counts per min per μg) synthesized by B77 virus-associated DNA polymerase was reannealed at 3.0 ng/ml in the presence of 4 mg of DNA per ml from normal duck embryos (Δ), normal Japanese quail embryos ($\blacktriangle$), two pools of normal chicken embryos ($\square$, $\circ$), and B77-transformed chick cells ($\bullet$). Reassociation was performed at 68 C in 0.6 M NaCl and was assayed by batch elution from hydroxyapatite (panel A) and by S1 nuclease resistance (panel B).

FIG. 3. Effect of avian DNAs upon the reassociation of B77 srDNA under relaxed hybridization conditions. B77 srDNA (2.2 ng/ml) was reassociated at 59 C in 1.0 M phosphate buffer (1.5 M Na^+) in the presence of 4 mg of calf thymus DNA per ml ($\blacktriangle$), duck embryo DNA (Δ), quail embryo DNA (∇), or chicken embryo DNA ($\circ$). Annealing was assayed by elution from hydroxyapatite columns with 0.12 M phosphate buffer and 0.4 M phosphate buffer at 50 C.

normal and infected chick cells by observing the effects of chick cell DNA upon the reassociation kinetics of srDNA. In contrast to our previous report that 10 to 15 copies of viral sequences were measured in normal or infected cells (26), we now found only 0.5 to 0.9 copies of these sequences (per diploid genome) in uninfected chicken embryo cells, and we observed an increment in copy number, to 1.9 to 2.3 copies, in the B77-transformed cells used in the experiment shown in Fig. 2. (Other infected cell populations have contained 2 to 8 copies per cell.) No copies were detected in quail or duck embryo cells. Identical results were obtained using either

fractionation on hydroxyapatite (panel A) or resistance to S1 nuclease (panel B) as a measure of reassociation. In an effort to resolve discrepancies with our earlier report, we also attempted to detect DNA related, but not identical, to ASV DNA in chicken or quail embryos by performing and assaying reassociation under the nonstringent conditions used for demonstrating relatedness between mouse and rat DNA (18). However, there was no effect of quail (or duck) embryo DNA upon reassociation of slowly reassociating ASV DNA when incubations were performed at 59 C in 1.0 M phosphate buffer and reassociation was assayed by fractionation on hydroxyapatite at 50 C (Fig. 3); the effect of chick DNA was identical to that observed under conventional conditions (Fig. 2).

(iii) **Kinetics of annealing of B77 cDNA to chick cell DNA.** We also assessed the number of copies of ASV-specific DNA in normal and transformed cells by studying the kinetics of annealing of B77 cDNA in comparison to the kinetics of annealing of unique-sequence chick DNA. The 30% of sequences in B77 cDNA capable of annealing to normal chick DNA under these conditions did so with a $C_0t_{1/2}$ of approximately 5×10^2 mol-s/liter, whereas unique-sequence DNA in the same reaction mixture reannealed with a $C_0t_{1/2}$ of 1.3×10^3 (Fig. 4A). (The $C_0t_{1/2}$ values are the C_0t values at which one-half of the observed maximal reaction occur; i.e., in Fig. 4A, the C_0t values at which 15% of the cDNA or 40% of the unique-sequence DNA is nuclease resistant.) This observation indicates that about 2.6 copies of the relevant sequences are present per haploid genome (or about 5 per diploid genome). B77

FIG. 4. Kinetics of annealing of ASV cDNA and unique-sequence chick DNA to DNA from normal chicken embryos and ASV-transformed chick cells. B77 cDNA (2×10^7 counts per min per μg) or SR-ASV cDNA (5×10^6 counts per min per μg) was incubated with DNA (5 mg/ml) from normal or infected chicken embryo cells at a ratio of 1,000 counts per min per mg of cell DNA. (This provides a mass ratio of 2×10^7 for cell DNA:B77 cDNA. Assuming the majority of the cDNA to be copied from 10% of the viral genome, a single copy of viral DNA per cell would provide a 100-fold excess of unlabeled to labeled viral DNA.) Unique-sequence DNA from chicken embryo cells labeled with [^{14}C]thymidine ($\times$) was also present in the reaction mixtures. Samples were removed at several times during the reactions and assayed for resistance of [^3H]cDNA and [^{14}C]unique-sequence DNA to S1 nuclease. The C_0t value at which one-half of maximal annealing occurred was determined and the number of copies per haploid genome was calculated from $(C_0t_{1/2} \text{ for cDNA}) / (C_0t_{1/2} \text{ for unique-sequence DNA})$. (A) Normal chicken embryo DNA, 2.6 copies (with B77 cDNA, $\circ$). (B) B77-transformed chick cell DNA, 4 copies (with B77 cDNA, $\bullet$). (C) Another preparation of B77-transformed chick cell DNA, 20 copies (with B77 cDNA, $\bullet$). (D) Prague B ASV-transformed chick cell DNA, 18 copies (with B77 cDNA, $\blacktriangle$). (E) SR-ASV transformed chick cells, 6 copies (with B77 cDNA, $\bullet$) or 10 copies (with SR-ASV cDNA, $\circ$).

cDNA annealed more rapidly with DNAs from a variety of ASV-infected cells than with DNA from uninfected cells (Fig. 4B-E). As expected from results presented above (e.g., Fig. 1), the extent of the reaction was also greater. There was considerable variation in copy numbers determined with DNAs from these infected cells; the numbers ranged from 4 to 20 per haploid genome. The copy numbers were independent of the ratio of cell DNA to cDNA used (although this affected the extent of annealing [Fig. 1]). The possibility that cDNA was hybridized to residual fragments of virus-specific RNA in the DNA from these virus-producing cells was excluded by treatment of portions of several DNA preparations (e.g., panels B and D) with 0.3 M NaOH at 37 C for 18 h, without effect upon the kinetics of cDNA annealing. Similar copy numbers for viral sequences in SR-ASV transformed cells were determined with B77 and SR-ASV cDNA (panel E); the lower specific activity of the SR-ASV cDNA reduced the ratio of cell DNA to cDNA, and the extent, but not the rate, of annealing. The number of copies of viral sequences measured in normal chicken embryos with this technique (about five) is more than the number (less than one) obtained by measuring the effect of chick DNA upon the

reassociation kinetics upon srDNA (Fig. 2 and 3). Likewise, the number of copies in ASV-transformed chick cells was two- to fivefold higher when measured by the rate of cDNA hybridization. Some reasons for these differences are considered below.

DISCUSSION

Several features of DNA-DNA hybridization have promoted its widespread use for detection of RNA tumor virus-specific DNA in cells: the availability of highly labeled virus-specific DNA synthesized *in vitro* by virion-associated RNA-directed DNA polymerases; the favorable rate of DNA-DNA, as opposed to RNA-DNA, duplex formation (4, 13); and the relative stability of DNA, compared with RNA, as a hybridization reagent. A major drawback to the use of DNA-DNA hybridization in this context is posed by the failure of the viral DNA polymerases to synthesize single- or double-stranded products which are uniform copies of the entire viral genome (9-11, 25, 29). Nevertheless, polymerase products have been used widely as reagents for measuring virus-specific DNA in cells (7, 8, 10, 17, 20, 26, 30), and several approaches are illustrated in this report.

(i) Measurement of the effect of unlabeled cell DNA upon the reassociation kinetics of double-stranded polymerase product (Fig. 2 and 3) (Varmus et al., *Comparative Leukemia Research*, in press; 8, 10, 11, 26-30) permits economical quantitation of viral DNA in cells, but it is limited in its usefulness by the limited representation of the genome in the hybridization probes (25). In addition, the absolute values for copy numbers are uncertain, since the calculations are based upon indirect measurements of the concentration of the labeled DNA and its molecular complexity. The molecular complexity of these reagents poses a particularly vexing problem. As estimated from its reassociation kinetics (6), the srDNA synthesized by ASV polymerase has a complexity of 6×10^6 daltons of double-stranded DNA (25, 28); in addition, about 30% of viral 70S RNA can hybridize to this fraction (5). These facts are consistent if the genome is composed of non-identical subunits of about 3.5×10^6 daltons (5). If, however, the genome proves to be polyploid (32), the complexity measurement by reassociation kinetics would appear to be a threefold overestimate. In that case, copy numbers would be threefold higher than we have calculated here, bringing the numbers from experiments in Fig. 2 and 4 into closer harmony.

(ii) Some of the uncertainties of copy number determination can be circumvented by measuring the rate of cDNA annealing to cell DNA in relation to the rate of reassociation of unique-sequence DNA (Fig. 4) (7, 20, 22; Varmus et al., *Comparative Leukemia Research*, in press). These results are independent of the concentration and complexity of the cDNA, but relatively large quantities of cell DNA are required for adequate analysis, and differentially labeled unique-sequence DNA is necessary for each host species under study. Again, use of the cDNA synthesized in vitro is plagued by its heterogeneity with respect to size and sequence representation.

(iii) By measuring the ability of a sample of cell DNA to anneal labeled cDNA at a high C_0t value (Fig. 1), the labor involved in kinetic analysis is greatly diminished; this method is particularly well suited to detection of viral DNA in analytical procedures or in large numbers of cell cultures. In addition, the procedure can be calibrated with DNA containing a known number of viral DNA copies and used as a quantitative as well as a qualitative assay (H. E. V., unpublished data). It should be noted that since cDNA is complementary to the viral genome, it is capable of measuring only DNA of genomic polarity (i.e., plus-strand DNA).

In our view, there are considerable liabilities

associated with all of the hybridization techniques now available for study of RNA tumor virus-specific nucleotide sequences in cells. The DNA-DNA annealing procedures are hampered for the reasons cited above. The best minimal estimate of the fraction of a viral genome present in cell DNA is currently obtained by hybridization of labeled 70S RNA to a vast excess of cell DNA (Table 2) (4, 13, 14-16, 17, 21, 27). However, the unfavorable kinetics of this reaction prevent its completion; moreover, the procedure is compromised by the large quantities of cell DNA required, the long incubation periods, difficulties in labeling RNA to very high specific activity, instability of the RNA, and the relative uncertainty of copy numbers without extensive analysis. RNA-driven annealing of labeled 70S RNA to cell DNA immobilized on filters offers some logistical advantages (1, 2, 19, 22), but the results may be suspect because of the extremely small fraction of the labeled RNA which anneals. We believe that many of the problems that beset current technology can be circumvented by the purification of sufficient quantities of unintegrated viral DNA from recently infected cells (Varmus et al., Cold Spring Harbor Symp. Quant. Biol., in press). The in vivo polymerase product, which is homogeneous in size (6×10^6 daltons) (Varmus et al., Cold Spring Harbor Symp. Quant. Biol., in press) and presumably representative of the complete viral genome, should conserve the advantages of DNA-DNA annealing procedures without the problems attached to the use of viral DNA synthesized in vitro.

The results with DNA from chicken cells presented in this report are generally consistent with the experience of several investigators using RNA-driven hybridization with DNA immobilized on filters (1, 2, 19, 22), RNA-DNA hybridization in solution with vast DNA excess (14-16, 21, 27) and, more recently, DNA-DNA annealing (20). Although there are minor differences in the reported values (which may be ascribed to real differences in the cells studied or to apparent differences generated by the techniques used), some general conclusions about ASV-specific DNA in normal and infected chick cells can be drawn. Normal cells, whether or not they express endogenous avian RNA tumor virus information, contain a low number of copies of viral DNA (1, 15, 19, 20, 26, 27) with sufficient information to code for several viral functions and, in at least some (if not all) cells, for production of an infectious virus (33). This endogenous virus has not yet transformed cells in culture or caused tumors in animals, leaving unsettled the issue of whether

virus-specific oncogenic information is present in normal cells. However, its genome has considerable homology with the genomes of ASV (16; our unpublished data), and therefore ASV hybridization reagents (70S RNA or polymerase products) are capable of detecting endogenous viral genes. However, the homologies are incomplete, and this may lead to underestimates of copy number with certain annealing techniques. For example, this may account for the fraction of a copy seen in normal chick cells (Fig. 2 and 3) and for the differences between copy numbers determined in chicken cells by the techniques used in this report.

After infection of chicken cells by ASV, the number of copies of ASV-specific sequences increases by a factor of about two to four (Fig. 2 and 4) (1, 2, 14, 19, 20). This result is consistent with the considerable biological data supporting Temin's provirus hypothesis (23), and with hybridization experiments performed with DNA from unnatural hosts for ASV (1, 21, 27-30). These cells (e.g., mammalian or duck cells) normally lack any ASV-specific sequences but acquire one or several copies of ASV DNA during infection.

Infection not only added viral DNA to chick cells but also introduced sequences not found in normal cells (Table 1). Shoyab et al. (21) and Neiman et al. (16) have drawn the same conclusion from different experimental approaches. It is not yet possible to state whether the newly introduced sequences include those coding for oncogenic functions; development of hybridization reagents specific for sequences deleted in the formation of transformation-defective viruses (31) may permit an answer to this critical question, which bears directly upon the oncogene hypothesis (24). In the interim, we may conclude that the genomes of common laboratory strains of ASV are incompletely represented in the DNA of normal chick cells, and that RNA-directed DNA synthesis during infection introduces some or all of these missing sequences into the cell genome.

No readily apparent explanation for the discrepancies between this and our earlier report has yet been found; the differences could conceivably be due (i) to the sequences represented in the polymerase products used as hybridization reagents (our earlier reagents were prepared with SR-ASV rather than B77 virus and are no longer available); (ii) to minor improvements in hybridization technology (e.g., better standardization of the hydroxyapatite fractionation (28), introduction of the S1 nuclease assay, or use of mineral oil overlay or sealed capillaries to avoid volume loss during incubation); or (iii) to differences in the cell popula-

tions studied. The last possibility seems unlikely in view of the similar results seen with three pools of chicken embryos and with several preparations of ASV-transformed cells and ASV-induced tumors. It is possible that our previous detection of ASV-specific DNA in a small pool of Japanese quail embryos may reflect the presence of such sequences in some embryos, but our current results (Fig. 2 and 3; Table 2) document that at least some embryos are devoid of ASV-like DNA.

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