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Synthesis and Integration of Viral DNA in Chicken Cells at Different Times After Infection with Various Multiplicities of Avian Oncornavirus

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To see if integration of the provirus resulting from RNA tumor virus infection is limited to specific sites in the cell DNA, the variation in the number of copies of virus-specific DNA produced and integrated in chicken embryo fibroblasts after RAV-2 infection with different multiplicities has been determined at short times, long times, and several transfers after infection. The number of copies of viral DNA in cells was determined by initial hybridization kinetics of single-stranded viral complementary DNA with a moderate excess of cell DNA. The approach took into account the different sizes of cell DNA and complementary DNA in the hybridization mixture. It was found that uninfected chicken embryo fibroblasts have approximately seven copies per haploid genome of DNA sequences homologous to part of the Rous-associated virus 2 (RAV-2) genome. Infection with RAV-2 adds additional copies, and different sequences, of RAV-2-specific DNA. By 13 h postinfection, there are 3 to 10 additional copies per haploid genome. This number can not be increased by increasing the multiplicity of infection, and stays relatively constant up to 20 h postinfection, when some of the additonal viral DNA is integrated. Between 20 and 40 h postinfection, the cells accumulate up to 100 copies per haploid genome of viral DNA. Most of these are unintegrated. This number decreases with cell transfer, until cells are left with one to three copies of additional viral DNA sequences per haploid genome, of which most are integrated. The finding that viral infection causes the permanent addition of one to three copies of integrated viral DNA, despite the cells being confronted with up to 100 copies per haploid genome after infection, is consistent with a hypothesis that chicken cells contain a limited number of specific integration sites for the oncornavirus genome.

When RNA tumor viruses infect cells, their genomes are transcribed to DNA (6, 31, 34, 39, 41). At least some of this DNA is integrated in the host cell genome (1, 20, 43). It is not known whether integration is restricted to a specific site or sites in the host DNA.

Whether the amount of viral DNA (vDNA) which is produced and finally integrated is different after high- or low-multiplicity infections, or at short or long times after infection, was an unanswered question. If cells contain a limited number of specific integration sites, an upper limit to the copies of vDNA integrated would be expected, regardless of the conditions of initial infection. Consequently, we decided to see if varying the multiplicity of infection (MOI) caused different amounts of vDNA to be produced and integrated at short times after infection and after passaging cells. The experimental system used was the infection of chicken cells with the leukosis virus Rous-associated virus 2 (RAV-2) (13). Due to the difficulty of maintaining transformed cells for a long period of time, a nontransforming virus was deliberately chosen.

The number of copies of vDNA was determined by liquid hybridization of a single-stranded probe of DNA complementary to virus RNA (cDNA), synthesized in a RAV-2 endogenous reaction, to cell DNA. This probe DNA measures the concentration of the strand of vDNA complementary to viral RNA. Liquid, rather than filter, hybridization was chosen since it can distinguish endogenous viral sequences found in uninfected chicken cells (17, 25, 41) from exogenous sequences, not homologous to vDNA, added after infection (25, 30, 34, 41). Knowing the large amount of cell DNA required by previous authors for liquid hybridization, and the large number of DNA samples required for this study, we used a different approach involving hybridization in a moderate, rather than a huge, excess of cell DNA. This approach is detailed in the Appendix. It

takes into account the difference in sizes of cDNA and cell DNA in the hybridization mixture, a problem often encountered due to the small size of avian cDNA (35, 37, 44).

RAV-2 RNA could have been used as a probe rather than cDNA. However, the high temperature of hybridization incubation introduces breaks in RNA. Its smaller size alters its hybridization kinetics, complicating analysis of copy number (26). This is not a problem with cDNA (see Materials and Methods).

Whether or not newly synthesized virus-specific DNA is integrated has been determined by its sedimentation pattern in alkaline sucrose gradients (1, 21). A modified version of the sedimentation technique allowed considerable technical simplification. By first lysing chicken cells with detergent, and then following with Pronase digestion and layering, enough DNA could be sedimented through one gradient to give, by subsequent hybridization, a profile of vDNA.

MATERIALS AND METHODS

Materials. Sarkosyl NL 97 was a gift from Geigy Industrial Chemicals, Ardsley, N.Y. Other sources of materials are given in Hayward and Hanafusa (16). S_1 nuclease isolation is described in the same reference. ^{3}H-labeled RAV-2 RNA (specific activity, approximately 3×10^6 counts/min per μg) was a gift from W. Hayward.

Cells and viruses. The experimental system used was the infection of chicken embryo cells (free from the expression of group-specific antigens and chicken helper factor; SPAFAS, Inc.) (15) with RAV-2 (13). Conditions of cell growth were described previously (14). High-titer RAV-2 was produced by growing cells, infected with RAV-2 in the presence of DEAE-dextran, in roller bottles with small amounts of medium (7 ml of Scherer's medium with 5% calf serum [14; called 5% medium] in Corning plastic roller bottles), and harvesting at 4-h intervals. The titer of the RAV-2 preparation used in almost all of the experiments described here was 10^9 interference inducing units (IIU)/ml for overnight infection, determined by end-point dilution. Titers of other RAV-2 preparations used were determined with a plaque assay (18).

Cells were infected in two ways. (i) Cells (4×10^6) were seeded in 90-mm plastic dishes in 2% medium (14). At 4 to 6 h after seeding, the medium was replaced with 3 ml of 2% medium containing virus and 72 μg DEAE-dextran. The dishes were then placed in an airtight chamber containing air with 5% CO_2, and rocked for 2 h at 37 C. The medium was replaced with 15 ml of 5% medium and the plates were incubated conventionally. (ii) Cells (3×10^7) were seeded in Corning plastic roller bottles in 35 ml of 2% medium. At 4 to 6 h later, the medium was replaced with 7 ml of 2% medium containing virus and 530 μg of DEAE-dextran. The bottles were rolled at 37 C for 2 h and the medium was replaced with 35 ml of 5% medium.

Infection of cells in roller bottles was as efficient as infection on plates, as determined by infective center assay with RSV (RAV-2) (Bryan strain of Rous sarcoma virus produced with RAV-2 as helper) (14). The 2-h infections used gave one-half the number of plaques in a plaque assay (18) as overnight infection. Therefore, the effective titer of a virus stock with 10^9 IIU/ml for overnight infection was 5×10^8 IIU/ml for 2 h of infection.

Size analysis of DNA by alkaline sedimentation. The sizes of single strands of DNA were measured by their sedimentation in 5 (5% sucrose [wt/vol], 0.9 M NaCl, 0.1 N NaOH, 0.003 M EDTA) to 18% (18% sucrose [wt/vol], 0.85 M NaCl, 0.15 N NaOH, 0.003 M EDTA) isokinetic alkaline sucrose gradients (19). On top of those gradients was a 0.2-ml layer of 0.45 N NaOH, 0.55 M NaCl, and 0.01 M EDTA to insure denaturation of the DNA layered. Bacteriophage f-1 DNA, which had been labeled by growth in the presence of deoxyadenosine and [^{3}H]thymidine, was used as a standard and sedimented at 18.7*S*. Had this not been a single-strand circle, it would have sedimented at 16.6*S* (9, 33). Using this as the sedimentation value for DNA of 1.8×10^6 daltons (23), molecular weights of the DNAs were calculated from their *S* values and the Studier relationship with $\alpha = 0.4$ (33).

Extraction and cleavage of cell DNA and chicken embryo DNA. Fibroblasts were collected by trypsinization. Embryos were fragmented with a tissue grinder after suspension in 6 ml of 0.1 M EDTA, 0.1 M NaCl, and 0.05 M Tris (pH 8.0). DNA was extracted by a modification of the Marmur procedure (22). RNA was digested with pancreatic RNase, and overnight shaking with chloroform was used for deproteinization. DNA was collected by ethanol precipitation.

DNA was cleaved by depurination caused by 70 C incubation at pH 4.2, followed by alkali denaturation (20). To DNA solutions, 1/19 volume of 2 M sodium acetate (pH 4.2) was added, and the mixtures were incubated for 47 min at 70 C. Then 1/14 volume of 3 N NaOH was added, and the solution was incubated for 20 min at 100 C. This treatment yielded fragments of DNA having a sedimentation profile with a peak fraction at 48,000 daltons (single-strand size).

Virus purification. The procedure for virus purification was described previously (16).

Isolation of viral RNA. The procedure for isolation of viral RNA was essentially the same as in reference 16.

Isolation of cDNA from an endogenous reaction. A 0.5-ml volume of virus from the final sucrose step gradient used in virus isolation was one part of a 3.1-ml reaction mixture which contained dATP, dGTP, and dCTP (each at 0.5 mM), [^{3}H]TTP (0.01 mM) (54 Ci/mmol, which should yield cDNA with a specific activity of 2.2×10^7 counts/min, at 25% counting efficiency), 150 μg of actinomycin D per ml, 50 mM $MgCl_2$, 0.2 M Tris (pH 8), 10 mM dithiothreitol, and 0.02% Nonidet P-40. The mixture was incubated at 37 C for 3.5 h. EDTA was added to 5 mM, Pronase to 200 μg/ml, and sodium dodecyl sulfate (SDS) to 0.1%, and the mixture was incubated for 30 min at 45 C. NaCl was added to 0.1 M and carrier yeast 4*S* RNA was added to 20 μg/ml, followed by an equal

volume of chloroform-isoamyl alcohol (24:1). The mixture was shaken for 15 min and the aqueous phase was precipitated with ethanol. The precipitate was pelleted and suspended in 0.2 ml of 0.45 N NaOH, 0.55 M NaCl, 10 mM EDTA (pH 13.3). This was layered onto a 5 to 18% (wt/vol) alkaline sucrose gradient (described above). Between 0.5 and 1.0 h after the pellet had been dissolved, centrifugation was begun for 19 h at 40,000 rpm, 20 C, in a Beckman SW50.1 rotor. Under these conditions of pH, any pieces of RNA should have been hydrolyzed to under 10 nucleotides (5). The gradient was fractionated and the resulting profile (Fig. 1A) showed that 26% of the total counts sedimented at molecular weights greater than 47,000. Most of the remaining DNA sedimented in a single band with a peak fraction molecular weight of 16,000. The high-molecular-weight DNA (≥47,000) was pooled and incubated at 100 C for 20 min to insure complete hydrolysis of any RNA present. (This rather harsh procedure should not produce DNA strands with sedimentation profiles having peak fractions smaller than molecular weight 48,000; see above.) It was then neutralized with HCl, diluted to 0.2 M Na^+, and ethanol precipitated with yeast 4S carrier RNA.

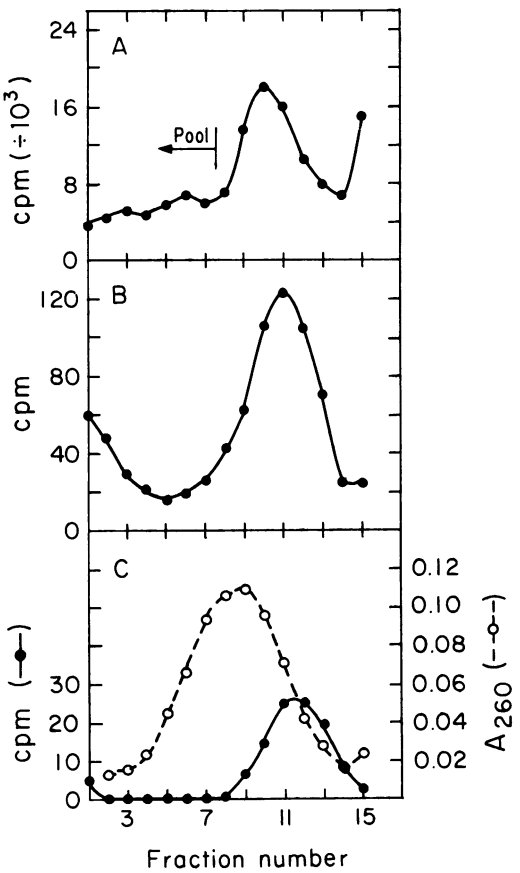

FIG. 1. *Sedimentation of [³H]cDNA in alkaline sucrose. Sedimentation is from right to left. (A) Size of cDNA after synthesis. Spun for 19 h at 40,000 rpm in a Beckman SW50.1 rotor at 20 C. The pooled region is molecular weights greater than 47,000. (B) Size of cDNA after purification. Spun for 15.3 h at 50,000 rpm in an SW65 rotor at 20 C. (C) Size of cDNA (●) and cell DNA (○) after preparation of hybrid mixture. Material in this gradient was from 2 μl of a hybridization mixture which had 5 mg of DNA per ml and 12 counts/min of cDNA per μg of cell DNA. Spun for 17 h at 40,000 rpm in an SW50.1 rotor at 20 C.*

To judge if the DNA was a single- or double-strand product of the endogenous reaction, the pellet was dissolved and incubated under conditions which would have allowed renaturation (0.6 M NaCl, 0.02 M Tris, 10 mM EDTA, 0.05% SDS, pH 7.3, 10^6 counts/min per ml [0.05 μg/ml], 68 C for 18 h; reference 42). A portion of the DNA was mixed with S_1 buffer (see below) and heat-denatured salmon sperm DNA (40 μg/ml). S_1 nuclease made 96 to 97% of the counts trichloroacetic acid soluble, indicating that there was very little self-annealing and, consequently, complementary DNA.

The cDNA was ethanol precipitated, pelleted, and then hybridized with RAV-2 RNA at an RNA:DNA weight ratio of 4.4:1 in an attempt to eliminate viral genome sequences excessively represented. The 0.35-ml hybridization mixture had 50% (vol/vol) formamide, 3 × SSC (standard saline citrate: 0.15 M NaCl, 0.015 M sodium citrate), 1 mM EDTA, 0.05% SDS, 2 μg of RAV-2 RNA per ml, and 450 μg of yeast 4S RNA per ml. It was incubated at 50 C for 65 h. After this incubation, 73% of the DNA was resistant to S_1 nuclease digestion, as opposed to 3 to 4% prior to incubation.

Hybridized and unhybridized DNA was separated by hydroxyapatite chromatography. DNA eluting in the position of duplex nucleic acid had 85% of the total counts. Phosphate was removed from this material by chromatography with Sephadex G-50. After collection, NaOH was added to 0.1 N and the DNA was incubated for 20 min at 100 C to hydrolyze viral RNA. The solution was then neutralized and yeast 4S RNA was added to 20 μg/ml. The DNA was ethanol precipitated and pelleted by centrifugation for 1 h at 75,000 × *g*.

A portion of the cDNA preparation was sedimented in alkaline sucrose. Surprisingly, the cDNA banded in two distinct regions (Fig. 1B). Approximately 70% of the cDNA was smaller than 47,000 daltons, with a peak at 16,000, and the remaining 30% had a range of sizes greater than 47,000. cDNA with such a heterogeneous size distribution could not be used as a hybridization probe since it would not have a unique hybridization rate constant (see Appendix). Depurination was used to selectively fragment large cDNA. This was conveniently achieved during preparation of the hybridization mixtures by mixing cDNA with cell DNA so that both were depurinated simultaneously. The cDNA sedimentation profile after this treatment, and during hybridization incubation, was a single peak with a maximum at 11,000 daltons (Fig. 1C; molecular weight of 11,000 is an average of four sedimentations). Eighty percent of the final cDNA preparation could be protected from S_1 nuclease digestion by hybridization with RAV-2 RNA.

[³H]cDNA from RAV-0 (provided by W. Hayward,

this laboratory), had the same size as RAV-2 cDNA after depurination. (RAV-0 is virus produced by uninfected chicken embryo fibroblasts from line 100 chicken [see reference 17].)

Hybrid preparation and analysis. After depurination and alkali treatment, the mixture of cDNA and cell DNA was concentrated by ethanol precipitation. Hybridization mixtures contained 0.6 M NaCl, 0.02 M Tris, 10 mM EDTA, 0.05% SDS, and DNA at a concentration in the range of 1.5 to 10 mg/ml, and were pH 7.4. Each mixture was divided into 7 to 12 samples which were sealed in capillary tubes and had volumes in the range of 3 to 15 μl. Each sample had 200 to 400 counts/min of cDNA. The capillaries were incubated at 68 C. Under these conditions, single-strand breaks were introduced in DNA at the rate of 0.03/10,000 daltons per 100 h (unpublished results), a rate too small to change the size of the reactants enough to alter hybridization kinetics (see Appendix).

Both the amount of probe DNA hybridized and the extent of cell DNA renaturation were measured for each capillary. The first step was to expel each sample from its capillary in nine times its volume of 0.3 M NaCl, and heat it at 60 C for 15 min or one-fourth the hybrid incubation time, whichever was shorter, to insure that the sometimes viscous hybrid solutions were dissolved. A 5-μl volume was removed and saved for cell DNA renaturation analysis. The remainder was mixed with 9 volumes of 25 mM potassium acetate, 0.3 M NaCl, 5 mM $ZnSO_4$, pH 4.4 ("S_1 buffer"). This was split and one-half was digested with S_1 nuclease for 1.5 h at 37 C. Both halves were then precipitated with trichloroacetic acid, collected on Whatman GF/C filters, and then counted. Counts were corrected for differential quench, measured by reconstruction experiments, caused by the different amounts of DNA on each filter.

A 0.165-ml volume of a solution of 1.15× SSC containing 65% formamide was aded to the 5-μl portion saved previously. In this solution, with final concentration of 1.18× SSC and 63% formamide, native cellular DNA, fractionated to 2×10^5 daltons by sonic oscillation, had a melting temperature of 47 C, as assayed by the increase in optical density at 270 nm as the temperature of the DNA solution was increased. Melting occurred in the temperature range of 30 to 58 C. Absorbance measurement could not be made at 260 nm because of the strong absorbance of formamide at that wavelength (4). For native DNA, the expression $(A_{58\ C} - A_{30\ C})/A_{58\ C}$ was equal to 0.255. The fraction of DNA renatured in hybrid samples was equal to the same expression divided by 0.255.

Hybrid C_0t values (concentration of DNA in moles of nucleotide per liter) were not corrected to standard salt conditions.

Hybridization of ^{3}H-labeled chicken DNA with unlabeled chicken DNA. Chicken embryo fibroblast DNA was labeled by including [^{3}H]thymidine, 0.5 μg/ml, and 0.16 μCi/ml, in the 5% medium. The ^{3}H-labeled chicken DNA was depurinated for different lengths of time and then mixed with unlabeled chicken DNA depurinated for 33 to 47 min.

Alkaline sucrose gradient analysis of integration. From 1.3×10^7 to 5×10^7 cells were collected by trypsinization, pelleted, and suspended in 3 ml of 0.1 M NaCl, 20 mM EDTA, 10 mM Tris (pH 7.5). The cells were again pelleted and resuspended in 0.7 ml of the above solution, and lysed with 0.08 ml of 10% (wt/wt) Sarkosyl. Predigested Pronase was added to 100 μg/ml (16 μl of 5 mg/ml), and the solution was incubated at 37 C for 1 h.

A 54-ml alkaline sucrose gradient was prepared in tubes for a Beckman SW25.2 rotor. The component solutions were: (i) 5% (wt/vol) sucrose, 0.003 M EDTA, 0.9 M NaCl, and 0.1 N NaOH (pH 12.2) and (ii) 26% (wt/vol) sucrose, 0.003 M EDTA, 0.8 M NaCl, and 0.2 N NaOH (pH 11.8). Calculations showed this should be an isokinetic gradient in an SW25.2 rotor. The gradients were overlaid with 3 ml of 0.01 M EDTA, 0.55 M NaCl, and 0.45 N NaOH (pH 13.3).

The cell lysate was layered on top of this gradient (the gentlest method of layering was to slowly pour this extremely viscous solution onto the gradient), and centrifugation at 20 C for 2.5 h at 25,000 rpm was begun approximately 1 h after layering.

The gradient was fractionated from the top, by pumping heavy sucrose in the bottom, into 12 to 24 fractions. To each was added 0.12 volume of 2 M sodium acetate (pH 4.2), bringing the pH to the range of 4 to 6. An equal volume of chloroform-isoamyl alcohol (24:1) was added to each tube. The fractions were mixed with a Vortex for several seconds and allowed to stand at room temperature for several hours. Those fractions which had substantial material at the interface had their aqueous phases extracted again with chloroform-isoamyl alcohol.

Each aqueous phase was precipitated with ethanol and pelleted at 9,000 × g for 40 min. Supernatants were decanted and the pellets were dried and then dissolved in 1 ml of 0.1× SSC containing 1 mM EDTA. cDNA was added such that the quantity [(cells layered/number of fractions)/counts per minute of cDNA] was equal to 8×10^3, unless otherwise noted. At that time 0.05 volume of 2 M sodium acetate (pH 4.2) was added, followed by 300 μg (0.1 ml) of salmon sperm DNA as carrier. The fractions were incubated at 70 C for 47 min. Then 0.25 volume of 1 N NaOH was added, followed by incubation at 100 C for 20 min. This step cleaved any cell DNA present in the fractions to the standard size for hybridization.

The fractions were neutralized with 0.17 vol of 1.5 M sodium acetate (pH 4.2) and precipitated with ethanol. They were then spun for 30 min at 1,800 × g, the supernatants were decanted, and the pellets were dried.

A 30-μl volume of a solution of 0.6 M NaCl, 0.02 M Tris, 10 mM EDTA, 0.05% SDS (pH 7.4) was added to each fraction. Incubation at 100 C followed to dissolve the pellets, and the solutions were placed in 50-μl capillaries. These were sealed and heated at 100 C for 2 min, and then incubated at 68 C for an amount of time such that the quantity [(cells layered/number of fractions) × (time in hours)] equaled 2.8×10^8, thereby achieving the same C_0t values in all cases.

Each sample was expelled in 0.7 ml of 25 mM

potassium acetate, 0.3 M NaCl, 5 mM $ZnSO_4$ (pH 4.4). S_1 nuclease analysis of the amount of cDNA hybridized followed as previously described.

RESULTS

DNA from uninfected chicken cells has sequences homologous to the RAV-2 genome (17). Consequently, before proceeding with hybridization experiments with DNA from infected cells, it was necessary to determine, with DNA from uninfected cells, the hybridization kinetics of cDNA and the maximum amount of cDNA which could be hybridized. These results are shown in Fig. 2A, using DNA isolated from chicken embryos or from chicken embryo fibroblasts which had been passaged several times. The data are plotted using the conventional log C_0t abscissa, where C_0 is the DNA concentration in moles per liter, and t is time in seconds (7). Values of C_0t have been normalized in this and all succeeding plots of this type, on the basis of the independently determined $C_0t_{1/2}$ for renaturation of unique sequence cell DNA, as described in the Appendix. The difference in the two curves shown here can not be considered significant. The small maximum hybridization, 15%, was of considerable interest since approximately 70 to 80% of the sequences in the RAV-2 genome are present in the RAV-0 genome, which is completely or nearly completely homologous to sequences in DNA from uninfected chicken cells (17). (RAV-0 is produced by uninfected line 100 chicken cells, but not by SPAFAS cells. Consequently SPAFAS cells may not contain the complete RAV-0 genome).

There were two possible explanations for the limited amount of hybridization shown in Fig. 2A. It could have been a result of either limited homology between the cDNA and uninfected cell DNA, or a result of insufficient excess of cell DNA sequences (see Fig. 11 in Appendix, also reference 32). Less stringent hybridization conditions, allowing hybridization of partially homologous regions of DNA, might have increased the amount of hybridization, but probably by no more than 30% (17). To distinguish between these possibilities, samples were made with a varying ratio of cDNA to cell DNA sequences, and each was hybridized to a C_0t of 10,000. The results are shown in Fig. 2B. The 15% maximum is not a result of the ratio of sequences, and must be due to limited homology between the cDNA and uninfected cell DNA. cDNA's of avian RNA tumor virus are often overrepresented in sequences not homologous to uninfected cell DNA, but homologous sequences usually constitute more than 15% of the cDNA (17). Enrichment for these nonhomologous sequences might have occurred in the initial molecular weight selection of cDNA in an alkaline sucrose gradient (see Materials and Methods). Whatever the reason, the result was quite fortuitous. It meant that values of hybridization greater than 15%, in experiments with DNA from infected cells, indicated that nonhomologous viral sequences had been added.

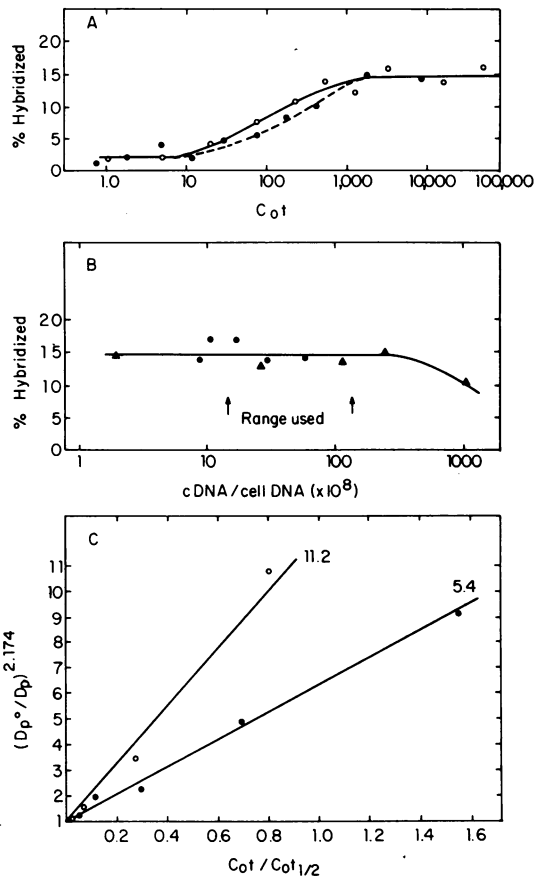

FIG. 2. *Hybridization of RAV-2 [^{3}H]cDNA to DNA from uninfected chicken cells. (A) Percent of cDNA hybridized is plotted versus DNA concentration (C_0, moles of nucleotide per liter) times time (t, in seconds). Symbols: ○, DNA from uninfected chicken cells in the fifth passage, hybridized with cDNA at a ratio of cDNA to cell DNA = 1.8×10^{-7}; ●, DNA extracted from 11-day-old chicken embryos, ratio of cDNA to cell DNA = 3.6×10^{-7}. (B) Samples with a varying ratio of cDNA to cell DNA were hybridized to a C_0t of 10,000. Different symbols indicate different experiments. (C) Copy number analysis of normalized data from panel A, plotted as described in the Appendix. D_p = concentration of unhybridized cDNA. $D_p°$ is concentration at $t = 0$. k_1/k_2 (rate constant for renaturation of cell DNA/rate constant for hybridization of cDNA) = 2.174. $C_0t_{1/2}$ is of unique sequence cell DNA. Symbols are the same as panel A. The numbers next to the lines are the initial slopes, which are equal to the number of copies per haploid genome of vDNA.*

The initial slope method for analysis of the number of copies of virus-specific sequences (Appendix) could not be used directly for a reaction in which only a portion of the cDNA could be hybridized. Consequently, the values of hybridization in Fig. 2A were normalized to 100%. Initial slope analysis of this normalized data is shown in Fig. 2C. $C_0t_{1/2}$ was evaluated from renaturation of unique sequence cell DNA, as described in the Appendix. The term D_p°/D_p is equal to [1/(1-fraction cDNA hybridized)]. There were 5 to 11 copies per haploid genome of endogenous vDNA sequences. The Appendix contains a discussion of the uncertainties in this type of analysis and of how much of the data can be used to determine the initial slope.

As an independent method of determining the number of copies of endogenous virus genes in chicken cells, RAV-0 [^{3}H]cDNA was hybridized with chicken embryo DNA. In uninfected cells, those cell DNA sequences to which RAV-2 cDNA hybridizes are also homologous to cDNA from RAV-0 (48). The results are shown in Fig. 3A. Hybridization proceeded to 35%. Since it was known that RAV-0 cDNA is almost completely homologous to chicken cell DNA (17), the limited amount of hybridization was assumed to be a result of the cell DNA being in moderate, rather than vast, excess over cDNA sequences. Figure 3B shows the initial slope analysis of this hybridization curve, and indicates five copies per haploid genome of RAV-0-specific sequences. Making the assumption that the copy numbers from Fig. 2C and 3B are different only because of the experimental uncertainties, an average of the three results gives a value of seven copies per haploid genome of endogenous vDNA in uninfected cells.

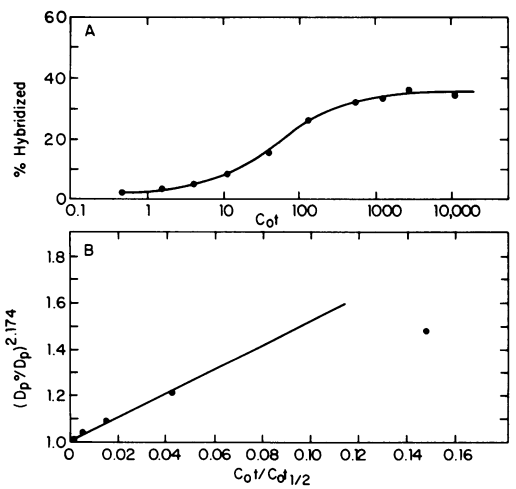

FIG. 3. *Hybridization of RAV-0 [^{3}H]cDNA to DNA extracted from 11-day-old chicken embryos. The cDNA had a sedimentation peak fraction at molecular weight 11,000. The ratio of cDNA to cell DNA was 3.4 × 10^{-7}. (A) Percent of cDNA hybridized plotted versus C_0t (moles of nucleotide per liter × incubation time in seconds). (B) Initial slope copy number analysis described in legend to Fig. 2C.*

The hybridization data to be presented give the total hybridization to endogenous vDNA present in cells prior to infection plus vDNA added as a result of infection. Two possibilities exist. (i) Infection results in the addition of viral sequences which were homologous and other sequences which were nonhomologous to endogenous vDNA. Suppose a cell had seven copies per haploid genome of endogenous vDNA. Infection resulting in the addition of two complete copies of viral DNA would have given a total of nine sequences of one portion of the viral genome and two of the other. (ii) Infection results in the addition of only those viral sequences not homologous to the endogenous vDNA.

The effect that each of these possibilities would have on the initial slope plot is shown in Fig. 4. It can be seen that, in either case, when small numbers of exogenous copies are added, the effect of the endogenous vDNA is to increase the initial slope by 0.6 to 0.7. Otherwise it does not seriously affect the analysis. The increase in slope is small, despite the presence of seven copies per haploid genome of endogenous vDNA, because only 15% of the cDNA can be hybridized by the endogenous vDNA. (For a detailed explanation, see the legend to Fig. 6.) For the additon of a large number of copies of vDNA, the change in slope resulting from endogenous vDNA is within the uncertainty of the analysis. Consequently, to calculate the number of vDNA sequences added by infection, 0.6 must be subtracted from the initial slope.

DNA was extracted from cells at various times after RAV-2 infection. RAV-2 infection was at multiplicities ranging from very low (0.025) to very high (70).

At 7 h postinfection, with MOI values of 1, 10, and 25, there was no increase in extent or kinetics of hybridization of cDNA as compared to Fig. 2A.

Results using DNA extracted from cells 13 h postinfection are shown in Fig. 5. For each curve in Fig. 5A, the maximum amount of hybridization was greater than 15%, indicating that viral sequences not homologous to endogenous vDNA have been introduced. Figure 5B shows that MOI values of 1.4, 1.5, and 14 gave initial slopes of 5.6, 7.1, and 9.4, respectively. Subtracting the correction for endogenous se-

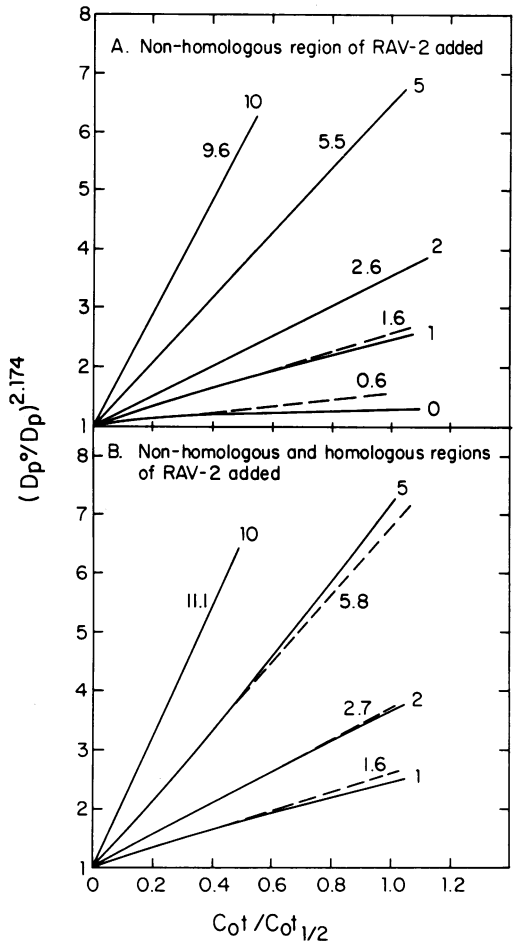

FIG. 4. *Effect of endogenous vDNA sequences on copy number analysis after RAV-2 infection. It was shown that DNA from uninfected cells can hybridize 15% of the RAV-2 cDNA (Fig. 2A). After subtracting the background level of hybridization and correcting for the observation that the maximum level of hybridization was 79% (Fig. 9), it was concluded that 17% of the cDNA was homologous to endogenous vDNA. Consequently, 83% of the cDNA was homologous to viral sequences not homologous to endogenous vDNA, which had been added after RAV-2 infection (unique exogenous sequences). Theoretical hybridization curves could be drawn for various additions of viral sequences; these curves were summations of the hybridization due to endogenous and unique exogenous sequences. The amount of hybridization from unique exogenous sequences was calculated from the explicit solution of the hybridization equation for the case of cell DNA in vast excess (see Appendix) and was multiplied by 0.83. This equation, as explained in the Appendix, describes the initial hybridization of cDNA, even when cell DNA is not in vast excess. Since the analysis of copy number depends on initial hybridization kinetics, it is proper to use this equation. The amount hybridized by endogenous sequences was taken from an average of the experimental data in Fig. 2A. To determine how much cDNA would be hybridized by endogenous vDNA if sequences homologous to endogenous vDNA were added because of infection, the data of Fig. 2A were translated along the x axis by an appropriate amount; e.g., if the number of sequences added was equal to the number in uninfected cells, the data were shifted so that C_0t values were reduced by 50%. These theoretical amounts of hybridization were plotted in the usual way for copy number analysis. The numbers at the ends of the lines are the numbers of copies of vDNA added. The numbers on the lines are the initial slopes. When a line deviates from straightness, the continuation of the initial slope is shown by a dashed line. The data from Fig. 2A were plotted in panel A (labeled "0" copies added), but it was not normalized as in Fig. 2B, and consequently it gives a line of small initial slope. In both cases described here, when a small number of copies of viral sequences was added, the endogenous sequences led to a slope which is 0.6 to 0.7 greater than the number of sequences added.*

sequences gives, respectively, 5.0, 6.5, and 8.8 copies of vDNA per haploid genome.

Figure 6 shows results for DNA extracted from cells at 42 h postinfection. Multiplicities of 0.025, 1.25, and 25 gave initial slopes of 3.6, 57, and 125, respectively.

In contrast to the results in Fig. 6, Fig. 7 shows that, after extended subculture, the number of copies of vDNA decreased to a rather small number.

Table 1 is a summary of Fig. 5, 6, and 7, and other hybridization results. Numbers in either the same row or the same column were from the same experiment.

One major question was whether vDNA introduced by infection was integrated into the cell DNA. To determine this, the sedimentation rate of vDNA from a chicken cell lysate was examined by hybridizing DNA from gradient fractions with [^{3}H]cDNA, as described in Materials and Methods. Results are shown in Fig. 8. Panel A shows the results of three gradients. One was the sedimentation of chicken cell DNA labeled with [^{3}H]thymidine. Another was the sedimentation of [^{3}H]DNA released from f-1 bacteriophage. Whole phage particles had been mixed with unlabeled chicken cells prior to making a lysate. The phage DNA sedimented in its characteristic position unaffected by the presence of chicken cell DNA. This suggested that unintegrated vDNA would sediment at a position characteristic of its molecular weight. The peak fraction of the distribution of cell DNA was at 4×10^7 daltons, calculated using the sedimentation position of f-1 DNA as a marker. Consequently, integrated viral DNA,

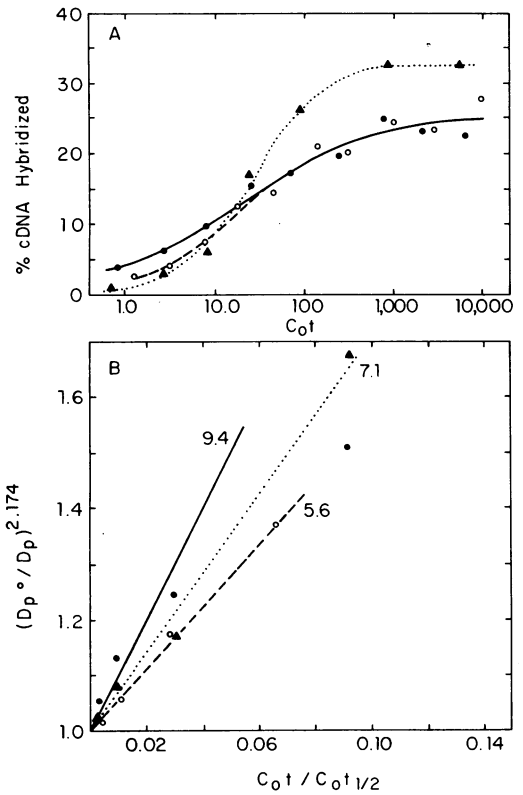

FIG. 5. *Hybridization of RAV-2 [^{3}H]cDNA to cell DNA (more precisely, to vDNA in the cell DNA) extracted at 13 h postinfection. Experiment 1: ○, MOI = 1.4; ●, MOI = 14 (because of the data scatter, the initial slope was drawn using the least-squares fit described in the Appendix). Experiment 2: ▲, MOI = 1.5. See Table 1 for cDNA to cell DNA ratio. (A) Percent of cDNA hybridized versus C_0t (moles of nucleotide per liter × incubation time in seconds). (B) Initial slope analysis as described in Fig. 2C. Copy number of vDNA added is initial slope minus 0.6, as described in Fig. 4 and in text.*

sedimenting with cell DNA, should have been well separated from unintegrated vDNA, whose single-strand molecular weight is approximately 3×10^6 or smaller (10, 11, 39), as expected from the complexity of viral RNA (2, 8, 45). One complication was that viral DNA in superhelical form sediments more quickly in alkali than its molecular weight would indicate (10, 39). However, if hybridization was due to superhelices instead of integrated vDNA, the distribution of vDNA would be quite narrow (10, 39), unlike the broad distribution of cell DNA. The position where superhelical DNA would have sedimented is indicated in the figure.

Panel A (Fig. 8) also shows the sedimentation of vDNA from lysates of uninfected chicken embryo fibroblasts. While most of the vDNA sedimented at molecular weights greater than 3×10^6, the peak of the sedimentation profile was at a molecular weight position slightly smaller than the expected 4×10^7. This could have resulted from excessive shear of DNA during handling of this particular lysate, from vDNA normally being integrated in pieces of cell DNA which are smaller than average size, or from anomalous slow sedimentation of the cell DNA in this particular gradient. Note that, as expected, the maximum amount of cDNA hybridized was close to 15%.

Panel B (Fig. 8) shows sedimentation of vDNA at 19 and 44 h after infection with a multiplicity of 2. The profile at 19 h shows that some vDNA has been integrated. It is possible that some of the apparently integrated DNA is due to superhelices. By 44 h postinfection, there was a substantial increase in the number of copies of vDNA (Table 1). The sedimentation profile at this time shows that a large portion of these new copies was unintegrated. A substantial portion of the unintegrated vDNA, at both 19 and 44 h postinfection, was smaller than 3×10^6 in molecular weight. Panel D shows this more clearly. vDNA complementary to cDNA ("plus" strand of vDNA) has sizes in the range

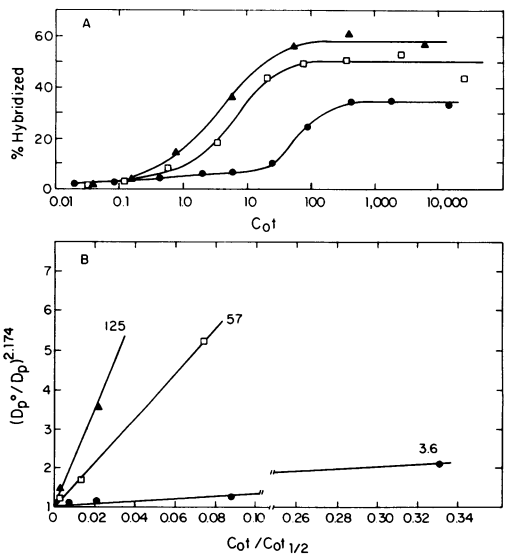

FIG. 6. *Hybridization of RAV-2 [^{3}H]cDNA to cell DNA extracted 42 h postinfection. Symbols: ●, MOI = 0.025; □, MOI = 1.25; ▲, MOI = 25. (A) Percent of cDNA hybridized versus C_0t (moles of nucleotide per liter × incubation time in seconds). (B) Initial slope analysis as described in Fig. 2C. Copy number of vDNA added is initial slope minus 0.6, as described in Fig. 4 and in text.*

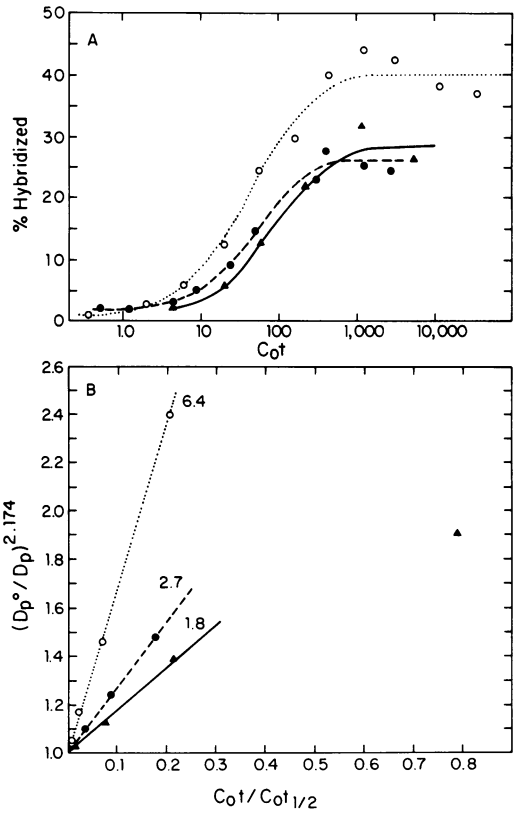

FIG. 7. *Hybridization of RAV-2 [³H]cDNA to cell DNA extracted three passages after infection. Experiment 1: ●, MOI = 0.025; ▲, MOI = 1.5. Experiment 2: ○, MOI = 70. (A) Percent of cDNA hybridized versus C_0t (moles of nucleotide per liter × incubation time in seconds). (B) Initial slope analysis as described in Fig. 2C. Copy number of vDNA added is initial slope minus 0.6, as described in Fig. 4 and in text.*

10^5 to 8×10^5 daltons. vDNA complementary to viral RNA ("minus" strand of vDNA), if it has a linear configuration, is 2.2×10^6 daltons. There was also an increase in the number of integrated copies at 44 h postinfection, as compared to 19 h.

Sedimentation of vDNA at many cell passages after infection is shown in panel C (Fig. 8). Compared to panel B, there is a large decrease in the amount of unintegrated vDNA. The fraction of vDNA integrated can be estimated by counting the number of fractions in which the amount of cDNA hybridized was greater than the amount in any fraction with molecular weight $\leq 3 \times 10^6$. Assuming that superhelices do not cause a significant amount of hybridization, these profiles indicate that at least 70% of the vDNA was integrated.

DISCUSSION

Variation in the amount of virus-specific DNA after infection of chicken fibroblasts with RAV-2 can be described as follows. Chicken cells have approximately seven copies per haploid genome of endogenous vDNA. Proviral DNA, with sequences different from endogenous vDNA, appears at 7 to 13 h postinfection. The amount of vDNA which can be introduced in a chicken cell by increasing the MOI probably has a maximum; in this paper an MOI of 1.4 resulted in five copies per haploid genome, whereas an MOI of 14 resulted in less than twice that number. The number of copies stays relatively constant up to 20 h postinfection. At this time, after an infection at a multiplicity of 2, some of the vDNA is integrated. Between 20 and 44 h postinfection, there is a burst of vDNA synthesis, with cells accumulating over 100 copies per haploid genome if the initial MOI was large enough to insure that every cell was infected. A large portion of this increase is unintegrated vDNA with a complete or nearly complete "minus" strand and a segmented "plus" strand. There is also a substantial increase of integrated vDNA, up to an amount at least equal to what is integrated after extended cell passage.

After the cells are subcultured once, there is a substantial decrease in the number of copies of vDNA in those cells infected at an MOI >1. There is a large increase in copies in cells infected at an MOI of 0.025. Presumably, virus spread was not completed in these cells until they were subcultured, when many underwent infection for the first time. Consequently, the amount of vDNA after subculture is similar to that in cells infected with an MOI >1 at 40 h after infection.

With continued cell transfer, the number of copies of vDNA decreases, regardless of the initial MOI. The results for the CK_5 and CK_7 cells (chicken cells in the 5th and 7th passages, respectively) show from one to six copies per haploid genome. To reach this level of copies, the cells must be extensively transferred. The range of numbers of copies in CK_5 cells (Table 1) may indicate that three transfers after infection is marginal for reaching a final constant number of copies. A reasonable hypothesis is that cells, regardless of the initial MOI, end up with one to three additional copies of vDNA per haploid genome as a result of RAV-2 infection. However, until copy number is analyzed more extensively in CK_7 and later passages, the final number of copies can only be restricted to one to six. The sedimentation profile of vDNA from extensively subcultured cells is similar to that

TABLE 1. *Number of copies of vDNA added to chicken embryo fibroblasts by RAV-2 infection*[a]

MOI	vDNA copies added by RAV-2 infection (no.)							
	13 h[b]	17.5 h[b]	20.5 h[b]	37 h[b]	42–44 h[b]	CK_3[c]	CK_5[c]	CK_7[c]
0.025					3.0 *8.9*	62 (10) *5.4*	5.8 (35) *2.0* 2.1 (27) *6.4*	
1.25 to 2.5	5.0[e] *4.5* 6.5 *8.6*	3.0 *7.5*	11 *11*	45 *13*	57 *3.3* 45 *8.8*	28 *8.6* 19[e] (17) *1.4*	1.2 *7.5*	1.5[d] (20) *(11)* 1.4[d] (30) *2.5*
14	8.8[f] *4.7*							
25					125 *3.4*	11 *3.0*	3.9 *3.4*	
70							5.8 *1.8*	

[a] Numbers shown here are slopes from copy number analysis corrected for endogenous vDNA by subtracting 0.6. Numbers in either the same row or column are from the same experiment. Numbers in parentheses give the total number of days from infection to cell lysis for DNA extraction. Numbers in italics are the weight ratios of cDNA to cell DNA, multiplied by 10^7.

[b] Hours postinfection.

[c] Chicken cells in the 3rd (CK_3), 5th (CK_5), and 7th (CK_7) passages.

[d] MOI = 1.

[e] MOI = approximately 0.25.

[f] MOI = 1.4 and 14.

of total chicken cell DNA, and indicates that at least 70% of the vDNA is integrated.

The copy numbers reported here must be qualified by the realization that a region of the viral DNA repeated a different number of times, but underrepresented in the cDNA, would not be detected.

The observation that chicken cells acquire a small amount of permanent copies of integrated viral genetic material as a result of RAV-2 infection, regardless of the initial MOI and despite their being confronted with up to 125 copies per haploid genome after infection, is consistent with a hypothesis that vDNA must be integrated to be maintained by a cell, and that the number of integration sites is limited. This conclusion assumes that the 125 copies per haploid genome can reach the nucleus.

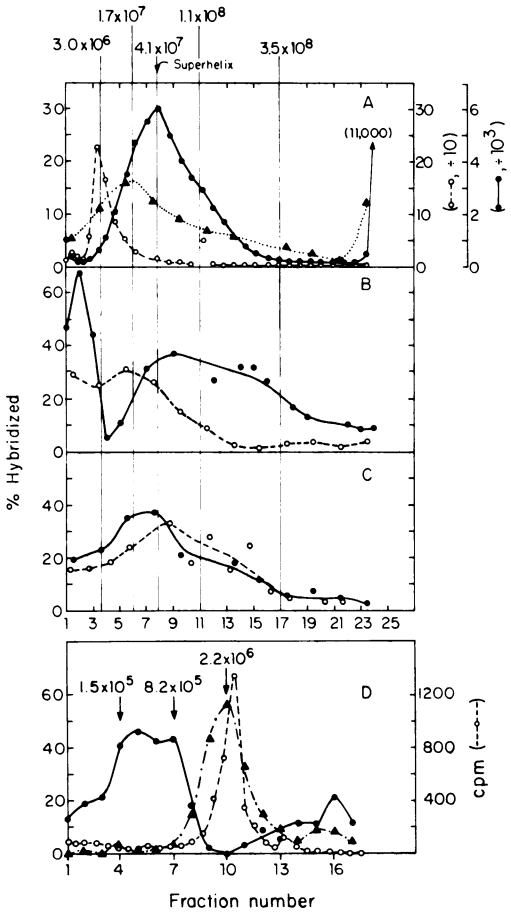

FIG. 8. *Sedimentation of vDNA, detected by hybridization with [³H]RAV-2 cDNA or [³H]RAV-2 RNA, in infected cells. The numbers at the top give single-strand DNA molecular weights calculated from the position of the f-1 marker DNA. "Superhelix" indicates the position to which superhelices of double-strand molecular weight 6 × 10⁶ would sediment (calculated from reference 39). (A) ³H-labeled DNA from 5 × 10⁷ chicken embryo fibroblasts, total fraction counted in toluene + Triton X-100 scintillation fluid, ordinate is counts per minute (●); lysate of f-1 phage with ³H-labeled DNA plus 5 × 10⁷ chicken embryo fibroblasts, counting same as in preceding description (○); sedimentation of vDNA from uninfected chicken embryo fibroblasts (▲). Fractions hybridized with [³H]cDNA. (B) Cells infected with MOI = 2 and lysed 19 h postinfection (○); cells infected with MOI = 2 and lysed 44 h postinfection (●). In both cases fractions hybridized with [³H]cDNA. (C) Cells infected with MOI = 0.25 and lysed three passages (14 days) postinfection (○); cells infected with MOI = 0.25 and lysed four passages (33 days) postinfection (●). In both cases fractions hybridized with [³H]cDNA. (D) Two gradients were run, each with 5 × 10⁷ cells, 44 h after MOI = 3.5. Parallel fractions were pooled. Fractions were purified, depurinated, and precipitated as described. They were then dissolved in 2 ml of 0.1 × SSC + 1 mM EDTA and were split. To one-half was added 1,000 counts/min of [³H]cDNA, to the other was added 1,000 counts/min of [³H]vRNA. For the [³H]cDNA, the rest of the procedure followed the text. The fractions with [³H]RAV-2 RNA were ethanol precipitated and the pellets were dissolved in 30 μl of 3× SSC, 50% formamide, and incubated at 50 C for 110 h. The fractions were diluted in 0.7 ml of 2 × SSC and split, and one-half was treated with pancreatic RNase A + RNase T_1. They were then precipitated with trichloroacetic acid and counted. A parallel gradient of f-1 phage with [³H]DNA, lysed in the same way as the cells, was also run. Symbols: ●, hybridized with [³H]cDNA; ▲, hybridized with [³H]RAV-2 RNA; ○, f-1 DNA, fractions counted in toluene + Triton X-100 scintillation fluid. Numbers on plots give the molecular weights of linear DNA molecules which sediment to these positions.*

This is the most extensive study to date of the effect of MOI and time after infection on the amount of integrated and unintegrated vDNA. Previous work with avian cells and viruses pertinent to this report can be summarized as follows. Using liquid hybridization with labeled viral RNA, Neiman et al. (25) and Wright and Neiman (48) found that uninfected chicken cells have one to four copies per cell of the RAV-0 endogenous chicken virus genome. DNA from tumors induced by the Prague strain of RSV and bursal lymphomas induced with field isolates of RAV have, besides new genetic sequences, approximately one additional copy of viral genes per cell.

Using filter hybridization with ³H-labeled avian myeloblastosis virus RNA, Shoyab et al. (30) found 10 copies of vDNA (of 3 × 10⁶-daltons single-strand size) per cell in uninfected chicken embryos. Avian myeloblastosis virus-induced leukemic myeloblasts have two to three times the number of copies of viral genes, besides having different genetic sequences (30, 31). With filter hybridization, Ali and Baluda (1) followed the time course of appearance of vDNA after Prague strain RSV infection of chicken embryo fibroblasts at a multiplicity of 4. vDNA in Hirt supernatants (putative unintegrated DNA; see also reference 20) reached a maximum at 36 to 72 h after infection, whereas vDNA in Hirt pellets (putative integrated DNA) at 50 h after infection was twice that in uninfected cells. At 36 to 72 h postinfection, there was approximately two to three times the amount of vDNA in the Hirt supernatant plus pellet as was found in leukemic myeloblasts, which had 20 copies of vDNA per cell (assuming a single-strand size of 3 × 10⁶ daltons). At 60 h postinfection, vDNA sedimented in alkali in the regions 18 to 28S and 80 to 122S, consistent

with the idea that some of the vDNA had been integrated and some remained free.

Using liquid hybridization with a B77 single-stranded DNA probe, Varmus et al. (41) found that uninfected chicken cells have 2.6 copies of viral sequences per haploid genome, a number which is uncorrected for the alteration of hybridization rate due to the small size of probe cDNA compared to cell DNA in the hybridization mixture (see Appendix and reference 46). Cells transformed by B77, Prague, and Schmidt-Ruppin RSV had 4 to 20 copies per haploid genome. The results presented by us suggest that these numbers would have decreased to one small number if the cells were passaged further. At 8 h postinfection, Varmus et al. (40) found in duck cells, which do not have endogenous viral sequences, an amount of vDNA produced proportional to MOI in the range of 0.4 to 8, which gave 0.14 to 2.7 copies per cell. The number of copies present after infection with B77 or Prague RSV increased with time to a maximum of six per cell (38), which the network assay (43) indicated were all integrated by 38 to 48 h postinfection (12, 38).

Schincariol and Joklik (28) claimed that, by 96 h after infection with Prague RSV, the number of viral genomes increased from one per haploid genome in uninfected cells to three. They did not see any additional different viral sequences resulting from the infection, or a large transient increase of vDNA.

Using filter hybridization, Rosenthal et al. (27) found that DNA from chicken cells infected with RSV(RAV-1), Schmidt-Ruppin RSV, or RAV-2 hybridized approximately twice as much labeled RNA from RSV(RAV-1), Schmidt-Ruppin RSV, RAV-1, and RAV-60, as did DNA from uninfected cells.

Direct comparison of the results reported here with those of previous authors is difficult, since different virus strains were used and many of the past results are with DNA extracted from tumors. In spite of this, there are more similarities than differences, excluding the work of Schincariol and Joklik (28), who did not report the addition of new genetic sequences after infection. Estimates of the number of sequences of endogenous vDNA per cell haploid genome vary from one to seven; estimates of the number of copies added after infection and extended cell passage or neoplastic growth vary from 1 to 15. There were no reports of anything other than small numbers such as these. The observation in this paper of a transient accumulation of large amounts of vDNA, part of which is integrated, is supported by Ali and Baluda (1) but not by Schincariol and Joklik (28).

There are three possible explanations of the large increase of vDNA at 40 h postinfection observed here. The DNA must come from either a new round of reverse transcription, synthesis of vDNA from a previously synthesized vDNA template, or secondary infection. Considering secondary infection, it is known that chicken cells infected with RAV-2 become refractory to infection with subgroup B virus at approximately the same time as the progeny virus production reaches its maximum level. Since the maximum virus production occurs at about 40 h, secondary infection could conceivably occur before this time and cause the increase seen here. There is one puzzling aspect of a secondary infection interpretation. The data in Table 1, for 13 h postinfection and MOI values of 1.4 and 14, probably indicate that there is a limit to the amount of vDNA which can be introduced in a cell during the initial infection. How, then, is it possible to introduce a large number of copies by secondary infection? A speculative answer might be that there are a limited number of virus receptor sites on the cell surface, and that a large percentage of a RAV-2 virus stock are defective particles, not capable of producing vDNA. Consequently, the amount of vDNA produced is small. Secondary infection might involve freshly produced virus particles, with few defectives, and would lead to the production of a large amount of vDNA.

If a new round of reverse transcription causes the increase in vDNA, perhaps using viral RNA transcribed from integrated vDNA, it would have to be regulated in some way to occur only between 20 and 40 h postinfection. This seems unlikely. Consequently, of the three hypotheses, we favor secondary infection or specific replication of vDNA. At this time both seem reasonable.

If only one copy of vDNA was permanently added to a cell after infection, it would be expected that the cell could express genetic information from only one virus. Temin (36) showed that chicken cells can express the information of two types of RSV. It was also shown (Kawai and Hanafusa, unpublished data) that chicken cells infected with a Schmidt-Ruppin RSV double mutant [ts-68(−)], which is temperature sensitive for transformation and defective for glycoprotein synthesis, and when held at the restrictive temperature, can be transformed by superinfection with wild-type Schmidt-Ruppin RSV. Chicken cells infected with three subgroups of avian leukosis virus become resistant to further infection with all three subgroups (unpublished observation, this laboratory). Also, the usual way to produce RSV(RAV) is to superinfect RSV-transformed cells with

RAV. RSV(RAV) and RAV are then both produced. These results would indicate there must be at least two to three copies of vDNA per cell or, if haploid genome segregation had occurred because of cell division, of vDNA per haploid genome.

In each of these cases all of the putative integration site(s) would have been occupied by the first virus before infection with the second. Therefore, the excision and replacement of proviral DNA, or the addition of viral DNA in tandem at the same site, may well be involved in infection of cells by multiple viruses.

APPENDIX

Analysis, by liquid hybridization with a cDNA probe, of the number of virus-specific gene sequences in chicken cells has had two problems.

The first was the small size of the cDNA probe. This caused the rate constant, k_2, for hybridization of cDNA to cell DNA to be smaller than the rate constant, k_1, for cell DNA renaturation (46). An obvious solution was to break the cell DNA until it was the same size as cDNA. However, this led to the additional problem that cell DNA with a sedimentation profile peak fraction smaller than 35,000 (single-strand molecular weight) did not renature with simple second-order kinetics (unpublished observation).

Wetmur (46) found that the hybridization rate constant was proportional to the square root of the size of the reacting DNA strands. If the strands were of unequal size, the smaller one determined the reaction rate. He predicted that, for very small DNA, k_2 might be proportional to the first power instead of the square root of the molecular weight. Since the size of the cDNA used in this study was smaller than the DNA used by Wetmur, it was necessary to measure its hybridization rate constant. To do this a model was used. This consisted of ^{3}H-labeled chicken cell DNA of varying size hybridized to unlabeled chicken cell DNA in excess which had been fractionated to give a sedimentation profile with peak fraction from 35,000 to 58,000 (single-strand molecular weight). This hybridization should be described by the following equations: $dD_1/dt = -k_1D_1^2 - k_2D_1D_2$; and $dD_2/dt = -k_2D_2^2 - k_2D_1D_2$; where D_1 is the concentration of unlabeled chicken DNA and D_2 is the concentration of labeled chicken DNA.

The ratio of $D_1°$ (initial concentration of D_1) to $D_2°$ was 60, a ratio high enough to insure that, at least initially, almost all of D_2 hybridized with D_1 did not renature. This was determined by solving the above equations numerically, with $D_1° = 1$, $D_2° = 1/60$, $k_1 = 1.0$, and k_2 having a range of values, and either including or not including the term $k_2D_2^2$ which described the renaturation of D_2. The solutions for hybridization of D_2 did not differ significantly, for k_2 as low as 0.17, until twice the time necessary for one-half of D_1 to renature. (The numerical solution followed the general outline of Strauss and Bonner [32], and used a Δt which was 1.3% of the accumulated time. To check if this Δt was sufficiently small, one solution, with $k_2 = 0.46$, also had $\Delta t = 0.1\%$. This caused no change in the kinetics of hybridization.)

Hybridization of D_2 was assayed by digestion with S_1 nuclease, whereas renaturation of D_1 was assayed by hyperchromicity measurements, as described in Materials and Methods. To determine whether these two techniques were equivalent, we hybridized ^{3}H-labeled chicken DNA to an excess of unlabeled chicken DNA, with all DNA fractionated to the same size. The results are shown in Fig. 9A. The renaturation curves obtained by the two methods did differ somewhat. Figure 9B shows that hybridization of unique sequence DNA, assayed by digestion with S_1 nuclease, follows second-order kinetics with a $C_0t_{1/2}$ of 270. The theoretical curve is a solu-

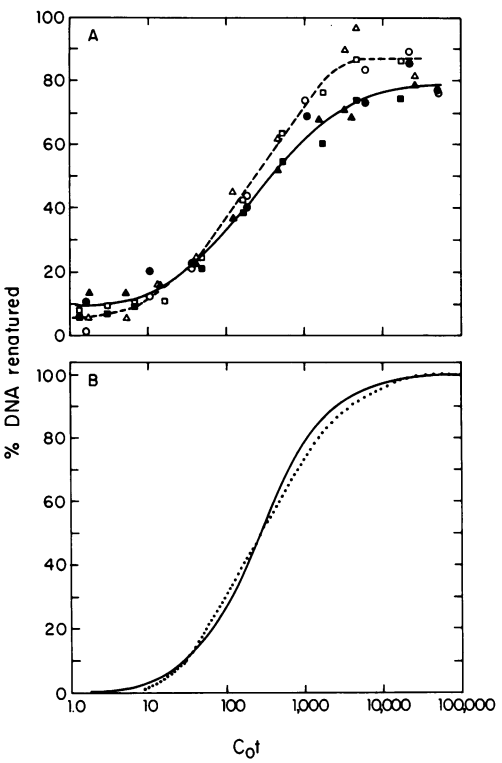

FIG. 9. *Renaturation of chicken embryo DNA. Percentage of DNA renatured is plotted versus DNA concentration times time. (A) Assayed by S_1 digestion (closed symbols) and by hyperchromicity measurements (open symbols). (B) Normalization of data from S_1 disgestion in Fig. 9A. The amount of DNA renatured was divided by 0.79, the maximum amount of hybridization. The normalized amount of DNA renatured by $C_0t = 8.0$ (0.152) was attributed to repetitive sequences and subtracted from amounts hybridized at higher C_0t values. The resulting numbers were then normalized by dividing by 0.848 (1 − 0.152). The dotted line resulted. The solid line is a theoretical renaturation curve for DNA which has been placed to have a $C_0t_{1/2} = 270$.*

tion to the above equations with $k_1 = k_2$. k_1 has been adjusted to give a $C_0t_{1/2}$ of 270. This curve is coincident with the explicit solution of $dD/dt = -k_1D^2$, as it should be. The $C_0t_{1/2}$ for unique sequence DNA, assayed by hyperchromicity, is 215 (Fig. 9A). These numbers were used as standards. Renaturation of cell DNA by hyperchromicity was measured for each hybridization curve. The C_0t values for hybridization of cDNA (in the plots of percent of hybridization versus C_0t) were multiplied by a factor of $215/(C_0t_{1/2}$ of unique sequence cell DNA).

Figure 10A to E shows hybridizations from the chicken DNA-^{3}H-labeled chicken DNA model system. The lines drawn through the data points are numerical solutions to the above set of equations with different values of k_2, but the same values of k_1, $D_1°$, and $D_2°$ as in Fig. 9B. The numerical solutions have been chosen to give the best fit up to a C_0t of 1,000, for reasons which will become clear. At higher values of C_0t, the amounts of hybridization were often lower than theoretically expected, especially for experiments with the smaller ^{3}H-labeled chicken DNAs.

Figure 10F is a summary of the variation of k_2/k_1 with the ratio of molecular weights of [^{3}H]DNA and unlabeled DNA. As shown, the relationship is consistent with that predicted by Wetmur (46). In the experiments described in this paper, the average ratio of molecular weights of cDNA and cell DNA was 0.23; therefore, k_2/k_1 was 0.46.

The second problem in copy number analysis concerned the uncertainty in the excess of virus-specific sequences in cell DNA to cDNA sequences. Above a certain ratio, usually called "vast excess," hybridization kinetics are the same as those for infinite excess. This is shown in Fig. 11A, which is a numerical solution to the following set of equations: $dD_1/dt = -k_1D_1D_2$; $dD_2/dt = -k_1D_1D_2 - k_2D_2D_p$; $dD_p/dt = -k_2D_2D_p$; where D_1 is the concentration of the vDNA strand not homologous to cDNA, D_2 is the concentration of the vDNA strand homologous to cDNA, and D_p is the concentration of viral se-

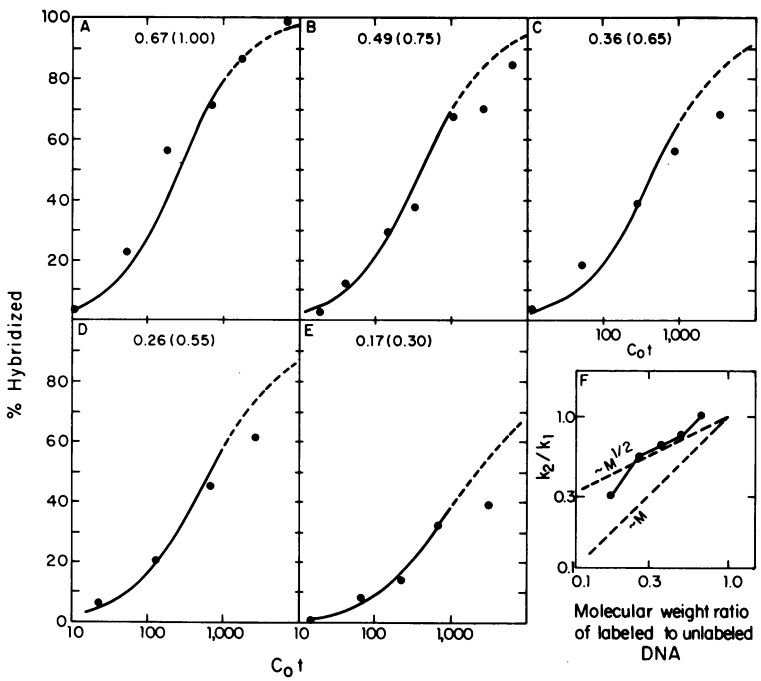

FIG. 10. *Variation of hybridization rate constant with size of reactants. (A-E) ^{3}H-labeled chicken DNA of varying size was hybridized to unlabeled chicken DNA at a weight ratio of 1:60. Results are plotted as percent of ^{3}H-labeled chicken DNA hybridized versus DNA concentration (C_0) times time. The amount of [^{3}H]DNA hybridized was analyzed as described in the text. The maximum fraction of S_1-resistant DNA produced in reactions with DNAs of equal size was 0.79 (Fig. 9A); consequently, the amounts hybridized were divided by this number. Hybridization due to repetitive sequences was subtracted as described in the legend to Fig. 9, and the data were normalized by dividing by 0.85, the fraction of unique sequence DNA in chicken cells (Fig. 9 legend). C_0t values were adjusted to standard conditions as described in the text. The numbers at the top of each panel are the ratio of molecular weights of ^{3}H-labeled DNA to unlabeled DNA, and the numbers in parentheses are the ratios k_2/k_1 in the numerical solution, which is the solid line through the data points. The portions of the curves drawn with solid lines indicate where an attempt has been made to fit the data points. Sizes of ^{3}H-labeled DNA in panels A to E are, sequentially, 29,000, 28,000, 13,000, 11,000, and 7,600 molecular weight. F, The dashed lines give the possible relationships between M_2/M_1 and k_2/k_1 described by Wetmur (46).*

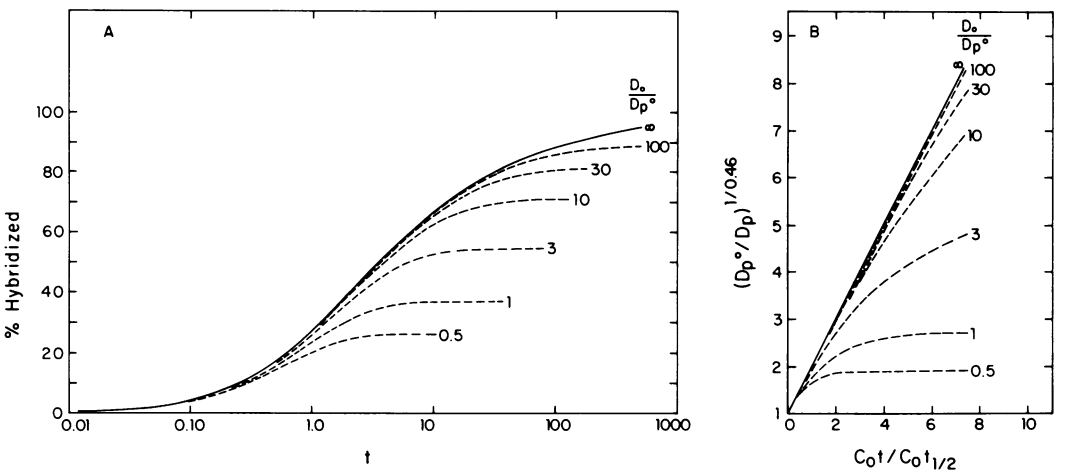

FIG. 11. *Theoretical variation of hybridization kinetics with change in ratio of cellular DNA to cDNA. (A) Numerical solution for D_p in equations described in text with $k_1 = 1.00$, $k_2 = 0.46$, $D_1^\circ = D_2^\circ = 1$, and D_p° variable. t is time (arbitrary units). Percent hybridized = 100 (1 − D_p). Numbers at the ends of lines are D_0/D_p°. The values of hybridization given by the numerical solution for the case of infinite excess of D_0 agree within 0.5% with the values given by the explicit solution for this case, which is given in the text. Similar numerical solutions have been reported by Strauss and Bonner (32). (B) Same data plotted as described in text. $C_0 = 1$, consequently r should equal 1.*

quences in cDNA. At t = 0, $D_1 = D_1^\circ = D_0$; $D_2 = D_2^\circ = D_0$; $D_p = D_p^\circ$. For the above equations, $k_1 = 1.00$, $k_2 = 0.46$, $D_1^\circ = D_2^\circ = 1$, and D_p is variable.

There are two problems with attempting to hybridize probe cDNA in a vast excess of cell DNA. First, since the probe is an uneven distribution of viral sequences, "excesses" are different for different sequences. Second, even if the probe was an equal sequence representation, hybridization in vast excess would necessitate an unwieldy amount of cell DNA.

An alternative approach could be developed because the initial kinetics of hybridization was the same irrespective of the excess of cell sequences (Fig. 11A). In a manner analagous to Melli et al. (24), the above equations could be solved explicitly in the case $D_0 = D_1^\circ = D_2^\circ \gg D_p^\circ$, to give $D_p^\circ/D_p = (1 + k_1 D_0 t)\exp(k_2/k_1)$. This equation describes the change of D_p at small t, regardless of the ratio D_0/D_p° (Fig. 11A).

Rearranging gives $(D_p^\circ/D_p)^{k_1/k_2} = 1 + k_1 D_0 t$.

For the virus-specific sequences under question, $D_0 = rC_0$ where C_0 is the concentration of unique sequence DNA and r is the number of copies of viral sequences per cell haploid genome. Also, k_1 may be computed independently, from the renaturation of unique sequence cell DNA: $dC/dt = -k_1C^2$, where C is the concentration of unique sequence DNA and C_0 is the concentration at t = 0.

Solving for the time $t = t_{1/2}$ when $C/C_0 = 1/2$, $k_1 = 1/C_0t_{1/2}$. Consequently $(D_p^\circ/D_p)^{k_1/k_2} = 1 + r\ (C_0t/C_0t_{1/2})$. Therefore, a plot $(D_p^\circ/D_p)^{k_1/k_2}$ versus $(C_0t/C_0t_{1/2})$ should have slope r. If the reaction does not have a vast excess of virus-specific cell sequences, the initial slope in this type of plot should still be equal to r, since the initial kinetics of hybridization is independent of the excess (Fig. 11A). To test this conclusion, the data of Fig. 11A were plotted in this way, with C_0 equal to 1. Fig. 11B shows that the initial slope of the family of lines is the same, regardless of the ratio D_0/D_p°.

If the initial slope is drawn using ordinate values from 1.0 to 1.3, the ratio of viral DNA sequences in cells to cDNA sequences can be as low as 0.5 without causing more than a 15% error in measurement of r (Fig. 11B). In the experiments described here, the weight ratio of cDNA to cell DNA varied from 1.5×10^{-7} to 11×10^{-7}, and one experiment had a ratio of 13×10^{-7}. Assuming that infected cells have at least one copy per haploid genome of vDNA, the weight ratio of vDNA to cDNA, in the mixture having a cDNA-to-cell DNA ratio of 13×10^{-7}, was ≥4.2 (using the weight of one strand of the vDNA). This ratio is 8.4 times what is minimally necessary, assuming the cDNA does not have overrepresented viral sequences. Since it does, the question which must be asked is how much of the cDNA can be viral sequences represented 8.4 times more than expected. An estimate of this can be calculated from the observation that a 4.4 times weight excess of RAV-2 RNA hybridizes 73% of a possible 80% of cDNA (90% of possible hybridization, if normalized; see Materials and Methods). Considering the case where one part of the viral genome is represented 8.4 or less times, and the other part 8.4 or more times, at most 20% of the cDNA can be represented more than 8.4 times, and still allow 90% of the cDNA to be hybridized by RAV-2. This 20% or less would not seriously affect the analysis of copy number. Consequently, the initial slope could be computed from data up to an ordinate value of at least 1.3. Sometimes data points with ordinate values >1.3, from experiments with a high ratio of cell DNA to cDNA, were on or near a straight line which was a

reasonable fit of data up to an ordinate value of 1.3. In these cases, all of these data points were used to determine the initial slope. An exception to this is the data for uninfected cell DNA. The data in Fig. 2B indicate that the excess of cell DNA to cDNA is great. Consequently, the slope should be drawn through most of the normalized data.

One conceivable problem with the initial slope analysis is the interpretation of hybridization data if different regions of the viral genome are repeated a different number of times in the cell DNA, or if a portion of the viral genome is not present at all. In this case the initial slope would represent hybridization of cDNA to partial viral sequences, and the number of these sequences would be underestimated (Fig. 12). Unless there are substantial differences in the repetition numbers, the hybridization curve will not be bimodal, especially when k_2/k_1 is smaller than 1.0 (Fig. 13). Consequently, interpretation of initial slope data must be qualified by the possibility of quantitative errors due to differing repetitiveness of different portions of the viral genome.

Since initial slope analysis usually involves levels of hybridization from 0 to 10 or 20%, the counts per minute in these hybrid samples digested with S_1 nuclease are quite low. The largest number is usually in the range of 12 to 30 counts/min. The uncertainty in the initial slope plot resulting from statistical fluctuations in scintillation counting was estimated as follows. The uncertainty in the slope of a line drawn through points with differing uncertainties can be evaluated using the procedure described

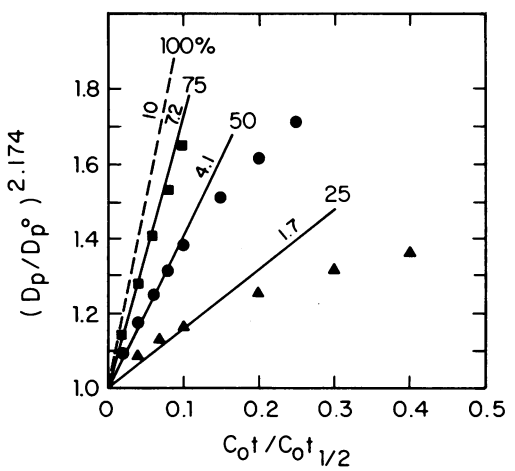

FIG. 12. *Effect of incomplete viral genome representation on analysis of copy number. The time course of hybridization of cDNA was computed, using the explicit solution for the case of infinite excess of cell DNA (Appendix), when only part of the viral genome was present, capable of hybridizing 25, 50, or 75% of cDNA. The copy number of viral DNA sequences was set at 10. k_1/k_2 = 1/0.46 = 2.174. Symbols are defined in text and legend to Fig. 2C. The numbers on the lines give the initial slope using ordinate values up to 1.3. The dashed line would be expected if the complete viral genome was present.*

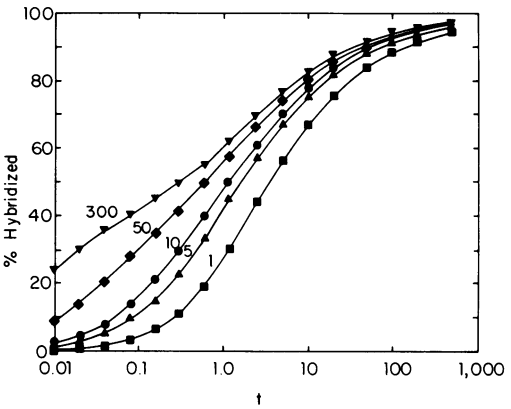

FIG. 13. *Hybridization of cDNA to sequences of differing repetitiveness. Using the explicit solution for hybridization of a single-stranded cDNA to cell DNA in vast excess, theoretical hybridization curves were drawn assuming 50% of the cDNA hybridized to sequences repeated one time, and the remaining 50% hybridized to sequences repeated different numbers of times (numbers given on the lines). k_1 = 1.0, k_2 = 0.46, t = time.*

by Bevington (3). This approach uses a least-squares fit through the data points, weighted for the uncertainty of each point. It was modified for use here since the intercept of the line in the initial slope plot was known to be 1. In this case, for a line described by the equation $y = mx + 1$ [$(y_i = (D_p°/D_p)^{2.174}$, $x_i = C_0t/C_0t_{1/2}$]

$$\sigma_m^2 = 1/\Sigma(x_i^2/\sigma_i^2) ,$$

where σ_m is the standard deviation of the slope, and

$$m = \sigma_m^2 \left(\Sigma \frac{x_i y_i}{\sigma_i^2} - \Sigma \frac{x_i}{\sigma_i^2} \right)$$

where σ_i is the standard deviation for the point y_i. To estimate the error due to the statistical uncertainty of scintillation counting, this was assumed to be the only source of error. A line of slope 1 was chosen as an example. Three points, with $C_0t/C_0t_{1/2}$ from 0.05 to 0.46, which had ordinate values from 1.05 to 1.46, were chosen. These represented percents of hybridization from 2.0 to 16.0, and it was assumed that these were calculated from net counts of 2.5 to 20.0 counts/min. It was also assumed that the samples were counted for 60 min (the usual experimental procedure) and the background was 7.5 counts/min. The uncertainty in $(D_p°/D_p)^{2.174}$ could be calculated, knowing that the standard deviation of the counts which provided the raw data was $(N)^{1/2}$ (N = total counts recorded). The standard deviation of the slope was calculated to be 0.045, or 4.5% of the value of the slope. Consequently, the statistical uncertainties in the counts used to compute the initial slope were not large enough to make the value of the slope unreliable.

An attempt was made to calculate the uncertainty in the slope from both statistical and experi-

mental error. Systematic errors are described in the next paragraph. Experimental error from subtraction of the fraction of single-strand DNA which was undigestible with S_1 nuclease was estimated to have $\sigma = 0.005$, and the division of the sample prior to S_1 digestion plus succeeding steps during trichloroacetic acid precipitation was estimated to have σ/(volume of sample) = 0.03. Incorporating these deviations in the above calculation, the standard deviation in the slope was increased from 4.5 to 7.3%, which is still well within tolerable limits.

There are two possible sources of systematic error in the copy number analysis. The first is the evaluation of $C_0t_{1/2}$ from renaturation of unique sequence cell DNA. A rough estimate of the maximum possible error from this step would be ±30%, so it was assumed that σ = 15%. A second source of error could be in the evaluation of k_2/k_1. Besides size, the guanine plus cytosine (G+C) content of the reacting species would affect this ratio (47). The G+C content of chicken cell DNA is different from that in viral RNA (29), and consequently presumably different from that in cDNA. This might cause an underestimate in k_2/k_1 of approximately 20%, and therefore an overestimate of copy number in the range of 15 to 20%. It was assumed that σ was 10% for this source of error. Summing the possible sources of error indicates that the initial slope has a σ equal to 35%. Computation of copy number by subtracting 0.6 introduces additional error due to the uncertainty in this value. This matters little where large copy numbers are concerned, but is important when computing smaller numbers. For instance, the copy number 1.2 in Table 1 could be, within one σ, anything from 0.4 to 2.0. Two times the standard deviation gives a range from 0.0 to 2.4. However, the number must be greater than 0 since, in this and all other experiments recorded in Table 1, the maximum amount of hybridization was greater than the maximum value for endogenous sequences alone.

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LITERATURE CITED

1. **Ali, M., and M. A. Baluda.** 1974. Synthesis of avian oncornavirus DNA in infected chicken cells. J. Virol. 13:1005-1013.
2. **Baluda, M. A., M. Shoyab, P. D. Markham, R. M. Evans, and W. N. Drohan.** 1974. Base sequence complexity of 35S avian myeloblastosis virus RNA determined by molecular hybridization kinetics. Cold Spring Harbor Symp. Quant. Biol. 39:869-874.
3. **Bevington, P. R.** 1969. Data reduction and error analysis for the physical sciences. McGraw-Hill, New York.
4. **Bishop, J. O.** 1972. Molecular hybridization of ribonucleic acid with a large excess of deoxyribonucleic acid. Biochem. J. 126:171-185.
5. **Bock, R. M.** 1967. Controlled partial hydrolysis of RNA, p. *In* L. Grossman and K. Moldave (ed.), Methods in enzymology, vol. 12. Academic Press Inc., New York.
6. **Boettinger, D., and H. M. Temin.** 1970. Light inactivation of focus formation by chicken embryo fibroblasts infected with avian sarcoma virus in the presence of 5-bromodeoxyuridine. Nature (London) 228:622-624.
7. **Britten, R. J., and D. E. Kohne.** 1968. Repeated sequences in DNA. Science 161:529-540.
8. **Duesberg, P., P. K. Vogt, K. Beeman, and M. Lai.** 1974. Avian RNA tumor viruses: mechanism of recombination and complexity of the genome. Cold Spring Harbor Symp. Quant. Biol. 39:847-858.
9. **Fareed, G. C., M. C. McKerlie, and N. P. Salzman.** 1973. Characterization of simian virus 40 DNA component II during viral DNA replication. J. Mol. Biol. 74:95-111.
10. **Gianni, A. M., D. Smotkin, and R. A. Weinberg.** 1975. Murine leukemia virus: detection of unintegrated double-stranded forms of the provirus. Proc. Natl. Acad. Sci. U.S.A. 72:447-451.
11. **Gianni, A. M., and R. A. Weinberg.** 1975. Partially single-stranded form of free Moloney viral DNA. Nature (London) 255:646-648.
12. **Guntaka, R. V., B. W. J. Mahy, J. M. Bishop, and H. E. Varmus.** 1975. Ethidium bromide inhibits appearance of closed circular viral DNA and integration of virus-specific DNA in duck cells infected by avian sarcoma virus. Nature (London) 253:507-511.
13. **Hanafusa, H.** 1965. Analysis of the defectiveness of Rous sarcoma virus. III. Determining influence of a new helper virus on the host range and susceptibility to interference of RSV. Virology 25:248-255.
14. **Hanafusa, H.** 1969. Rapid transformation of cells by Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 63:318-325.
15. **Hanafusa, H., T. Hanafusa, S. Kawai, and R. E. Luginbuhl.** 1974. Genetic control of expression of endogenous virus genes in chicken cells. Virology 58:439-448.
16. **Hayward, W. S., and H. Hanafusa.** 1973. Detection of avian tumor virus RNA in uninfected chicken embryo cells. J. Virol. 11:157-167.
17. **Hayward, W. S., and H. Hanafusa.** 1975. Recombination between endogenous and exogenous RNA tumor virus genes as analyzed by nucleic acid hybridization. J. Virol. 15:1367-1377.
18. **Kawai, S., and H. Hanafusa.** 1972. Plaque assay for some strains of avian leukosis virus. Virology 48:126-135.
19. **Khoury, A. T., and R. A. Deering.** 1973. Sedimentation of DNA of *Dictyostelium discoideum* lysed on alkaline sucrose gradients: role of single-strand breaks in gamma ray lethality of sensitive and resistant strains. J. Mol. Biol. 79:267-284.
20. **McConaughy, B. L., and B. J. McCarthy.** 1967. The interaction of oligodeoxynucleotides with denatured DNA. Biochim. Biophys. Acta 149:180-189.
21. **Markham, P. D., and M. A. Baluda.** 1973. Integrated state of oncornavirus DNA in normal chicken cells and cells transformed by avian myeloblastosis virus. J. Virol. 12:721-732.
22. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3:208-218.
23. **Marvin, D. A., and B. Hohn.** 1969. Filamentous bacterial viruses. Bacteriol. Rev. 33:172-209.
24. **Melli, M., C. Whitfield, K. V. Rao, M. Richardson, and J. O. Bishop.** 1971. DNA-RNA hybridization in vast DNA excess. Nature (London) New Biol. 231:8-12.
25. **Neiman, P. E., H. G. Purchase, and W. Okazaki.** 1975. Chicken leukosis virus genome sequences in DNA from normal chick cells and virus induced bursal

lymphomas. Cell 4:311-319.
26. **Neiman, P. E., S. E. Wright, and H. G. Purchase.** 1974. Studies of the interrelationship of chicken leukosis virus and host cell genomes by RNA-DNA hybridization. Cold Spring Harbor Symp. Quant. Biol. 39:875-883.
27. **Rosenthal, D. N., H. L. Robinson, W. S. Robinson, T. Hanafusa, and H. Hanafusa.** 1971. DNA in uninfected and virus-infected cells complementary to avian tumor virus RNA. Proc. Natl. Acad. Sci. U.S.A. 68:2336-2340.
28. **Schincariol, A. L., and W. K. Joklik.** 1973. Early synthesis of virus-specific RNA and DNA in cells rapidly transformed with Rous sarcoma virus. Virology 56:532-548.
29. **Shoyab, M., and M. A. Baluda.** 1973. Separation of DNA sequences complementary to the RNA of avian myeloblastosis virus from chicken DNA by alkaline cesium chloride sedimentation. J. Virol. 12:534-537.
30. **Shoyab, M., M. A. Baluda, and R. Evans.** 1974. Acquisition of new DNA sequences after infection of chicken cells with avian myeloblastosis virus. J. Virol. 13:331-339.
31. **Shoyab, M., R. M. Evans, and M. A. Baluda.** 1974. Presence in leukemic cells of avian myeloblastosis virus-specific DNA sequences absent in normal chicken cells. J. Virol. 14:47-49.
32. **Strauss, N. A., and T. I. Bonner.** 1972. Temperature dependence of RNA-DNA hybridization kinetics. Biochim. Biophys. Acta 277:87-95.
33. **Studier, F. W.** 1965. Sedimentation studies of the size and shape of DNA. J. Mol. Biol. 11:373-390.
34. **Sweet, R. W., N. C. Goodman, J. R. Cho, R. M. Ruprecht, R. R. Redfield, and S. Spiegelman.** 1974. The presence of unique DNA sequences after viral induction of leukemia in mice. Proc. Natl. Acad. Sci. U.S.A. 71:1705-1709.
35. **Taylor, J. M., A. J. Faras, H. E. Varmus, W. E. Levinson, and J. M. Bishop.** 1972. Ribonucleic acid directed deoxyribonucleic acid synthesis by the purified deoxyribonucleic acid polymerase of Rous sarcoma virus. Characterization of the enzymatic product. Biochemistry 11:2343-2351.
36. **Temin, H. M.** 1961. Mixed infection with two types of Rous sarcoma virus. Virology 13:158-163.
37. **Tereba, A., L. Skoog, and P. K. Vogt.** 1975. RNA tumor virus specific sequences in nuclear DNA of several avian species. Virology 65:524-534.
38. **Varmus, H. E., J. M. Bishop, and P. K. Vogt.** 1973. Synthesis and integration of Rous sarcoma virus-specific DNA in permissive and non-permissive hosts, p. 373-379. *In* C. F. Fox and L. Silvestri (ed.), Virus research. Academic Press Inc., New York.
39. **Varmus, H. E., R. V. Guntaka, C. T. Ding, and J. M. Bishop.** 1974. Synthesis, structure and function of avian sarcoma virus-specific DNA in permissive and non-permissive cells. Cold Spring Harbor Symp. Quant. Biol. 39:987-996.
40. **Varmus, H. E., R. V. Guntaka, W. J. Fan, S. Heasly, and J. M. Bishop.** 1974. Synthesis of viral DNA in the cytoplasm of duck embryo fibroblasts and in enucleated cells after infection of avian sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 71:3874-3878.
41. **Varmus, H. E., S. Heasly, and J. M. Bishop.** 1974. Use of DNA-DNA annealing to detect new virus-specific DNA sequences in chicken embryo fibroblasts after infection by avian sarcoma virus. J. Virol. 14:895-903.
42. **Varmus, H. E., W. E. Levinson, and J. M. Bishop.** 1971. Extent of transcription by the RNA-dependent DNA polymerase of Rous sarcoma virus. 1971. Nature (London) New Biol. 233:19-21.
43. **Varmus, H. E., P. K. Vogt, and J. M. Bishop.** 1973. Integration of deoxyribonucleic acid specific for Rous sarcoma virus after infection of permissive and non-permissive hosts. Proc. Natl. Acad. Sci. U.S.A. 70:3067-3071.
44. **Verma, I. M., N. C. Menth, and D. Baltimore.** 1972. Covalent linkage between ribonucleic acid primer and deoxyribonucleic acid product of the avian myeloblastosis virus deoxyribonucleic acid polymerase. J. Virol. 10:622-627.
45. **Weissman, C., J. T. Parsons, J. W. Coffin, L. Rymo, M. A. Billeter, and H. Hofstetter.** 1974. Studies on the structure and synthesis of Rous sarcoma virus RNA. Cold Spring Harbor Symp. Quant. Biol. 39:1043-1056.
46. **Wetmur, J. G.** 1971. Excluded volume effects on the rate of renaturation of DNA. Biopolymers 10:601-613.
47. **Wetmur, J. G., and N. Davidson.** 1968. Kinetics of renaturation of DNA. J. Mol. Biol. 31:349-370.
48. **Wright, S. E., and P. E. Neiman.** 1974. Base-sequence relationships between avian ribonucleic acid endogenous and sarcoma viruses assayed by competitive ribonucleic acid-deoxyribonucleic acid hybridization. Biochemistry 13:1549-1554.