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Detection of Avian Tumor Virus RNA in Uninfected Chicken Embryo Cells

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Uninfected chicken embryo cells were analyzed for the presence of viral ribonucleic acid (RNA) by molecular hybridization with the single-stranded deoxyribonucleic acid (DNA) product of the RNA-dependent DNA polymerase contained in avian sarcoma-leukosis virions. Viral RNA was detected in all cells which contained the avian tumor virus group-specific antigen and the virus-related helper factor. The amounts of viral RNA in these cells ranged from approximately 3 to 40 copies of viral-specific sequences per cell. In general, the viral RNA content correlated with the level of helper activity in the cells. Cells infected with Rous-associated virus 2 contained 3,000 to 4,000 copies of viral RNA per cell. RNA from these infected cells hybridized with nearly 100% of the viral ^{3}H-DNA. By contrast, a maximum of less than 50% hybridization was obtained with RNA from the uninfected helper-positive cells, suggesting that not all of the viral RNA sequences were present in these cells. No viral RNA was detected in cells which lacked group-specific antigen and helper activity. Under the conditions used in these studies, less than 0.3 viral genome equivalents of RNA per cell would have been detected.

Two products specific to avian leukosis-sarcoma viruses are present in uninfected cells derived from a majority of chicken embryos from leukosis-free flocks, although mature virus particles are not detectable in these cells (10, 23, 25, 43). The formation of these viral products—the group-specific (gs) antigen and a component of the viral envelope—is genetically determined, with presence being dominant (31, 45). Presumably because the latter product can complement a defective function of Bryan strain Rous sarcoma virus (B-RSV), these chicken cells provide a helper activity for this particular virus. The level of expression of both the helper function and gs antigen varies quite widely among cells derived from different embryos (25). Cells from a minority of embryos contain no detectable viral products. A new type of leukosis virus, Rous-associated virus 60 (RAV-60), can be isolated from the viral product-positive cells following infection with other known leukosis or sarcoma viruses (24). This virus contains genetic information, apparently derived from the host cell, which codes for the two viral products described above.

Further studies, using a very sensitive technique, showed that cells derived from all of these embryos, including those which lacked detectable viral products, were capable of producing RAV-60 following infection with other leukoviruses (25). The presence of viral genetic information in all types of cells was also demonstrated by molecular hybridization analysis, which showed that viral deoxyribonucleic acid (DNA) sequences are present in similar amounts in uninfected cells both positive and negative for the viral products (3, 34, 40). The presence of viral DNA in cells which lack viral functions suggests that the expression of the viral genes is blocked by a specific control mechanism. However, it is also possible that cells which lack these two viral products contain incomplete or partially defective viral genomes.

To gain additional information about the expression of viral genes, we have examined uninfected chicken embryo cells for the presence of viral ribonucleic acid (RNA) by molecular hybridization. Viral product-positive cells would be expected to contain viral RNA, though perhaps at very low levels. Cells which lack detectable viral products might also contain viral RNA, since the two functions which can be assayed may not adequately reflect the expression of the viral genes. If

the expression of viral functions is regulated by some control mechanism, the presence or absence of viral RNA in viral product-negative cells would depend on whether control is mediated at the level of transcription or translation.

Virus-specific RNA was detected by molecular hybridization with ^{3}H-labeled viral DNA, which was synthesized using the endogenous RNA template and RNA-dependent DNA polymerase contained in sarcoma-leukosis virions (2, 38). This method has been used successfully for the detection of viral RNA in infected cells of both avian and mammalian origin (9, 16, 19, 26, 30). Hybrid formation was detected by analysis with the single-strand-specific S-1 nuclease of *Aspergillus oryzae* (1, 30, 37, 41). This technique permits the use of high concentrations of RNA, thus increasing the sensitivity of the assay. The conditions used in this study are capable of detecting less than 0.3 viral genome equivalents of RNA per cell.

MATERIALS AND METHODS

Materials. Deoxyribonuclease I (ribonuclease-free) and hyaluronidase were obtained from Worthington Biochemical Corp.; crude α-amylase (*A. oryzae*), trypsin, and diethylaminoethyl (DEAE)-cellulose were from Sigma Chemical Co.; Pronase and actinomycin D were from Calbiochem; hydroxylapatite (hypatite C) was from Clarkson Chemical Co.; Nonidet P-40 (NP-40) was from Shell Chemical Corp.; transfer RNA (tRNA) (Baker's yeast) and salmon sperm DNA were from Schwarz/Mann; poly(rA) and poly(dT) from Biopolymers, Inc.; and ^{3}H-thymidine triphosphate (TTP) was from New England Nuclear Corp.

Cells and viruses. Viruses used in this study were Rous-associated virus 2 (RAV-2), RAV-60, and the Schmidt-Ruppin strain of Rous sarcoma virus subgroup D (SR-RSV). Conditions for growth and infection of cells and characteristics of viruses have been described previously (21, 22, 24, 42). RAV-2 and SR-RSV were grown in chick cells which lack the gs antigen and helper activity, and RAV-60 was prepared in quail embryo cells. The SR-RSV used in these studies contained spontaneously appearing nontransforming virus (equivalent to RAV-50), which was detected by the plaque assay of Kawai and Hanafusa (28). The ratio of focus-forming to plaque-forming units in this preparation was approximately 3:1.

Classification of chicken embryo cells. The titer of gs antigen in each embryo was determined by complement fixation (15, 27, 31), using the viscera which were removed during the preparation of primary cultures (25). Embryos have been classified into three categories, gs^-, gs_L (low), and gs^+ (see Table 1). The presence of helper factor activity was determined by assaying for the production of infectious B-RSV(f) following infection with the defective B-RSV(−) in the presence of ultraviolet (UV)-inactivated Sendai virus (Method A), or with B-RSV(RAV-2) (Method B) (25). Embryos have been classified h^-, h_L (low), h_H (high) or h_E (extremely high), based on the relative titer of infectious virus in these assays (see Table 1). All embryos were tested by both methods.

Four relatively distinct classes of embryos have been used in these studies (Table 1). Embryos of type gs^-h^-, gs^-h_L, and gs^+h_H correspond to cell types C/O', C/O'-L, and C/O, respectively, and have been described in detail previously (25). Embryos of type gs_Lh_E, which are relatively rare, contain low levels of gs antigen and produce infectious RSV(f) at extremely high levels. All embryos used in this study are resistant to viruses of subgroup E. Thus, they are more properly classified in a new genetic category of C/E (24, 32), rather than C/O as reported in earlier publications.

Purification of viruses. Virus stocks for RNA extraction and DNA synthesis were prepared from infected secondary cultures of chicken embryo cells which lack both gs antigen and chick cell helper factor activity. Culture fluids of fully infected cells were collected at 12-hr intervals, pooled, and centrifuged at 8,000 × *g* for 20 min. The supernatant fluid was centrifuged for 2 hr at 19,000 rpm in an International type 19 rotor. The pellet was suspended in TEN buffer (0.01 M tris(hydroxymethyl)aminomethane [Tris], *p*H 7.4; 0.001 M ethylenediaminetetracetic acid [EDTA]; 0.15 M NaCl) containing 10 μg per ml of hyaluronidase and centrifuged at low speed to remove cell debris. The pellet was resuspended in a small volume of TEN plus hyaluronidase, incubated for 10 min at 38 C, and again centrifuged at low speed. The supernatant fluids from these low-speed centrifugations were pooled and treated for 20 min at 37 C with 20 μg/ml of pronase which had been predigested for 2 hr at 37 C to remove nucleases. The viruses were further purified by equilibrium density centrifugation, using a linear gradient of 15 to 50% (w/w) sucrose in TEN buffer. Virus in a single band ($\rho \simeq 1.16$) was pooled, diluted with TEN, and concentrated by centrifugation onto a layer of 50% sucrose overlaid with 20% sucrose. This material was used for RNA extraction or DNA synthesis.

Extraction of RNA. Viral RNA was prepared by sodium dodecyl sulfate (SDS)-phenol extraction at room temperature and concentrated by ethanol

TABLE 1. *Classification of chicken embryo cells*

Cell type	Titer of gs antigen[a]	Titer of RSV(f) at day 5[b]
gs^-h^-	0	0
gs^-h_L	0	10–200
gs^+h_H	8–16	1,000–3,000
gs_Lh_E	2–4	10,000–30,000

[a] Expressed as the reciprocal of the terminal antigen dilution which gives more than a +3 reaction in the complement fixation test.

[b] Determined by Method A (see reference 25).

precipitation. Yeast tRNA (200 μg/ml) was used as a carrier. Viral 70s RNA was purified by rate zonal centrifugation, using a linear gradient of 15 to 30% glycerol in TEN buffer. For isolation of 35s RNA, phenol-extracted viral RNA was heated for 3 min at 70 C in 0.01 × SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) prior to centrifugation. Centrifugation was for 90 min (70s RNA) or 150 min (35s RNA) at 50,000 rpm in an SW50.1 rotor. Fractions containing 35s or 70s viral RNA were pooled, dialyzed for 6 hr against 2 × SSC and stored at −20 C. When necessary, RNA was concentrated by lyophilization. Whole cell RNA was prepared from secondary cultures 4 to 5 days after transfer. Cells were removed from culture plates by trypsinization (0.5 mg/ml, 10 min at 38 C) and washed with Tris-saline. RNA was extracted by the hot phenol method, as described by Scherrer (36). After phenol extraction the RNA was precipitated with ethanol and resuspended in a buffer solution containing 0.01 M Tris (pH 7.0), 0.01 M NaCl, and 0.001 M $MnCl_2$. The sample was treated with 10 μg/ml of deoxyribonuclease (ribonuclease-free) for 60 min at 4 C. Predigested Pronase (50 μg/ml) was then added, and the sample was incubated for 30 min at 37 C. The RNA was then repurified by SDS-phenol extraction at room temperature and precipitated with ethanol. The pellet was resuspended in 2 × SSC containing 0.1% SDS and stored at −20 C. Cellular 4 to 10s RNA was prepared by rate zonal centrifugation of this RNA, using a linear gradient of 15 to 30% glycerol in TEN buffer. RNA concentrations were determined by optical density at 260 nm. The 260/280 ratio was greater than 2 for all preparations.

Synthesis and purification of Viral DNA. ^{3}H-DNA was synthesized from detergent-treated virions in the presence of actinomycin D. The reaction mixture contained 20 mM Tris (pH 8.1), 5 mM $MgCl_2$, 15 mM dithiothreitol, 0.03% NP40, 0.5 mM each of deoxyadenosine triphosphate, deoxyguanosine triphosphate, and deoxycytidine triphosphate; 0.0005 mM ^{3}H-TTP (51 Ci/mmole), 200 μg/ml of actinomycin D, and purified virus (0.5 to 1 mg/ml of viral protein). The mixture was incubated for 3 hr at 38 C, followed by addition of EDTA to a concentration of 10 mM. DNA was purified by SDS-phenol extraction at room temperature, dialyzed overnight against 0.01 × SSC, and treated with 0.5 N NaOH for 20 hr at 37 C. The sample was dialyzed overnight against 0.05 M $(Na)PO_4$ buffer (pH 7.8) with two changes. A minor fraction of this DNA (8 to 12% in different preparations) was resistant to digestion by the single-strand-specific S-1 nuclease of *A. oryzae*, even after treatment with alkali or incubation at 100 C. (Nuclease digestion was performed in 0.3 M NaCl, as described for analysis of RNA-DNA hybrids. Under these conditions the S-1 nuclease is completely active against very low concentrations of single-stranded DNA [30].) These properties suggest the presence of some structures containing intramolecular regions of complementarity which rapidly reanneal following denaturation. These structures were not further characterized, however, and their significance is not clear. The nuclease-resistant material was effectively removed from the DNA preparation by fractionation on hydroxylapatite. DNA was applied to a column (1 by 8 cm) of hydroxylapatite equilibrated with 0.05 M $(Na)PO_4$ (pH 7.8) at 55 C. The DNA was eluted at the same temperature, using a linear gradient of 0.05 to 0.4 M $(Na)PO_4$ buffer. Fractions containing single-stranded ^{3}H-DNA (eluting at less than 0.18 M PO_4) were pooled, dialyzed against water, and concentrated by lyophylization. The lyophilized DNA was resuspended in 2 × SSC-0.1% SDS, and stored at −20 C. This DNA, which constituted approximately 70% of the ^{3}H-DNA applied to the column, was 97 to 98% hydrolyzed by S-1 nuclease. The DNA prepared in this way contained more than 60% of the viral sequences as indicated by hybridization experiments of the type described by Duesberg and Canaani (12), using DNA-RNA ratios as high as 250:1. (The specific activity of the ^{32}P-RNA was 10^7 counts per min per μg, based on the specific activity of the ^{32}P used in the growth medium during virus production.) However, the majority (70–80%) of this ^{3}H-DNA represented only a small fraction (ca. 15–20%) of the viral genome, as indicated by the kinetics of the annealing reaction in this study (*unpublished results*). Similar results have been reported for viral DNA products synthesized in the absence of actinomycin D (17, 39).

Isolation of S-1 nuclease. S-1 nuclease was purified by ammonium sulfate precipitation and DEAE-cellulose column chromotography, as described by Ando et al. (1). The starting material was crude α-amylase from *A. oryzae*. Enzyme preparations obtained from the DEAE-cellulose column were stored on ice, or at −20 C in 50% glycerol. The activity against double-stranded DNA with these preparations was less than 0.1% of the single-strand activity, under the conditions used in the hybridization analysis (*see below*).

Hybridization. The annealing mixture contained 0.3 M NaCl, 0.03 M sodium citrate, 0.1% SDS, 37% formamide, 0.005 μg per ml of ^{3}H-labeled DNA (except where indicated), and RNA as indicated in legends. The specific activity of the ^{3}H-DNA was 1.8×10^7 counts per min per μg. The reaction was performed at 45 C, using either 5 or 10 μliters of annealing mixture in sealed 50-μliter capillary tubes. For C_rt analysis of cellular or viral RNA samples, a series of annealing reactions was performed, using a single RNA concentration and varying the incubation time. In most cases, samples were frozen following incubation and stored at −80 C until incubation was completed for all samples in the series. The extent of hybridization was analyzed by digestion with the single-strand-specific S-1 nuclease. Annealing mixtures were diluted into 0.1 ml of a buffer solution containing 0.025 M potassium acetate (pH 4.4), 0.005 M $ZnSO_4$, 0.3 M NaCl, 60 μg/ml of double-stranded salmon sperm DNA, and 10 units/ml of S-1 nuclease. (One unit of nuclease is equal to that amount which hydrolyzes 1 μmole of nucleotide per min under the conditions described here,

using 250 μg/ml of denatured salmon sperm DNA as substrate.) The native salmon sperm DNA was included during the nuclease treatment of the hybrids to saturate any double-strand-specific nuclease which might be present in the S-1 nuclease preparation. The samples were incubated for 60 min at 38 C, and precipitated with cold 10% trichloroacetic acid, using tRNA (150 μg/ml) as a carrier. The hybridized ^{3}H-DNA was collected on nitrocellulose filters as acid-insoluble material, and the radioactivity was determined by scintillation spectrometry. In duplicate experiments, hybridization values were in agreement within a range of ±5%.

A low level of self-annealing (up to 2-3% above background) was observed when ^{3}H-DNA was incubated for long periods of time in the absence of RNA. To correct for this time-dependent self-annealing, and for varying background levels with different DNA preparations, control mixtures containing only ^{3}H-DNA were incubated in parallel with DNA-RNA hybridization mixtures. All data have been corrected for the background levels obtained in control samples incubated for comparable periods of time. This control level was less than 5% of the total ^{3}H-DNA. A positive control mixture, containing both cellular RNA (20 mg/ml) and viral RNA (1 μg/ml), was also incubated under annealing conditions to test each RNA sample for the presence of ribonuclease or other inhibitors. In every case, greater than 90% hybridization was obtained with these positive controls. Hybridization between viral ^{3}H-DNA and RNA from helper-positive cells was completely abolished by pretreating the RNA sample with ribonuclease. Thus, the virus-specific sequences which annealed to the ^{3}H-DNA probe were contained in RNA, not in DNA.

The half-C_rt (*see below*) for hybridization between RAV-2 ^{3}H-DNA and RAV-2 RNA was 8×10^{-2} mole·sec/liter, using the conditions described above. In 2 × SSC (without formamide), values of 10^{-1} (60 C) and 2×10^{-2} (68 C) were obtained.

RESULTS

Specificity of the hybridization assay. RAV-2 ^{3}H-DNA was synthesized in the presence of actinomycin D, using detergent-treated RAV-2 virions as a source of enzyme and RNA template (*see above*). This DNA hybridized almost completely with 70*s* or 35*s* RNA from RAV-2 (98 and 93%, respectively) (see Table 2). Thus, essentially all of the ^{3}H-DNA prepared in this way is complementary to the viral plus (+)-strand RNA. The RAV-2 ^{3}H-DNA also hybridized extensively with 70*s* RNA from two other leukoviruses, RAV-60 and SR-RSV (96 and 88% hybridization, respectively). This demonstrates that a considerable amount of cross-homology is present in the RNAs of these three related viruses.

RAV-2 ^{3}H-DNA was tested for hybridization with heterologous RNA from 3T3 and hamster embryo cells to determine the degree of specificity in the annealing reaction. The level of hybridization with these RNAs was less than 2% above background (Table 2). (The background level in the experiments was approximately 3%.) Low levels of hybridization were also found with poly(rA) (0.6%) and with 4 to 10*s* RNA (0.5%) isolated from chicken embryo cells of the type used for the virion preparations. These latter controls were included to test specifically for the possibility that a portion of the ^{3}H-DNA might have been transcribed from the adenine-rich sequences of the viral RNA (18, 20, 29, 35), or from low-molecular-weight cellular RNA which might be present in the virion (5, 13, 14, 33). Such transcripts would hybridize with the adenine-rich sequences of cell messenger RNA (mRNA), and with the 4 to 10*s* RNA in the cell, thus interfering with our analysis of viral-specific RNA in these cells. The results described above demonstrate, however, that transcripts of this type do not constitute a significant fraction of the ^{3}H-DNA. The absence of sequences in the ^{3}H-DNA which were complementary to adenine-rich regions of the viral RNA was further confirmed by the fact that excess poly(dT) did not compete in the annealing reaction between RAV-2 ^{3}H-DNA and RAV-2 RNA (see Table 2). Approximately 98% hybridization was obtained in the presence of poly(dT) at a concentration 120-fold in excess of viral RNA and 120,000-fold in excess of RAV-2 ^{3}H-DNA.

TABLE 2. *Test for hybridization of RAV-2 ^{3}H-DNA with polynucleotides of various types*

Polynucleotide	Concn (μg/ml)	% RAV-2 ^{3}H-DNA hybridized[a]
3T3 RNA	3,000	1.1
Hamster RNA	3,000	1.0
Hamster RNA	20,000[b]	1.5
Poly(rA)	200	0.4
Poly(rA)	600	0.6
Poly(dT)	600	0.4
4-10*s* RNA from gs$^-$h$^-$ chicken cells	500	0.5
RAV-2 70*s* RNA	5	98
+ poly(dT)	200	97
+ poly(dT)	600	98
RAV-2 35S RNA	5	93
RAV-60 70S RNA	5	96
SR-RSV 70S RNA	5	88

[a] Reaction mixtures were incubated for 150 hr.

[b] The kinetics of hybrid formation are not affected by this high RNA concentration. The presence of 20 mg/ml of heterologous RNA did not affect either the rate or extent of the annealing reaction between RAV-2 RNA and RAV-2 ^{3}H-DNA (see Fig. 2).

Characteristics of the annealing reaction. Hybridization data can be conveniently represented on a semilogarithmic graph, plotting the percent DNA hybridized against the product of initial RNA concentration × time (C_rt) (8, 30). The parameter C_rt, suggested by Birnstiel et al. (4), is analogous to C_ot (DNA concentration × time) introduced by Britten and Kohne (8) to describe DNA-DNA interactions. With this representation, the curves obtained for a given polynucleotide species will be identical over a wide range of RNA concentrations since the incubation time (t) required to reach a given level of hybridization is inversely proportional to the initial RNA concentration (C_r) when RNA is in sufficient excess. The C_rt value at which 50% maximal hybridization is attained (half-C_rt) is characteristic of the hybridizing RNA species and is roughly proportional to its genetic complexity (4, 6).

When the RNA sequences homologous to the radiolabeled DNA are present in a mixture of RNA species (e.g., cellular RNA), a C_rt curve can be constructed by calculating C_rt values based on total RNA concentration. The curve obtained in this way will be displaced to higher C_rt values, in proportion to the dilution of the homologous RNA species with heterologous RNA. The concentration of the hybridizing RNA can be calculated by comparing the half-C_rt obtained for the RNA mixture with that obtained using only RNA homologous to the DNA probe.

The kinetics of hybrid formation between RAV-2 ^{3}H-DNA and RAV-2 RNA were studied over a 10,000-fold range of viral RNA concentrations to determine the limits of sensitivity of our assay (see Fig. 1). At concentrations of 0.75 (curve A, open circles) to 100 μg/ml (X) the points fall on the same curve, reaching a maximum of nearly 100% hybridization. The linear portion of this curve, extrapolated to 0 and 100%, covers approximately 2 logs of C_rt, as expected for an ideal second-order reaction (8). Viral RNA was readily detectable at levels as low as 0.012 μg/ml (curve D). However, the curve obtained with this low RNA concentration differed considerably from the ideal represented by curve A, and less than 25% of the ^{3}H-DNA was hybridized at the longest incubation time tested. These non-ideal kinetics are a result of the low-input ratio of RNA to DNA, which is only 2.4:1 in these samples (curve D). The RNA-DNA ratio appears to be extremely critical in these experiments, presumably because of the heterogeneity of the DNA probe. Although the ^{3}H-DNA used in these experiments contains sequences homologous to at least 60% of the viral RNA, the majority of the DNA is homologous to only a limited region (15–20%) of the viral genome (see above). At low RNA-DNA ratios many of the DNA species would be in considerable excess over the homologous RNA sequences, whereas other DNA species would not. Thus, the kinetics of the annealing reaction would be heterogeneous because of the different reaction rates of the various DNA species. The heterogeneity of the DNA is reflected in the decreased slope of curve D, and, to a lesser extent, in the slopes of curves C and B, in which RNA concentrations of 0.10 and 0.23 μg/ml were used. Apparently, even at a ratio of 45:1 (curve B), some of the RNA sequences are not in sufficient excess.

It seemed possible that the hybridization values obtained with RNA-DNA ratios of 45:1 or less might be artificially low due to the long incubation times (up to 500 hr) required at these RNA concentrations. To test this possibility,

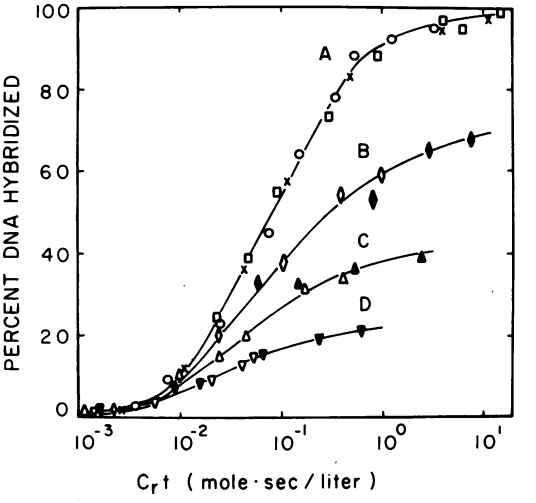

FIG. 1. *Hybridization of RAV-2 ^{3}H-DNA with RAV-2 RNA. Hybridization was performed as described in Materials and Methods, using RNA-DNA ratios of 150 to 20,000 (curve A), 45 (curve B), 20 (curve C), and 2.4 (curve D). ^{3}H-DNA concentrations were 0.060 μg/ml (▼), 0.038 μg/ml (▲, ♦), or 0.005 μg/ml (all others). Viral RNA was used at concentrations of 100 μg/ml (X), 9.0 μg/ml (□), 0.75 μg/ml (○), 0.225 μg/ml (◇), 1.70 μg/ml (◆), 0.10 μg/ml (△), 0.75 μg/ml (▲), 0.012 μg/ml (▽), and 0.144 μg/ml (▼). Annealing reactions were performed in the presence of heterologous RNA from hamster embryo cells (□) or yeast tRNA (others) at a concentration of 20 mg/ml. C_rt values and RNA-DNA ratios were calculated from viral RNA concentrations and thus do not reflect the presence of heterologous RNA.*

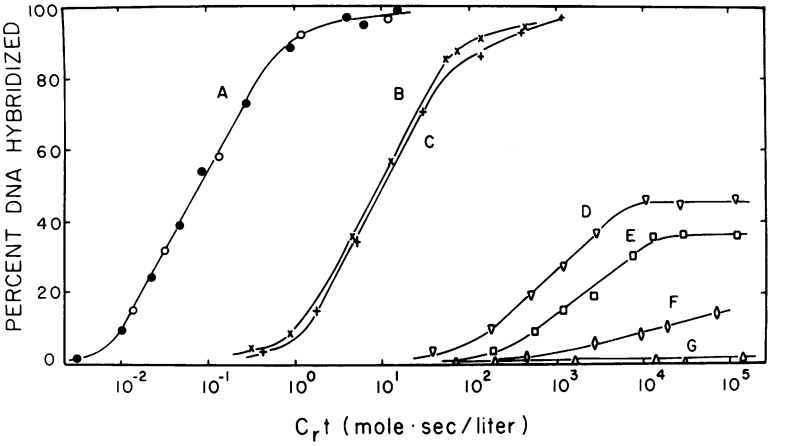

FIG. 2. *Hybridization of RAV-2 ^{3}H-DNA with RNA isolated from infected and uninfected chicken embryo cells. ^{3}H-DNA was hybridized with RNA isolated from the following sources: RAV-2 virus (curve A); RAV-2-infected cells of type gs^-h^- (curve B); and gs^+h_H (curve C); uninfected cells of type gs_Lh_E (curve D), gs^+h_H (curve E), gs^-h_L (curve F), and gs^-h^- (curve G). RNA concentrations were 9.0 μg/ml of viral RNA (curve A) in the presence (●) or absence (○) of whole cell RNA (20 mg/ml) isolated from hamster embryo cells; 3 mg/ml of cellular RNA (curves B and C); and 20 mg/ml of cellular RNA (curves D-G). Approximately 1,000 counts per min of ^{3}H-DNA were used in each hybrid reaction. C_rt values for curve A were based on viral RNA concentration; all others were calculated from total cellular RNA concentration.*

samples were prepared containing higher concentrations of both RNA and DNA, but maintaining the same input ratios used in the experiments above. The incubation times required to reach a given C_rt value will be decreased in proportion to the increase in RNA concentration. (The increase in DNA concentration will not affect the C_rt curves. The data are normalized to DNA concentration by expressing the hybridization level in terms of percent DNA hybridized.) The values obtained at these higher nucleic acid concentrations (Fig. 1, closed symbols) fell on the same curves obtained with similar RNA-DNA ratios at lower concentrations and longer incubation times (open symbols). Thus, it seems unlikely that hybridization levels were significantly affected by nucleic acid degradation or other artifacts resulting from prolonged incubation.

Very high RNA concentrations can be used in the annealing mixture without affecting the kinetics of the reaction. The C_rt curve obtained with RAV-2 RNA in the presence of 20 mg of heterologous RNA/ml from hamster cells (Fig. 2, curve A, closed circles) was identical to that obtained in the absence of heterologous RNA (open circles). Neither whole cell RNA, as mentioned above, nor low-molecular tRNA (*data not shown*) affected the rate or extent of the reaction at a concentration of 20 mg/ml, and both have been used as heterologous RNA in experiments described below.

Hybridization analysis of RNA isolated from infected and uninfected chicken embryo cells. When RNA isolated from RAV-2-infected cells was tested for the presence of viral RNA by hybridization with RAV-2 ^{3}H-DNA (Fig. 2, curves B and C), nearly 100% hybridization was obtained, demonstrating that essentially all of the nucleotide sequences which were represented in the viral DNA were present in the infected cells. The half-C_rt values obtained with these infected cell RNA samples were 0.9 and 1.1×10^1 mole·sec/liter, as compared with 8×10^{-2} mole·sec/liter for purified RAV-2 RNA (curve A). Based on these half-C_rt values, we have calculated that approximately 0.7 to 0.9% of the infected cell RNA is viral specific, in good agreement with previously published reports (9, 16, 19). This would correspond to approximately 3,000 to 4,000 viral RNA copies per cell, based on a value of 1.7×10^{-11} μg of RNA per viral genome (11) and an estimated 8×10^{-6} μg of total RNA per cell. The slight differences in the C_rt curves obtained with the infected helper-positive and helper-negative cells are not significant with this assay.

No viral-specific RNA was detected in uninfected chicken embryo cells which lacked gs antigen or helper activity (Fig. 2, curve G), even at C_rt values of greater than 10^5. However, hybrid formation was detected with RNA from all cells which contained these viral functions (curves D-F). The levels of hybridization with RNAs from cells with high levels of helper activity (gs^+h_H and gs_Lh_E) reached maxima of

approximately 36 and 45%, respectively. The fact that these curves reached plateau levels of less than 100% suggests that many of the viral sequences represented in the ^{3}H-DNA are not present in the RNA from these cells. Low but significant levels of hybridization were obtained with RNA from cells with low helper activity (gs^-h_L cells) (curve F). However, no plateau value was reached at the level of sensitivity used in these experiments.

A total of 14 embryos of different types were examined for the presence of viral RNA (see Table 3). No hybrid formation (<2%) was detected with RNA from any of five embryos which lacked gs antigen and helper activity. Of nine helper-positive embryos, all contained detectable levels of viral RNA. The maximum levels of hybridization and the half-C_rt values for embryos of each type were similar to those shown in Fig. 2.

To exclude the possibility that some of the hybridization observed with cellular RNA was due to the presence of host cell sequences in the ^{3}H-DNA preparation, a sample of ^{3}H-DNA was pre-annealed to viral 35*s* RNA and then treated with S-1 nuclease. This treatment would eliminate any DNA sequences which would not hybridize with the viral RNA. The DNA-RNA complexes which remained after nuclease treatment were extracted with SDS-phenol and treated with NaOH to hydrolyze the RNA. This preannealed ^{3}H-DNA was then used in hybridization experiments with RNA from each of the cell types tested above. Levels of hybridization obtained with the preannealed DNA (Table 3) were essentially the same as those obtained with DNA which had not been preannealed. Slightly lower values (0.4 and 0.7% versus 1.2 and 1.7%) were obtained with the RNA from gs^-h^- cells. However, values of less than 2% above background are not significant under the conditions used in these experiments. We conclude that the hybridization obtained using RAV-2 ^{3}H-DNA represents virus RNA-specific sequences.

Estimation of viral RNA content in uninfected cells. Although the amount of RAV-2 RNA in infected cells was calculated by com-

TABLE 3. *Hybridization of RAV-2 ^{3}H-DNA with RNA from uninfected cells*

Source of RNA	Embryo no.	Half-C_rt[a]	% DNA hybridized	
			RAV-2 ^{3}H-DNA[b]	Preannealed RAV-2 ^{3}H-DNA[c]
3T3 cells			1.1[d]	0.7
Hamster embryo cells			1.5	
Chick cells:				
gs^-h^-	1510		1.2[d]	0.7
gs^-h^-	1537		1.5[d]	
gs^-h^-	1603		0.8	
gs^-h^-	1613		1.1	
gs^-h^-	1622		1.7	0.4
Chick cells:				
gs^-h_L	1541	ND[e]	13	12
Chick cells:				
gs^+h_H	1488	1.5×10^3	36[d]	39
gs^+h_H	1539	1.8×10^3	35[d]	37
gs^+h_H	1542	ND[f]	34[d]	
gs^+h_H	1602	2.1×10^3	39	
gs^+h_H	1621	1.8×10^3	36	
gs^+h_H	1624	0.7×10^3	48	
Chick cells:				
gs_Lh_E	1601	0.6×10^3	46	45
gs_Lh_E	1620	0.7×10^3	45	

[a] C_rt value at which 50% of the plateau level of hybridization was attained.

[b] Maximum hybridization level obtained; analyses were extended to C_rt values of 0.5 to 1.0×10^5 using an RNA concentration of 20 mg/ml, except where indicated.

[c] Determined at a C_rt value of 0.5×10^5. ^{3}H-RAV-2 DNA was preannealed to viral 35*s* RNA and then treated with S-1 nuclease. The DNA-RNA complexes which remained after nuclease treatment were phenol extracted and then treated with NaOH to hydrolyze the RNA.

[d] Analyses extended to C_rt values of 0.2 to 0.4×10^5 using 4 to 5 mg/ml of RNA.

[e] Not determined; analysis was not extended to high enough C_rt values to reach a plateau level.

[f] Not determined; hybridization levels were determined only at high C_rt values.

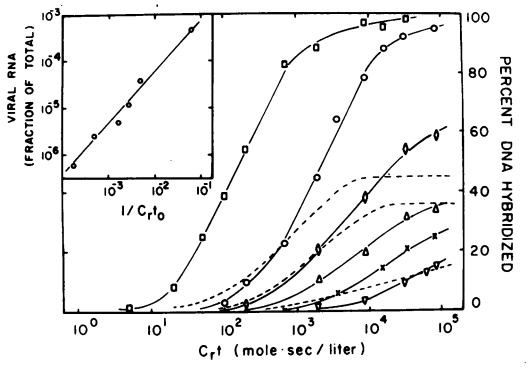

FIG. 3. *Analysis of hybridization levels in reconstruction experiments using heterologous RNA and various amounts of viral RNA. Reaction mixtures contained 0.005 μg/ml of RAV-2 ^{3}H-DNA, 20 mg/ml of heterologous RNA from hamster embryo cells* (□), *gs$^-$h$^-$ chicken embryo cells* (X), *or yeast tRNA* (○, ◇, △, ▽); *and RAV-2 RNA at concentrations of 9.0 μg/ml* (□), *0.75 μg/ml* (○), *0.225 μg/ml* (▽), *0.10 μg/ml* (△), *0.05 μg/ml* (X), *and 0.012 μg/ml* (◇). *These amounts of viral and heterologous RNA would be equivalent to approximately 210* (□), *18* (○), *5* (◇), *2.4* (△), *1.2* (X), *and 0.29* (▽) *copies of viral RNA per cell (see text). C$_r$t values in the experiments were calculated from total RNA concentrations, rather than from viral RNA concentration as in Fig. 1. C$_r$t curves for uninfected cell RNA, shown in Fig. 2 (curves D, E, F), have been superimposed (broken lines) to facilitate comparison with the curves obtained in these reconstruction experiments. The presence of 20 mg/ml of heterologous RNA, whether from hamster cells, gs$^-$h$^-$ chicken cells, or yeast tRNA, does not affect the kinetics of the annealing reaction. (Inset) Relationship between relative viral RNA concentration and C$_r$t$_0$ values obtained in reconstruction experiments. C$_r$t$_0$ values were determined from the C$_r$t curves shown above, by extrapolating the linear portion of each curve to the base line (i.e., to 0% hybridization). The linear portions used for this analysis were taken from points 3-6 on the first two curves* (□, ○) *and points 2-4 on the remaining curves.*

paring the half-C$_r$t values with that of RAV-2 RNA, this method might not be valid for uninfected cells containing much lower concentrations of viral RNA. To evaluate results obtained with uninfected cell RNA, we have plotted a series of C$_r$t curves based on reconstruction experiments in which RAV-2 ^{3}H-DNA was hybridized with mixtures containing various low amounts of viral RNA, and 20 mg/ml of heterologous RNA (see Fig. 3). At low RNA-DNA ratios the half-C$_r$t does not accurately reflect the initial concentration of viral RNA, since the kinetics of hybrid formation are affected by the change in viral RNA concentration as the reaction progresses. However, a C$_r$t value which more nearly reflects the initial reaction rate can be obtained by extrapolating the C$_r$t curve to the base line. This extrapolated value, which we shall call C$_r$t$_0$, appears to be a more valid parameter for comparison of hybridization data at low RNA-DNA ratios. When 1/C$_r$t$_0$ was plotted against the relative concentration of viral RNA in the reconstruction experiments, a nearly linear relationship was obtained, even at the lowest RNA concentrations used (see inset, Fig. 3). Thus, instead of half-C$_r$t values, we have used these C$_r$t$_0$ values for estimating the amount of viral RNA in uninfected cells.

A second problem in estimating the amount of viral RNA in uninfected cells arises from the fact that only a fraction of the viral RNA sequences were detected in these cells. Both the plateaus and the slopes of the C$_r$t curves for uninfected cell RNA (Fig. 3, broken lines) were significantly lower than those obtained at comparable C$_r$t values with RAV-2 RNA (solid lines) in the reconstruction experiments. The precise fraction of viral RNA sequences present in the uninfected cells cannot be determined because of the heterogeneity of the ^{3}H-DNA (see Discussion). For this reason, the concentration of viral RNA in these cells cannot be calculated. However, the average number of copies of virus-specific sequences can be estimated from these data. The rate of hybridization is directly proportional to the number of the copies of unique RNA sequences in the reaction mixture which hybridize with the DNA, irrespective of the molecular weight of the RNA (4, 6). The number of copies of viral RNA sequences in uninfected cells was estimated from C$_r$t$_0$ values, using the relationship between C$_r$t$_0$ and relative viral RNA concentration shown in Fig. 3 (inset) as a standard. One genome equivalent of viral RNA per cell corresponds to approximately 2.1×10^{-6} μg of viral RNA per μg of total RNA, based on a value of 1.7×10^{-11} μg of RNA per viral genome (11) and an estimated 8×10^{-6} μg of total RNA per cell. Based on these calculations we have obtained values of 36, 15, and 3 copies of viral-specific RNA per cell for gs$_L$h$_E$, gs$^+$h$_H$, and gs$^-$h$_L$ cells, respectively. These numbers represent average values for those sequences which hybridize specifically with the RAV-2 ^{3}H-DNA. The range of values for the four embryos of gs$^+$h$_H$ type was 14 to 20 copies per cell; values of 36 and 40 copies were obtained for the two embryos of type gs$_L$h$_E$. One embryo of type gs$^+$h$_H$ (embryo 1624) gave a value similar to those obtained for embryos of type gs$_L$h$_E$ (38 copies per cell).

TABLE 4. *Comparison of hybridization levels using ^{3}H-DNA from RAV-2 and SR-RSV*

Source of RNA	Embryo no.	% DNA Hybridized[a]	
		RAV-2 ^{3}H-DNA	RSV ^{3}H-DNA
Hamster embryo cells		1.5	1.4
Chicken cells:			
gs^-h^-	1603	0.8	1.1
gs^-h^-	1622	1.7	1.3
gs^-h_L	1541	13	11
gs^+h_H	1621	36	31
gs^+h_H	1624	47	36
gs_Lh_E	1620	46	40
RAV-2		98	69
SR-RSV		88	97

[a] Determined at a C_rt value of 0.5×10^5 for cellular RNA; hybridization levels for viral RNA are maximum plateau values.

Hybridization of cell RNA with ^{3}H-DNA from SR-RSV virions. Approximately 98% of the ^{3}H-DNA synthesized from SR-RSV virions hybridizes with SR-RSV RNA, but a maximum of only 69% hybridization was obtained with RAV-2 RNA (see Table 4). Thus, a significant fraction of the RSV ^{3}H-DNA represents sequences which are not present in RAV-2 RNA. These additional sequences may include viral genes involved in the transformation process.

It seemed possible that some of these additional sequences might be present in cells which do not contain RNA homologous to the RAV-2 ^{3}H-DNA. For this reason, samples of RNA from cells of each type were tested for hybridization with RSV ^{3}H-DNA. As in the previous experiments, no viral-specific RNA was detected in the gs^-h^- cells (see Table 4). Significant levels of hybridization were observed with RNA isolated from cells which contained gs antigen or helper activity. The values obtained with the RSV ^{3}H-DNA were somewhat lower than those obtained with RAV-2 ^{3}H-DNA. This difference would be expected if the additional sequences in the RSV DNA were not present in uninfected cells.

DISCUSSION

Previous investigators have detected virus-specific RNA in infected chicken cells, but not in uninfected cells (9, 16). The failure to detect viral RNA in the uninfected cells may have been due to limits in sensitivity under the conditions employed. However, cells used in the previous studies were not examined for gs antigen or helper activity, so the results cannot be directly compared with those reported here. Recently, Leong et al. (30) have briefly described preliminary observations suggesting the presence of viral RNA in some normal chicken cells.

The sensitivity of the hybridization analysis is primarily determined by three factors: incubation time, RNA concentration, and specific activity of the radiolabeled DNA. We have tried to optimize the conditions for this assay by performing the reaction at a relatively low temperature (45 C) in the presence of formamide, thus permitting prolonged incubation without serious thermal degradation of RNA (4, 7). The use of S-1 nuclease to analyze hybrid formation provides several advantages over other methods (30, 37, 41). The assay is relatively simple, permitting the handling of large numbers of samples; low and consistent background levels are obtained; and high concentrations of RNA can be used, thus increasing the sensitivity of the assay. RNA concentrations of up to 20 mg/ml were used in our experiments. This high concentration did not affect the kinetics of the annealing reaction. The conditions used in these studies permit the detection of less than 0.3 viral genome equivalents/cell, as demonstrated in reconstruction experiments (Fig. 3).

In our studies we have used only single-stranded DNA complementary to the viral RNA. Thus, the results presented here do not apply to minus-strand RNA. Reports from other laboratories indicate that only the plus strand of viral RNA is synthesized in virus-infected cells (9, 30). One serious limitation to the use of virion-synthesized DNA is the heterogeneity of the DNA product. Essentially all of the viral sequences are transcribed into DNA (12, 17). However, the transcription is nonsymmetrical, with the majority of DNA representing a small fraction (5-25%) of the viral genome (17, 39). Thus, viral sequences which are represented at very low levels in the ^{3}H-DNA may not be detected with this assay. Likewise, the plateau levels of hybridization obtained with helper-positive cell RNA do not necessarily reflect the fraction of viral RNA present in these cells.

No viral RNA ($<<0.3$ copies per cell) was detected in uninfected cells which lacked gs antigen or helper activity. However, significant levels of viral RNA were found in all cells which contained either of these viral functions. The amount of RNA in helper-positive cells, ranging from 3 to 40 copies per cell, correlated reasonably well with the level of helper activity. Only a fraction of the viral RNA sequences appeared to be present in these cells, however. This was indicated both by the low plateau levels and by the decreased slope of the C_rt curves for cellu-

lar RNA as compared with those for RAV-2 RNA at similar C_rt values (see Fig. 3). The analysis of RNA from cells with low helper activity was not carried to a high enough C_rt value to determine a plateau level. However, the slope obtained with this sample was lower than that for comparable reconstruction curves, suggesting that a similar plateau would be obtained if the assay were extended to higher levels of sensitivity. Several possibilities can be offered to explain these results. (i) A significant portion of the viral genes in these cells may be repressed or expressed at levels too low to be detected in this assay. (ii) The viral genes in these cells may contain significant regions which are not homologous with the RAV-2 ^{3}H-DNA. However, similar plateau levels were obtained with ^{3}H-DNA from SR-RSV. Furthermore, a high degree of cross-homology was observed between RAV-2 ^{3}H-DNA and RNA from two other leukoviruses, SR-RSV and RAV-60. The RAV-60 preparation was derived from cells of the type studied here and presumably contains viral genetic material from the host cell. (iii) The cells may contain incomplete viral genomes. This could explain why spontaneous production of virus particles is never observed in these cells. RAV-60 is recovered only after infection with other leukosis or sarcoma viruses. Furthermore, the induction of virus production has not been observed in these cells by treatment with various chemical or physical agents (T. Hanafusa, *personal communication*). Cells used in these studies appear to differ in this respect from those studied by Weiss et al. (44), which produced low levels of virus following treatment with chemical and physical agents.

The average number of viral RNA copies in uninfected cells or whether it is induced by the mately 1% of that in infected cells. This could mean either that 1% of the cells contain viral RNA at high levels, equal to that in infected cells, or that all cells contain low levels of viral RNA. No virus-specific RNA sequences were detected in uninfected helper-negative cells. However, the presence of one cell per 10^5 which contained viral RNA in amounts equal to that in the infected cells would not be detected at the levels of sensitivity used in this assay.

The presence of helper activity in chicken cells is determined by a biological assay which involves infection with B-RSV. It is not known whether this function is normally expressed in uninfected cells or whether it is induced by the infecting virus. The hybridization analysis used in these studies does not permit identification of the individual RNA species which code for this function. However, the correlation observed between helper activity and viral RNA levels in uninfected cells suggests that the viral genes are expressed even in the absence of viral infection.

Previous experiments have demonstrated that helper-negative cells from these chicken flocks contain viral DNA which can be detected both by molecular hybridization (34) and by rescue of RAV-60 following superinfection (25). The absence of detectable levels of viral RNA in these cells suggests that viral gene expression is controlled at the transcription level. However, a mechanism which involves rapid breakdown of viral RNA cannot be excluded.

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