Acquisition of New DNA Sequences After Infection of Chicken Cells with Avian Myeloblastosis Virus

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DNA-RNA hybridization studies between 70S RNA from avian myeloblastosis virus (AMV) and an excess of DNA from (i) AMV-induced leukemic chicken myeloblasts or (ii) a mixture of normal and of congenitally infected K-137 chicken embryos producing avian leukosis viruses revealed the presence of fast- and slow-hybridizing virus-specific DNA sequences. However, the leukemic cells contained twice the level of AMV-specific DNA sequences observed in normal chicken embryonic cells. The fast-reacting sequences were two to three times more numerous in leukemic DNA than in DNA from the mixed embryos. The slow-reacting sequences had a reiteration frequency of approximately 9 and 6, in the two respective systems. Both the fast- and the slow-reacting DNA sequences in leukemic cells exhibited a higher T_m (2 C) than the respective DNA sequences in normal cells. In normal and leukemic cells the slow hybrid sequences appeared to have a T_m which was 2 C higher than that of the fast hybrid sequences. Individual non-virus-producing chicken embryos, either group-specific antigen positive or negative, contained 40 to 100 copies of the fast sequences and 2 to 6 copies of the slowly hybridizing sequences per cell genome. Normal rat cells did not contain DNA that hybridized with AMV RNA, whereas non-virus-producing rat cells transformed by B-77 avian sarcoma virus contained only the slowly reacting sequences. The results demonstrate that leukemic cells transformed by AMV contain new AMV-specific DNA sequences which were not present before infection.

DNA sequences complementary to the RNA of avian myeloblastosis virus (AMV) and other avian leukosis viruses (ALV) are present in all apparently normal chicken cells (3, 6, 19, 25, 26, 29, 35). The production of ALV of subgroup E either spontaneously, or by induction with various chemical and physical agents, suggests that some cells derived from normal chicken embryos contain an entire copy of the ALV genome (17, 37). Other chicken embryos whose cells express either the ALV group-specific (gs) antigen or a glycoprotein which complements the replication of defective Rous sarcoma virus (chicken helper factor) contain at least partial copies of ALV genomes (3, 6, 19, 29). After infection with AMV, the amount of viral-specific DNA sequences increased in leukemic cells, kidney tumor cells, and nontransformed virus-producing cells (3, 4, 6). Definitive evidence for the acquisition of new genetic information after infection with avian oncornaviruses has been obtained in rat cells which did not contain DNA sequences complementary to avian oncornaviruses before infection but which did contain such sequences after transformation by B-77

avian sarcoma virus (3). Similar results were also obtained with rat cells and mouse cells transformed with Rous sarcoma virus (RSV) (18, 36).

In our previous studies (3, 6), the DNA sequences complementary to AMV or RSV were detected by hybridization of denatured cellular DNA immobilized on filters with an excess of viral RNA. Although this RNA-DNA hybridization revealed quantitative differences in the content of oncornavirus-specific sequences between normal and leukemic chicken cells, it could not establish what proportion of the AMV genome was represented in both types of cells. However, both quantitative and qualitative differences in viral-specific sequences can be detected by hybridization of viral RNA in solution with an excess of cellular DNA (24). Therefore, we have used DNA-RNA hybridization in DNA excess to investigate the nature of the DNA sequences complementary to AMV, RNA present in (i) gs antigen-positive, non-virus-producing chicken embryos, (ii) in gs antigen-negative, non-virus-producing chicken embryos, (iii) in virus-producing leukemic chicken myeloblasts,

(iv) in normal rat cells, and (v) in non-virus-producing rat cells transformed by B-77 avian sarcoma virus (1, 8, 9, 14, 16, 24). The results revealed both qualitative and quantitative differences between the viral-specific DNA sequences present in uninfected chicken cells and those in AMV-induced leukemic cells. In confirmation of earlier results (3), B-77-transformed rat cells acquired avian sarcoma virus-specific DNA sequences which were not present in untransformed rat cells.

MATERIALS AND METHODS

Virus. Avian myeloblastosis virus, strain A, of the Bureau of Animal Industries, subgroup B (Vogt classification) was used.

Embryonated chicken eggs. Chickens of the White Leghorn strain, cross K-137, were obtained from Kimber Farms, Pomona, Calif. The background incidence of spontaneous leukosis in this strain was found to be 8.2% by observation of untreated chickens for 8 to 12 months after hatching (2). Chicken embryonated eggs from parents homozygous for the absence of gs antigen (gs negative) were kindly supplied by R. E. Luginbuhl, the University of Connecticut College of Agriculture and Natural Resources, Storrs, Conn., and were hatched in our facilities. When K-137 embryos were tested for congenital infection with leukosis virus by the oncornavirus production assay method (5), about 15 to 20% were found to be positive. However, we have not found virus producers among 24 gs antigen-negative chicken embryos which we tested.

Virus production assay. Cell cultures were prepared from the thigh muscles of 19- to 20-day-old individual embryos and tested for ALV production by incubation for 10 h in modified Eagle medium containing 5% dialyzed chicken serum, 5% dialyzed calf serum, 10^{-6} M thymidine, and 10 μCi of [^{3}H]uridine per ml (24 to 31 Ci/mmol). The labeled virions were detected as described earlier (5).

Transformed embryonic rat cells. Embryonic rat cells, transformed with the B-77 strain of avian sarcoma virus (1), were kindly supplied by P. K. Vogt and cultivated in modified Eagle medium with 5% calf serum.

Leukemic myeloblasts. Leukemic myeloblasts were obtained from the peripheral blood of acutely leukemic chicks (28).

^{3}H-labeled 70S AMV RNA. The preparation and purification of ^{3}H-labeled AMV and the isolation of ^{3}H-labeled 70S RNA from purified AMV virions were described previously (6).

Preparation of DNA for liquid hybridization. The extraction and purification of DNA from normal and leukemic tissues were performed as before (6). The purified DNA was dialyzed extensively against 0.1 × SSC (1 × SSC = 0.15 M sodium chloride + 0.015 M sodium citrate) and 0.001 M EDTA to remove traces of contamination of metal ions. DNA fragments of 9 to 10S were obtained by shearing for 5 min at 0 C in a Waring blender; fragments of 6.4S were obtained by ultrasonic disruption for 5 min at 0 C with a model

1000 insonator from Precision Cells. The sedimentation coefficient of the fragments was determined by alkaline sucrose velocity sedimentation.

Preparation of E. coli cRNA with homologous DNA. Purified *Escherichia coli* DNA-dependent RNA polymerase prepared by the method of Burgess (11) was purchased from Miles Laboratories, Ind. The reaction mixture contained the following: 0.04 M Tris-hydrochloride (pH 8.0), 0.01 M MgCl$_2$, 0.25 mM dithiothreitol, 0.15 M KCl, 0.5 mg of bovine serum albumin per ml, 0.15 mM each of GTP, ATP, and CTP, 0.15 mM of [^{3}H]UTP (16.6 Ci/mmol), and 40 μg of enzyme and 10 μg of *E. coli* DNA in 0.5 ml. The mixture was kept at 37 C for 20 h. DNA was then digested with DNase (RNase-free from Worthington, further purified by glycerol gradient velocity sedimentation) at a concentration of 20 μg/ml for 20 min at 37 C. After adding yeast RNA (1 mg) and sodium dodecyl sulfate (SDS) (0.5% final concentration), the reaction mixture was passed through a Sephadex G-50 column (1.8 by 32 cm) in TE buffer (0.02 M Tris-hydrochloride, pH 7.4, and 1 mM EDTA) plus 0.1% SDS. The first peak was collected and extracted twice with TE buffer-saturated phenol, and the RNA was precipitated with ethanol. From the specific activity of [^{3}H]UTP and the guanine-cytosine content of *E. coli* DNA (50%), the homologous *E. coli* complementary RNA (cRNA) should have a specific activity of 1.9 × 10^7 disintegrations per min per μg.

^{3}H-35S AMV RNA. [^{3}H]uridine- and [^{3}H]cytidine-labeled 70S RNA was converted into 35S RNA by heating at 70 C for 3 min in 0.01 M Tris-0.001 M EDTA buffer, pH 7.4. After addition of NaCl to a concentration of 0.1 M, the RNA was fractionated on a 5 to 20% sucrose velocity gradient and the ^{3}H-labeled 35S AMV RNA was isolated.

DNA-RNA hybridization on cellulose nitrate filters. The procedures for immobilizing denatured cellular DNA on filters and for hybridizing ^{3}H-labeled 70S RNA in excess with the immobilized DNA have been described (3).

Liquid hybridization of ^{3}H-labeled 70S RNA in DNA excess. The hybridization mixture contained 3 to 4 mg of cellular DNA per ml sheared into 9 to 10S fragments (average molecular weight, 436,000) or into 6.4S fragments (average molecular weight, 162,000), sonically treated (average size of 8 to 10S) ^{3}H-labeled 70S AMV RNA (specific activity 10^6 counts per min per μg), and 0.1% SDS in 0.4 M phosphate buffer (pH 6.8) or 4 × SSC. The hybridization was carried out in tightly silicone-stoppered tubes. After boiling for 3 min in a water-ethylene glycol bath, the mixture was quickly transferred to a bath at 60 C. Samples of 0.25 ml were removed at different time intervals and diluted to a DNA concentration of 50 μg/ml in a final salt concentration of 2 × SSC. The samples were divided into two portions, one portion was incubated with pancreatic RNase A (15 μg/ml) and ribonuclease T1 (15 U/ml) for 1 h at 37 C. The other portion was incubated in the absence of RNases. Cold trichloroacetic acid (50%) was added to give a final concentration of 5% to precipitate the undegraded RNA. After 30 min or longer at 0 to 4 C, the precipitate was collected on cellulose nitrate filters (Millipore Corp.), washed with 50 ml of 5% cold trichloroacetic acid,

dried, and counted. Chicken DNA was replaced by mouse DNA in the control tubes. The zero time values obtained by immediate quenching of the denatured mixture were deducted from the experimental values. The acid-precipitable RNase-resistant ^{3}H-RNA counts in a mixture of ^{3}H-70S RNA and mouse DNA remained at background level at all time intervals.

Reassociation of denatured DNA. DNA renaturation in 0.4 M phosphate buffer was monitored by separation of single- and double-stranded DNA on hydroxylapatite columns (7). Samples of 0.1 ml (except at Cot of 10 for which the sample was 0.2 ml) were removed at given time intervals, diluted with ice-cold water to a final phosphate buffer concentration of 0.05 M, and applied to a hydroxylapatite column at 60 C. The column was washed with 2 bed volumes of 0.05 M phosphate buffer. Single-stranded DNA was eluted with 0.14 M phosphate buffer, and double-stranded DNA was eluted with 0.4 M phosphate buffer (7). The concentration of DNA in single-stranded or in double-stranded fractions was calculated from the absorbance at 260 nm after making a correction for the hyperchromic shift of denatured DNA.

RESULTS

Liquid hybridization of E. coli DNA in excess with homologous cRNA. The analytical complexity (20) of the *E. coli* genome is the same as its molecular weight, 2.7×10^9, since *E. coli*, like other prokaryotes, does not contain reiterated DNA sequences (10, 13). The guanine-cytosine content of *E. coli* DNA is 50% (34). The plot of the fraction of *E. coli* cRNA which hybridized in 0.4 M phosphate buffer (pH 6.8) with denatured homologous DNA versus log Cot (Cot = concentration of nucleotides in moles per liter multiplied by the reaction time in seconds) gives a monophasic curve that represents the hybridization of unique RNA and DNA sequences (Fig. 1). Of the total input RNA, 58% was rendered RNase resistant at a Cot of 5,000 mol s liter^{-1}. The normalized data (8) corrected for RNA hybridized at Cot zero and for RNA not hybridized at Cot infinity show that the Cot$^h_{1/2}$ for the unique *E. coli* cRNA sequences was 22 mol s liter^{-1}.

Liquid hybridization of 70S AMV RNA with excess DNA from leukemic myeloblasts or normal K-137 chicken embryos. Figure 2 shows the fraction of 70S viral RNA rendered RNase resistant after different time intervals (expressed as Cot values) of hybridization with leukemic DNA (curve 2) or with K-137 chicken embryonic DNA (curve 3). For comparison, the reassociation curve of denatured chicken DNA has been included in this figure (curve 1). As reported for other vertebrate DNAs (10), the DNA reassociation curve as a function of log Cot is biphasic, indicating the presence of reiterated and unique sequences in chicken DNA. About
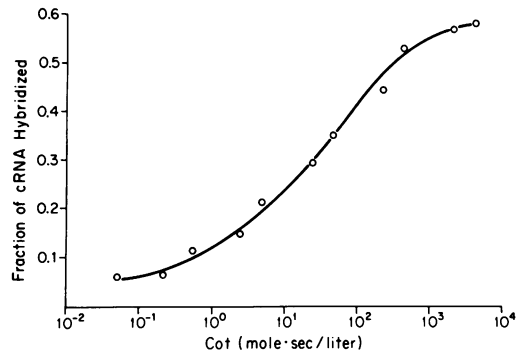

FIG. 1. *Kinetics of hybridization of E. coli DNA with homologous cRNA synthesized in vitro with the E. coli DNA-dependent RNA polymerase system. The hybridization of E. coli DNA sheared into 6.4S fragments with homologous cRNA was carried out under conditions described in the text. To obtain Cot values between 10^{-2} and 10^1, lower DNA concentrations (0.1 to 1.0 mg per ml) and lower salt concentrations (0.05 M to 0.12 M phosphate buffer, pH 6.8) were used. Appropriate corrections were made to present the Cots for 0.18 M equivalent monovalent cation concentration according to Wetmur and Davidson (38).*
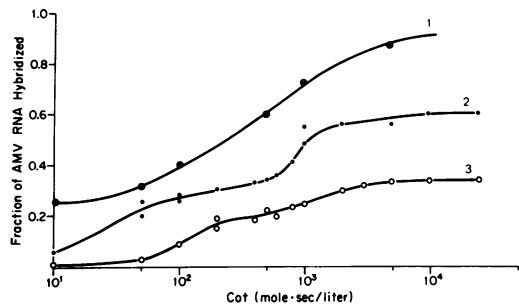

FIG. 2. *Kinetics of reassociation of chicken DNA and hybridization of 70S AMV RNA with leukemic DNA, or with DNA from 24 pooled K-137 chicken embryos. Denatured normal chicken DNA sheared into 9 to 10S fragments was reassociated in 0.4 M phosphate buffer. The hybridization of 70S viral RNA in DNA excess was carried out in 4 × SSC as described in Materials and Methods. Curve 1, Reassociation of denatured chicken DNA; curve 2, Leukemic DNA (●); curve 3, K-137 chicken embryo DNA (○).*

25% of the total chicken DNA renatured rapidly with a Cot$_{1/2}$ of about 0.03 mol s liter^{-1}. The remaining chicken DNA reassociated slowly with a Cot$_{1/2}$ of 475 mol s liter^{-1}. The reassociation profile of DNA from leukemic chicken myeloblasts was similar to that of normal chicken embryo DNA.

The leukemic DNA was isolated from several leukemic chicks, and the K-137 DNA was prepared from 24 chicken embryos, which, although apparently normal, had not been tested

for ALV production due to congenital infection. In all likelihood, 15 to 30% were congenitally infected by an ALV other than AMV. Therefore, this K-137 DNA represents a mixture of DNAs from nonproducing and ALV-producing embryos. As expected from theoretical considerations, the rate of hybridization of AMV RNA with the leukemic or normal DNA was slower than the rate of renaturation of DNA. The kinetics of DNA-RNA hybridization showed biphasic components representing fast- and slow-hybridizing DNA sequences in both the normal and the leukemic DNA preparations. However, the leukemic DNA appeared to contain twice as many virus-specific sequences as did the K-137 embryonic DNA. Whereas 60% of the AMV RNA was rendered RNase resistant with leukemic myeloblast DNA, only 34% was rendered RNase resistant with K-137 chicken embryo DNA. Approximately equal proportions of the AMV RNA were hybridized to the fast-reacting and to the slow-reacting sequences in the DNA from leukemic cells or from the mixed embryos.

The normalized (8) kinetics of hybridization of the fast-reacting and slow-reacting parts of the curves of Fig. 2 are shown in Fig. 3 and are compatible with a second-order reaction. Both types of virus-specific sequences were more numerous in the leukemic DNA than in the K-137 embryonic DNA. The $Cot^h{}_{1/2}$ values of the fast-reacting sequences in DNA from leukemic cells or from the mixture of congenitally infected and normal K-137 chicken embryos can be estimated to be approximately 40 and 120 mol s liter^{-1}, respectively. The $Cot^h{}_{1/2}$ values for the slow-reacting DNA sequences can be estimated to be approximately 1,000 and 1,500 mol s liter^{-1} for the leukemic myeloblasts and for the mixed K-137 chicken embryos, respectively.

The molecular weight of the chicken genome is 1.44×10^{12} and 25% of the total chicken DNA is reiterated, representing families of DNA sequences with 10 to 10^6 copies. Assuming that the reiterated DNA is equally divided among the various families, the analytical complexity of chicken DNA would be $[75 + (25/6) (10^{-1} + 10^{-2} + 10^{-3} + 10^{-4} + 10^{-5} + 10^{-6})] (1.44/100) \times 10^{12}$ or approximately 1.09×10^{12}. Therefore, the reiteration frequency of the fast-reacting sequences in DNA from leukemic cells and from the K-137 chicken embryonic cells can be estimated to be approximately 200 and 75 copies per cell genome, respectively (24). The slow-reacting viral-specific DNA sequences in the leukemic DNA have an estimated reiteration frequency of 9 copies per cell genome, whereas in the DNA mixture from congenitally infected
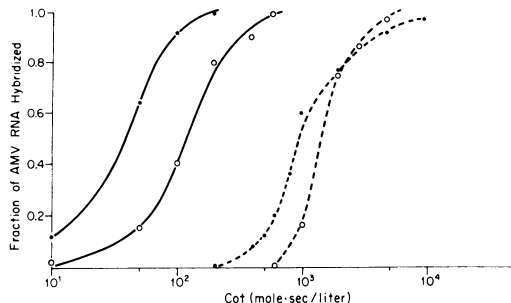

FIG. 3. *Normalized kinetics of hybridization of 70S AMV RNA with leukemic DNA, or with K-137 DNA. The fast and slow phases of the hybridization curves in Fig. 2 were separated into two curves and normalized according to Bishop (8) after correcting for the fraction of RNA which is RNase resistant at zero Cot and for the fraction of RNA which did not hybridize at infinite Cot. Symbols:* ●, *leukemic DNA;* ○, *K-137 chicken embryo DNA;* ——, *fast reacting sequences;* - - -, *slowly reacting sequences.*

and normal embryos they have an estimated reiteration frequency of 6 copies per genome. Similar values for the reiteration frequency of fast and slow sequences in leukemic DNA have been obtained in three experiments. The reproducibility of the determination in normal cells can be judged from results in Table 1. When tested by the filter hybridization technique in viral RNA excess (3), the same DNAs from the leukemic myeloblasts and the K-137 chicken embryos contained, respectively, 21 and 12 copy equivalents of the 36S viral RNA subunit per cell.

In experiments not shown, the maximal amount of RNA hybridized increased with increasing temperatures, showing an optimum at 65 C for both the normal and the leukemic DNA. If the temperature was further increased, the amount of RNA in RNA-DNA hybrids decreased. Under similar experimental conditions, the AMV RNA hybridized to mouse DNA remained constant, reflecting nonspecific binding. The temperature optimum for AMV RNA hybridization with DNA from leukemic or normal cells was 26 and 24 C, respectively, below the T_m of the respective DNA-RNA hybrids (see Fig. 7).

Liquid hybridization of AMV RNA with excess DNA from individual non-virus-producing chicken embryos. The K-137 DNA used in the previous hybridization experiments was extracted from 24 pooled chicken embryos and, as mentioned earlier, some 15 to 30% were probably congenitally infected and ALV producers. To determine whether all normal chickens contain both fast- and slow-reacting viral-specific sequences, AMV RNA was hy-

TABLE 1. *Hybridization of 70S or 35S AMV RNA with DNA from individual nonproducer chicken embryos*[a]

DNA	Viral RNA	Viral RNA hybridized (%)		Reiteration frequencies		Viral equivalents/cell[b]
		Cot 300	Cot 25,000	Fast	Slow	
K-137 embryo						
1	70S	3.0	37	44.4	4.2	ND[c]
2		3.0	36	44.4	4.2	ND
3		9.5	34	74.0	1.7	ND
4	35S	7.0	31	44.4	5.9	10.7
5		5.0	25	88.8	3.6	10.6
6		7.0	26	44.4	5.9	10.7
gs-negative embryo						
1	70S	5.0	28	74.0	2.2	ND
2		4.0	30	59.2	3.0	ND
3		4.5	21	59.2	2.5	ND
4	35S	3.5	32	98.7	3.9	9.2
5		5.0	35	88.8	3.9	7.4
6		4.5	31	88.8	4.0	7.4

[a] DNA was extracted and purified from 20-day-old chicken embryos which had been tested for the absence of ALV production. About 3 to 5 mg of purified DNA was obtained from each embryo and was sheared into 6.4S fragments. The DNA-RNA hybridization in DNA excess has been described in Materials and Methods. The viral RNA concentration was adjusted in such a way that the cellular DNA-to-viral RNA ratio was the same for every embryo. The data were normalized and plotted to determine the $Cot^h_{1/2}$.

[b] The number of DNA equivalents per cell of the viral 35S RNA subunit was determined by the filter hybridization technique.

[c] ND, Not done.

bridized with DNA from separate non-virus-producing 19- to 20-day-old chicken embryos. DNA extracted from six separate non-virus-producing K-137 chicken embryos and from six separate gs antigen-negative, non-virus-producing (Conn.) chicken embryos was sheared into 6.4S fragments and hybridized individually either with 70S or 35S viral RNA. The results (Fig. 4 and 5 and Table 1) indicate that these DNAs appeared to contain both types of viral-specific DNA sequences but fewer of the fast-reacting sequences than the K-137 DNA tested earlier. Only one-fifth to one-tenth of the hybridizable viral RNA was hybridized by the fast-reacting sequences. The maximum amount of input AMV RNA rendered RNase resistant varied between 30 and 36% for the different K-137 embryos, and was between 21 and 35% for the different gs-negative embryos. There was no detectable difference if 35S AMV RNA was used instead of 70S RNA. The reiteration frequencies of the fast- and the slow-reacting DNA sequences calculated from the $Cot^h_{1/2}$ values were similar in the gs-negative Conn. embryos and in the K-137 embryos which are mostly gs positive (albeit, they were not tested for gs antigen in this experiment) (Table 1). The reiteration frequencies of the slow sequences were remarkably close to the number per cell genome of DNA equivalents of the 35S AMV
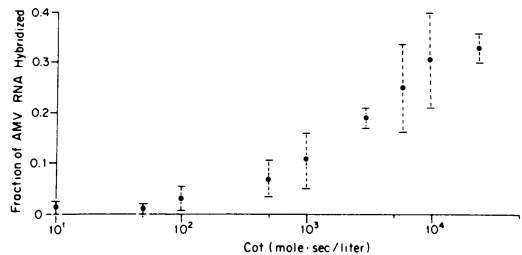

FIG. 4. *Hybridization of AMV RNA with DNA from individual non-virus-producing K-137 chicken embryos. About 3 to 5 mg of purified DNA was obtained from each one of six 20-day-old chicken embryos which had been tested for the absence of ALV production. The DNA-RNA hybridization was carried out in an excess of DNA sheared into 6.4S fragments. The viral RNA concentration was adjusted in such a way that the cellular DNA-to-viral RNA ratio was the same for every embryo. DNA from three separate embryos was hybridized with 70S AMV RNA and DNA from the other three separate embryos was hybridized with 35S AMV RNA. The mean and standard deviation of the fraction of RNA hybridized (six determinations) was plotted against log Cot.*

subunit determined by the filter hybridization technique in RNA excess.

Hybridization of AMV RNA with DNA from normal rat cells and from rat cells transformed by B-77 avian sarcoma virus. The kinetics of hybridization of AMV RNA with
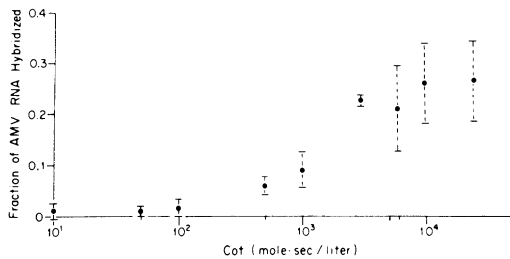
FIG. 5. Hybridization of AMV RNA with DNA from individual non-virus-producing gs-negative chicken embryos. The experiment was carried out as in Fig. 4.

DNA sheared into 6.4S fragments from normal rat cells or from rat cells transformed by B-77 avian sarcoma virus (RB-77 cells) showed (Fig. 6) that normal rat cell DNA did not contain nucleotide sequences complementary to AMV RNA, but that the RB-77 cells did contain such sequences. This confirms the findings obtained by the filter hybridization technique (3). Also, the DNA from RB-77 cells appeared to contain only the slow-reacting virus-specific sequences which have a $Cot^h{}_{1/2}$ value of approximately 5,000 mol s liter^{-1} and therefore a reiteration frequency of approximately 2. Only 16% of the input AMV RNA became RNase resistant at a Cot of 25,000 mol s liter^{-1}.

Thermal dissociation of DNA-RNA hybrids. The thermal stability of DNA-RNA hybrids is a function of the size and the base composition of the two nucleic acid strands and of the proportion of complementary base pairs in the hybrids (23). Therefore, the thermal dissociation curves and the 50% dissociation temperature (T_m) of hybrids formed between 70S AMV RNA and DNA from AMV-producing leukemic cells or from non-virus-producing K-137 or gs-negative, normal chicken embryonic cells were compared to determine the extent of base pairing in these various hybrids. In addition, the hybrids formed with the different DNAs were separated into two classes: (i) hybrids formed at a Cot of 400 which represent mostly the fast-hybridizing sequences, and (ii) hybrids formed at a Cot of 10,000 which represent all the hybridizable sequences. At a Cot of 400, 19.1% of the AMV RNA was rendered RNase resistant by leukemic DNA, 11.5% was rendered resistant by the K-137 DNA, and 8.6% was made resistant by the gs-negative Conn. DNA. At a Cot of 10^4, the percentages of AMV RNA rendered RNase resistant were 60.2% with leukemic DNA, 41.5% with K-137 DNA, and 39.3% with gs-negative DNA. The DNA-RNA hybrids formed in liquid between AMV RNA and a large excess of leukemic or normal cellular

DNA (Fig. 7) demonstrated a high thermal stability indicating that long sequences of complementary base pairs were involved (12, 21, 22, 27, 30). Similar results were obtained earlier by the filter hybridization technique with immobilized denatured cellular DNA and a large excess of AMV RNA (6). As found in our previous study, the total hybrids formed by the normal cell DNAs have a slightly lower T_m (89 C) than that of the total hybrids (91 C) formed with leukemic cell DNA. Similarly, the fast-reacting sequences in leukemic DNA formed hybrids with AMV RNA that had a slightly higher T_m (89 C) than the T_m of the hybrids (87 C) formed by the fast-reacting sequences of the normal DNAs. There was no significant difference between the DNAs from K-137 embryos and from gs-negative embryos. If the total hybridizable sequences are corrected for the presence of the fast-reacting sequences, the slowly hybridizable DNA sequences (Fig. 7, broken lines) are found to dissociate with a T_m of 93 C for the leukemic DNA and with a T_m of 91 C for the normal DNAs. Therefore, the T_m of the RNA-DNA hybrids formed by the fast or slow viral-specific sequences is 2 C higher in leukemic DNA than in normal DNA. Similar findings have been reported for Rous sarcoma virus by Neiman (25). This indicates that the viral-specific DNA sequences synthesized after infection with AMV have slightly more complementarity to AMV RNA than the endogenous DNA sequences complementary to AMV RNA present in normal cells before infection.

DISCUSSION

In agreement with our earlier findings obtained by the filter hybridization technique in RNA excess, 70S, or 35S, AMV RNA hybridizes more with DNA from leukemic chicken myeloblasts than with DNAs from normal non-virus-
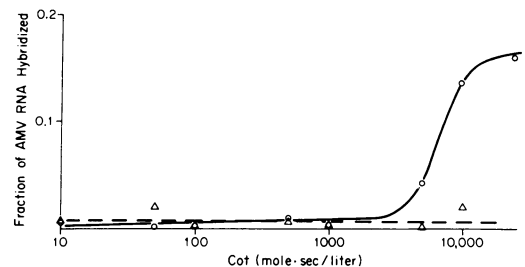

FIG. 6. Hybridization of 70S AMV RNA with DNA from normal or B-77 sarcoma virus-transformed rat embryonic cells. The method of DNA-RNA hybridization was the same as that described in Fig. 2. Symbols: O, transformed rat cells DNA; Δ, normal rat DNA.
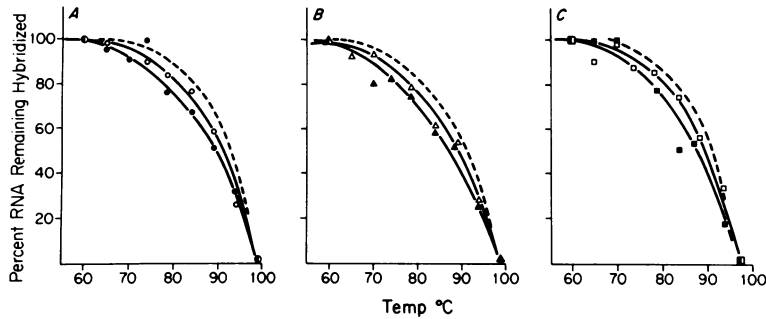
FIG. 7. *Thermal denaturation of DNA-RNA hybrids. Two vials each containing 12 mg of DNA (6.4S frag-ments) and 8,000 counts/min of AMV RNA (1.7 × 10^6 counts per min per μg) in 3 ml of 0.4 M phosphate buffer (pH 6.8), plus 0.1% SDS were hybridized at 60 C. One vial was hybridized to a Cot of 400 mol s $liter^{-1}$ and the other was hybridized to a Cot of 15,000 mol s $liter^{-1}$. Each reaction mixture was then diluted to 40 ml with cold distilled water and 2 × SSC to make the final salt concentration 2 × SSC. Duplicate 2-ml samples were re-moved, heated for 10 min at the indicated temperature, and immersed in ice water. The samples were further diluted with 2 × SSC to lower the DNA concentration to 50 μg/ml. One-half of each sample was treated with RNases A and T1 as described in Materials and Methods while the other half served as untreated control. All samples were then acid-precipitated and dried, and the radioactivity was determined. A: O, total AMV-specific DNA sequences in leukemic DNA; ●, fast-reacting AMV-specific DNA sequences in leukemic DNA. B: △, total AMV-specific DNA sequences in DNA from non-infected K-137 chicken embryos; ▲, fast-reacting AMV-specific sequences in DNA from non-infected K-137 chicken embryos. C: □, total AMV-specific DNA sequences in DNA from gs antigen-negative chicken embryos; ■, fast-reacting AMV-specific DNA sequences in DNA from gs antigen-negative chicken embryos; -----, slowly reacting AMV-specific sequences calculated by subtracting the fast-reacting sequences from total sequences data.*

producing, gs antigen-positive or -negative chicken embryos (5, 8, 33). In AMV-producing leukemic cells and in congenitally infected ALV-producing chicken embryos, there are two classes of viral-specific DNA sequences, distin-guished by the rapidity of their hybridization with viral RNA. Similar results have been recently reported for RSV and for Rous asso-ciated virus (O) (RAV[O]) (25, 26). The leuke-mic DNA appears to contain twice as many fast and slow AMV-specific sequences than does DNA from K-137 embryos, some of which were congenitally infected and produced some type of ALV. This reflects a lesser concentration of both types of AMV-specific sequences in the K-137 DNA than in leukemic cells transformed by AMV. This could be due either to the presence of partial ALV genomes, in DNA form, in normal cells, or to a limited homology between the genome of AMV which was used as a probe and the genomes of the endogenous integrated ALV and of the congenitally transmitted ALV. It should be pointed out that the RNA probe used may have contained sequences homologous to several ALV, since no steps were taken to avoid induction of endogenous ALV nor congenital infection in the preparation of the AMV stocks. A third alternative, the presence of fewer DNA equivalents undistinguishable from AMV DNA, can be ruled out because, as determined by both the filter and the liquid hybridization tech-

niques, the leukemic DNA contained approxi-mately twice as many viral DNA equivalents as did the normal DNA. Such a difference in viral DNA concentrations, and consequently in DNA-RNA ratios in the hybridization mixture, would not be sufficient to decrease the rate and the maximal level of hybridization by a factor of 2 (8). The limitations of the experimental proce-dures and the possible heterogeneity of the RNA probe do not allow a precise quantitation of the fast- and slow-hybridizing DNA sequences. Their nature and relationship to the number of viral genome equivalents present per cell is under investigation.

Since AMV RNA is presumably replicated via a DNA intermediate, leukemic myeloblasts which produce AMV should contain an entire DNA transcript of the AMV RNA. However, in our experiments, only 60 to 76% of the input RNA was rendered RNase resistant with leu-kemic DNA even at a Cot of 30,000 mol s $liter^{-1}$. The inability to render the viral RNA 100% resistant to RNase may be explained by the experimental conditions. Because of the tre-mendous increase in the viscosity of the hybridi-zation mixture at Cots of 10,000 mol s $liter^{-1}$ or above, there was a reduced rate of the collision of the reactants. Also, the overall rate of the reassociation of DNA is faster than the rate of DNA-RNA hybridization (8, 9), and, conse-quently, the ratio of denatured DNA to RNA

decreases with increasing Cot. In addition, at least part of the viral DNA is integrated in regions of the chicken DNA which are reiterated approximately 1,200 times and reassociate at a relatively fast rate (Evans, Baluda, and Shoyab, unpublished data). These factors tend to decrease the effectual DNA to RNA ratio at high Cots, thereby causing a decrease in the maximal level of hybridization (8). Therefore, 60 to 76% of RNase resistance may conceivably be considered the maximal level of hybridization under these conditions. Under identical conditions, the maximum proportion of AMV RNA hybridized with DNAs from normal embryos varied between 21 and 36%. Thus, it appears that only 35 to 60% of the AMV RNA can maximally hybridize to DNA from normal cells. As mentioned earlier, this could result from the presence of either incomplete DNA copies of some ALV genome or of complete DNA copies of some ALV genome which is only partially homologous to AMV. In support of the latter explanation, it has been reported that 70% of the RNA from RAV(O), which is a subgroup E virus representative of the endogenous integrated virus spontaneously produced by some normal chicken embryos, can be rendered RNase resistant in similar hybridization experiments with DNA from normal chicken cells (26).

The thermal denaturation curves of the DNA-RNA hybrids suggest that there is a difference in base composition between the hybridizing sequences in leukemic DNA and those in normal DNA. Therefore, the kinetics of hybridization, the maximal level of hybridization, and the thermal denaturation indicate that the viral-specific sequences in leukemic cells are qualitatively and quantitatively different from the viral-specific sequences present before infection with AMV.

It remains to be seen whether the newly synthesized viral-specific DNA sequences are alone responsible for the changes leading to malignancy or are responsible in conjunction with oncogenic information already present in the normal cells before infection. Rat cells transformed by B-77 sarcoma viruses acquire only the slowly reacting sequences. With one exception (32), mammalian cells transformed by avian sarcoma viruses do not release the avian virus (1), but they contain avian oncornavirus-specific DNA sequences (5, 42), and infectious virus can be rescued by fusion of transformed rat cells with chicken cells (14, 33). It is possible, therefore, that the slow-reacting DNA sequences may contain the information necessary for oncogenesis. However, the AMV

RNA used as a probe may not share homology with the fast-reacting B-77 specific sequences.

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