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Widespread Presence, in Chickens, of DNA Complementary to the RNA Genome of Avian Leukosis Viruses

(DNA–RNA hybridization/DNA intermediate/avian myeloblastosis virus)

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ABSTRACT **DNA–RNA hybridization experiments have demonstrated the widespread presence in chickens of DNA complementary to the RNA of avian myeloblastosis virus. All apparently normal chicken embryos, or adult chickens that were tested, contained viral DNA in amounts ranging from 1.7 to 4.6 viral genome equivalents per cell. Embryos that were negative or positive for the group-specific antigen of avian leukosis viruses contained the same amount of viral DNA. Embryos from a strain of chickens free of leukosis viruses of groups A and B that develop 40% fewer spontaneous leukotic tumors than the original strain contained an average of 2.1 viral DNA equivalents per cell, whereas the original strain contained 3.2 viral DNA equivalents. By comparison, leukemic cells and cells infected with avian myeloblastosis virus or Rous sarcoma virus contained between 4 and 13 viral DNA equivalents. This study provides direct biochemical evidence for the presence in normal chicken cells of avian leukosis virus DNA that is inheritable and that represents either complete or incomplete viral genomes.**

The RNA genome of avian leukosis viruses (ALV) appears to be replicated through a DNA intermediate (1–7, see also refs. in 3). Previous findings from this laboratory have established that viral DNA complementary to RNA from avian myeloblastosis virus (AMV) was present not only in cells transformed by ALV but also, to a smaller extent, in DNA preparations from pooled, apparently normal, chicken embryos (1, 3, 4). The specificity of the hybridization procedure and the nature of the DNA–RNA hybrids formed exclude the possibility that the hybrids result from short, accidentally similar, nucleotide sequences of the two nucleic acids. In this study, DNA was extracted from individual, apparently normal, chicken embryos and adult chickens and tested for its capacity to hybridize with AMV-RNA for determination of the proportion of chickens that contain viral DNA and the concentration of viral DNA per cell. Surprisingly, every chicken tested contained viral DNA.

MATERIALS AND METHODS

Viruses. Schmidt–Ruppin strain of Rous sarcoma virus (SR-RSV), D classification, and AMV-BAI strain A, B classification, were used.

Embryonated Chick Eggs. Embryonated eggs from four different types of chickens were used: (*i*) Chickens of the White Leghorn strain, cross K-137, obtained from Kimber Farms, Pomona, Calif. The background incidence of "spontaneous" leukosis in this strain was determined to be 8.2% by observation of untreated chickens for 8–12 months after hatching (8). 90% of the tumors consisted of visceral lymphomatosis and 8% were kidney tumors (nephroblastomas). (*ii*) Subline SPF-K-137 of White Leghorn chickens. These chickens were kept isolated in a specific pathogen-free environment and were bred by closed flock mating since 1961 by Dr. Walter Hughes at Kimber Farms, Niles, Calif. These chickens were selected specifically for the absence of contamination by group A leukosis viruses; they were also found to be free of group B leukosis viruses when tested in several laboratories. According to Dr. Hughes, this flock develops lymphoid tumors of the viscera with an incidence of only 5%. (*iii*) C/B (resistant to avian leukosis viruses of group B) White Leghorn chickens (Heisdorf-Nelson Farms, Redmond, Wash.). Frozen embryos of these chickens were kindly supplied by Dr. R. Friis, University of Washington, Seattle. (*iv*) Pooled normal or SR-RSV-transformed fibroblasts from White Leghorn chicken embryos were kindly supplied as frozen cells by Professor H. Temin, University of Wisconsin, Madison, Wis.

Rat Embryonic Cells. Frozen rat embryonic cells, normal or transformed with strain B-77 of avian sarcoma virus, were kindly supplied by Dr. H. Temin. The transformed rat cells produced B-77 virus after cocultivation or fusion with chicken cells (9).

Leukemic Myeloblasts. Purified myeloblasts were obtained from acutely leukemic chicks (10).

Cellular DNA. Cellular DNA was extracted, purified, denatured, and trapped on 0.45-μm nitrocellulose Millipore filters as described (3).

^{3}H-Labeled 71S AMV-RNA. The preparation and purification of ^{3}H-labeled AMV and of 71S RNA from purified AMV viruses have been described (3, 10).

DNA–RNA Hybridization. The method of hybridization of ^{3}H-labeled 71S RNA with DNA immobilized on nitrocellulose filters has been described (1).

Virus Production Test. Cell cultures from individual embryos were tested for ALV production by incubation of the cells for 10–16 hr in modified Eagle's medium containing 5% dialyzed calf serum, 5% dialyzed chicken serum, 1 μM thymi-

Abbreviations: ALV, avian leukosis viruses; AMV, avian myeloblastosis virus; SR-RSV, Schmidt–Ruppin strain of Rous sarcoma virus; SSC, 0.15 M NaCl–0.015 M sodium citrate; gs, group specific (antigen); COFAL, complement fixation for avian leukosis (test).

dine, and 10 μCi/ml of [5-^{3}H]uridine (24–31 Ci/mmol) (10); labeled virions were detected as described (10).

RESULTS

I. Presence of viral DNA in infected and in normal cells

The presence of DNA complementary to AMV-RNA in cells transformed by ALV and in cells from uninfected embryos is demonstrated in Fig. 1. The specificity of the DNA-RNA hybridization reaction, already documented in previous publications (1–4), is shown by the data obtained by hybridization of ^{3}H-labeled 71S AMV-RNA with DNA from normal mouse or rat embryonic cells. These DNAs bound only 20 cpm more than blank filters that were treated exactly like the other filters. In other similar experiments, DNA from *Escherichia coli* or from mouse leukemic cells that were transformed by Gross leukemia virus also failed to hybridize with AMV-RNA. However, DNA from rat embryonic cells transformed by B-77 sarcoma virus hybridized to a considerable extent with the AMV-RNA. Irrespective of the amount of cellular DNA trapped on a filter, DNA from cells made leukemic by AMV hybridized with 1.7 times more AMV-RNA than did DNA from pooled, untreated chicken embryos. DNA from chicken embryo fibroblasts transformed by SR-RSV hybridized with six times more AMV-RNA than did DNA from the uninfected control cells.

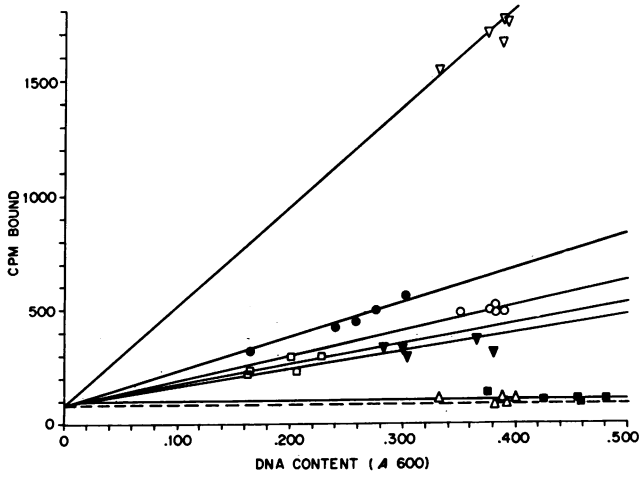

FIG. 1. Hybridization of ^{3}H-labeled 71S AMV-RNA to various DNAs. The radioactivity (cpm) bound per filter was plotted versus the DNA content of the filter, expressed as absorbance (A) at a wavelength of 600 nm of the chromophore from the diphenylamine reaction (3). No correction has been made, and the background has not been subtracted. The sources of the DNA trapped on the filters are: ▽, fibroblasts from Wisconsin White Leghorn chicken embryos (pool) transformed by SR-RSV; ●, leukemic myeloblasts from leukemic K-137 chicks (pool); ○, rat embryonic fibroblasts transformed by B-77 avian sarcoma virus; □, normal K-137 chicken embryos (pool); ▼, normal Wisconsin White Leghorn chicken embryos (pool); △, normal mouse embryos; ■, normal rat embryonic fibroblasts. The five blank filters, without DNA, were treated exactly like the other filters (*dashed line*). Hybridization was performed for 10 hr at 70° with five filters per vial in 1.5 ml of hybridization mixture that contained, per ml: 2.3 × 10^6 cpm of ^{3}H-labeled 71S AMV-RNA (4.3 × 10^5 cpm/μg), 2.3 mg of unlabeled chicken embryo RNA, and 0.05% of sodium dodecyl sulfate in 4 × SSC (0.60 M NaCl–0.060 M sodium citrate). An absorbance of 0.640 at 600 nm corresponds to 100 μg of DNA, as determined by appropriate standards.

II. Determination of number of viral DNA equivalents per cell in reference DNA

The cellular concentration of viral DNA in a given DNA preparation was calculated from the ratio between the amount of ^{3}H-labeled 71S AMV-RNA hybridized to the DNA investigated and the amount of ^{3}H-labeled 71S AMV-RNA hybridized, under identical conditions, to a reference DNA of known viral DNA content. This ratio is equal to the ratio of the viral DNA concentrations, since the amount of [^{3}H]RNA hybridized by a given DNA depends only upon the viral DNA concentration under the hybridization conditions used. These conditions are: (*a*) the same ^{3}H-labeled AMV-RNA is used at the same concentration with each DNA to be tested; (*b*) the amount of [^{3}H]RNA is in large excess over that of viral DNA in the hybridization mixtures, and the [^{3}H]RNA concentration remains essentially constant throughout the reaction time; (*c*) ^{3}H-labeled AMV–RNA is fragmented into relatively small pieces (about 2.5 × 10^5 daltons) (2); (*d*) from the shape and the ratio of the curves in Fig. 2, there is no apparent loss of DNA–RNA hybrids from the filter.

For reference, one leukemic and one normal DNA preparation were used, and their viral DNA content was determined at saturation (Fig. 2). DNA was obtained from leukemic cells pooled from six leukemic chicks and from normal cells pooled from twelve untreated chicken embryos. These pooled DNA

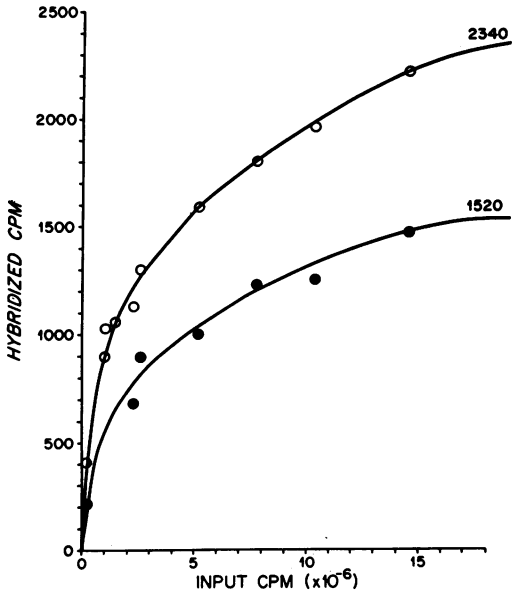

FIG. 2. Saturation curves for pooled leukemic myeloblast DNA (*upper curve*) from six K-137 leukemic chicks and for normal DNA (*lower curve*) from 12 pooled normal 10-day-old K-137 chicken embryos. The cpm hybridized per 100 μg of DNA were plotted versus the concentration of input ^{3}H-labeled 71S AMV-RNA. Hybridization was performed for 10 hr at 70° with five experimental DNA–filters and two mouse DNA–filters per vial in 1.6 ml of hybridization mixture containing [^{3}H]AMV-RNA (5.7 × 10^5 cpm/μg), 2.5 mg of mouse embryo RNA per ml, and 0.05% sodium dodecyl sulfate in 4 × SSC. The mean amount of DNA per filter was 38 and 44 μg for normal and leukemic DNAs, respectively. Cpm bound by mouse DNA–filters were deducted as background.

TABLE 1. *Quantitative data pertinent to hybridization experiments*

Weight of one viral RNA genome:	2.0×10^{-11} μg
Weight of DNA per chicken-cell genome:	2.4×10^{-6} μg
Number of genomes per 100 μg of chicken DNA:	4.17×10^{7}
Specific activity of ^{3}H-labeled AMV-RNA:	5.7×10^{5} cpm/μg
Radioactivity, hybridized at saturation per 100 μg of DNA, corresponding to one viral DNA equivalent per chicken genome:	475 cpm

preparations should contain whatever varieties of viral DNA species may be present in normal cells. A best-fit curve drawn through the experimental points gives plateaus at 2340 and 1520 cpm for DNA from leukemic and normal cells, respectively (11). At the saturation level, as well as at seven other different input RNA concentrations, the ratio of cpm hybridized by leukemic DNA to cpm hybridized by normal DNA was constant with a mean of 1.54 and a standard deviation of 0.065. Thus, the viral DNA content of either DNA, from normal or leukemic cells, can be determined from the viral DNA content of the other DNA within a 95% confidence limit of ±8% (i.e., ±2 SD), irrespective of the ^{3}H-labeled AMV-RNA concentration. With the quantitative parameters listed in Table 1, it can be calculated that the leukemic DNA contains 4.95 viral DNA equivalents per leukemic cell and the normal DNA contains 3.2 equivalents per normal cell.

III. Cellular concentration of viral genome equivalents in apparently normal embryos and in leukemic chicks

DNA–filters were prepared with DNA extracted from individual whole 10- to 13-day-old chicken embryos or from leukemic myeloblasts of individual acutely leukemic chicks, 14–31 days after infection. DNA on these filters and reference DNA were hybridized with ^{3}H-labeled 71S AMV-RNA; the viral DNA equivalents per cell were calculated as described in Section II.

As shown in Table 2, all the embryos from strain K-137 contained viral DNA; the mean was 3.2 viral genome equivalents per cell, with a range from 2.1 to 4.5 equivalents. Embryos with high viral DNA content may have been virus producers due to congenital transmission of ALV by viremic hens (12), but they were not tested for virus production. Many other normal embryos, with up to 3.5 viral genome equivalents, were tested and did not produce virus.

The SPF-K-137 embryos that were not contaminated with ALV of groups A and B contained viral DNA but in reduced amount; they had a mean cellular concentration of 2.1 viral DNA equivalents with a range from 1.7 to 2.7 equivalents. Therefore, the selection for absence of contamination by ALV of groups A and B that may have led to a 40% reduction in the incidence of "spontaneous" leukosis also led to a reduction in the number of viral DNA equivalents per cell.

The cellular concentration of viral DNA in different K-137 leukemic chicks varied from 3.9 to 6.7 viral equivalents, with a mean of 5.8. The only leukemic SPF-K-137 chick tested contained 13.1 viral DNA equivalents per cell.

After *in vitro* infection with ALV, fibroblasts from two SPF-K-137 chicken embryos contained more viral DNA (8.7 and 11.6 equivalents per cell) than infected K-137 fibroblasts (from 4.7 to 7.1 viral equivalents with a mean of 5.2). The presence of a greater amount of viral DNA in the SPF chickens might be linked to their greater susceptibility to AMV than the original K-137 strain; it has been repeatedly observed that they show an earlier and greater incidence of acute leukemia and their leukemic cells produce more AMV.

IV. Absence of correlation between expression of ALV group-specific (gs) antigen and cellular concentration of viral DNA

Since the presence of gs-antigen in normal cells is thought to result from the partial expression of ALV genomes (13), it was possible that gs-antigen-negative embryos might not contain viral DNA. Therefore, two C/B gs-antigen-negative embryos from two sets of siblings, kindly supplied by Dr. R. Friis, as well as one gs-antigen-negative K-137 embryo, were tested. The results (Table 3) show that gs-antigen-negative embryos contained as much viral DNA as their gs-antigen-positive siblings, and that there was also no correlation between the amount of viral DNA present in a chicken embryo and the COFAL titer. Thus, either gs-antigen expression is controlled by a separate set of regulatory genes, or the viral DNA present in gs-antigen-negative cells lacks a functional gs-antigen gene, e.g., it might be an incomplete viral genome.

V. Amount of viral DNA in ALV-infected cultured cells

These experiments were performed to find out whether virus replication alone caused an increase in cellular viral DNA concentration or whether morphological transformation of the host cell was also required. Chicken embryonic fibroblasts infected with AMV become virus producers but are not morphologically transformed and continue to divide as fast, and

TABLE 2. *Cellular concentration of viral genome equivalents in individual chicks or chicken embryos*

	Number of viral DNA equivalents per cell*												
Source of DNA	1	2	3	4	5	6	7	8	9	10	11	12	13
Untreated embryos:†													
K-137 (Pomona)		8	15	9									
SPF-K-137 (Niles)		9	1										
Leukemic chicks:‡													
Pomona				3	4	2	1						
Niles													1
Cells infected *in vitro*:§													
Pomona					4	1	2						
Niles									1			1	

* Calculated from cpm hybridized under identical conditions by experimental DNA or by reference DNA with a known viral DNA content. The number of viral DNA equivalents is given ±0.5 equivalent, e.g., 2 = 1.5–2.5. The actual determinations fall within a 4 SD range of ±8%.

† DNA was extracted from whole 10- to 13-day-old chicken embryos.

‡ DNA was extracted from leukemic myeloblasts obtained from acutely leukemic chicks by exsanguination 14 to 33 days after infection.

§ Chicken embryo fibroblast cultures were prepared from 10-day-old chicken embryos and infected with AMV or SR-RSV at a multiplicity of 2–6 infectious units per cell, 3–6 days later. DNA was extracted from about 60 cultures 1–2 weeks after infection.

for the same number of generations, as noninfected cultures (unpublished results). In contrast, infection of chicken embryonic fibroblasts with SR-RSV results in rapid morphological transformation and virus production. Cell cultures from each of three K-137 embryos that had been shown to be free of congenitally transmitted ALV were either kept uninfected, infected with AMV, or infected with SR-RSV. Between the 7th and 15th day after infection, each culture was tested for virus production and for viral DNA content. The noninfected cultures did not produce virus, whereas those infected with AMV or SR-RSV did. The cultures infected with SR-RSV were also typically transformed. As shown in Table 4, the uninfected cultures contained 2.1–2.5 viral equivalents per cell even though they did not produce ALV, whereas the other cultures, whether infected with AMV or SR-RSV, contained 4.7–7.1 equivalents per cell. Therefore, viral DNA can be present in apparently normal cells that do not make virus; infection with AMV or SR-RSV increases the viral DNA concentration whether or not the cells are transformed.

DISCUSSION

Before this study, two types of untreated chicken embryos, with respect to viral DNA content, might have been thought to exist: (*i*) those which, because of congenital transmission of ALV by viremic hens, are virus producers and should contain as much viral DNA as experimentally infected chickens, and (*ii*) embryos that are not infected with ALV and should not contain viral DNA. Surprisingly, every one of at least 50 apparently normal chicken embryos that were individually tested contained viral DNA in amounts ranging from 1.7 to 4.6 viral genome equivalents per cell. Some of these embryos did not produce ALV and even some were gs-antigen negative. In addition, six healthy egg-laying adult hens that were tested in other experiments were also found to contain 2–3 viral equivalents per cell. By comparison, leukemic chicks or cells infected *in vitro* with AMV or SR-RSV had 4–13 viral DNA equivalents per cell.

The viral DNA that is present in apparently normal cells probably corresponds to complete viral genomes or large parts of them. As demonstrated (1, 4, 11, 14), the DNA–RNA hybrids formed consist always of long nucleotide sequences, and the average nucleotide composition of the RNA eluted from the hybrids formed with DNA from some normal chicken embryos is nearly identical to that of the input AMV-RNA (11). The best direct evidence, so far, that indicates that viral DNA represents complete or partial DNA copies of ALV genomes, is the acquisition of 3.4 viral DNA equivalents by rat embryonic cells infected with B-77 avian sarcoma virus. Before infection, such cells contained only 0.1 viral equivalent, i.e., 29 cpm above background (see Fig. 1). Also, viral DNA in leukemic cells that are transformed by AMV, or in cells transformed by SR-RSV, had the same average nucleotide composition as AMV or SR-RSV, respectively (2). It appears, therefore, that the increase in viral DNA, from 2–3 to 5–6 equivalents, after infection does not result from duplication of previously existing DNA that is naturally homologous to AMV-RNA. SPF chickens that are free of ALV of groups A and B and that develop 40% fewer spontaneous tumors than the original K-137 strain, contain significantly less viral DNA per cell, i.e., 2 instead of 3 viral equivalents. This suggests that viral DNA in normal cells may also be responsible for tumor induction.

The findings reported in this article agree with other reports, which attribute certain biological activities in normal cells to the expression of ALV genes. (*i*) The presence of an antigen that crossreacts with antibody against the ALV group-specific antigen (13); (*ii*) the synthesis of a small number of apparently noninfectious C-type virions in the pancreas and liver of leukosis-free embryos (15) and in the pancreas and ovary of healthy adult hens (16); (*iii*) the helper function without which RSV(0) cannot replicate (13); (*iv*) the production of RAV (Rous-associated virus)-60 that is induced by infection with RSV (17), and probably by the same mechanism, the often noted contamination of pure ALV stocks by other leukosis viruses; (*v*) the induction of ALV production by methylcholanthrene,

TABLE 3. *Lack of correlation between gs-antigen expression and number of viral DNA equivalents per cell*

Embryo*	COFAL titer†	Viral DNA equivalents
2469 A	0	2.6
2469 B	1:2	2.8
2469 C	1:4	3.3
2469 D	1:2	3.5
2456 B	1:2	3.7
98 E	1:4	3.7
98 F	0	3.4
R-8 K1	0	2.4
R-8 K2	1:4	2.6

* Frozen embryos from hens 2469, 2456, and 98 were kindly supplied by Dr. R. Friis. They were C/B White Leghorns from Heisdorf-Nelson Farms, Redmond, Wash. R8 K1 and R8 K2 were chicken embryonic fibroblast cultures from two separate 9-day-old K-137 (Pomona) embryos. The DNA was extracted after the cells had been cultured for 10 days.

† COFAL microtests were performed with hamster anti-SR-RSV D serum, 2 units of complement, and 2 units of hemolysin, by Dr. R. Friis (0 = no gs-antigen detected).

TABLE 4. *Amount of viral DNA in cells infected in vitro*

Embryo* number	Days after infection	Cpm hybridized per 100 μg of DNA: Control	AMV infected	SR-RSV infected
1	7	489 ± 31† (2.2)‡	1572 ± 35 (7.1)	
	14			1436 ± 108 (6.5)
2	7	462 ± 25 (2.1)	1483 ± 45 (6.7)	
	15			1206 ± 70 (5.5)
3	9	560 ± 17 (2.5)	1039 ± 52 (4.7)	
	15			1056 ± 50 (4.8)

* Chicken embryonic fibroblast cultures were prepared from eviscerated and decapitated 10-day-old K-137 embryos. At time of culture transfer, 3 days later, secondary cultures were infected with AMV (leukemic plasma) at a multiplicity of about 6 or with SR-RSV at a multiplicity of about 2 infectious units per cell.

† Mean and standard deviation of 5 DNA–filters.

‡ The number in parentheses indicates the number of viral genome equivalents per cell.

aging, or x-irradiation (13); and (*vi*) the spontaneous production of ALV in originally virus-free cultures (11, 18).

It is possible that some chickens contain only partial copies of the viral genome, e.g., the helper function for RSV(0) can be expressed in cells that are gs-negative or which cannot be induced to produce RAV(0). Therefore, the finding that the guanine + cytosine content of viral DNA in some normal embryos differs slightly from that of AMV-RNA (4) may indicate a partial copy with a nucleotide composition different from that of the entire genome. On the other hand, some embryos must contain complete copies of ALV genomes, since ALV production can be induced in their explanted cells and, as mentioned earlier, the average nucleotide composition of the viral DNA in some normal embryos is nearly identical to that of AMV-RNA. The elucidation of the origin of viral DNA in normal chicken embryos may provide a clue to the role of RNA tumor viruses in oncogenesis.

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NOTE ADDED IN PROOF

Recently, Rosenthal *et al.* (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2336–2340, have confirmed our findings that uninfected chicken cells contain DNA that is complementary to RNA from avian tumor viruses and that the amount of complementary DNA increases after infection with an avian tumor virus. They also showed that RNAs from different avian tumor viruses have base sequences indistinguishable by DNA–RNA hybridization and that uninfected chicken cells that cannot be induced to release RAV-60 contain as much DNA complementary to viral RNA as uninfected cells that can be induced to release the virus.

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