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Lack of Infectivity of the Endogenous Avian Leukosis Virus-Related Genes in the DNA of Uninfected Chicken Cells

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The infectivity of the avian leukosis virus-related genes in the DNA of four genetically distinct types of chicken cells was determined. Infectious DNA of Rous-associated virus-0 (RAV-0) was obtained from V^- chicken cells which were experimentally infected with RAV-0 and from V^+tvb^s chicken cells, which spontaneously produced RAV-0 and were sensitive to exogenous RAV-0 infection. However, infectious DNA of RAV-0 was not obtained from uninfected V^- chicken cells or from V^+tvb^r chicken cells, which spontaneously produced a low titer of RAV-0 but were resistant to exogenous RAV-0 infection. No detectable amplification of the RAV-0-related DNA sequences in the V^+tvb^s cells was found by hybridization of RAV-0 ^{125}I-labeled RNA to the DNAs of V^+tvb^s and uninfected V^- cells. These results indicate that the endogenous avian leukosis virus-related genes in uninfected V^- and V^+tvb^r cells differ from the RAV-0 proviruses in RAV-0-infected V^- and V^+tvb^s cells. The lack of infectivity of the DNA of V^+tvb^r cells is consistent with the hypothesis that the endogenous RAV-0 genome in V^+tvb^r cells is linked to a *cis*-acting control element, which results in its inefficient expression.

The DNAs of apparently all uninfected chicken cells contain nucleotide sequences that are homologous to some nucleotide sequences of the virion RNA of avian leukosis viruses (4, 25, 27, 30, 35). Uninfected cells of the majority of chickens, which are V^-, do not produce avian leukosis virus particles, although they may contain proteins related to some avian leukosis virus virion proteins (7, 11, 16-19, 29, 37). In contrast, uninfected cells of a few inbred lines of chickens, which are V^+, spontaneously produce a nontransforming subgroup E avian leukosis virus, Rous-associated virus-0 (RAV-0) (10, 36). However, the DNAs of both V^- and V^+ chicken cells contain nucleotide sequences that are homologous to most of the nucleotide sequences of RAV-0 RNA (25, 27).

We have studied the endogenous avian leukosis virus-related genes in uninfected chicken cells by comparing the infectivity of DNAs extracted from uninfected and RAV-0-infected donor cells. Since the assay of infectious DNA measures biological activity (9, 23), this assay might detect differences between the avian leukosis virus-related genes of genetically distinct types of chicken cells, which are not detectable by nucleic acid hybridization. In addition, since purified DNA preparations were used, the infectivity of the donor DNAs was not affected by regulatory proteins or RNAs which might control the expression of the avian leukosis virus-related genes in the donor cells.

Four genetically distinct types of chicken cells were used as possible donors of infectious DNA of RAV-0: (i) uninfected V^- cells; (ii) V^+ cells which were resistant to exogenous RAV-0 infection as a result of the absence of receptors required for RAV-0 penetration (V^+tvb^r cells); (iii) V^+ cells which were sensitive to exogenous RAV-0 infection (V^+tvb^s cells); and (iv) V^- cells which were experimentally infected with RAV-0 [V^-(RAV-0) cells]. The results indicate that the endogenous avian leukosis virus-related genes in both V^- and V^+ chicken cells differ from the RAV-0 proviruses in cells exogenously infected with RAV-0.

MATERIALS AND METHODS

Cells and viruses. Cells were grown by standard procedures in modified Eagle minimal essential medium containing 20% tryptose phosphate broth (ET medium) and supplemented with calf and fetal bovine sera. Fertile chicken eggs were purchased from Spafas, Norwich, Conn., Sunnyside Hatcheries, Oregon, Wis., and Hyline, Dallas Center, Iowa. Fertile line 100 and line 6 chicken eggs (10) were a gift of L. B. Crittenden, U.S. Department of Agriculture, Beltsville, Md. RAV-0-producing, line 7 chicken embryo fibroblasts (36) were a gift of P. E. Neiman, Univer-

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sity of Washington, Seattle, Wash. RAV-0-producing line 15I chicken embryo fibroblasts (17) were a gift of H. Hanafusa. The Rockefeller University, New York, N.Y. Fertile ringneck pheasant eggs were obtained from the Poynette Game Farm, State of Wisconsin Department of Natural Resources. Fertile Orlopp turkey eggs were obtained from the Wilmar Poultry Co., Wilmar, Minn.

Chicken embryo fibroblasts were tested for sensitivity to infection with subgroup B and E avian leukosis viruses, for avian leukosis virus production by assay of sedimentable DNA polymerase activity in supernatant media, for *V* gene allele by co-cultivation with ringneck pheasant or turkey cells (10), for chick helper factor (chf) as described by Hanafusa et al. (method B) (21) and by Weiss et al. (39), and for avian leukosis virus group-specific antigen by complement fixation. (The sensitivity of the cells to infection with avian leukosis virus subgroups other than B and E is not relevant to the experiments described and, therefore, is not presented.) Spafas and Sunnyside chicken embryo fibroblasts were C/E, V^-, chf negative, and avian leukosis virus group-specific antigen negative. Hyline (WC line b2 histocompatibility) chicken embryo fibroblasts were C/O, V^-, chf negative, and avian leukosis virus group-specific antigen negative. Line 6 chicken embryo fibroblasts were C/E, V^-, chf positive, and avian leukosis virus group-specific antigen positive. Line 100 chicken embryo fibroblasts were either C/O (tvb^s) or C/BE (tvb^r) and were V^+. Line 7 and line 15I chicken embryo fibroblasts were C/O (tvb^s) and V^+.

Ringneck pheasant cells and turkey cells were sensitive to infection with subgroup E avian leukosis viruses, negative for a helper factor (13, 20), and negative for avian leukosis virus group-specific antigen.

RAV-0 was harvested from cultures of V^+tvb^s line 100 chicken embryo fibroblasts.

Extraction of infectious DNA. DNA was extracted either from cultures of chicken embryo fibroblasts or from individual 8-day-old chicken embryos. When DNA was extracted directly from embryos, a small portion of tissue was used to establish cultures of chicken embryo fibroblasts, which were tested for expression of avian leukosis virus gene functions as described above. The remainder of the embryo was suspended in SSC (0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), dispersed by gentle Dounce homogenization, and used for DNA extraction.

The DNA extraction procedure was the same as described in detail previously (9). Cells were lysed with 0.5% sodium dodecyl sulfate, and the extract was deproteinized by digestion with Pronase (250 μg/ml) and three extractions with chloroform-isoamyl alcohol (24:1). DNA was precipitated with ethanol, dissolved in SSC, treated with RNase A (100 μg/ml), redigested with Pronase, extracted four to six times with chloroform-isoamyl alcohol, precipitated with ethanol, dissolved in SSC, and stored at −70 C. The A_{260}:A_{280} ratios of all DNA preparations were 1.8 to 1.9. The average molecular weights of DNAs extracted by this procedure were approximately 50 million (9).

Assay of infectious DNA of RAV-0. Recipient cultures of C/E (Spafas) or C/O (Hyline) chicken embryo fibroblasts were prepared as previously described (9). When C/E cells were used as recipients, ringneck pheasant cells or Orlopp turkey cells, which were sensitive to infection with subgroup E avian leukosis viruses, were added to the cultures 1 to 3 days after DNA treatment. Ringneck pheasant cells or turkey cells were not used directly as recipient cells for assay of infectious RAV-0 DNA, since infection of these cells with avian leukosis virus DNAs was much less efficient than infection of chicken cells (9; unpublished data).

Infectious DNA was assayed either using the DEAE-dextran method, as previously described (9), or using the calcium method originally described by Graham and Van der Eb (14, 15). When the calcium method was used, the infectious DNA preparation was mixed with salmon sperm DNA (Calbiochem, Los Angeles, Calif.) to a final DNA concentration of 10 μg/ml in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-buffered saline (15). Calcium chloride (1.25 M) was added to a final concentration of 0.125 M, and a precipitate was allowed to form for 10 to 20 min at room temperature. Aliquots (0.5 ml) of the suspension were added to recipient cell cultures in 60-mm petri dishes, which contained 5 ml of ET medium supplemented with 4% fetal bovine serum. After 4 h of incubation at 37 C, the medium was changed to fresh ET medium supplemented with 3% fetal bovine serum and polybrene (5 μg/ml).

The calcium method and the DEAE-dextran method were equally sensitive for assay of infectious avain leukosis virus and reticuloendotheliosis virus DNAs (unpublished data). However, the calcium method was less toxic to the recipient cells than the DEAE-dextran method, particularly when more than 1 μg of DNA per culture was used.

DNA-treated cells were cultured in ET medium supplemented with 1 to 4% fetal bovine serum and polybrene (5 μg/ml) for 2 to 3 weeks, including two transfers, after DNA treatment. Production of RAV-0 was determined either by assay of sedimentable DNA polymerase activity in the supernatant medium of the DNA-treated cell cultures or by assay of a sample of the supernatant medium of the DNA-treated cell cultures in ringneck pheasant cells. RAV-0 infection of the pheasant cells was assayed by measurement of sedimentable DNA polymerase activity 10 to 14 days after infection.

It was usually necessary to cultivate DNA-treated cells for approximately 3 weeks before detectable amounts of progeny RAV-0 were produced. In contrast, detectable amounts of other avian leukosis viruses were produced within 1 week after DNA treatment (9). The longer period of cultivation required to detect progeny RAV-0 was probably the result of the low specific infectivity of RAV-0 compared to other avian leukosis viruses (18; unpublished data).

The assay of infectious RAV-0 DNA was quantitated by end point dilution of the donor DNA as previously described (9).

Assay of sedimentable DNA polymerase. Virus was concentrated from 10 ml of cell-free supernatant

medium and disrupted with 0.1 ml of Nonidet P-40 disruption buffer (26). Disrupted virus (25 μl) was added to 100 μl of reaction mixture, which contained 0.02 M Tris-hydrochloride (pH 8.0), 0.025 M KCl, 0.02 M $MgCl_2$, 0.4 mM EDTA, 10 mM dithiothreitol, 0.1 mM dATP, 0.075 mM ATP, 2.5 μg of phosphoenolpyruvate, 0.5 units of pyruvate kinase, 1.25 μg of poly(dA-dT) (P.-L. Biochemicals), and 3.8 μCi of [^{3}H]TTP (40 Ci/mmol, New England Nuclear Corp, Boston, Mass.). Samples (25 μl) were removed after 0, 30, and 60 min of incubation at 37 C and were assayed for acid-precipitable radioactivity. The activity of the RAV-0 DNA polymerase was approximately 20-fold higher with poly(dA-dT) in the reaction mixture described above than with poly(rA)·$(dT)_{12-18}$ in the reaction mixture described by Aaronson et al. (1) (unpublished data). In addition, the activity of the RAV-0 DNA polymerase was approximately threefold higher with poly(dA-dT) than with activated calf thymus DNA (unpublished data).

Nucleic acid hybridization. RAV-0 ^{125}I-labeled RNA was hybridized to an excess of chicken cell DNA as described by Kang and Temin (25).

Preparation of high-molecular-weight DNA. DNA was subjected to electrophoresis for 3 h at 60 V in 0.7% agarose gels which contained ethidium bromide (9). Lambda DNA (molecular weight, 30×10^6), obtained from W. Szybalski, was used as a marker. DNA with molecular weights greater than 30×10^6 was eluted from the gels in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-buffered saline, and the ethidium bromide was extracted with isoamyl alcohol.

RESULTS

RAV-0 production by donor cells. The amounts of RAV-0 produced by the four types of cells that were used as possible donors of infectious RAV-0 DNA were determined by assaying culture fluids for infectious RAV-0 and for sedimentable DNA polymerase activity (Table 1). Uninfected V^- chicken cells did not produce detectable amounts of RAV-0 either when grown alone (Table 1) or when co-cultivated for 2 weeks with ringneck pheasant or turkey cells (unpublished data). In contrast, RAV-0 production by V^+tvb^r cells, V^+tvb^s cells, and RAV-0-infected V^- cells [V^-(RAV-0) cells] was detected by assaying culture fluids either for DNA polymerase activity or for infectious RAV-0 (Table 1).

As previously reported by Crittenden et al. (10), the amount of RAV-0 produced by V^+ line 100 chicken cells was controlled by the sensitivity of the cells to exogenous infection with RAV-0. The amount of RAV-0 produced by V^+tvb^r line 100 cells, which were resistant to exogenous RAV-0 infection, was approximately 10^3-fold less than the amount of RAV-0 produced by V^+tvb^s line 100 cells, which were sensitive to exogenous RAV-0 infection (Table 1). The amounts of RAV-0 produced by V^+tvb^s line 7 and line 15I chicken cells were similar to the amount of RAV-0 produced by V^+tvb^s line 100 cells (unpublished data). The amount of RAV-0 produced by V^-(RAV-0) cells was similar to the amount of RAV-0 produced by V^+tvb^s cells (Table 1). Therefore, the high titer of RAV-0 produced by V^+tvb^s cells, as compared

TABLE 1. *Production of RAV-0 by cells used as possible donors of infectious RAV-0 DNA*[a]

Chicken cells	RAV-0 production	
	DNA polymerase activity (counts/min per h per culture)	Infectious units/culture
V^-[b]	<5	<5
V^+tvb^r[c]	10^2 (10–10^3)	50 (5–500)
V^+tvb^s[c]	10^5 (5×10^4–5×10^5)	5×10^4 (2×10^4–2×10^5)
V^-(RAV-0)[d]	3×10^4	ND[e]

[a] Cultures of the indicated types of chicken embryo fibroblasts were tested for RAV-0 production by assay of the supernatant media for sedimentable DNA polymerase activity or by plating serial 10-fold dilutions of the supernatant media on ringneck pheasant cells. The ringneck pheasant cells were cultured for approximately 2 weeks, and the supernatant media were assayed for sedimentable DNA polymerase activity to determine the end point of infectious RAV-0.

[b] V^- chicken embryo fibroblasts were obtained from Spafas, Sunnyside, Hyline, and line 6 embryos. Thirteen individual embryos were tested.

[c] Line 100 chicken embryo fibroblasts were classified as V^+tvb^r or V^+tvb^s by their sensitivity to infection with subgroup B and E avian leukosis viruses (10). DNA polymerase activities are average values from nine V^+tvb^r and six V^+tvb^s embryos. Titers of infectious RAV-0 are average values from five V^+tvb^r and two V^+tvb^s embryos. The range of values is indicated in parentheses.

[d] V^- C/O chicken cells (Hyline) were infected with RAV-0 harvested from cultures of V^+tvb^s line 100 cells. The RAV-0-infected V^- cells [V^-(RAV-0) cells] were transferred once before assay of the supernatant medium for sedimentable DNA polymerase activity.

[e] ND, Not done.

to V^+tvb^r cells, was probably the result of exogenous infection of the V^+tvb^s cells with RAV-0 (10).

In spite of the 10^3-fold difference in the amount of RAV-0 produced by V^+tvb^r cells and V^+tvb^s cells, the specific infectivity (infectious units per DNA polymerase activity) of the RAV-0 produced by V^+tvb^r cells was similar to the specific infectivity of the RAV-0 produced by V^+tvb^s cells (Table 1). The specific infectivity of RAV-0 from both V^+tvb^s and V^+tvb^r cells was approximately 1 infectious unit per count/min per h of DNA polymerase activity (Table 1), whereas the specific infectivity of RAV-60 was approximately 10^3 infectious units per count/min per h of DNA polymerase activity (unpublished data).

Assay of infectious RAV-0 DNA. DNA extracted from V^+tvb^s line 100 chicken cells was infectious for both C/O and C/E recipient cells (Fig. 1). Fifty percent infective dose values of approximately 1 μg of DNA were obtained with both recipients. The infectivity of the DNA extracted from V^+tvb^s line 100 cells, therefore, appeared to be slightly lower than the infectivity of DNAs extracted from cells infected with other avian leukosis viruses, which corresponded to 50% infective dose values of approximately 0.1 μg of DNA (9; unpublished data). The kinetics of infection with DNA extracted from V^+tvb^s line 100 cells appeared to be one-hit (Fig. 1), as previously reported for DNA extracted from cells infected with Rous sarcoma virus (9).

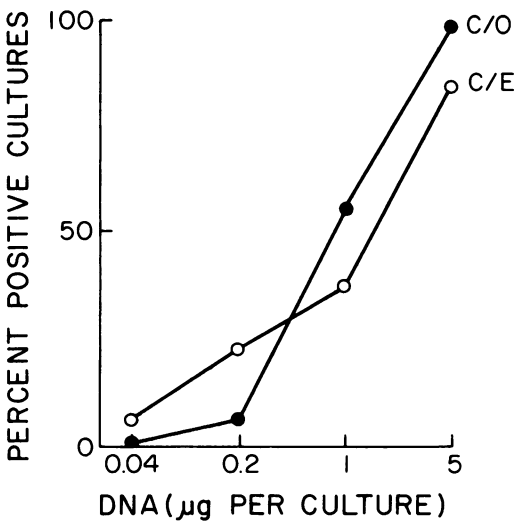

FIG. 1. *Assay of infectious DNA of RAV-0. Cultures of C/O (●) or C/E (○) chicken embryo fibroblasts were treated with fivefold serial dilutions of DNA extracted from V^+tvb^s line 100 chicken embryo fibroblasts. Approximately 15 cultures of C/O cells and 40 cultures of C/E cells were treated with each dose of DNA. Ringneck pheasant or turkey cells were added to the C/E cells to amplify the replication of progeny RAV-0.*

The host range of the progeny virus obtained after treatment of C/E recipient cells with DNA extracted from V^+tvb^s line 100 cells is illustrated in Table 2. The progeny virus was infectious for C/O chicken cells and for ringneck pheasant cells, but not for C/E chicken cells. Similar results were obtained with three different progeny virus isolates. Therefore, the progeny viruses belonged to avian leukosis virus subgroup E, as did the parental RAV-0 (10).

Infectivity of DNAs from different cells. DNAs were extracted from the four types of chicken cells described in Table 1 and were assayed for infectious DNA of RAV-0. The V^- chicken cells used as DNA donors included cells that were negative for expression of avian leukosis virus group-specific antigen and chick helper factor, as well as cells that were positive for these avian leukosis virus-related gene products. Qualitatively similar results were obtained whether DNAs were extracted directly from embryos or from cultures of chicken embryo fibroblasts.

The DNAs of uninfected V^- cells and of V^+tvb^r cells were not infectious (Table 3). No infectivity was detected in assays of DNAs of 12 different V^- embryos or of eight different V^+tvb^r embryos. A total of 36 recipient cultures were treated with 5 μg each of V^- DNA, and a total of 63 recipient cultures were treated with 5 μg each of V^+tvb^r DNA.

In contrast, infectious DNA of RAV-0 was detected in assays of the DNAs of all V^+tvb^s

TABLE 2. *Host range of progeny virus from C/E cells treated with V^+tvb^s cell DNA*[a]

Virus	DNA polymerase activity (counts/min per h per culture)		
	C/O cells	Ph/BD cells[b]	C/E cells
Parental	8×10^3	4.0×10^3	<50
Progeny	3×10^3	1.7×10^4	<50

[a] Cultures of C/O chicken cells, C/E chicken cells, or ringneck pheasant cells (Ph/BD) were exposed to virus harvested from V^+tvb^s line 100 chicken cells (parental virus) or from C/E chicken cells, which were positive for virus production after treatment with 1 μg of DNA extracted from V^+tvb^s line 100 chicken cells (progeny virus). Supernatant media were harvested 8 and 10 days after infection and were assayed for sedimentable DNA polymerase activity.

[b] Resistant to infection with avian leukosis virus subgroups B and D.

TABLE 3. *Assay of different chicken cells for infectious DNA of RAV-0*[a]

Donor DNA	Fraction of positive donor embryos[b]	Fraction of positive recipient cultures[c]	
		DNA (1.0 μg)	DNA (5.0 μg)
V^-[d]	0/12	0/22	0/36
V^+tvb^r[e]	0/8	0/25	0/63
V^+tvb^s[f]	8/8	38/98	46/84
V^-(RAV-0)[g]	1/1	1/8	7/8

[a] DNAs were extracted from different types of chicken cells and were assayed for infectious DNA of RAV-0 using C/O and C/E chicken cells as recipients. Similar results were obtained with both types of recipient cells.

[b] Number of individual embryos that yielded infectious DNA of RAV-0 over the total number tested. A minimum of four recipient cultures were treated with DNA of each embryo.

[c] Number of recipient cultures that were positive for infection with RAV-0 over the total number treated with each dose of DNA. Data obtained with both C/O and C/E recipients are included.

[d] Six Spafas, one Sunnyside, one Hyline, and four line 6 embryos. The line 6 embryos were positive for chick helper factor and for avian leukosis virus group-specific antigen; the others were negative.

[e] Line 100 embryos.

[f] Six embryos of line 100, one of line 7, and one of line 15I.

[g] V^- chicken cells (Hyline) experimentally infected with RAV-0 as described in the legend to Table 1.

embryos and of V^-(RAV-0) cells (Table 3). Fifty-five percent of the recipient cultures treated with 5 μg each of V^+tvb^s DNA and seven of eight recipient cultures treated with 5 μg each of V^-(RAV-0) DNA were positive for RAV-0 infection.

The infectivity of DNAs extracted directly from some V^+tvb^s line 100 embryos appeared to be lower than the infectivity of DNAs extracted from cultures of V^+tvb^s line 100 chicken embryo fibroblasts (unpublished observations). This difference may be the result of less exogenous RAV-0 infection in the cells of 8-day-old embryos than in the chicken embryo fibroblasts, which had been cultured for at least 3 weeks before being used for DNA preparation. Data obtained with V^+tvb^s DNAs of relatively low infectivity, which were extracted directly from embryos, are included in Table 3, whereas the data presented in Fig. 1 were obtained with V^+tvb^s DNAs extracted from cultures of chicken embryo fibroblasts. This difference accounts for the lower percentage of positive cultures obtained after treatment with 5 μg of V^+tvb^s DNA in the experiments presented in Table 3 (55%), as compared to those presented in Fig. 1 (89%).

The infectivity of V^-(RAV-0) and V^+tvb^s DNAs and the lack of infectivity of V^- and V^+tvb^r DNAs indicate that infectious DNA of RAV-0 was obtained only from cells exogenously infected with RAV-0.

Quantitation of RAV-0 DNA sequences by nucleic acid hybridization. One hypothesis that would account for the lack of infectivity of the endogenous avian leukosis virus-related DNA in uninfected V^- and V^+tvb^r chicken cells is that the amount of avian leukosis virus-related DNA in these cells was below the limits of detection of the infectious DNA assay. According to this hypothesis, exogenous infection with RAV-0 resulted in a sufficient quantitative increase in the amount of RAV-0 DNA in V^-(RAV-0) and V^+tvb^s cells to permit detection of infectivity. Infectivity was detected with as little as 0.04 μg of DNA extracted from V^+tvb^s line 100 cells (Fig. 1), but not with 5 μg of DNA extracted from uninfected V^- or V^+tvb^r cells (Table 3). In addition, the specific infectivity of the DNA of V^+tvb^s cells was approximately 1 infectious unit/μg of DNA (Fig. 1), whereas the specific infectivities of the DNAs of V^- and V^+tvb^r cells were less than 0.01 infectious unit/μg of DNA (Table 3). Therefore, at least 100-fold amplification of the RAV-0 DNA in V^+tvb^s line 100 cells was necessary to account for the difference in infectivity between the DNA of V^+tvb^s cells and the DNAs of V^- and V^+tvb^r cells.

This hypothesis was tested by hybridization of RAV-0 ^{125}I-labeled RNA to an excess of DNA extracted from V^+tvb^s line 100 chicken embryo fibroblasts or from uninfected V^- embryos (Fig. 2). The $C_0t_{1/2}$ for the hybridization of RAV-0 RNA to the DNA of V^+tvb^s line 100 chicken embryo fibroblasts was 4.5×10^3 mol·s per liter, whereas the $C_0t_{1/2}$ for the hybridization of RAV-0 RNA to the DNA of V^- Spafas embryos was 3.2×10^3 mol·s per liter. These results indicated a slightly higher concentration of RAV-0-related DNA sequences in the DNA of uninfected V^- Spafas embryos than in the DNA of V^+tvb^s line 100 chicken embryo fibroblasts, although this difference was too small to be considered significant. The concentration of RAV-0-related DNA sequences in the DNA of V^- cells appears to correspond to a frequency of only one or two copies per haploid genome (22, 27, 28). Therefore, the V^+tvb^s line 100 chicken embryo fibroblasts also appear to contain only one or two copies of the RAV-0 DNA

sequences per haploid genome, and we conclude that the hypothesis of amplification does not account for the difference in infectivity between the DNA of V^+tvb^s cells and the DNAs of uninfected V^- and V^+tvb^r cells.

Infectivity of integrated RAV-0 DNA. Since infectious RAV-0 DNA was obtained only from cells which were exogenously infected with RAV-0, we tested the hypothesis that only unintegrated forms of RAV-0 DNA were infectious by assaying the infectivity of high-molecular-weight DNA extracted from V^+tvb^s line 100 cells.

DNA with molecular weights greater than 30 million was isolated by agarose gel electrophoresis of V^+tvb^s line 100 cell DNA. This high-molecular-weight DNA preparation did not contain unintegrated avian leukosis virus DNA, which has a molecular weight of 6 million (34; E. Fritsch, personal communication). The infectivity of this high-molecular-weight DNA, assayed in C/E recipient cells, corresponded to a 50% infective dose value of 0.8 μg of DNA. Since this infectivity was the same as the infectivity of unfractionated V^+tvb^s cell DNA (Fig. 1), it appears that integrated RAV-0 DNA in V^+tvb^s cells was infectious.

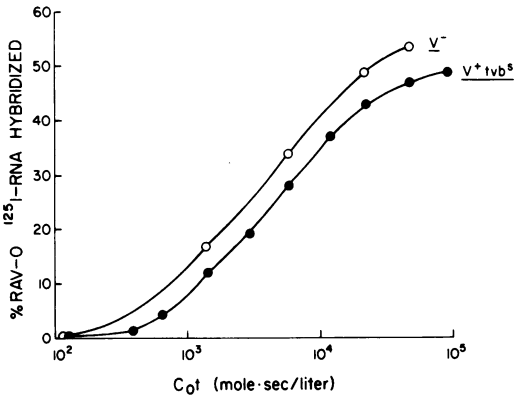

FIG. 2. *Kinetics of hybridization of RAV-0 RNA to chicken cell DNAs.* ^{125}I*-labeled 60 to 70S RNA of RAV-0 was hybridized to DNAs extracted from* V^- *Spafas chicken embryos* (○) *or from* V^+tvb^s *line 100 chicken embryo fibroblasts* (●) *as described by Kang and Temin (25). Hybridization mixtures (25 μl) consisted of 10 mg of DNA per ml and 3,000 counts/min (approximately 0.3 ng) of RAV-0[*^{125}I*]RNA in buffer containing 1 M NaCl. Samples were incubated at 63 C for different times, and the extent of hybridization was determined by digestion with ribonuclease (25).* C_0t *values were corrected to a monovalent cation concentration of 0.18 M using standards for DNA-DNA reassociation (6).*

DISCUSSION

Infectious DNA of RAV-0 was obtained from RAV-0-infected V^- cells and V^+tvb^s cells, but it was not obtained from either uninfected V^- cells or V^+tvb^r cells. Both V^+tvb^r and V^+tvb^s cells spontaneously produced RAV-0, but they differed in their sensitivity to exogenous RAV-0 infection (10, 32). The V^+tvb^r cells were resistant to exogenous RAV-0 infection and, therefore, contained only endogenous RAV-0 genetic information. The V^+tvb^s cells, however, were sensitive to exogenous RAV-0 infection and, therefore, were presumably exogenously infected with RAV-0 as a result of exposure to the RAV-0 which they spontaneously produced. The high titer of RAV-0 produced by V^+tvb^s cells, as compared to V^+tvb^r cells, probably resulted from this exogenous RAV-0 infection. Therefore, infectious DNA of RAV-0 was obtained from cells that were exogenously infected with RAV-0, but not from uninfected V^- or V^+tvb^r cells, which contained only endogenous avian leukosis virus-related genes. We have previously reported that uninfected chicken cells also do not have infectious DNAs of a transforming avian leukosis virus or of a reticuloendotheliosis virus (8).

Scolnick and Bumgarner (31) have reported that murine sarcoma virus-infected BALB/c mouse cells have infectious DNA of an endogenous xenotropic murine leukemia virus. Similarly, infectious DNA of an endogenous cat virus has been obtained from the CCC line of cat cells (31; M. Nicolson and R. McAllister, personal communication). However, infectious DNAs of endogenous viruses were not obtained from NIH mouse cells, NZB mouse cells, cat liver, or baboon liver (31; E. Scolnick, personal communication). Therefore, most mammalian cells, like most chicken cells, do not appear to contain infectious DNAs of endogenous viruses.

The difference in infectivity between the DNAs extracted from RAV-0-infected and uninfected chicken cells was not accounted for by a quantitative increase in the amount of RAV-0 DNA or by the presence of unintegrated RAV-0 DNA in the RAV-0-infected cells. (However, the presence or absence of an additional RAV-0 provirus resulting from external infection could not be determined from this experiment.) In addition, the lack of infectivity of the DNAs extracted from uninfected chicken cells does not appear to be a direct result of inefficient expression of the endogenous avian leukosis virus-related genes, since infectious DNA of Rous sarcoma virus was obtained from Rous sarcoma virus-infected, non-virus-producing rat cells (9). Therefore, it appears that the endogenous

avian leukosis virus-related genes in uninfected V^- and V^+tvb^r cells are different from the RAV-0 proviruses in RAV-0-infected cells. (An earlier model of Bentvelzen and Daams [5] also suggested the possibility of differences between endogenous virus-related genes in uninfected cells and proviruses in virus-infected cells.)

Since uninfected V^- chicken cells do not produce RAV-0, it is not clear whether or not the endogenous avian leukosis virus-related genes in V^- chicken cells contain all of the information required for RAV-0 replication. Nucleic acid hybridization studies indicate that the DNA of V^- chicken cells contains nucleotide sequences homologous to most of the nucleotide sequences of RAV-0 RNA (22, 25, 27, 28), but these experiments do not exclude the possibility that the RAV-0-related genes in V^- cells represent a defective or incomplete RAV-0 genome. Weiss et al. (38) have reported induction of avian leukosis viruses by treatment of V^- chicken cells with chemical and physical carcinogens, but the extremely low efficiency of this process does not exclude the possible occurrence of mutational and/or recombinational events. Therefore, three hypotheses might account for the lack of infectivity of the endogenous avian leukosis virus-related genes in V^- chicken cells: (i) the endogenous avian leukosis virus-related genes do not contain all of the information required for RAV-0 replication; (ii) the endogenous avian leukosis virus-related genes are not present as an intact RAV-0 genome; or (iii) the endogenous avian leukosis virus-related genes are linked to a region of cell DNA that interferes with their infectivity (see below).

Since the amount of RAV-0 produced by V^+tvb^r cells is much lower than the amount of RAV-0 produced by V^+tvb^s cells (10, 32) (Table 1), it appears that the endogenous RAV-0 genome in V^+tvb^r cells is inefficiently expressed. However, since RAV-0 is produced by V^+tvb^r line 100 cells (10, 32) (Table 1), it appears that the V^+tvb^r line 100 cells contain a complete endogenous RAV-0 genome. In addition, the specific infectivity of the RAV-0 produced by V^+tvb^r cells is the same as that of the RAV-0 produced by V^+tvb^s cells (Table 1). A model which accounts for the inefficient expression and the lack of infectivity of the endogenous RAV-0 DNA in V^+tvb^r cells is presented in Fig. 3. Our hypothesis is that the endogenous RAV-0 genome in V^+tvb^r cells is linked to a *cis*-acting control element, which results in its inefficient expression. This hypothesis accounts for the lack of infectivity of the DNA extracted from

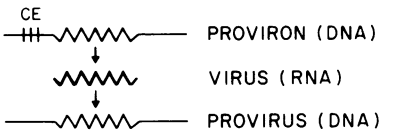

FIG. 3. *Model of the endogenous RAV-0 proviron in V^+ chicken cells. The DNA of uninfected V^+ chicken cells contains an endogenous RAV-0 proviron (top) which consists of the RAV-0 genome (zig-zag line) linked to a cis-acting control element (CE). Transcription of the RAV-0 genome results in production of RAV-0 RNA (middle) and infectious RAV-0. Exogenous infection by RAV-0 results in formation of a RAV-0 DNA provirus (bottom) which consists of the RAV-0 genome without the cis-acting control element.*

V^+tvb^r cells, since the control element is transferred to the recipient cells together with the donor RAV-0 genome. The donor RAV-0 DNA, linked to the *cis*-acting control element, is inefficiently expressed in the recipient cells and, therefore, is not infectious.

We propose that stable RNA transcripts of the endogenous RAV-0 DNA in V^+ cells do not include the control element, so that the RAV-0 which is spontaneously produced by uninfected V^+ cells contains a RAV-0 RNA genome consisting only of the RAV-0 genes (Fig. 3). Exogenous infection of a sensitive cell, for example, a V^+tvb^s cell, results in formation of a RAV-0 provirus, no longer associated with the control element which is linked to the endogenous RAV-0 genome. Since the RAV-0 provirus is not linked to the *cis*-acting control element, it is expressed more efficiently than the endogenous RAV-0 genome, and DNA from cells that contain the RAV-0 provirus is infectious.

Uninfected V^- cells could also contain a complete RAV-0 genome linked to a *cis*-acting control element (hypothesis 3, above). However, since V^- cells do not produce RAV-0, the expression of the endogenous RAV-0 genome in V^- cells would have to be controlled much more stringently than in V^+ cells. This difference could result either from differences in the *cis*-acting control elements, or in *trans*-acting regulatory proteins or RNAs.

We have called the endogenous genetic unit of a RAV-0 genome linked to a *cis*-acting control element a "proviron," in contrast to a RAV-0 "provirus," which is formed as a result of exogenous infection. The proposed linkage of the endogenous RAV-0 genome to a *cis*-acting control element is analogous to the structure of the bacterial operon. Evidence supporting the hypothesis that *cis*-acting control elements are linked to structural genes of eukaryotes has

been obtained in genetic studies of yeast (12, 33), fungi (2, 3, 24), and *Drosophila melanogaster* (A. Chovnick et al., Genetics, **80:** S3, 1975). If our hypothesis is correct, the RAV-0 proviron is an example of this type of gene control in vertebrates.

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