Transfection of Chicken Embryo Cells with DNA Extracted from Avian Virus-Producing Neoplastic Cells

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DNA isolated from avian virus-producing leukemic myeloblasts induced the production of viruses, but not morphological transformation, in cultivated chicken fibroblasts. The recovered virus had the same biological characteristics as the original avian myeloblastosis virus (AMV) and produced myeloblastosis and nephroblastomas when injected into chickens. Neutralization experiments with chicken anti-AMV-BAI strain A sera showed an antigenic community between the DNA-transfected virus and the original virus. Virus induced in fibroblasts after treatment with DNA from a viral nephroblastic nephroblastoma line only gave nephroblastoma when injected into chicken. Treatment of chicken embryo cells with DNA extracted from normal chicken embryos did not induce viral production.

Prior to 1970 little information was available on the replication mechanisms of RNA tumor viruses, even though other infectious viruses with a single-stranded RNA genome demonstrated a double-stranded replicative form in infected cells. It was not obvious that the single-stranded RNA of oncornaviruses passed through a double-stranded state while replicating. This fact, and the prevention of infection by inhibition of DNA synthesis, led Temin (18) to the hypothesis of a DNA provirus. The subsequent finding of viral reverse transcriptase capable of using the single-stranded RNA genome of oncornaviruses as template for DNA synthesis permitted an explanation of the general pattern of oncornavirus replication (1, 19). If Temin's assumption that the genetic information of RNA cancer viruses persists in infected cells as DNA is correct, it should be possible to isolate the proviral DNA from transformed cells and to use it to infect and transform normal cells. The results obtained with DNA extracted from rat XC cells previously transformed by the Prague strain of Rous sarcoma virus, which gave rise to sarcoma virus production and cell transformation when introduced into permissive chicken embryo cells, suggest that cells transformed by Rous sarcoma virus do indeed contain a DNA provirus which can be isolated in an infectious state (5).

In a preliminary note we showed that treatment of chicken embryonic cells with DNA from

avian-transformed and virus-producing leukemic cells induced virus production without morphological transformation, and, when injected into chickens, the virus produced myeloblastic leukemia (13). The leukemic cells used for DNA extraction were transformed by the avian myeloblastosis virus (AMV) BAI strain A (originally given by Baluda and propagated in lymphomatosis-free brown Leghorn chickens) which induces *in vivo* not only myeloblastosis but also nephroblastomas. In this report we will present experiments showing that DNA extracted from leukemic myeloblasts and applied to chicken embryo cells in culture induces an identical virus to the AMV-BAI strain A which, when injected *in vivo*, induces nephroblastomas as well as leukemia. We also investigated the action of DNA extracted from cells transformed by a virus (viral nephroblastic nephroblastoma [DNV]) isolated from the AMV stock and only responsible for nephroblastomas.

MATERIALS AND METHODS

Preparation of DNA from leukemic or normal cells. DNA was extracted from leukemic cells collected at the terminal stage of leukemia from the blood of chickens infected with the AMV-BAI strain A or from chicken embryo cells prepared by trypsinization of 11-day-old embryos. The Marmur procedure (14) was utilized with an additional Pronase treatment (Worthington Biochemicals Corp., 500 μ g/ml, 3 h at 37 C, after treatment with boiled Worthington pancreatic RNase, 50 μ g/ml, 30 min at 37 C). After

the isopropanol step DNA fibers were treated successively with 70, 80, and 90% ethanol for 3 min each; they were then kept in 75% ethanol for 1 day, redissolved in sterile 0.015 M NaCl plus 0.015 M sodium citrate, and stored at 4 C.

Preparation of DNA from DNV. A transmissible nephroblastoma line has been developed in chickens. The origin of this nephroblastoma was described elsewhere (11). Briefly, a leukemia appeared in a hamster after inoculation of AMV-producing chicken cells and was successfully transmitted to hamster, and a transplantable tumor line HP was established. Avian leukemia gs antigen was found in these tumor cells, and AMV-like particles were observed by electron microscopy in early passage material (21).

The HP tumor was examined for HaLV gs antigen by complement fixation and immunodiffusion and was negative by both tests (3). Inoculation of HP tumor cells, associated with normal chicken embryo fibroblasts, induced nephroblastomas in chickens. Transmission of one such nephroblastoma was possible with either cells or cell-free extracts in 1- to 6-day-old chicks. The virus produced, DNV, transforms the nephrogenic cells *in vivo*. A malignant transformation was observed only in kidney cells (12). The DNA virus was passed for 27 generations in lymphomatosis-free brown Leghorn chickens, a strain sensitive to AMV, but neither leukemias nor any other neoplasias were observed.

DNA from DNV cells, obtained from the tumor by trypsinization, was prepared in the same way as above.

Contamination of DNA preparations by RNA was tested immunologically by agar diffusion (10). Antipoly I.poly C serum which reacts with single- or double-stranded RNA was used. No RNA contamination of DNA was detected. With this method, 0.1 μ g of RNA can be detected, so the maximal RNA contamination was less than 1%. The average molecular weight of DNA was determined by band-forming centrifugation in a Spinco analytic ultracentrifuge. A portion of the DNA was treated for 1 h at 37 C with Worthington RNase-free DNase (made up in Tris-hydrochloride, 1 M, pH 7.0; $MgCl_2$, 1 M) at a DNase-DNA ratio of 1:40.

Treatment of cultured chicken cells with DNA from normal or neoplastic cells. The brown Leghorn strain of chicken, type C/O lymphomatosis-free, initially supplied by J. Carr and then bred at the Institut Gustave-Roussy, was used in all experiments. Procedures for the culture of chicken embryo cells and the treatment with DNA were the same as those previously described (13, 6). In short, primary cultures originally derived from 11-day-old chicken embryos were prepared in 250-ml plastic flasks (Falcon) in 20 ml of Dulbecco's modification of Eagle medium (Long Island BA) supplemented with 20% decomplexed calf serum. After 24 h of incubation at 37 C in a humidified CO_2 incubator, the cultures were rinsed with 0.14 M NaCl, 0.05 M Tris, pH 7.4, and pretreated with 10 ml of the same solution containing 100 μ g of DEAE-dextran per ml (Pharmacia) for 15 min at 37 C. This solution was then replaced by DNA (40 μ g in 2.5 ml of 0.14 M NaCl, 0.05 M Tris). After 15

min of contact at 37 C, the DNA was replaced by 20 ml of Eagle medium supplemented with 5% decomplexed calf serum and 10% tryptose phosphate broth. This treatment was repeated one or two times after one or two passages.

Detection and assay of virus production in transfected cells. Virus production was detected by electron microscopic observation of cells and by assay of supernatant fluids from confluent cultures for DNA polymerase. Cells were fixed with glutaraldehyde and stained with uranyl-acetate for electron microscopic examination. DNA polymerase was assayed in the supernatant, previously centrifuged at low and medium speeds (2,000 and 10,000 $\times g$), by a two-step assay using a synthetic template. After preincubation with detergent (Brij 58, 0.05%) and β -mercaptoethanol (150 mM), the enzymatic reaction was done in 10 mM KCl, 50 mM NaCl, 17 mM $MgCl_2$, 67 mM Tris, pH 8.3, polydeoxyribosethymidilic acid-polyribosadenylic acid double-stranded complex, 0.025 optical density unit (determined at 260 nm) per ml and 0.37 μ M 3H -dTTP (specific activity = 24,000 counts per min per pmol) in a final volume of 0.1 ml. Incubation was for 1 h at 37 C, and the acid-precipitable fraction was put on a Whatman GF filter, rinsed, dried, and counted in a liquid scintillation counter. Supernatants of nonproductive cultures give 100 to 200 counts/min, and those of infected cultures incorporated up to 20,000 counts/min.

To have an estimate of the amount of virus, a reaction with a standard plasma containing a known number of virus particles was included with each assay. Biological activity of the DNA-treated culture was tested by intraperitoneal injection of 24-h-old chickens with the virus-containing culture fluid. Negative cultures were kept for 1 month.

Neutralization of virus. Antiserum was prepared in chickens by injections of active AMV-BAI strain A (20). Virus was mixed with a 1:100 dilution of antiserum and, after 30 min of contact at room temperature, was injected into 24-h-old chickens. Controls were injected with the same quantity of untreated virus.

RESULTS

Recovery of virus in chicken embryo cells treated with DNA from neoplastic myeloblasts. In the first series of experiments, randomly growing chicken embryo cells were treated with DNA extracted from virus-producing myeloblasts previously transformed by AMV. After 14 days and two or three DNA applications, virus particles were observed in the electron microscope in two out of three experiments (Table 1). Virus was absent in cells treated under the same conditions with DNA that had been preincubated with DNase (13).

Further investigations showed that virus particles appeared after a single leukemic DNA treatment, and repeated treatments were not necessary. In a series of three experiments in which chicken embryo cells were treated only

TABLE 1. *Recovery of virus from chicken embryo cells treated with neoplastic myeloblast DNA*

Expt	No. of DNA treatments	Virus recovery	Time interval between first DNA treatment and virus detection (days)
1	2	+	14
2	3	0	(-)
3	3	+	14
4	1	+	8
5	1	+	8
6	1	+	12

once with DNA (average molecular weight = 12×10^6), virus particles were detected by the DNA polymerase assay in the supernatant 8 (2 out of 3) and 12 days after DNA application (Table 1). Comparing the DNA polymerase activity with the standard leukemic plasma, the supernatant of confluent DNA-treated cells contained about 5×10^8 virus particles/ml. Morphological transformation was not observed.

Fibroblasts infected with one infectious particle per 10^5 cells (as determined by ATPase activity (15) and assuming one infectious particle per 1,000) produced sufficient AMV to provide detectable DNA polymerase activity at 4 days and a high activity at 8 days.

Biological and antigenic characterization of the virus recovered after treatment with DNA from neoplastic myeloblasts. Supernatant from confluent virogenic chicken embryo cell cultures was injected intraperitoneally into 10 newly hatched chickens. The appearance of leukemic cells was observed on blood smears. The existence of AMV in the plasma was studied by assaying the ATPase activity according to Mommaerts et al. (15). Blood smears revealed typical myeloblastic leukemic cells. The plasma was virogenic at 8×10^9 to 6×10^{11} particles/ml as determined by ATPase activity. Nephroblastomas identical to those induced with AMV-BAI strain A and described by Ishiguro et al. (9) were discovered by post mortem examinations. Results are summarized in Table 2.

One of the leukemic plasmas was injected into 3-day-old chickens for immunological characterization. In the control group 10 out of 10 injected chickens died of leukemia. In contrast, among 10 birds injected with the same amount of virus neutralized by chicken anti-AMV-BAI strain A serum (see Materials and Methods), only one developed a myeloblastosis, and nine

survived infection 6 months without leukemia. This indicates immunological similarity between the DNA-induced virus and AMV-BAI strain A.

Treatment of chicken embryo cells with DNA extracted from DNV nephroblastoma. Chicken embryo cells were exposed to DNA extracted from nephroblastoma cells by the same methods as used above. Four applications of DNA were employed in one experiment and only two in a second experiment. Virus was detected by DNA polymerase assay in the supernatant and/or by electron microscopic observation of the cells at 9 and 16 days, respectively, after the first DNA treatment. Injections of the supernatant into newly hatched chickens produced nephroblastomas after 2 to 4 months in six out of eight animals in the first experiment and five out of five in the second. Subsequent cell passages in vivo of these nephroblastomas always produced nephroblastomas. Tumors were mostly bilateral and histologically appeared as typical nephroblastomas differentiating into nephron elements as well as cartilaginous and osteoid cells (21). Weekly blood smears failed to give any indication of leukemia.

Treatment of chicken embryo cells with DNA extracted from normal kidney or total chicken embryos. Experiments were undertaken to establish whether the application of DNA from normal homologous chicken embryo cells could lead to the production of information for oncornaviruses. The methods of treatment were identical to those of the previous experiments; however, in one experiment the number of DNA treatments greatly exceeded that used with DNA from leukemic cells. The results of five experiments are summarized in Table 3.

In contrast to chicken embryo cells in which virus appeared 8 to 12 days after a single application of leukemic DNA, no viral produc-

TABLE 2. *Neoplasias induced by virus recovered from chicken embryo cells after exposure to leukemic cell DNA*

Chick	Leukemia	Nephroblastoma	Days after infection
1	+	0	51
2	+	0	57
3	+	0	60
4	0	0	62
5	+	+	71
6	0	0	84
7	0	+	102
8	0	+	109
9	0	+	129
10	0	+	153

TABLE 3. *Chicken embryo cells treated with DNA from normal kidney or total chicken embryo*

Expt	No. of treatments with DNA	Virus recovery	No. of passages
1	3	0	9
2	3	0	8
3	8	0	20
4	2	0	7
5	4	0	7

tion was discovered even after long-term culture (5 to 6 weeks) and eight applications of DNA (average molecular weight = 8×10^6) from normal chicken embryos. It must be pointed out that the normal embryos were obtained from a RIF-free flock (type C/O) and that C-particles have never been observed in our laboratory in control cultures of fibroblasts derived from these embryos.

DISCUSSION

AMV-BAI strain A induces myeloblastosis and nephroblastomas in chickens. In vitro, AMV efficiently transforms only the hematopoietic cells, and the transformed cells are virogenic (2). Upon infection of fibroblasts with AMV, only the virogenic information is expressed and the virus-producing cells are not morphologically transformed.

Treatment of chicken embryo cells with DNA extracted from leukemic or nephroblastic cells induces virus production, but not morphological transformation. The induced virus has the same biological characteristics as the original virus when injected into chickens, and either produces leukemia and nephroblastomas when induced by myeloblast DNA or only DNV nephroblastomas when induced by DNV nephroblastoma DNA. Thus, this DNA is virus specific and not simply a consequence of transformation. Thus, treatment of RIF-free chicken fibroblasts with cellular DNA extracted from virus-producing leukemic cells triggers the formation of a virus responsible for both leukemia and nephroblastoma, but treatment of the same cells with cellular DNA from nephroblastic nephroblastoma cells triggers the formation of virus that can induce nephroblastoma only.

Our data are in agreement with the findings that transfecting DNA from cells transformed by different strains of Rous sarcoma virus induces the production of virus identical to the original virus, and thus indicates that this is a general phenomenon for the avian oncornaviruses (5-7, 13, 16, 17). All these results support the assumption that avian virus-

induced neoplastic cells contain a DNA transcript of the viral genome. However, we do not yet know if this DNA is integrated into the cell genome.

In 1969, a viral oncogene hypothesis proposed (8) that cells of most or all vertebrates contain the genome of C-type RNA viruses that are vertically transmitted from parents to offspring. C-particles have, in fact, been found in normal chicken embryonic cells (4). Our experiments failed to confirm the oncogene hypothesis, at least in the chicken strain used. Neither virus production nor transformation was observed in cells treated with DNA from normal embryos or kidney cells even in long-term culture. Perhaps a more sophisticated experimental model is necessary for expression of virus genetic information, if such information exists in normal cells.

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