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STUDY OF AVIAN ADENOVIRUS DNA INFECTIVITY IN CHICK EMBRYOS

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Summary. — Inoculation of CELO adenovirus deproteinized DNA into the allantoic cavity of 9-day-old chick embryos (CE) induced the synthesis of infectious viral particles. The produced virions appeared to be identical with CELO adenovirions in terms of morphology, electrophoretic and immunochemical properties of hexon major capsid protein and also of DNA dot-hybridization. High infectivity of CELO DNA (minimal infective dose equaled 40 ng) may be also related, at least in part, to the absence of deoxyribonuclease activity in the allantoic fluid (AF).

Key words: adenoviruses; infectious DNA; DNA minimal infective dose; chick embryos; allantoic fluid; recombinant DNA

Introduction

Animal and plant viruses of many groups have infective nucleic acids; this phenomenon consists of the production of standard virions resulting from the introduction of viral genome into permissive cells in the form of deproteinized DNA or RNA. In pilot experiments for the demonstration of RNA infectivity, total nucleic acids have been extracted from the cells or organs infected with RNA-containing viruses (Picornaviridae, Togaviridae), and nucleic acid preparations have been administered into mouse organs (Franklin *et al.*, 1959; Sokol *et al.*, 1959).

In further studies of viral DNA and RNA infectivity, a better controlled system — permeabilized cell culture into which nucleic acids had been transfected has been used (Green and Wold, 1980). Infectious adenovirus DNA has been first detected during infection of permissive cell culture (Graham *et al.*, 1974) and since that it has been followed only in cell cultures, DNA transfection in the presence of calcium phosphate gel being most frequently employed (Flint, 1982). Recently, the infectivity of adenovirus DNA has been used for production of infectious progeny after recombinant DNA administration (Chinnadurai *et al.*, 1978) and also in genetic experiments for DNA recombination (Shiroki *et al.*, 1983) or marker rescue (Galos *et al.*, 1979).

The main drawbacks of transfection to permeabilized cells are the low specific activity of DNA, the complicated special pretreatment of cells before transfection and the necessity of plaque production under agar overlay. Meanwhile, in vitro cell system is not the only possible tool for viral nucleic acids infectivity measurement.

Even in the earliest papers CE, commonly used in virology, have been successfully employed for transfection of picornavirus RNA (Monntain and Alexander, 1959). In this paper we describe the production of high infectivity chicken adenovirus type I DNA (gal I; Chick embryo lethal orphan virus — — CELO as revealed by its administration into CE allantoic cavity.

Materials and Methods

Viruses. Avian adenovirus CELO, strain Phelps kindly supplied by T. S. Denisova was propagated in CE (Denisova *et al.*, 1979) and the virions were purified with the use of a conventional technique (Green and Pino, 1963). Simian adenovirus type 7 (SA7) was prepared and purified as described in Ponomareva *et al.* (1979).

Production of viral DNA preparations. CELO DNA was isolated by phenol deproteinization after protein hydrolysis with proteinase K (van Der Eb, 1969); the DNA complex with the terminal protein was prepared by deproteinization and gel filtration in guanidine chloride (Challberg *et al.*, 1980). For maximal purification of CELO DNA from possible contamination with trace amounts of CELO virions, centrifugation in CsCl equilibrium density gradient (1.60— —1.80 g/cm^3) has been carried out for 30 hr at 105,000×g. DNA preparation was applied on top of the gradient and fractions were collected from below after puncturing the bottom of the test tube. SA7 virus DNA was prepared as described earlier (Ponomareva *et al.*, 1979).

Labelling and hybridization of DNA preparations. CELO adenovirus DNA was labelled with ^{32}P in a slightly modified nick translation reaction (Maniatis *et al.*, 1975). DN ase was added at a concentration 10 times as low as that recommended for this technique (0.01 μg/ml). The incubation was carried out at 15 °C for 30 min. Specific activity of CELO DNA preparations equaled $0.5-1\times10^6$ cpm/min/μg. Dot-blot hybridization of DNA preparations immobilized on nitrocellulose filters with CELO ^{32}P-DNA was carried out as described by Thomas (1980). γ-^{32}P label was introduced with the help of γ-^{32}P-ATP-ase and polynucleotide kinase into a 17-base pair oligodeoxyribonucleotide whose synthesis had been described elsewhere (Lunin *et al.*, 1983).

Electrophoresis. DNA preparations were electrophoresed in 1 % agarose gel (Osterman, 1981); the synthetic oligonucleotides — in 20 % polyacrylamide gel (PAG) with autoradiographic detection (Osterman, 1983). Electrophoretic analysis of adenovirus structural proteins was carried out in 12 % PAG according to the method of Osterman (1983) as modified in our paper (parallel application of heated and unheated samples, Khilko *et al.*, 1983). The proteins were stained with ammonia silver complexes (Osterman, 1983).

Immunochemistry. Immunochemical identification of CELO proteins was carried out with the use of rocket immunoelectrophoresis in 1 % agarose gel (Osterman, 1983) containing rabbit antibodies against purified CELO virions; electrophoregrams obtained were stained in the same manner as the proteins in PAG.

Electron microscopy of CELO virions was made on carbon formvar-coated grids contrasted for 1 min in glow discharge with uranyl acetate (Horne *et al.*, 1975).

Transfection. For transfection, deproteinized DNA preparations or CELO DNA-protein complex preparations were diluted in sterile saline, and administered via individual glass capillaries into the allantoic cavity of 9-day-old CE; the embryos were incubated for 72—96 hr at 37 °C. After the embryos were chilled, the AF was collected under sterile conditions and concentrated by sedimentation onto the CsCl solution layer by ultracentrifugation. The resulting concentrated AF was analysed with the use of above-mentioned methods and then purified according to the scheme proposed for virions (Green and Pino, 1963).

Minimal infectious dose of CELO DNA was determined as follows: appropriate dilutions of DNA preparation (10, 20, 40, 80, 160, 320, 640 ng and 1 μg per embryo) were administered to

9-day-old CE, 5 embryos per each CELO DNA dilution. After 72 hr the AF was taken and tested for CELO virions. In the event of negative result, AF was passaged in CE. After 4 passages, CELO virions were detected by both, electron microscopy and immunologically in the CAF of all the 5 embryos inoculated with 40 ng of CELO DNA.

Results and Discussion

After the introduction of CELO adenovirus deproteinized DNA preparations into the allantoic cavity of CE, particles whose size and morphology were typical of CELO adenoviruses (Fig. 1) were detected in concentrated AF. The experiments with inoculation of saline or adenovirus SA7 DNA preparations (which cannot reproduce in the avian cells) allowed us to rule out the possibility that adenovirions detected in concentrated AF are not descendants of CELO DNA, but are produced by induction caused either by the process of injection itself, or by the injection of nonspecific DNA. In these experiments, concentrated AF appeared to contain no adenovirions as evident from electron microscopic data as well as from electrophoretic and immunochemical analysis of proteins. To exclude the possibility of embryo infection with contaminating virions contained in CELO DNA preparations, two control experiments have been carried out.

The injection of pancreatic DNase-treated CELO DNA preparations into the embryos failed to cause the appearance of adenovirions in concentrated AF. Meanwhile, after administration of CELO DNA additionally purified in CsCl equilibrium density gradient, adenovirions were detected in concentrated AF. In the latter experiment, infectious activity of the gradient DNA fraction was observed only in peak fractions with 1.713 g/cm^3 density which corresponds to CELO DNA density, but not at the top of the gradient where CELO virions with 1.345 g/cm^3 buoyant density must have been present (Laver *et al.*; 1971) (Fig. 2).

Infectious activity of CELO DNA-induced virions was equal to the infectious activity of the original virus (10^8—10^9 virions/ml concentration caused 50 % CE letality). For characterization of adenovirions produced by transfection of CE with CELO DNA we used several methods of DNA and protein

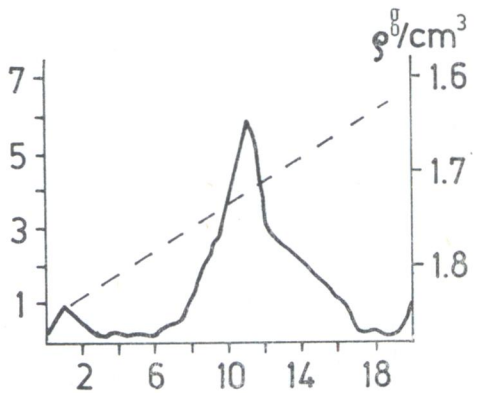

Fig. 2.
Centrifugation of ^{32}P-labelled CELO DNA in CsCl equilibrium density gradient
Abscissa: fraction number; left ordinate: radioactivity (counts × 10^3/min); right ordinate: density constant (ρ) g/cm^3

analyses. Concentrated AF DNA was hybridized with CELO ^{32}P-DNA (Fig. 3). This hybridization has not been observed in control samples containing DNA from concentrated AF of uninfected embryos or of embryos injected with hydrolyzed CELO DNA. Insignificant hybridization of ^{32}P--DNA with simian adenovirus SA7 DNA (Fig. 3-I) indicated a certain homology between genomes of these viruses belonging to 2 different genera of family Adenoviridae (Norrby *et al.*, 1976). Similar data have been reported by Allestrom and coworkers (1982) for human adenovirus type 2 DNA. Concentrated AF proteins were electrophoretically analysed in PAG. We have utilized the unique ability of the major adenovirus structural hexon protein to modify electrophoretic mobility depending on the temperature of protein dissociation (Khilko *et al.*, 1983). Fig. 4 (tracks 1 and 2) shows electrophoregrams of CELO structural proteins after denaturation at 100 °C and after mild dissociation in the same solution at 20 °C. In the former case CELO hexon monomeric chains with molecular mass 115 kD were observed (in addition to other viral proteins) and in the latter — trimers, i.e. native hexon capsomers were found (Khilko *et al.*, 1983). In the concentrated AF collected after the transfection of embryos with CELO DNA preparations bands with mobility of monomers (track 3) and trimers (track 4) have been detected under the same conditions.

Immunochemical analysis of AF preparations obtained after transfection of embryos with CELO DNA has detected specific antigens not present in control samples. Quantitative analysis of CELO DNA infective activity has shown that minimal infectious dose equals 40 ng of CELO DNA per embryo. The fact that such a low DNA dose is maintained in the embryos and remains active indicates that either CELO DNA has an increased resistance to nuclease or AF has essentially no deoxyribonucleases. Complete hydrolysis of CELO DNA by pancreatic nuclease allowed us to refuse the first suggestion. The second suggestion has been verified in the course of incubation of 9-day--old chick embryo AF in the tests for endodeoxyribonuclease (Fig. 5) and exodeoxyribonuclease activities. As seen from Fig. 5, the superhelical DNA of pBR_{322} plasmid remains intact after 2-hr incubation with AF and is not converted into open rings or linear molecules. This indicates that AF does not contain even traces of endonuclease activity, since one nick in superhelical DNA molecule is enough for its conversion into an open ring. AF has no exonuclease activity either, as evident from the stability in its presence of 5-end-labelled ^{32}P-oligodeoxyribonucleotide. This result can be accounted for by either complete absence of DNases in AF or by the presence of a potent inhibitor; the latter is hardly likely, however, as AF appeared to be unable to inhibit hydrolysis by restriction endonucleases. Therefore, high infective activity of CELO DNA observed in experiments with CE transfection can be accounted for (at least partially) the stability of injected DNA molecules in AF, since infectivity of adenoviral DNA in cell cultures is increased owing to the use of DNA complex with terminal protein (Goodherst, 1971) or to DNA treatment by some agents, DEAE-dextran included. However, the methods mentioned failed to cause a significant rise of DNA infectivity: virion yield was not significantly increased.

It can be seen from our data that we have demonstrated infective activity of CELO chicken adenovirus DNA by transfection of CE. Available literature does not have reports of transfection of this system by DNA preparations of any adenoviruses or viruses of other families or groups. The described phenomenon allows to utilize CE as a simple, practicable and highly efficient system in different experiments with avian adenovirus DNA infectivity. In particular, the method of virion preparation by adenovirus DNA injections into CE allantoic cavity seems promising in the framework of avian adenovirus genetic studies and in experiments with gene engineering using avian adenovirus recombinant DNA.

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Explanation of Figures (Plates XXV—XXVII):

Fig. 1. Electron microscopy of concentrated AF collected after injection of CELO adenovirus DNA into 9-day-old chick embryos. Magn. 120,000×.

Fig. 3. Dot-blot hybridization of the samples tested with ^{32}P-labelled CELO DNA probes.
I: 1 — immobilization site of concentrated AF prepared from uninfected 12-day-old CE; 2 — immobilization site of concentrated AF prepared from saline-infected embryos; 3— — concentrated AF from embryos infected by DNase-hydrolyzed CELO DNA; 4 — concentrated AF from embryos infected by SA7 adenovirus DNA; 5 — immobilization site of pBR 322 plasmid DNA (100 ng); 6 — concentrated AF from CELO virus-infected embryos; 7 — concentrated AF from CELO DNA-infected embryos; 8 — immobilization site of CELO DNA (1 ng); 9 — immobilization site of CELO DNA (10 ng); 10 — immobilization site of CELO DNA (100 ng).
II: 1 — immobilization site of alkali-pretreated virions DNA; 2 — CELO DNA; 3 — SA7 adenovirus DNA; 4 — pBR 322 plasmid DNA; 5 — concentrated AF prepared by infection of CE with CELO adenovirus and alkali-pretreated before immobilization; 6 — concentrated AF from CELO DNA-infected embryos; 7 — the same concentrated AF as in 5, but pretreated with proteinase K before immobilization; 8 — the same concentrated AF as in 6 pretreated with proteinase K.

Fig. 4. Electrophoresis in 12 % PAG of concentrated AF prepared from CELO virus-infected or CELO DNA-infected CE.
1 — CELO-virus-infected concentrated AF has been applied on the gel after heating in boiling water bath in the presence of sodium dodecyl sulphate; 2 — the same preparation applied on the gel without heating; 3 — CELO DNA-infected concentrated AF pretreated as in 1; 4 — the same preparation as in 3 applied on the gel without heating.

Fig. 5. Electrophoresis in 1 % agarose gel of pBR 322 plasmid DNA incubated in AF of 9-day-old CE.
1 — 0 min at 37 °C; 2 — 30 min at 37 °C; 3 — 60 min at 37 °C; 4 — 90 min at 37 °C; 5 — — 120 min at 37 °C; 6 — 10 — pBR 322 DNA incubated in sterile saline for the same time and at same temperature.