POSSIBLE NUCLEASE ACTIVITY OF THE T-ANTIGEN OF VIRUS SV-40

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A preparation of the T-antigen of virus SV-40 was isolated from an extract of golden hamster tumors by precipitation with ammonium sulfate followed by fractionation on DEAE-cellulose. Despite 100-fold purification of the preparation it contained traces of cell proteins as impurities. Treatment of calf thymus DNA with the preparation of T-antigen in the presence of magnesium ions considerably reduced the viscosity of the DNA solution during the first hour of incubation. The T-antigen, if inactivated by heating, or the analogous fraction from normal hamster tissues had no such action. During centirfugation in a sucrose gradient the sedimentation constant of hamster DNA was reduced after treatment with T-antigen from 28 S to 16 S, corresponding to a reduction of about 4-5 times in the molecular weight of the DNA. It can be concluded from these results that the partially purified preparation of T-antigen of virus SV-40 possesses endonuclease activity.

KEY WORDS: virus carcinogenesis; T-antigen of virus SV-40; nuclease activity.

One of the central problems in virus carcinogenesis is the study of the mechanisms of integration of virus DNA with the genetic apparatus of the cell. It has been postulated that oncogenic viruses have a system to incorporate their DNA actively into the DNA of the host cell and that the key enzyme of this system is one coded by a virus possessing the properties of an endonuclease; from the time of its appearance it must belong to the "early" virus proetins and immunologically it must be identical to the T-antigen [3].

The possible nuclease activity of the T-antigen of virus SV-40 was investigated.

EXPERIMENTAL METHOD

A 10% extract of hamster tumors, induced by virus SV-40, was used as the source of the T-antigen. The T-antigen was purified under the control of the complement fixation test (CFT) modified for Takachi's micromethod. The sera of hamsters with tumors, with a titer of not less than 1:40-1:80, were used as the immune sera. The basic test was carried out in the cold, for 18 h at 4°C, using the serum in a titer of 1:4 with the addition of 1.5 and 2 doses of complement. The T-antigen was purified in two stages.

A. Preliminary Purification of the T-antigen by Successive Precipitation of Proteins with Ammonium Sulfate [4]. The 10% extract, after centrifugation at 105,000 g for 1 h, was used for purification. Dry ammonium sulfate was added to the protein extract slowly in the proportion of 0.14 g to 1 ml of solution, with constant mixing in the cold for 30 min. The precipitate was removed by centrifugation, the supernatant was collected, and the same quantity of ammonium sulfate was again added to it, after which the precipitation procedure was repeated. The precipitates obtained after the first and second procedures were

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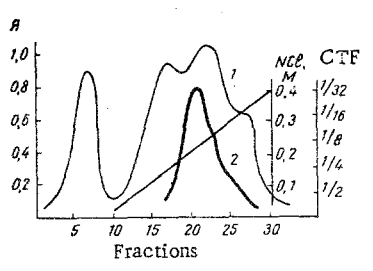

Fig. 1

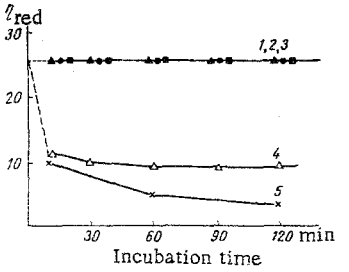

Fig. 2

Fig. 1. Fractionation of T-antigen on DEAE-cellulose: 1) protein (A 280); 2) T-antigen (titer in CFT).

Fig. 2. Changes in viscosity of DNA treated with preparation of T-antigen and pancreatic DNAase:) inactivated T-antigen; 2) fraction of normal tissue analogous to T-antigen; 3) viscosity of original DNA; 4) active preparation of T-antigen; 5) pancreatic DNAase.

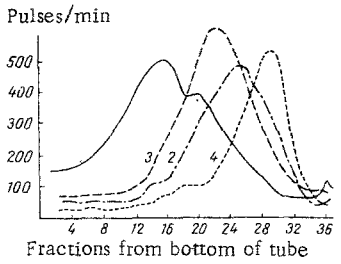

Fig. 3. Changes in sedimentation of DNA after treatment with preparation of T-antigen. Centrifugation in sucrose gradient: 1) neutral – original DNA; 2) DNA + I-antigen; 3) alkaline – original DNA; 4) DNA + T-antigen.

dissolved in $^1/_{10}$ of the initial volume of 0.14 M NaCl solution. The fractions were labeled as follows: fraction I) 0-20% saturation with ammonium sulfate, fraction II) 20-40% saturation, fraction III) not precipitated at 40% saturation.

The fractions were dialyzed overnight against solutions of the required ionic strength (for the CFT, 0.14 M NaCl solution; for fractionation on a column with DEAE-cellulose, 0.02 M).

B. Ion-Exchange Chromatography of DEAE-Cellulose [5]. The following solutions were used: for equilibrating the DEAE-cellulose and applying the protein – a 0.02 M sodium-phosphate buffer, pH 6.5 (solution 1); for preparing the concentration gradient – a 0.4 M NaCl solution, 0.02 M sodium-phosphate buffer, pH 6.5 (solution 2). From 20 to 50 mg protein from fraction II was applied to a column measuring 9 × 1.5 cm, the column was washed with 30 ml of solution 1, after which the proteins were eluted with a smooth NaCl concentration gradient (30 ml of each solutions 1 and 2). Fractions of 3 ml were collected. In some experiments a linear-stepwise gradient was used, so that the resolving power of the method was increased; the gradient was interrupted in the zone 0.15 M NaCl. The protein concentration in the resulting fractions was determined spectrophotometrically, the NaCl concentration with the IRF-23 refractometer, and the CFT was carried out in order to detect T-antigen in them. The purity of the preparations of the T-antigen was verified by electrophoresis in polyarylamide gel: 7.5% gel, pH 9.5, current to the tube 3 mA, time 2 h.

DNA was obtained from calf thymus for the viscosimetric tests by the usual method with sodium dodecylsulfate and extraction with a mixture of chloroform and butyl alcohol. H^3-labeled DNA was obtained by the same method from transplantable hamster cells of line KhRO. The cells were grown in Eagle's medium with 10% serum in the presence of thymidine-H^3 in a dose of 4-6 μCi/ml. To obtain DNA molecules homogeneous in size, gel filtration was carried out through a column with Sepharose 2B (size of column 35 × 1.2 cm, 0.01 M tris-buffer, 0.14 M NaCl, pH 7.8). The fractions collected contained about 50 μg/ml DNA with a specific activity of 5000 pulses/min/μg.

To detect hypothetical nuclease activity of the "early" virus proteins the action of T-antigen on the DNA was investigated by two methods: 1) viscosimetry; 2) sedimentation.

1. The decrease in viscosity of the DNA solutions, which reflects the decrease in molecular weight of the DNA, was studied in the presence of T-antigen or nucleases. Fractions containing the maximal quantity of T-antigen were chosen for the investigation; part of the solution of T-antigen was inactivated by heating to 56°C for 30 min (control). The incubation mixture was prepared in the proportion of 1 μg DNA in the presence of Mg^{++} ions (5×10^{-3} M), pH 7.8. Incubation was carried out directly in the capillary viscosimeter, placed in a water bath at 37°C, so that dynamic observations were possible. The relative vis-

cosity was determined experimentally, the reduced viscosity (η red) was calculated, and the graph of reduced viscosity versus incubation time was plotted.

2. The sedimentation of the DNA was investigated during centrifugation in a sucrose gradient after treatment with T-antigen. Double-stranded breaks in the DNA could be determined by sedimentation in a neutral gradient, single-stranded breaks only in an alkaline gradient; consequently, the sedimentation of H^3-labeled DNA was studied in both neutral and alkaline gradients. The DNA was treated with the T-antigen preparations as follows. To a sample of DNA-H^3 in a volume of 0.1-0.15 ml, containing not more than 5-8 μg DNA, 0.1 ml of a preparation of T-antigen containing 30-50 μg protein was added. To each sample one drop of 0.025 M $MgCl_2$ was added. All the solutions were made up in tris-buffer, pH 7.8. The samples were incubated for 2 h at 37°C or at 0°C (control) and centrifuged in 5-20% sucrose density gradients at neutral and alkaline pH values. The volume of the gradient was 4.8 ml; the conditions of centrifugation: SW50 rotor, 44,000 rpm, 180 min. The fractions were collected two drops at a time from the bottom of the tube on filter papers, fixed and washed with TCA solution, after which the radioactivity of the samples was counted in a scintillation spectrometer. The sedimentation constants and molecular weight of the double-stranded DNA were calculated by the formula of Bergey and Hershey and by the appropriate equations [1].

EXPERIMENTAL RESULTS

Fractionation of the T-antigen with ammonium sulfate showed that practically all the T-antigen was precipitated at 20-40% saturation (fraction II); fraction I contained only traces of T-antigen and fraction III contained none whatever. Only fraction II was therefore used for the subsequent purification.

A typical graph of ion-exchange chromatography of the T-antigen on DEAE-cellulose is shown in Fig. 1. Altogether 41.2 mg protein of fraction II in a volume of 16 ml was applied to the column. Fractions 1-10 contained protein not adsorbed on DEAE-cellulose and eluted with 0.02 M phosphate buffer. Fractions from 11 to 30 were collected during the run through of NaCl solution in a smoothly increasing concentration from 0.02 to 0.4 M. With the aid of the CFT, T-antigen was found in fractions from 18 to 26, the largest amount in fractions 20 and 21, in which its titer was 1:32. The beginning of elution of the T-antigen under these conditions began with 0.18 M NaCl and reached a maximum with 0.21-0.23 M. No T-antigen was detected in the other fractions. To estimate the degree of purification of the T-antigen from cell proteins, the fraction with the maximal content of antigen was studied by electrophoresis in polyarylamide gel. Staining revealed four bands of proteins. Calculations showed that the method used yielded a preparation of T-antigen with an approximately 100-fold degree of purification, although it was not homogeneous: it contained at least three cell proteins.

A study of the action of the T-antigen preparation on DNA by the viscosimetric method showed that the viscosity of DNA incubated with T-antigen was reduced by more than half after 10 min (Fig. 2). T-antigen, inactivated by heat, had no such property. For comparison, the results of treatment with pancreatic DNAase, an enzyme with the properties of endonuclease [2], is given; the enzyme was used in a proportion of 1 μg to 200 μg DNA. The action of T-antigen was exhibited only in the presence of Mg^{++} ions. The decrease in the viscosity of the DNA observed cannot be explained by complex formation between the DNA and proteins, for the viscosity of the pure DNA was indistinguishable from the viscosity of the DNA treated either with inactivated T-antigen or with native T-antigen in the absence of magnesium ion. The observed decrease in viscosity can only be explained by the formation of breaks in the DNA molecules.

The problem of whether the nuclease activity thus discovered belongs to the T-antigen or not remained unsolved, for cell nucleases could have been eluted at the same time. The same procedure of purification was accordingly carried out with tissue extracts from a healthy hamster (liver, kidneys). The results (Fig. 2) show that the fractions of normal tissues analogous to the preparation of T-antigen did not change the viscosity of the DNA solution.

Degradation of DNA also was observed during the study of the action of T-antigen on it by the method of sedimentation in neutral and alkaline sucrose gradients. Treatment of DNA-H^3 with the preparation of T-antigen led to a distinct shift of the peaks toward the lighter fractions (Fig. 3). DNA samples incubated with T-antigen at 0°C contained DNA fragments analogous to the original untreated DNA. The sedimentation constant of the untreated DNA in these experiments was about 28 S, but after treatment with T-antigen it fell to 16 S, corresponding to a decrease of 4-5 times in the molecular weight of the double-stranded DNA; this suggests induction of breaks in both strands of the DNA. The same pattern was observed also on sedimentation of the denatured DNA in an alkaline gradient.

It can be concluded from the results of viscosimetry and sedimentation analysis that the partially purified preparation of T-antigen possesses undoubted nuclease activity. To judge from the breaking up of the DNA molecules into relatively large fragments, this activity is endonuclease in character. However, a final conclusion on this matter can be drawn only after it has been shown whether the mononucleotide fraction is increased after treatment of DNA with the T-antigen.

The preparation of the T-antigen thus obtained still contained certain cell proteins as impurities, and the data on its biological activity given here must thus be regarded as still preliminary. The absence of nuclease activity in a fraction obtained from the normal cell of an animal of the same species, analogous to T-antigen, is an important but not an unambiguous argument in support of the nuclease activity of the "early" proteins of SV-40 virus. A final conclusion must await the obtaining of a homogeneous preparation of the T-antigen.

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