GENE MAPPING OF SV40: THE BIOLOGICAL ACTIVITY OF SPECIFIC VIRAL DNA FRAGMENTS PRODUCED BY CLEAVAGE WITH HAEMOPHILUS PARAINFLUENZAE RESTRICTION ENDONUCLEASE

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1. Introduction

The oncogenic simian virus 40 (SV40) induces tumor antigen (T-antigen) synthesis in permissive and non permissive cells. Intranuclear T-antigen appears after the initiation of the early SV40 mRNA synthesis and prior to the onset of DNA replication [1]. Since T-antigen seems to be involved in the regulatory mechanism of both cellular and viral DNA replication and in late viral gene expression [2,3], its further characterization is of particular interest.

Recently we have shown that T-antigen is a virus-coded and not a cell-coded protein [4,5]. In order to define the region of the viral genome required for T-antigen synthesis we prepared DNA fragments by digesting SV40 DNA component I (DNA I) with Haemophilus parainfluenzae I (Hpa I) and parainfluenzae II (Hpa II) restriction endonucleases. The isolated DNA fragments were transferred into permissive monkey cells (TC7) by means of our microinjection technique. T-antigen and viral capsid protein (V-antigen) synthesis were assayed in these cells.

2. Materials and methods

2.1. *Cell culture and virus*

For all experiments TC7 cells, a sub-line of the CV1 line of African green monkey kidney cells, and SV40 strain 777 were used.

2.2. *Preparation of SV40 DNA I*

Cultures of confluent TC7 cells were infected with plaque purified virus at a multiplicity of 0.1 plaque forming units per cell. After an adsorption period of 2 h, cells were washed and covered with serum free Eagle's medium. DNA was extracted from the cells by the selective extraction method of Hirt [6]. SV40 DNA I was purified as described elsewhere [3].

2.3. *Preparation of SV40 DNA fragments*

100 μg of SV40 DNA I were incubated with a mixture of Hpa I and Hpa II restriction enzymes (isolated as described elsewhere [7]) for 2 h at 37°C in 800 μl reaction mixture consisting of 0.01 M Tris–HCl (pH 7.4), 0.01 M $MgCl_2$, 0.006 M KCl, 0.1 M NaCl, 0.001 M dithiothreitol and 160 μl enzyme solution (1–2 μl of Hpa I and Hpa II solution digest completely 1 μg SV40 DNA as determined by electrophoresis of the DNA products, see below). The reaction was terminated by addition of EDTA to 0.05 M. DNA fragments were deproteinized by extraction with 1 vol chloroform/isoamylalcohol (24:1) and concentrated by precipitation with 2 vol of ethanol and 1/10 vol 1 M NaCl.

2.4. *Separation of SV40 DNA fragments by gel electrophoresis*

The fragments were separated by electrophoresis in agarose gels containing 1.4% agarose [8]. Electrophoresis was performed in E-buffer (0.036 M Tris–HCl, 0.03 M NaH_2PO_4, 0.001 M EDTA, pH

7.7, without ethidium bromide) for 90 min at 80 V and room temperature. In control gels the DNA fragments were visualized by staining the gels with ethidium bromide (3–5 μg/ml for 10 min). Bands containing DNA fragments were cut out and DNA was then recovered from the gel pieces by electrophoresis. For injection experiments the separated fragments were dialyzed against diluted injection buffer [5], vacuum dried and dissolved to a final concentration of 0.2 mg DNA per ml of injection buffer.

2.5. *Microinjection of SV40 DNA fragments*

The transfer of the DNA fragments into the recipient cells (grown on glass-slides) was performed under a phase-contrast microscope at a 400-fold magnification using glass-capillaries having a diameter of 0.5 μm at the tip [9]. The volume transferred per recipient cell was between 10^{-11} and 2×10^{-11} ml.

2.6. *Detection of SV40 T- and V-antigen*

T- and V-antigen synthesis were assayed by the indirect immunofluorescent technique. Cells (grown on glass-slides) were fixed in a mixture of acetone–methanol (2:1) for 10 min at −20°C and subsequently incubated for each antigen-antibody reaction in a humid chamber at 37°C for 45 min with extensive washing (phosphate buffer) after each staining step. Fluorescence was examined under dark-field u.v. illumination (Zeiss). SV40 T-antiserum (hamster) was kindly supplied by M. Fogel, Rehovot, and SV40 V-antiserum by G. Brandner, Freiburg.

3. Results

3.1. *T-antigen synthesis induced by microinjection of the SV40 DNA fragment B*

Restriction endonuclease Hpa I inserts three and Hpa II one double strand cleavage into SV40 DNA I. The physical order of these fragments on the SV40 DNA is shown in fig.1 [8,10]. In order to obtain direct experimental evidence as to whether one of these SV40 DNA fragments carries the information for T-antigen, SV40 DNA I was cleaved with a mixture of both restriction enzymes under

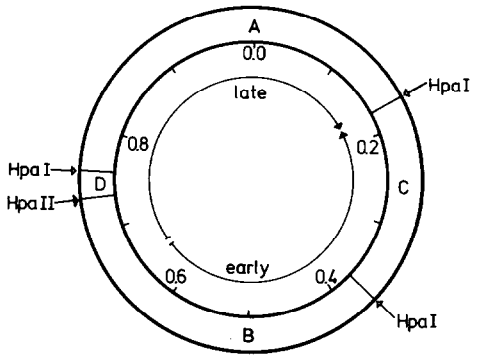

Fig.1. Hpa I and Hpa II restriction endonucleases cleavage map of the SV40 genome. The 00 position is defined as the site cleaved by Eco RI restriction endonuclease. The early and the late region and the transcription direction are indicated.

conditions described in materials and methods. Following isolation of the fragments by agarose gel electrophoresis (fig.2), each fragment was introduced into TC7 cells by our microinjection technique. After the transfer of the fragments, cells were further cultivated in serum free Eagle's medium at 37°C and T-antigen synthesis was assayed for by the indirect immunofluorescence technique.

T-antigen synthesis was only observed after microinjection of the fragment B. This fragment, which corresponds to 36% of the SV40 genome (fig.1), induced in about 60% of the recipient cells intranuclear T-antigen formation within the first 24 h after microinjection. Injection of fragments A, C and D independently or as a mixture did not induce T-antigen synthesis in TC7 cells.

3.2. *Time course of T-antigen formation*

As demonstrated in fig.3, T-antigen formation was first detectable about 10 h after microinjection of fragment B. At this time 3% of the recipient cells exhibited a weak intranuclear T-antigen fluorescence. Thereafter the percentage of T-antigen positive cells as well as their intranuclear fluorescence intensity increased, resulting in a maximum of 60% positive cells at 20–24 h post microinjection. The number of T-antigen positive cells decreased subsequently so that T-antigen was no longer detectable 80 h after microinjection.

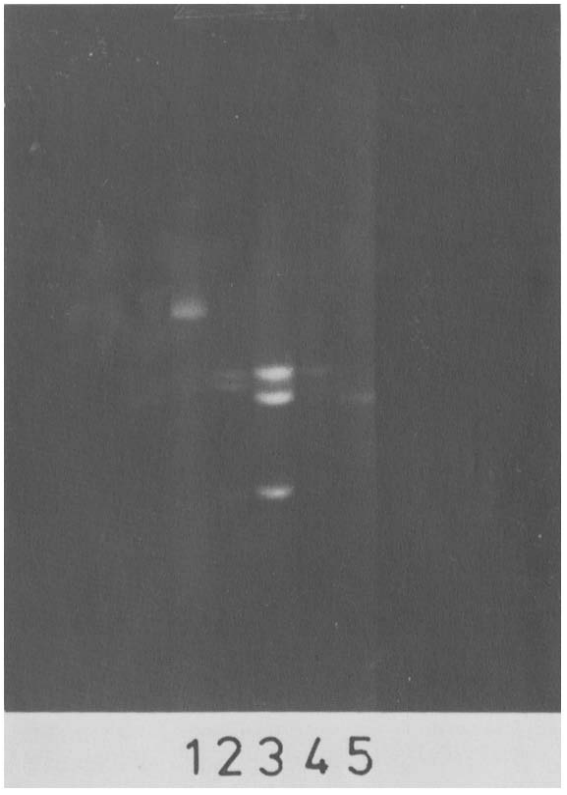

Fig.2. 1.4% agarose gel electrophoresis of SV40 DNA and DNA fragments: (1) SV40 DNA I with small amounts of DNA II; (2) SV40 DNA fragments generated by Hpa I restriction endonuclease digestion; (3) SV40 DNA fragments A, B and C generated by Hpa I and Hpa II restriction endonucleases digestion (fragment D is eluted from the gel); (4) fragment A (from Hpa I-Hpa II restriction endonucleases digestion); (5) fragment B (from Hpa I-Hpa II restriction endonucleases digestion). After staining in ethidium bromide gels were photographed using Polaroid type 107 film and Kodak no. 23A red filter; exposure time 15 sec.

3.3. *Microinjection of SV40 DNA fragments and V-antigen synthesis*

To determine whether V-antigen synthesis too is inducible by microinjection of an SV40 DNA fragment generated by Hpa I-Hpa II restriction endonucleases cleavage, isolated fragments or a mixture of all four fragments were transferred into TC7 cells as described above. We could not detect V-antigen synthesis in any of these experiments. Microinjection of a mixture of the three fragments

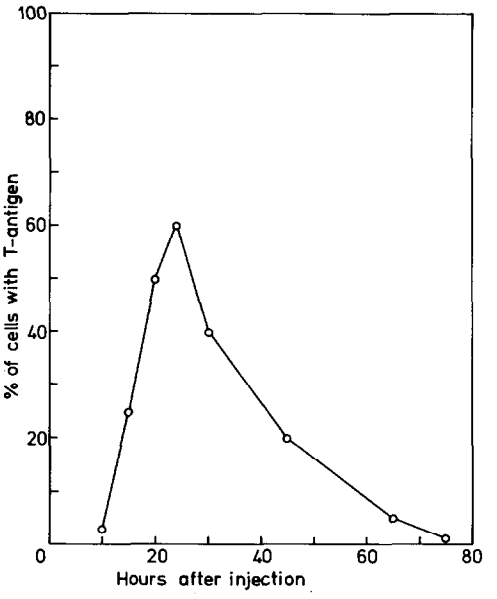

Fig.3. Time course of T-antigen formation in TC7 cells. Each time point is based on a count of 30 injected cells.

obtained by digestion of SV40 DNA I with Hpa I restriction endonuclease alone as well failed to induce V-antigen synthesis.

4. Discussion

SV40 T-antigen is a protein coded for by the early region of the SV40 genome. Mol. wt estimations for T-antigen, based on its electrophoretic mobility, range between 70 000 and 100 000 daltons [11,12]. In this investigation it was shown that intranuclear SV40 specific T-antigen fluorescence could be demonstrated in a high percentage of TC7 cells which had been microinjected with the B-fragment. This fragment was generated by complete digestion of SV40 DNA I with Hpa I and Hpa II endonucleases and then purified by gel electrophoresis. The isolated fragments are not contaminated with uncleaved DNA since microinjection of only one complete SV40 DNA molecule per TC7 cell should induce T- and V-antigen synthesis in 100% of the recipient cells [3].

Fragment B (0.375–0.735) corresponds to 36% of the total genome and contains about 60%

(0.375–0.655) of the early genome region (0.175–0.655) (fig.1). This early part of fragment B carries information for a polypeptide with a mol. wt of 40 000 to 50 000. Our results demonstrate that this polypeptide contains the determinant group of the T-antigen and that the C-terminal part of the entire early protein (T-antigen) is not required for the T-antigen-antibody reaction. This assumption is in good agreement with the hypothesis that T-antigen is a 'pleiotropic effector' carrying the various regulatory functions and the specifity for the immunological reaction on distinct sites of the molecule [2]. Further microinjection experiments should reveal whether this polypeptide coded by fragment B exerts regulatory functions as well (e.g. stimulation of DNA synthesis), and whether it is able to induce stable transformation.

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References

[1] Tooze, J. (ed.) (1973) The molecular biology of tumor viruses, Cold Spring Harbor Laboratory, New York.
[2] Weil, R., Salomon, C., May, E. and May, P. (1975) Cold Spring Harb. Symp. quant. Biol. 39, 381.
[3] Graessmann, A., Graessmann, M. and Mueller, C. (1975) submitted for publication.
[4] Graessmann, A., Graessmann, M., Hoffmann, E., Niebel, J., Brandner, G. and Mueller, N. (1974) FEBS Lett. 39, 249.
[5] Graessmann, M. and Graessmann, A. (1975) submitted for publication.
[6] Hirt, B. (1967) J. Mol. Biol. 26, 365.
[7] Lauppe, H. F. (1975) Doctoral dissertation, Univeristy of Heidelberg.
[8] Sharp, P., Sugden, B. and Sambrook, J. (1973) Biochemistry 12, 3055.
[9] Graessmann, M., Graessmann, A., Hoffmann, E., Niebel, J. and Pilaski, K. (1973) Mol. Biol. Reports 1, 233.
[10] Khoury, G., Howley, P., Nathans, D. and Martin, M. (1975) J. Virology 15, 433.
[11] Del Villano, B. and Defendi, V. (1973) Virology 51, 34.
[12] Tegtmeyer, P., Schwartz, M., Collins, J. and Rundell, K. (1975) J. Virology 16, 168.