MAPPING OF A DNA-BINDING DOMAIN OF SIMIAN VIRUS 40 T-ANTIGEN USING NON-DEFECTIVE ADENOVIRUS 2–SIMIAN VIRUS 40 HYBRID VIRUSES

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Received 26 March 1982

1. Introduction

The Simian virus 40 (SV40) large tumor antigen (T-Ag), a multifunctional phosphoprotein of ~92 000 M_r (review [1]) binds specifically to the origin of replication of SV40 DNA [2–5] and non-specifically to other types of DNA [6–9]. Several lines of evidence suggest that SV40 T-Ag is composed of functionally distinct domains. Many of these observations were obtained using adenovirus type 2 (Ad2)–Simian virus 40 hybrid viruses which code for a variety of T-Ag related proteins of different lengths (review [1]). In attempting to localize a non-specific DNA-binding region in the polypeptide chain of T-Ag, we compared the calf thymus DNA-binding properties of SV40 T-Ag from SV40-transformed human cells and T-Ag related polypeptides coded for by Ad2$^+$ND2 and Ad2$^+$ND4. These proteins share the C-terminal end of large T-Ag and range from 42 000 M_r to that of intact T-Ag (92 000 M_r). These experiments and several other observations suggest a DNA-binding domain inside the polypeptide chain of T-Ag located between maximally 0.53 and 0.39 SV40 DNA map units.

2. Materials and methods

2.1. *Infection, radiolabeling and extraction of cells*

Ad2$^+$ND2 and Ad2$^+$ND4 stocks, originally obtained from A. M. Lewis jr (National Institutes of Health, Bethesda MD) were prepared as in [10] (titer of both virus stocks: 5×10^8 plaque forming units [p.f.u.]/ml). HeLa S_3 cells grown in minimal essential medium (MEM) were infected at a multiplicity of 100 PFU/cell. Late in infection (24 h p.i.) 4×10^6 cells were labeled with 30 μCi [^{35}S]methionine (Amersham) in 1 ml methionine-free DMEM for 2 h. SV40-transformed human cells (SV80) (4×10^6) were labeled with 30 μCi [^{35}S]methionine for 2 h. After labeling, 4×10^6 cells were harvested, and lysed for 30 min on ice with 0.4 ml extraction buffer (0.5% non-ionic detergent NP40, 10 mM Tris–HCl, 0.1 M NaCl, pH 9.0) as in [11]. Homogenates were centrifuged at 105 000 × g for 30 min and the supernatants were used as extracts for DNA-binding experiments.

2.2. *Analysis of DNA-binding properties of SV40 T-Ag and related proteins*

DNA-binding experiments were performed as in [11]. Briefly, 0.4 ml cell extracts (pH 9.0) were incubated either directly with 30 mg calf thymus DNA-cellulose (PL Biochemicals) or after careful pH-adjustment with 0.1 M acetic acid to pH 6.0 or pH 7.3 shortly before incubation (30 min, 4°C). After washing with the corresponding buffer, the DNA-cellulose was eluted twice with 0.4 ml elution buffer (10 mM Tris–HCl (pH 9.0), 0.8 M NaCl, 0.5% NP40). The combined eluates and supernatants were analyzed for DNA-bound and -unbound SV40-specific proteins, respectively, by immunoprecipitation with 10 μl rabbit anti-SDS-T serum [12] and 150 μl preswollen protein A–Sepharose (Pharmacia) (60 min, 0°C). Protein A–Sepharose beads were washed extensively with 10 mM Tris–HCl (pH 7.2), 0.5% NP40, and eluted (200 μl 50 mM NH_4HCO_3, 1% SDS, 1% 2-mercaptoethanol, 30 min on ice). After lyophilization, immune complexes were dissolved (5 min, 100°C) in 20 μl sample buffer (65 mM Tris–HCl (pH 6.8), 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue) and analyzed by SDS–polyacrylamide gel electrophoresis. Labeled proteins were detected by fluorography [13].

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00145793/82/0000–0000/$02.75

3. Results and discussion

3.1. *DNA-binding of SV40-specific proteins encoded by the hybrid viruses Ad2⁺ND4 and Ad2⁺ND2*

In a first attempt to map a DNA-binding region on the polypeptide chain of SV40 large T-Ag, we analyzed Ad2⁺ND4 encoded SV40-specific proteins. [^{35}S]-Methionine-labeled extracts of Ad2⁺ND4 infected HeLa cells were incubated at pH 6.0, 7.3 or 9.0 with calf thymus DNA-cellulose. DNA-unbound (u) and -bound (b) SV40-specific proteins were immunoprecipitated from the DNA–cellulose supernatants and eluates, respectively, with rabbit anti-SDS-T serum and analyzed by SDS–polyacrylamide gel electrophoresis (fig.1A). The control immunoprecipitate from the total cell extract showed almost all the SV40-specific Ad2⁺ND4 proteins: 56 000 M_r protein, 60 000 and 70 000 M_r protein families, and the 92 000 M_r protein. SV40-specific proteins larger than the 56 000 M_r protein showed a DNA-binding activity increasingly similar to that of SV40 T-Ag, i.e., at pH 6.0 the 60 000 M_r and 70 000 M_r family proteins and the 92 000 M_r protein were found predominantly bound to DNA–cellulose, whereas the 56 000 M_r protein reproducibly had a weaker DNA-binding activity. At pH 7.3, only the 92 000 M_r protein showed some binding activity. In this experiment, the SV40-encoded 42 000 M_r protein was barely detectable, but after longer exposure it was found only in the unbound fraction at all pH-values tested.

To define more precisely the DNA-binding properties of the 56 000 and the 42 000 M_r proteins, we repeated this type of experiment under identical conditions using radiolabeled extracts of HeLa cells infected with Ad2⁺ND2 which codes for only two T-Ag related fragments, the 56 000 M_r (0.44–0.17 SV40 DNA map units, MU) and 42 000 M_r (0.39–0.17 SV40 DNA MU) proteins [14]. At all pH-values the

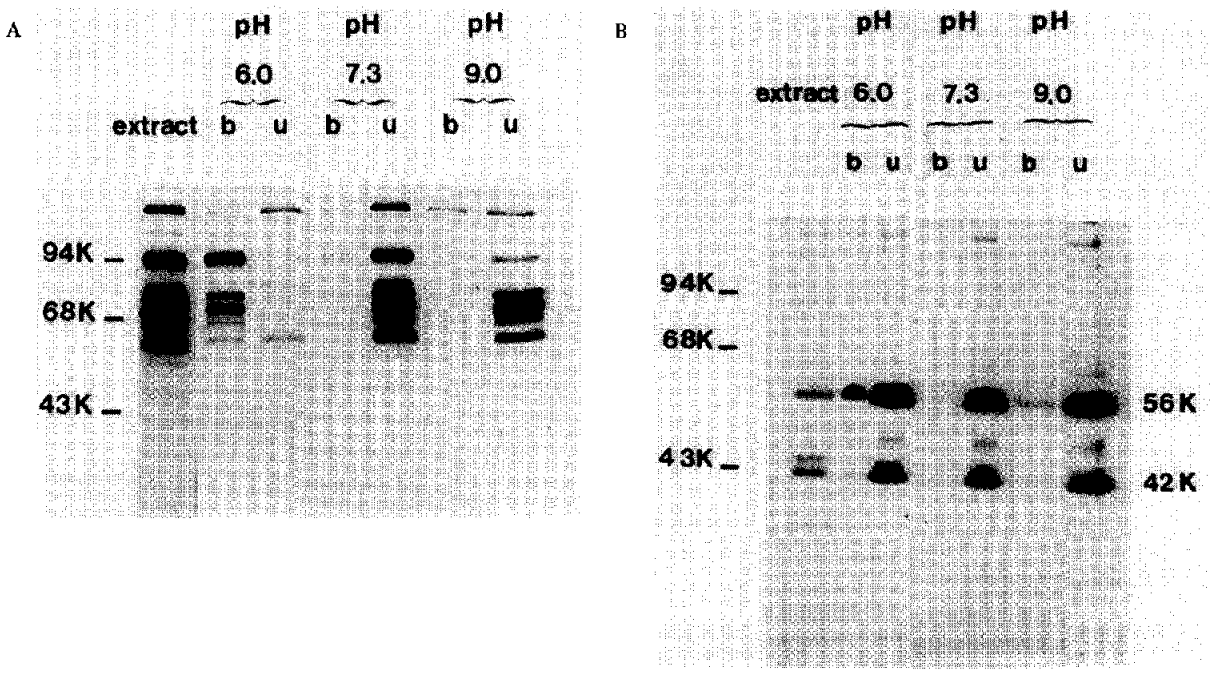

Fig.1. Analysis of DNA-binding properties of SV40 T-Ag related proteins encoded by Ad2⁺ND4 and Ad2⁺ND2. HeLa S_3 monolayer cells were infected with Ad2⁺ND4 or Ad2⁺ND2 and labeled for 2 h with 30 μCi [^{35}S]methionine/4 × 10^6 cells. Aliquots of cell extracts corresponding to 4 × 10^6 cells were incubated with 30 mg calf thymus DNA-cellulose at pH 6.0, 7.3 and 9.0. Unbound (u) and bound (b) SV40 T-Ag related proteins were immunoprecipitated from DNA-cellulose supernatants and eluates, respectively, and analyzed on 12.5% (A) or 10% (B) SDS–polyacrylamide slab gels. The fluorograms of the gels are shown. The left panels of (A) and (B) show the proteins immunoprecipitated from aliquots of the original extracts. ^{14}C-Radiolabeled M_r markers (Amersham) are indicated on the left of the figure: phosphorylase *a* (94 000 M_r), bovine serum albumin (68 000 M_r) and ovalbumin (43 000 M_r). (A) Ad2⁺ND4 (56 000, 60 000 and 70 000 M_r families, 92 000 M_r protein, 100 000 M_r adenovirus protein); (B) Ad2⁺ND2 hybrid virus-encoded proteins are indicated on the right of picture (56 000 and 42 000 M_r).

42 000 M_r protein remained unbound in the DNA–cellulose supernatant while the 56 000 M_r protein showed again some binding activity at pH 6.0 (fig.1B). The differences between the binding properties of the 42 000 and the 56 000 M_r fragments confirm the results obtained with Ad2$^+$ND4 and suggest that the C-terminal end of a DNA-binding region might be located inside the polypeptide chain of T-Ag corresponding to 0.39 MU (fig.3).

3.2. *Binding of Ad2$^+$ND2 hybrid virus encoded proteins to DNA in the presence of SV40 large T- and small t-antigen*

To compare the DNA-binding activities of SV40 large T-Ag and little t-antigen directly with those of both the 56 000 and 42 000 M_r proteins, we mixed extracts from [^{35}S]methionine labeled SV40-transformed cells (SV80) and Ad2$^+$ND2-infected HeLa cells at a 1:1 ratio and incubated these mixtures with DNA-cellulose as described above. Fig.2 demonstrates that the binding behavior of intact SV40 T-Ag was

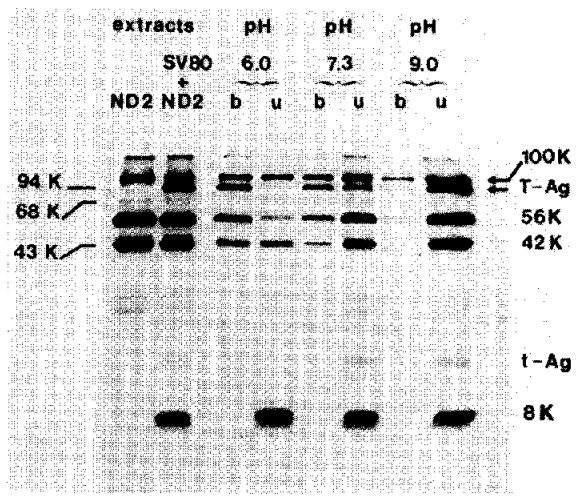

Fig.2. Analysis of DNA-binding properties of SV40 T-Ag related proteins (56 000 and 42 000 M_r) encoded by Ad2$^+$ND2 in the presence of SV40 T-Ag expressed in SV40-transformed SV80 cells. [^{35}S]Methionine-labeled cell extracts from HeLa S_3 cells infected with Ad2$^+$ND2 and from SV80 cells were mixed at a ratio of 1:1. DNA-binding experiments using aliquots of the mixed cell extracts corresponding to 4 × 10^6 cells and gel electrophoresis were performed as described for fig.1. The ^{14}C-radiolabeled M_r markers are indicated on the left of the picture. The following virus-encoded proteins are indicated on the right of the picture: T-Ag, t-Ag, 8000 M_r (SV40-encoded proteins in SV80 cells); 56 000, 42 000 M_r (Ad2$^+$ND2 encoded T-Ag related proteins); 100 000 M_r (Ad2$^+$ND2 encoded adenovirus 100 000 M_r protein).

similar to that in [11], i.e., it bound efficiently to DNA-cellulose at pH 6.0 and more weakly at pH 7.3, while at pH 9.0 most of it remained unbound in the supernatant after DNA-cellulose incubation. At pH 6.0 the 56 000 M_r protein showed a stronger DNA binding behavior than that observed in the absence of T-Ag in fig.1B. Even the 42 000 M_r protein showed some weak binding activity at pH 6.0 in the presence of T-Ag from SV80 cells. This control experiment supports the results obtained from fig.1A,B. Additionally, the mixing experiment suggests that possibly due to aggregation properties, intact T-Ag may influence the binding properties of the 56 000 and 42 000 M_r proteins.

SV40 small t-antigen has an N-terminal region of 82 amino acids (0.65–0.59 MU) in common with large T-Ag [15]. As expected, small t-antigen observed in all lanes containing SV40 large T-Ag occurred only in unbound fractions. The low-M_r t-antigen (8000 M_r) in [16] corresponds to the common N-terminal region of both small and large T-Ag, and it did not bind to DNA-cellulose (fig.2). These observations confirm the results in [8,16].

These results and those from other laboratories using:

(i) Known T-Ag peptide fragments [8,17] and SV40-specific proteins encoded by defective adeno-SV40 hybrid viruses [18];
(ii) Temperature-sensitive (tsA) or SV40 deletion mutants (review [1]) and applying;
(iii) Microinjection of early SV40 DNA fragments [19] support the idea that some of the multifunctional properties of T-Ag are explainable by a correlation between certain polypeptide regions and distinct functions.

Fig.3 shows the DNA-binding domain mapped inside the polypeptide chain of T-Ag between, maximally, 0.53–0.39 SV40 DNA MU. These results propose that according to the SV40 DNA mapping data of

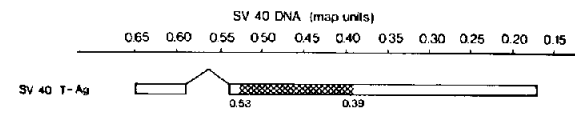

Fig.3. Mapping of the DNA-binding region of SV40 T-Ag according to SV40 DNA map units. The physical map of the early region of the SV40 DNA is shown in the top line [1]. The interruption indicates the position of the 0.59/0.54 intron. The hatched section shows the DNA-binding domain.

Ad2⁺ND2 and Ad2⁺ND4 [14] the right-end of a domain necessary for the non-specific DNA-binding properties of T-Ag can be mapped between 0.42–0.39 SV40 DNA map units. Additionally, our results agree with observations which allow one to restrict the left-end of the domain at ~0.53 MU: an 84 000 M_r protein, which has the N-terminal part in common with full length T-Ag, but misses a C-terminal region between 0.25–0.17 MU, shows a DNA-binding affinity comparable to full length T-Ag [17]; the 33 000 M_r protein encoded by the SV40 deletion mutant d1 1001 (0.64–0.43 MU) binds to DNA with a high affinity [20]; SV40 small t-antigen (0.64–0.54 SV40 MU) in our hands and in other laboratories [8,16] had no DNA-binding affinity; an 82 000 M_r in vitro translation product of early SV40 mRNA (0.53–0.17 MU) has a normal DNA-binding behavior [8].

The non-specific DNA-binding domain suggested here may be associated with the function of T-Ag to induce host cell DNA synthesis [21]. By using a number of cloned fragments with deletions in various positions of the SV40 DNA, only the region of T-Ag encoded between 0.51–0.42 SV40 DNA MU, i.e., inside the non-specific DNA-binding domain, is absolutely necessary for the induction of cellular DNA synthesis [21].

Acknowledgements

We appreciate the excellent technical assistance of Ms Asta Paule, Mr Peter Hirschkorn and Mrs Manuela Benedikt-Palme and thank Mrs Madge B. Henning for the help with the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (He 882/4 and De 212/3).

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