Extraction of SV40 T-antigen from lysates of transformed cells

J.A. Lenstra, R.A.M.M. Smits and H. Bloemendal

Department of Biochemistry, University of Nijmegen, Geert Grooteplein Noord 21, 6525 EZ Nijmegen, The Netherlands

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Subcellular fractions from SV-40 transformed hamster lens cells, prepared by chemical extractions, were tested for the presence of T-antigen by immunoautoradiography. Most of the T-antigen was present in the nucleus and was resistant to extraction by 2 M NaCl, indicating an association with the nuclear matrix. Another part of the T-antigen was, under certain conditions, resistant to extraction of the cells with a non-ionic detergent. This T-antigen could be solubilized by Ca^{2+} at low temperature, conditions that also cause a specific depolymerization of microtubules.

Transformed hamster lens cell *T-antigen*

1. INTRODUCTION

Like products of oncogen expression, SV40 T-antigen is capable of inducing and maintaining malignant cell transformation [1]. Studies of its mode of action [2] have focussed on its DNA binding capacity, associated enzymatic activities and its intracellular localization. Its presence in the nucleus and on membranes as well as its association with the salt-resistant nuclear matrix has been well documented [3–7]. On the basis of extraction of whole cells with the non-ionic detergent NP-40, it was postulated that T-antigen was also present in a 'nucleoplasmic' fraction, but not in the cytoplasm [6]. However, the presence of cytoplasmic T-antigen was demonstrated [8], which cosedimented with mRNP particles and was liberated by RNase treatment.

Here, we use cell fractionation by chemical extractions [9] and immunoautoradiography to localize T-antigen in transformed hamster lens cells. As in [5,6], we found that most of the nuclear T-antigen occurred in the nucleus and was resistant to salt extraction, confirming an association with the nuclear matrix. Another part of the T-antigen was released by Ca^{2+} from the non-ionic detergent-resistant structure if cells had been lysed under specific conditions. This cannot be explained readily by an association with mRNP particles [8] or its presence in a nucleoplasmic fraction [6], but suggests an interaction with microtubules.

2. MATERIALS AND METHODS

SV40-transformed hamster lens cells were maintained in suspension culture as in [10]. Hamster SV40 tumor serum was raised by injecting 10^7 cells into 6–8-week-old Syrian gold hamsters, collected after 8 weeks and stored at −80°C, yielding a titer with immunofluorescence of about 64.

Immunofluorescence was carried out by fixing cells with acetone at −20°C, incubation with tumor serum (30 min at 37°C) and with protein A coupled to fluorescein (Pharmacia, 1 mg/ml) in phosphate-buffered saline, 1 h at 4°C). Indirect immunoprecipitation with tumor serum (20 μl/10 μCi lysate) and protein A–Sepharose (Pharmacia) was carried out as in [11].

Subcellular fractionation was carried out by scaling up the methods in [9]. Transfer of proteins, separated by gel electrophoresis [12], to nitrocellulose (Schleicher and Schull) was performed as in [13] with a voltage gradient of 10 V/cm for 15–22 h, resulting in complete transfer of all pro-

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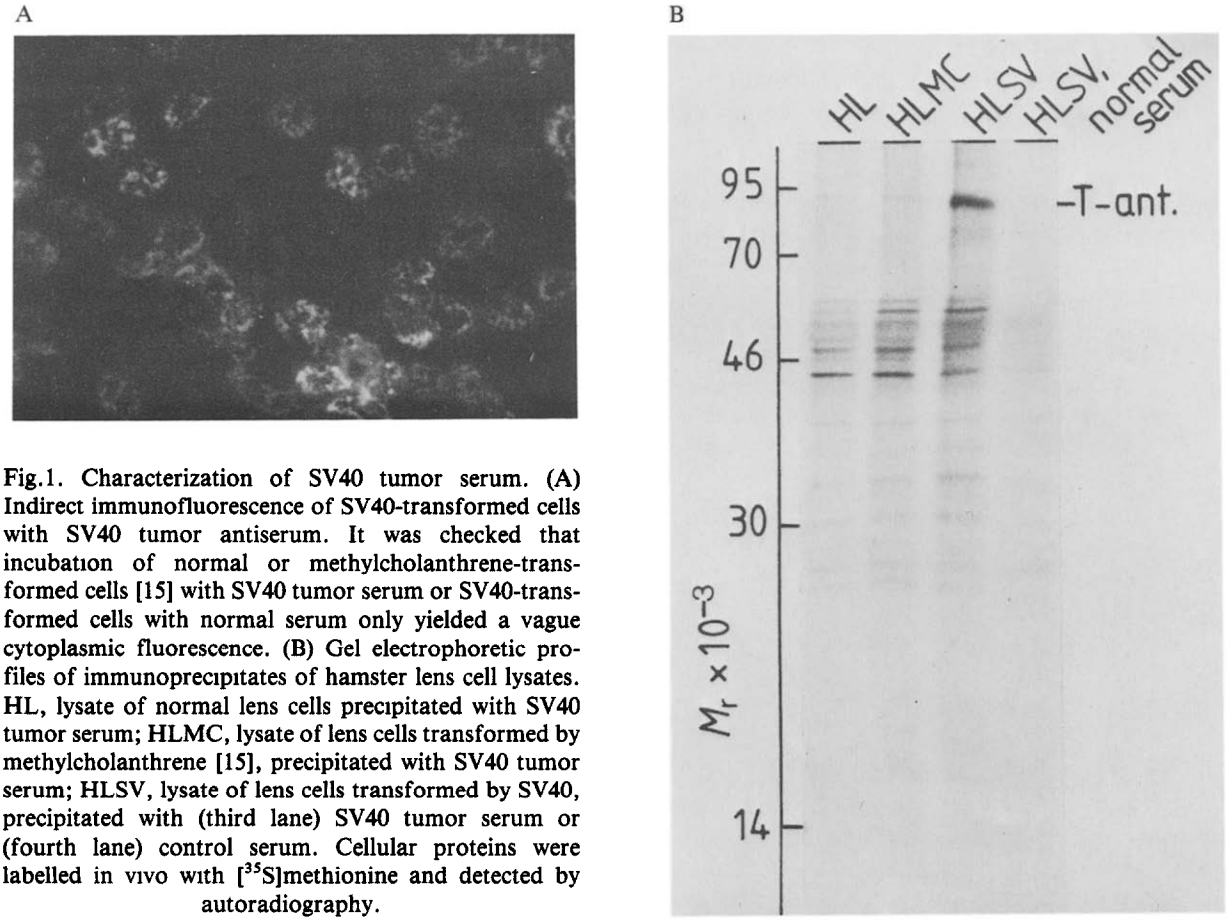

Fig.1. Characterization of SV40 tumor serum. (A) Indirect immunofluorescence of SV40-transformed cells with SV40 tumor antiserum. It was checked that incubation of normal or methylcholanthrene-transformed cells [15] with SV40 tumor serum or SV40-transformed cells with normal serum only yielded a vague cytoplasmic fluorescence. (B) Gel electrophoretic profiles of immunoprecipitates of hamster lens cell lysates. HL, lysate of normal lens cells precipitated with SV40 tumor serum; HLMC, lysate of lens cells transformed by methylcholanthrene [15], precipitated with SV40 tumor serum; HLSV, lysate of lens cells transformed by SV40, precipitated with (third lane) SV40 tumor serum or (fourth lane) control serum. Cellular proteins were labelled in vivo with [^{35}S]methionine and detected by autoradiography.

teins of up to 40 kDa and almost complete transfer of larger proteins. Immunoautoradiography of nitrocellulose sheets was carried out by preincubation in 0.2% (w/v) bovine serum albumin, 0.35 M NaCl, 10 mM Tris–HCl (pH 7.5), 0.1 mM phenylmethylsulfonyl fluoride for 3 h at room temperature, placing the sheets in polyethylene bags with 0.1 ml/cm^2 sheet, 0.5% (w/v) sodium deoxycholate, 1% (w/v) Triton X-100, 0.3% (w/v) gelatine, 0.15 M NaCl, 10 mM Tris–HCl (pH 7.5), 0.1 mM phenylmethylsulfonyl fluoride, addition of 1 μl antiserum/cm^2, sealing and rocking overnight at room temperature, followed by washing 3 times with the same buffer, incubation with 1 μCi [^{125}I]protein A (labeled by chloramine-T treatment) in 25 ml of this buffer per sheet for 3 h at room temperature, washing 3 times with water and autoradiography between two intensifying screens. Exposure time was about 4 days.

3. RESULTS

The SV40 tumor antiserum was characterized by immunofluorescence and immunoprecipitation. In agreement with [6,14], immunofluorescence (fig.1A) revealed a granular nuclear pattern, presumably due to binding of antiserum to large T-antigen. Immunoprecipitation of cell lysates with tumor antiserum (fig.1B) demonstrated the presence of T-antigen (94 kDa) exclusively in SV40-transformed cells. The 20-kDa T-antigen was not detected (cf. [16]). The band in the 55-kDa region, slightly more pronounced with both transformed cell lines, might represent the non-viral middle T-antigen [17], but this protein was not detected by immunoautoradiography (see below).

To avoid unequal solubilization of subcellular fractions during immunoprecipitation, we tested

the detection of T-antigen by lysing samples in SDS-containing sample buffer, electrophoresis, transfer to nitrocellulose and immunoautoradiography with tumor antiserum and [^{125}I]protein A. Fig.2 shows that by this technique the presence of T-antigen in SV40-transformed cells could be demonstrated. The SV40-specific faint band in the 20-kDa region (arrow) may represent SV40 T-antigen. However, except for occasional artificial staining of histone bands in other immunoautoradiographs, only T-antigen was detected reproducibly; therefore, in the other figures only the 90–110 kDa region is shown.

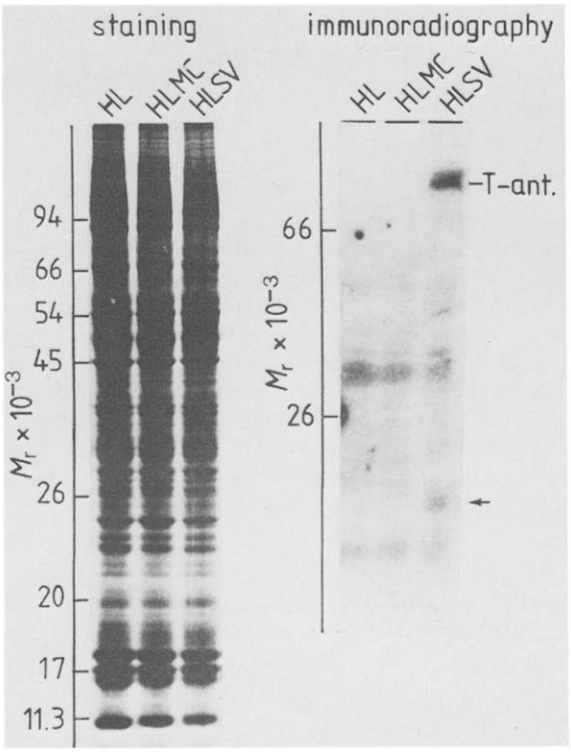

Fig.2. Gel electrophoretic staining patterns and immunoautoradiography of hamster lens cell lysates. HL, normal lens cells; HLMC, lens cells transformed by methylcholanthrene; HLSV, lens cells transformed by SV40. Lysates of 0.3 × 10^6 cells per sample were separated on two parallel SDS-polyacrylamide gels; proteins in one gel were detected by staining with Coomassie brilliant blue in the second gel by immunoradiography with SV40 tumor serum after transfer to a nitrocellulose sheet.

To localize SV40 T-antigen within the cell, we prepared subcellular fractions corresponding to the main structural components of cultured cells, water-soluble proteins, membranes, microfilaments with other deoxycholate-soluble proteins, intermediate filaments, microtubules and nuclei. It was found earlier [9] by two-dimensional gel electrophoresis that these fractions together account for nearly all cellular proteins.

Typical one-dimensional gel-electrophoretic profiles [9] are shown in fig.3A, together with the relevant parts of the immunoautoradiographs. Two fractions contained detectable amounts of T-antigen, the nuclear and the microtubular fractions. From the intensities of the bands and the amount of material applied to the gel, it could be estimated that the microtubular fraction contained about one-quarter of the amount of T-antigen in the nucleus.

The nuclear localization, in accord with the immunofluorescence, was analyzed further by extraction of nuclei with 2 M NaCl (fig.3B). This treatment solubilized the chromatin, containing the histones, and left non-chromatin (or nuclear matrix) together with the T-antigen in the pellet. Extraction with 0.4 M $(NH_4)_2SO_4$ [18] instead of 2 M NaCl yielded fractions with the same complementary protein profiles (not shown), but a chromatin fraction containing about half the amount of T-antigen of the non-chromatin fraction (cf. [5,6]). It may be concluded that a considerable part, if not all, of the T-antigen is associated with the salt-resistant nuclear-matrix fraction.

The microtubular fraction was prepared by lysis of cells with 0.5% Triton X-100 under microtubule-stabilizing conditions (50% glycerol, 10% dimethyl sulfoxide, absence of Ca^{2+}, room temperature) and extracting the residue with a buffer containing $CaCl_2$ without glycerol or dimethyl sulfoxide (see [9]). Apparently, this liberated T-antigen from the Triton X-100-resistant structure together with the tubulins (55 kDa) and part of the cytoplasmic actin (43 kDa, fig.3A, microtubule fraction). Solubilization of actin filaments alone by deoxycholate extraction of a Triton X-100-resistant residue (prepared by cell lysis at 4°C, which depolymerizes microtubules) did not liberate any T-antigen (fig.3A, microfilament fraction).

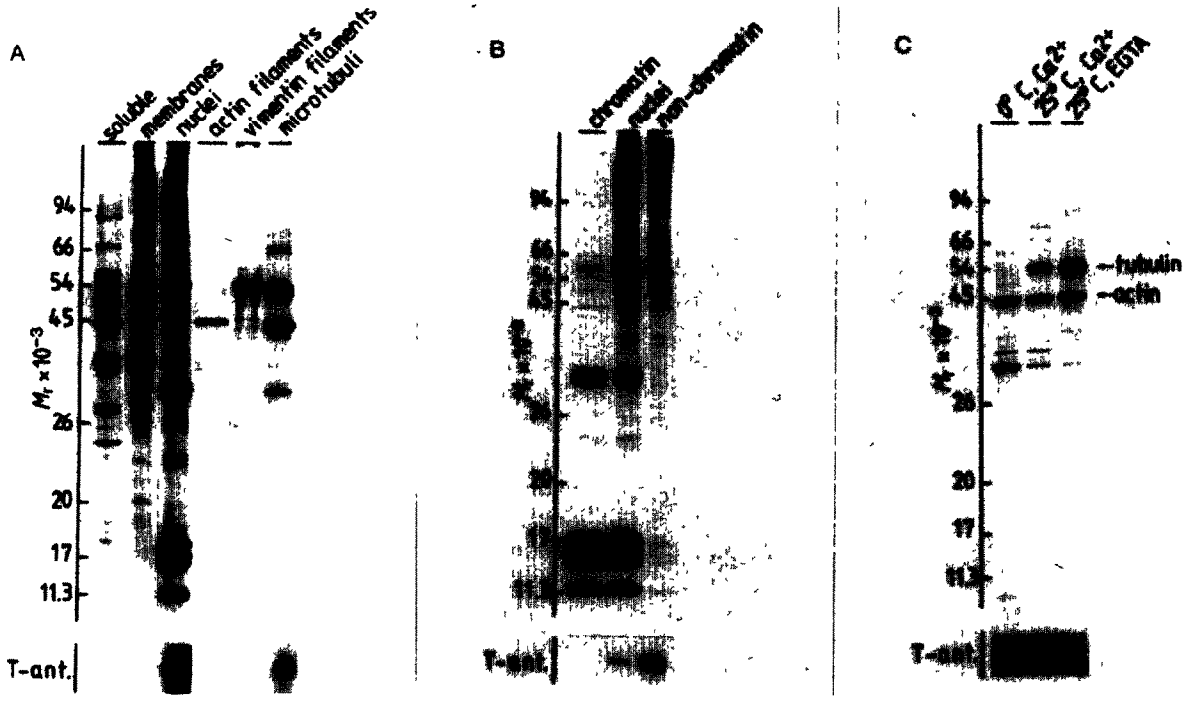

Fig.3. Staining and immunoautoradiography of fractions of SV40-transformed hamster lens cells. To detect low levels of T-antigen, from each fraction as much as possible without overloading was applied on both gels; these amounts were derived from 0.5×10^6 cells (soluble proteins), 60×10^6 cells (membranes), 4×10^6 cells (nuclei), 36×10^6 cells (actin filaments), 10×10^6 (vimentin filaments), 20×10^6 cells (microtubules) and 10×10^6 cells (chromatin and non-chromatin), respectively. (A) Subcellular fractions. The staining patterns may be compared with the results from [9]. (B) Subnuclear fractions. DNase (30 kDa), which had been used to degrade DNA, can be seen together with the histones, in the staining patterns of nuclei and chromatin. (C) Ca^{2+} extracts of residues after cell lysis under microtubule-depolymerizing conditions (0°C, 5 mM $CaCl_2$), intermediate conditions (25°C, 5 mM $CaCl_2$) and microtubule-stabilizing conditions (25°C, 2 mM EGTA). Other conditions were as described for the microtubular preparation in [9]. The amounts were derived from 24×10^6, 20×10^6 and 8×10^6 cells, respectively.

To determine whether the extraction of T-antigen from the non-ionic-detergent-resistant structure correlated with the depolymerization of microtubules, we examined the proteins released by the microtubule-extraction buffer after modulating the conditions of cell lysis. Partial depolymerization of microtubules by lysis in the presence of dimethyl sulfoxide and glycerol at 25°C, but with Ca^{2+} instead of EGTA (fig.3, middle lane) resulted in a lower yield of actin, tubulin as well as T-antigen when compared to normal conditions (25°C, EGTA lane most to the right, judged from the amounts of cells used to obtain comparable staining patterns). Lysis with Ca^{2+} at 0°C (lane most to the left) resulted in a fraction containing actin, but no tubulin and without T-antigen. Evidently, like polymerization of microtubules, the interaction of T-antigen with the Triton X-100-resistant structure depends on the absence of Ca^{2+} and on elevated temperature.

4. DISCUSSION

Extraction of nuclei with non-ionic detergents and high concentrations of salt leaves a residual structure, the nuclear matrix [18,19], to which replicating DNA is attached [20]. Association of T-antigen to this structure, in agreement with [5,6], may be viewed in connection with the tight specific binding of T-antigen to the origin of replication of SV40, the role of T-antigen in the replication of viral or cellular DNA [2] and hence, its role in cell

transformation (cf. [21]).

In addition, T-antigen may be extracted by Ca^{2+} from the Triton X-100-resistant structure, provided the cells are lysed at room temperature in the absence of Ca^{2+} and in the presence of dimethyl sulfoxide and glycerol. Since microtubules are specifically depolymerized by Ca^{2+} and cold [22], this may suggest an association with microtubular structures. Preparation of microtubules by repeated polymerization and depolymerization [23] yielded a fraction containing (predominantly) actin, tubulin and also T-antigen (not shown), arguing against a nuclear origin of the Ca^{2+}-extracted T-antigen and confirming a possible interaction with cytoskeletal elements.

Interestingly, an association of T-antigen with microtubules would explain the findings of authors in [24], who demonstrated that T-antigen stabilized tubulins in various SV40-transformed cell lines and proposed a central role of microtubules in the pleiotropic process leading to cell transformation.

Presumably, the T-antigen in the microtubular fraction accounts for at least part of the T-antigen found in a nucleoplasmic fraction [6], which was prepared by NP-40 extraction of whole cells in the cold and probably contained, besides cytosolic and soluble nuclear proteins, solubilized microtubular proteins. Recently, authors in [8] confirmed the occurrence of T-antigen in the cytoplasm. On the basis of sedimentation studies an association with messenger ribonucleoproteins was proposed. However, this would not explain our data, especially the combined effect of Ca^{2+} and cold (fig.3C). Clearly, more detailed studies, e.g., by combining chemical extractions, sedimentation techniques and optical methods, are needed to identify more accurately the subcellular structures associated with SV40 T-antigen.

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