Monoclonal Antibodies Against Simian Virus 40 Tumor Antigens: Analysis of Antigenic Binding Sites, Using Adenovirus Type 2-Simian Virus 40 Hybrid Viruses

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The antigenic binding sites of two monoclonal antibodies are located in the COOH-terminal region (clone 412) and probably in an internal region (clone 7) of simian virus 40 large T antigen. A third monoclonal antibody (clone 122), which has been shown to bind nonviral T antigen, does not react with HeLa cells infected with nondefective adenovirus type 2 (Ad2)-simian virus 40 hybrid viruses Ad2$^+$ND1, Ad2$^+$ND2, or Ad2$^+$ND4.

Cells infected or transformed by simian virus 40 (SV40) express two virus-coded but nonvirion proteins, large T antigen and small t antigen, and also have enhanced amounts of a cellular protein here referred to as nonviral T antigen (NVT). Large T and small t antigens (96,000 [96K] and 20K daltons, respectively) share a common NH$_2$-terminal amino acid sequence but have unique COOH-terminal regions (2, 12, 13). NVT antigens (48 to 58K daltons) have been detected in cells transformed by various agents and appear to be species-specific cellular proteins which form tight complexes with large T antigen in SV40-transformed cells (5, 7, 9). At least one of these tumor antigens, large T antigen, seems to be a pleiotropic molecule since it performs multiple functions both in lytic infection and in transformation (see reference 17 for review).

Antibodies which specifically recognize distinct antigenic domains would greatly facilitate the characterization and functional analysis of the tumor antigens. Gurney et al. (5) recently described the isolation of three monoclonal antibodies directed against SV40 tumor antigens, two (clones 7 and 412) reacting with large T antigen and one (clone 122) reacting with NVT antigens from five mammalian species. We report here further analysis of the antigenic binding sites of these three monoclonal antibodies, using three nondefective adenovirus type 2 (Ad2)-SV40 hybrid viruses (Ad2$^+$ND1, Ad2$^+$ND2, and Ad2$^+$ND4) which direct the synthesis of overlapping COOH-terminal fragments of SV40 large T antigen (10).

Culture medium containing monoclonal antibodies was harvested from cultures of cloned antibody-producing hybrid cells growing in the Dulbecco and Vogt modification of Eagle me-

dium supplemented with 20% fetal calf serum (5). Hybridoma serum was obtained by cardiac puncture of BALB/c mice bearing tumors induced by cloned antibody-producing cells (5). Mouse SV40 tumor serum was produced in 10- to 12-week-old male BALB/c mice by subcutaneous inoculation of 5 × 10^5 SV40-transformed BALB/c mouse cells. (The SV40-transformed mouse cells, line VLM [20], were a generous gift from M. Tevethia and S. S. Tevethia.) Sera were collected at a tumor size of approximately 1 to 2 cm at 6 weeks after inoculation and pooled. Media and sera were made 1 to 4 mM NaN$_3$ and stored at 2°C or at −25°C.

Seed stocks of Ad2 and the nondefective Ad2$^+$-SV40 hybrid viruses Ad2$^+$ND1, Ad2$^+$ND2, and Ad2$^+$ND4 were obtained from A. M. Lewis, Jr. Stocks of Ad2, Ad2$^+$ND1, and Ad2$^+$ND2 were prepared in HeLa monolayer cells (obtained from Cold Spring Harbor Laboratory), and stocks of Ad2$^+$ND4 in monkey CV1 cells were grown in the Dulbecco and Vogt modification of Eagle medium supplemented with 10% fetal calf serum. The titers of the virus stocks used in these experiments were 10^9 PFU/ml for Ad2, Ad2$^+$ND1, and Ad2$^+$ND2 and 10^8 PFU/ml for Ad2$^+$ND4. Stocks were assayed on HeLa monolayer cells as described by Williams (19).

To identify hybrid virus large T fragments containing the antigenic binding sites of the monoclonal antibodies, we compared the proteins precipitated by the monoclonal antibodies and by SV40 tumor serum from Nonidet P-40 detergent extracts of hybrid virus-infected HeLa cells (Fig. 1). Monoclonal antibody from clone 412 (mcl 412) and mouse tumor serum precipitated the same proteins from extracts of all three hybrid virus-infected cells (Fig. 1). Monoclonal antibody from clone 7 (mcl 7) precipitated none

of the Ad2$^+$ND1 28k protein, only small amounts of the Ad2$^+$ND2 42K and 56K proteins and of the Ad2$^+$ND4 42K, 56K, and 94K proteins, but large amounts of the Ad2$^+$ND4 proteins in the 60K and 74K molecular-weight range (Fig. 1). Similar results were obtained with Nonidet P-40 extracts of Ad2$^+$ND4-infected monkey cells (Deppert, unpublished data). Monoclonal antibody from clone 122 (mcl 122) precipitated no detectable SV40-specific proteins from extracts of the three hybrid virus-infected cells (Fig. 1).

For comparison, the proteins precipitated by the monoclonal antibodies and by SV40 tumor serum from Nonidet P-40 detergent extracts of

SV80 cells were included in the same gel (Fig. 1). As previously shown (5), all three monoclonal antibodies precipitated large T antigen but none of the three monoclonal antibodies precipitated small t antigen (Fig. 1). However, mcl 122 precipitated much less large T antigen, compared with NVT antigen (Fig. 1) than usually seen (5), and the other antibodies (tumor serum, mcl 412, and mcl 7) precipitated so little NVT antigen that the NVT bands were only visible on the gel shown in Fig. 1 after longer exposure. Both results point to dissociation of the majority of the large T antigen-NVT antigen complexes in the SV80 extract used here. Another interesting
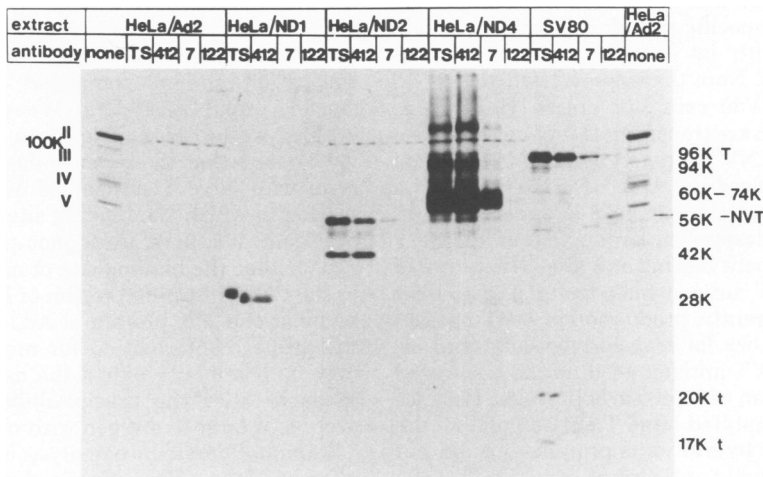
FIG. 1. *Immunoprecipitation of SV40-specific proteins from ^{35}S-labeled extracts of hybrid virus-infected HeLa and SV80 cells. HeLa S3 cells grown in suspension (5×10^7 total) were infected with 1 ml of undiluted virus stocks per 10^7 cells. After an absorption period of 20 min, the cells were diluted with growth medium (final concentration, 2×10^5 cells per ml). At 24 h (Ad2$^+$ND1-infected cells) or 32 h postinfection (Ad2-, Ad2$^+$ND2-, and Ad2$^+$ND4-infected cells), the cells were washed twice with 40 ml of Earle salt solution, resuspended at 5×10^6 cells per ml, and labeled for 1 h in Eagle medium minus methionine containing 20 μCi of [^{35}S]methionine (Amersham/Buchler, Braunschweig, Federal Republic of Germany; specific activity, 700 to 1,300 Ci/mmol) per ml and 5% calf serum. After the labeling period, the labeling medium was removed, and the cells were washed once in 40 ml of Eagle minimum essential medium. A dense culture of SV80 cells (16) growing on a 100-mm dish was labeled for 2 h at 37°C with 1 mCi of [^{35}S]methionine (Amersham Corp.; 1,060 Ci/mmol) in 2 ml of methionine-free Dulbecco-modified Eagle medium supplemented with 10% calf serum. At the end of the labeling periods, approximately 10^7 labeled hybrid virus-infected cells or labeled SV80 cells were lysed with 1 ml of lysis buffer (150 mM NaCl, 20 mM Tris, pH 8.0, 1% Nonidet P-40) by the method of Crawford and O'Farrell (3). A protease inhibitor, 1 mM phenylmethylsulfonylfluoride, was present during extraction of infected HeLa cells. Microfuge supernatants were centrifuged for 30 min at 100,000 × g in a type 50 rotor (Beckman) at 4°C, and the supernatants (Nonidet P-40 extracts) were stored at −70°C. Samples (25 to 100 μl) of the extracts were combined with either 10 μl of tumor serum or 500 μl of culture fluid from clone 412, 7, or 122. The culture fluid samples contained monoclonal antibody at equivalent titers since each reached background levels in the enzyme-linked immunosorbent assay for anti-T antibodies (5) only after 50- to 100-fold dilution. Antibody-bound polypeptides were precipitated with Formalin-fixed S. aureus Cowan I, eluted with sodium dodecyl sulfate, and separated by sodium dodecyl sulfate gel electrophoresis on a 7.5 to 15% polyacrylamide gradient slab gel (5). After electrophoresis for 1 h at 60 V and about 4 h at 120 V, the gel was prepared for fluorography as described by Bonner and Laskey (1). SV40 antigens (96K large T, NVT, and both 20K and 17K small t) as well as SV40-specific hybrid virus proteins (94K, 60 to 74K, 56K, 42K and 28K) are marked on the right side of the figure; Ad2-specific proteins (II, 100k, III, IV, and V) are marked on the left side of the figure. The material which migrated more slowly than hexon (II) in the ND4 lanes is of unknown origin and composition.*

observation made during these experiments was that mcl 412 immunocomplexes reacted only very weakly with protein A-Sepharose but reacted readily with fixed *Staphylococcus aureus*. The other two monoclonal antibodies were not tested with protein A-Sepharose. The reason for the different reactivity of protein A-Sepharose and *S. aureus* in binding mcl 412 immunocomplexes is not understood.

The COOH-terminal overlapping fragments of large T antigen synthesized by the hybrid viruses Ad2+ND1, Ad2+ND2, and Ad2+ND4 plus the NH_2-terminal shared sequence of small t antigen divide the large T polypeptide into five regions (Fig. 2). mcl 122 did not precipitate any of the SV40-specific proteins, a result which is consistent with its specificity for host-coded NVT antigen. Note that mcl 122 detected NVT antigen in SV80 cells but not in HeLa cells, although both are transformed human cells. Also note that no NVT antigen detectable with mcl 122 was induced in Ad2+ND4-infected HeLa cells (Fig. 1) or monkey cells (Deppert, unpublished data) despite the production of a large T antigen fragment lacking only the NH_2-terminal 7% of large T antigen (map units, 0.62 to 0.65; Fig. 2). Apparently, production of NVT antigen is not obligatory for transformed cells, and induction of NVT antigen need not be associated with expression of SV40 early proteins. Because mcl 412 precipitated large T antigen and all the SV40-specific hybrid virus proteins but did not precipitate small t antigen, we conclude that the binding site for mcl 412 is located within the COOH-terminal 26% of large T antigen (map units, 0.17 to 0.28; Fig. 2). Because mcl 7 precipitated large T antigen and the SV40-specific

proteins of Ad2+ND2 and Ad2+ND4 but did not precipitate small t antigen or the 28K protein of Ad2+ND1, we tentatively conclude that the binding site of mcl 7 is located within the internal 37% of large T antigen adjacent to the COOH-terminal region (map units, 0.28 to 0.44; Fig. 2). However, unlike mcl 412, mcl 7 did not precipitate all SV40-specific protein species equally well. The 60 to 74K proteins of Ad2+ND4 were precipitated much more efficiently by mcl 7 than larger or smaller SV40-specific proteins, suggesting a role for conformation in binding by this antibody. Although the inefficient precipitation of large T antigen by mcl 7 was previously attributed to mcl 7 having a low affinity for large T antigen (5), large T antigen was not lost from mcl 7 during rinsing of immune complexes (Harrison and Gurney, unpublished data). We suggest that mcl 7 binds well to large T antigen in one conformation and that these molecules are in equilibrium with large T antigen of different conformation in which the binding site is inaccessible or absent. We therefore cannot exclude the possibility that the binding site of mcl 7 is actually in the COOH-terminal region of large T antigen but that the 28K protein of Ad2+ND1 is not in the proper conformation for mcl 7 to bind. It may be possible to adjust the assay conditions so as to alter the precipitability of the 28K protein or large T antigen with mcl 7.

Immunofluorescence analysis of hybrid virus-infected HeLa cells with the three monoclonal antibodies gave results which agreed completely with the immunoprecipitation data: mcl 412 and mouse tumor serum stained cells infected by all the hybrid viruses; mcl 7 stained Ad2+ND2 and
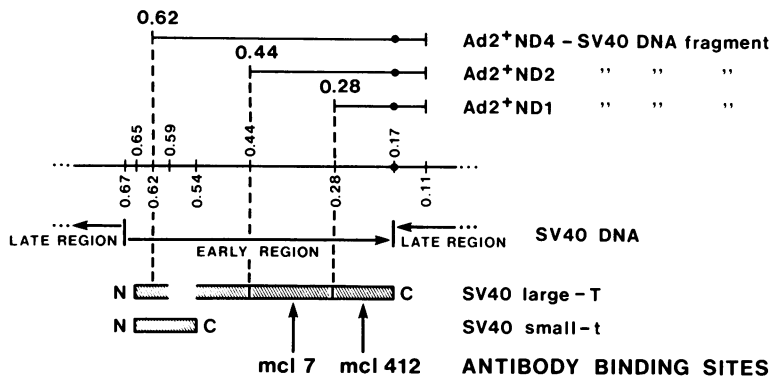

FIG. 2. *Location of monoclonal antibody binding sites on the SV40 large T polypeptide by use of Ad2+-SV40 hybrid viruses Ad2+ND1, Ad2+ND2, and Ad2+ND4. The positions of the early and late regions on the SV40 DNA map are derived from Reddy et al. (14). Arrows indicate the direction of transcription. The NH_2 termini of SV40 large T and small t antigens were positioned by the method of Paucha et al. (12). Mapping of Ad2+-SV40 hybrid virus SV40 DNA fragments on the SV40 DNA was performed according to Henry et al. (6) and Morrow et al. (11) for Ad2+ND1 and Ad2+ND2 and according to a more recent study by Westphal et al. (18) for Ad2+ND4.*

Ad2⁺ND4 but not Ad2⁺ND1 infected cells; and mcl 122 gave no specific staining (Fig. 3). In addition, the three monoclonal antibodies stained SV80 cells whether or not the cells had been heated before fixation, indicating that all three antibodies bound heat-stable antigenic determinants. Of these three monoclonal antibodies, only mcl 122 precipitated sodium dode-
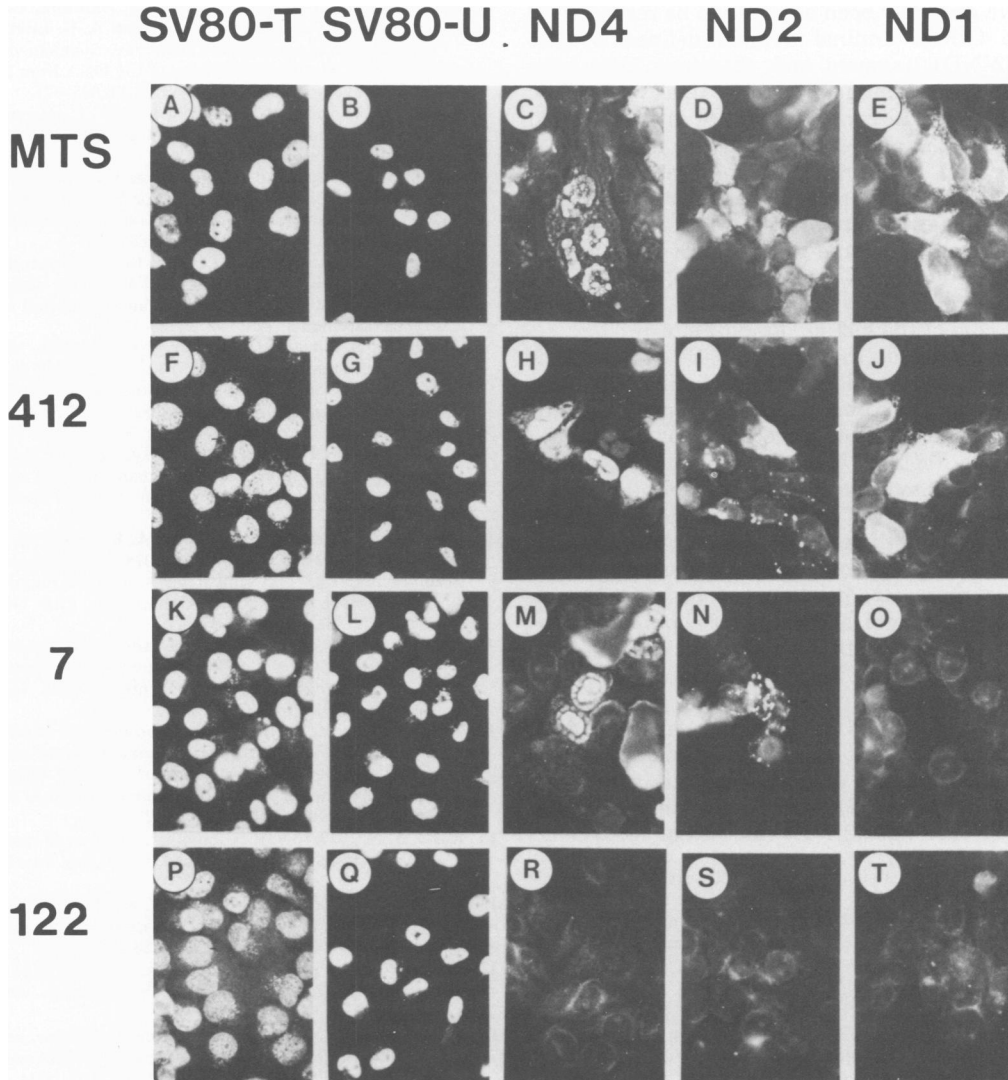

FIG. 3. *Analysis of SV80 cells and Ad2⁺-SV40 hybrid virus-infected HeLa cells for SV40 T and U antigens by immunofluorescence microscopy with mouse SV40 tumor serum or monoclonal antibodies 412, 7, and 122. SV80 cells growing on glass coverslips were either fixed directly (SV80-T) or were rinsed twice with phosphate-buffered saline and heated at 50°C for 30 min in a wet chamber to inactivate T antigen (8) before fixation (SV80-U). HeLa monolayer cells growing on glass cover slips were infected with Ad2⁺ND1 (ND1), Ad2⁺ND2 (ND2), or Ad2⁺ND4 (ND4) and fixed 32 h later. Cells were fixed with acetone–ethanol, 2:1, for 20 min at 4°C, air dried, and exposed to serum or culture fluid followed by fluoresceinated goat anti-mouse immunoglobulin G antibody (5). Cells were viewed with a Leitz microscope equipped with epifluorescent illumination and photographed with a 53× oil-immersion objective. SV80 cells and hybrid virus-infected HeLa cells stained with mouse SV40 tumor serum (MTS) at a dilution of 1:50 (A to E), with undiluted culture medium from hybrid clone 412 cells (412) (F to J), with serum from hybridoma clone 7 tumor-bearing mice (7), diluted 1:20 (K to O), or with undiluted culture fluid of hybridoma clone 122 cells (122) (P to T). The same magnification was used for all photographs.*

cyl sulfate gel-purified material whose preparation included boiling in sodium dodecyl sulfate (5). The extent of the heat-stable domain of NVT antigen detected with mcl 122 is unknown. The heat-stable determinants of large T antigen have generally been assumed to be restricted to the COOH-terminal domain defined by the Ad2+ND1 fragment and, therefore, have been called the U antigen (4, 8, 15). If, as we suspect, mcl 7 recognizes a binding site in the internal region of large T antigen present in Ad2+ND2 42K and 56K proteins but absent from the Ad2+ND1 28K protein, then heat-stable antigenic determinants must also reside in that internal region of large T antigen. Even within the COOH-terminal U antigen region, some of the determinants may be sensitive to heat. This is not true of the mcl 412 binding site.

We conclude that the Ad2+-SV40 hybrid viruses are a suitable tool for preliminary analysis of the antigenic binding sites of monoclonal antibodies against SV40 tumor antigens. Antibodies specific for large T antigen are quickly distinguished from antibodies specific for other molecules, such as NVT antigen. In addition, a preliminary determination of binding site location on the large T polypeptide is obtained at the same time. Interpretation of negative results with this system is limited by the potential conformational effects discussed above. Although the complicated DNA deletions and unusual mRNA splicing patterns which arise during growth of Ad2+ND4 on HeLa cells (18) may complicate the interpretation of binding sites in the central region of large T antigen (map units, 0.50 to 0.60; Fig. 2), results with HeLa cells can be checked in Ad2+ND4-infected monkey cells.

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