Immunocytochemical Localization of Simian Virus 40 T Antigen with Peroxidase-Labeled Antibody Fragments

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T antigen was localized in simian virus 40 lytically infected and transformed cells by Fab' antibody conjugates at the ultrastructural level. This virus-specific protein appeared in the cytoplasm of permissive cells as early as 3 h after infection. At later time intervals, the T antigen was localized in both the cytoplasm and nucleus and finally (24 h) in the nucleus. These results suggest a synthesis of T antigen on cytoplasmic ribosomes, with subsequent transfer to the nucleus.

T antigen is a virus-specific protein detectable in nuclei of cells transformed and infected with simian virus 40 (SV40). Recent investigations indicate that synthesis of the protein may be controlled by the viral A gene, thus conceivably regulating viral DNA replication or the initiation of cell transformation (2, 10, 16). Immunofluorescence and ferritin-labeled antibody techniques reveal T antigen localized in the nuclei of interphase infected cells, with no association to condensed chromosomes (6, 14, 15). Cytoplasmic staining for T antigen is generally dismissed as nonspecific (1, 6, 14). Ultrastructural localization of T antigen is thwarted by the inadequate cellular penetration of conventional antibody tracers with sedimentation coefficients of 7S. Nuclear staining for T antigen is thus obtainable at a cost of cellular and cytoplasmic detail (1, 6, 14). This study reports the use of labeled antigen-binding antibody fragments to determine the ultrastructural distribution of T antigen in both SV40 lytically infected and SV40-transformed cells. Cellular ultrastructural detail is preserved because of the enhanced penetration of the lower-molecular-weight tracer and gentler fixation techniques. This technique permits a sequence study of T antigen cytoplasmic synthesis and transport to the nucleus. Advantages of this technique for the localization of tissue antigens have been given in previous publications by Nakane (12) and Kraehenbuhl et al. (5).

T antigen resulting from the interaction of SV40 virus with a transformed cell line isolated from SV40-infected Syrian hamster brain cells and with CV-1 cells was identified in these studies. Nutrient media for the hamster cultures consisted of Ham F-12 medium with 15% inactivated fetal calf serum and penicillin and streptomycin. CV-1 cells were maintained in minimal essential medium supplemented with 5% fetal calf serum. The RH-911 strain of SV40 virus titering 10^7 PFU/0.1 ml on CV-1 cells was used in all experiments (7).

Antiserum against SV40 T antigen was prepared from hamsters bearing subcutaneous tumors induced with SV40-transformed cells. This antiserum has been compared to a commercially available product from Flow Laboratories, Inc. (Rockville, Md.), and our preparation has been tested for and found to be free of U antibody. These experiments were performed by heat inactivating the tissue sections, which removed the labile T antigen, and no staining was detected with the light or electron microscope. A gamma-G fraction titering 1:320 by the indirect peroxidase-labeled antibody method was passed through a Sephadex G-25 column after salting out with ammonium sulfate. Fab' fragments were prepared by the method of Grey and Kunkel (3), using pepsin at a concentration of 1% with a digestion time of 16 h at 37 C, and purified through a Sephadex G-100 column. Hamster Fab' antibody fragments against SV40 T antigen were coupled to horseradish peroxidase by the method of Nakane and Kawaoi (13).

SV40-transformed clonal cells and CV-1 cells grown in chamber slides were fixed in situ with periodate-lysine-paraformaldehyde solution for 1 h at 4 C (9). Cell fixation with paraformaldehyde was compared to acetone and glutaraldehyde and found to be superior for detection and preservation of SV40 T antigen. After fixation, cells were washed in phosphate-buffered saline with 1% sucrose for 12 h at 4 C and exposed to a conjugate of peroxidase-labeled Fab' fragments (0.14 mg of Fab' conjugate per ml) for 4 h at room temperature. Excess unbound antibody
Fig. 1. Localization of SV40 T antigen in transformed Syrian hamster brain cells and permissive CV-1 cells. Marker in each figure, 1 μm. (A) Reaction product distribution (arrows) within the nucleus of a transformed hamster brain cell. This pattern was observed in all transformed nuclei. (B) CV-1 cells, 3 h postinfection. Reaction product localized on rough endoplasmic reticulum and free polyribosomes. No nuclear localization of T antigen was evident, but the outer nuclear membrane was positively stained. (C) CV-1 cells, 6 h postinfection. Reaction product was localized in both cytoplasm and nucleus. (D) CV-1 cells, 24 h postinfection. Nucleus was markedly positive, and nucleoli and cytoplasm were devoid of stain.
conjugate was removed with five changes of phosphate-buffered saline over a total of 2 h. Cells were postfixed for 30 min with 2.5% glutaraldehyde in 0.05 M phosphate buffer, pH 7.4. The fixed cells were placed in freshly prepared substrate of 0.05 M Tris-chloride buffer (pH 7.6) containing 0.03% 3,3'-diaminobenzidine tetrachloride and 0.005% hydrogen peroxide for 5 min at room temperature. Cells were postfixed with 2% osmium tetroxide solution (0.05 M phosphate buffer, pH 7.4) for 2 h. The reaction products of peroxidase were sufficiently osmiophilic to provide electron density to visualize cell detail without counterstain. Cells were washed with phosphate-buffered saline and embedded in a mixture of Epon and Araldite after dehydration in graded ethanol. Ultrathin sections cut on a Porter-Blum micromote were observed and photographed by a JEM-100 electron microscope.

Two different controls have been used in this study: (i) transformed cells treated as above and reacted with peroxidase-labeled Fab' fragments obtained from normal hamster sera, and (ii) uninfected cells processed exactly as above. Control cells were uniformly negative for T antigen. Polymva T antibody was unable to block the SV40 T antibody (8). Electron microscopic observations of SV40-transformed hamster brain cells provided resolution of the distribution of T antigen without sacrifice of cellular ultrastructure. The osmiophilic reaction product was localized almost exclusively to the nucleus, whereas nucleoli were devoid of stain (Fig. 1A).

Cytoplasmic synthesis and transport of T antigen to the nucleus was followed by a timed-sequence study of SV40 infection in CV-1 cells. In these studies, 20 to 40 cells were analyzed at each time point. Three hours after infection with SV40 virus at a multiplicity of 120, a definite positive stain for T antigen appeared in the endoplasmic reticulum in 2 to 3% of the cells (Fig. 1B). The reaction product was on both the membrane-bound ribosomes and the free polyribosomes. Six hours after infection the percentage of cytoplasmic-positive cells increased, and the nuclei showed a faint stain for the protein (Fig. 1C). Twelve hours after infection (22% positive nuclei), a faint staining pattern was apparent in the nuclei. Eighteen hours after infection, T antigen was clearly evident in the nucleus, and the cytoplasm was clear. Cytoplasm was completely clear of T antigen 24 h (96% positive nuclei) after infection with nuclei, except for nucleoli, definitely stained (Fig. 1D). Virus-like structures resembling immature SV40 virions appeared 24 h after infection. As viruses matured, intensity of nuclear reaction for T antigen was markedly reduced.

By application of this sensitive immunocytochemical technique, initiation of T antigen synthesis in permissive cells occurred as early as 3 h after infection, with transport to the nucleus by 24 h postinfection. The fluorescent antibody technique has indicated that T antigen is detectable in nuclei of permissive cells as early as 6 to 10 h after infection with SV40 virus (4). The prompt appearance of T antigen after exposure to SV40 virus suggests an interaction of viral and cytoplasmic proteins at the translational level as an initiator of synthesis. The appearance of T antigen is known to precede the onset of viral DNA synthesis and capsid protein. These morphological studies indicate that the protein-synthesizing apparatus of the cytoplasm is diverted to the manufacture of a new virus-specific protein.

Further studies will be directed in two areas. First, we are utilizing lower multiplicities of virus (0.1 to 1 PFU cell) to determine whether premature cell lysis may have blocked further rounds of T antigen synthesis. Secondly, a nonpermissive cell, Chinese hamster (7), will be studied with this technique for appearance and distribution of T antigen. These studies demonstrate the experimental advantages of the antigen-binding fragment technique for cytochemical localization of synthesis and disposition of unique cellular antigens. Though the role of T antigen remains obscure, studies of its production, transport, and subcellular localization may provide an understanding of the critical processes involved in both the replication of viral DNA and cell transformation. Recently, a report appeared utilizing the peroxidase techniques for the localization of the SV40 capsid antigen (11). Further studies with the electron microscope and the peroxidase technique may enable a more complete understanding of critical and viral-related events in the permissive and nonpermissive cell.

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LITERATURE CITED


