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SV40 T antigen and the exocytotic pathway

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A chimeric gene consisting of DNA coding for the 15-amino acid signal peptide of influenza virus hemagglutinin and the C-terminal 694 amino acids of SV40 large T antigen was inserted into a bovine papilloma virus (BPV) expression vector and introduced into NIH-3T3 cells. Cell lines were obtained that express high levels (~5 x 10^6 molecules/cell) of the chimeric protein (HA-T antigen). The biochemical properties and intracellular localization of HA-T antigens were compared with those of wild-type T antigen. Wild-type T antigen is located chiefly in the cell nucleus, although a small fraction is detected on the cell surface. By contrast, HA-T antigen is found exclusively in the endoplasmic reticulum (ER). During biosynthesis, HA-T antigen is co-translationally translocated across the membrane of the ER, the signal peptide is cleaved and a mannose-rich oligosaccharide is attached to the polypeptide (T antigen contains one potential N-linked glycosylation site at Asn^{154}). HA-T antigen does not become terminally glycosylated or acylated and little or none reaches the cell surface. These results suggest that T antigen is incapable of being transported along the exocytotic pathway. To explain the presence of wild-type T antigen on the surface of SV40-transformed cells, an alternative route is proposed involving transport of T antigen from the nucleus to the cell surface.

Key words: T antigen/signal sequence/surface antigens/BPV vectors/exocytosis

Introduction

The large T antigen of SV40 (mol. wt. 94 000) is sufficient to transform rodent cells, whether or not they are established in culture, to the malignant state (see Tooze, 1980). It is also required for the replication of SV40 DNA during productive infection of permissive simian cells (Tegtmeyer, 1972) and for the excision of the viral genome from the integrated state (Botchan *et al.*, 1980; Gluzman, 1981). Newly-synthesized T antigen is transported rapidly from the cytoplasm to the nucleus and virtually all lines of SV40-transformed cells display a pattern of intense nuclear fluorescence when they are fixed, permeabilized and stained with specific anti-T sera (Pope and Rowe, 1964). However, a small proportion of T antigen is also presented on the cell surface, where it can be detected by a variety of other immunological and biochemical techniques (see for example, Lanford and Butel, 1979; Soule and Butel, 1979; Soule *et al.*, 1980; Deppert *et al.*, 1981; Tevethia, 1983; Luborsky and Chandrasekan, 1980; Santos and Butel, 1982; Klockmann and Deppert, 1983a, 1983b; Gooding *et al.*, 1984; Ball *et al.*, 1984). The relative contributions of each of these two forms of T antigen to the establishment and maintenance of the transformed state is unknown.

Two theories have been proposed to explain how T antigen may reach the cell surface. Lange-Mutschler *et al.* (1981) suggest that T antigen released from dead cells is then passively adsorbed to the surface of live cells in the culture. However, there are several lines of evidence against this idea: (i) expression of surface T antigen requires active protein synthesis (Gooding *et al.*, 1984); (ii) lines of cells that express similar amounts of nuclear T antigen display very different levels of surface T antigen; (iii) not all antigenic determinants are exposed on surface T antigen (Deppert and Walter, 1982; Ball *et al.*, 1984; Gooding *et al.*, 1984), a result suggesting specific orientation of the molecule rather than random adsorption; (iv) T antigen on the cell surface is acylated whereas that in the nucleus is not (Klockmann and Deppert, 1983a, 1983b). Acylation is a widespread post-translational modification of a variety of *bona fide* membrane proteins of both viral and cellular origin (Schmidt, 1982). These results, although not conclusive, are difficult to reconcile with the idea of passive absorption of T antigen to cell surfaces. The second theory postulates that both surface and nuclear T antigen are synthesized from the same pool of mRNA (Gooding *et al.*, 1984) and that the completed protein molecules enter alternate transport pathways – one of which leads to the nucleus, the other to the cell surface. Although this idea is attractive, it leaves unsolved the mechanism by which T antigen could negotiate two pathways that are so fundamentally different in character. Transport of proteins into the cell nucleus occurs through nuclear pores (Gurdon, 1970; reviewed by Paine, 1982; de Robertis, 1983) while their presentation on the cell surface requires translocation across a membrane. There is no evidence that T antigen has this ability. By contrast to typical mammalian transmembrane proteins, T antigen carries no hydrophobic signal sequence (Blobel and Dobberstein, 1975) at or near its N terminus. In fact it lacks any sequence of hydrophobic or uncharged amino acids long enough to span a membrane. Furthermore, unlike most *bona fide* transmembrane or secretory proteins (Milstein *et al.*, 1972), surface T antigen does not seem to be derived from a larger precursor. It is particularly puzzling that parts of both the N- and C-terminal domains of the protein are exposed on the outer surface of the cell and are accessible to antibodies, while the more central portion of the molecule is not (Ball *et al.*, 1984; Deppert and Walter, 1982; Gooding *et al.*, 1984). There is currently no theory to explain how considerable portions of a largely hydrophilic protein can be translocated across a membrane, nor where in the cell this process might occur.

To approach this problem, we decided to test whether it would be possible to increase the efficiency with which T antigen could be transported to the cell surface by equipping it with a hydrophobic signal peptide at its N terminus. In the initial stages of synthesis of conventional eukaryotic membrane and secretory

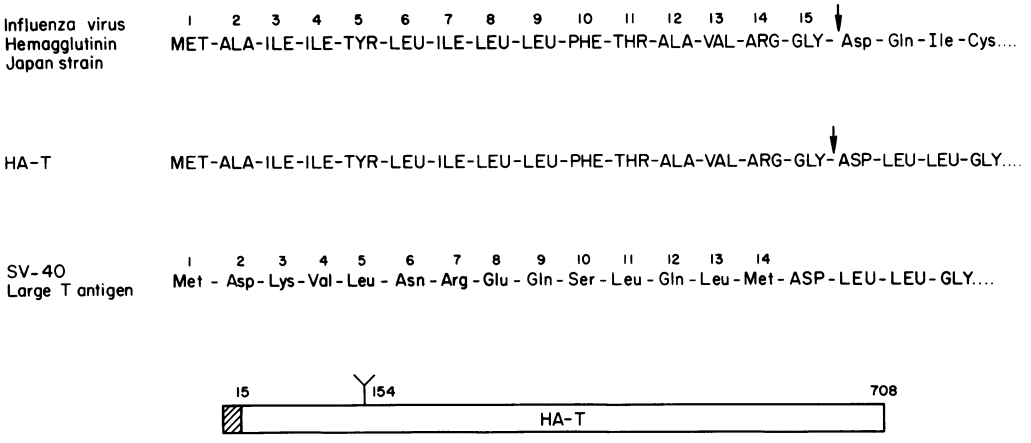


Fig. 1. Structure of a chimeric protein (HA-T) that contains sequences of influenza virus hemagglutinin and SV40 large T antigen. The primary amino acid sequences of the amino termini of influenza virus hemagglutinin (HA) (top line) and SV40 large T antigen (third line) are shown. The second line shows the predicted sequence of the chimeric molecule (HA-T) which consists of the 15 amino-terminal amino acids of HA (the signal peptide) and the 694 carboxy-terminal amino acids of SV40 large T antigen. Vertical arrows indicate the sites of cleavage by signal peptidase. Amino acids that are common to HA, T antigen and HA-T are shown in upper case. The drawing at the bottom of the figure is a schematic representation of the HA-T polypeptide, showing the amino-terminal signal sequence and the location of a potential site for N-linked glycosylation (153-Asn-Arg-Thr). Before cleavage with signal peptidase, the protein is 708 amino acids in length.

proteins, the signal peptide associates with a signal recognition particle (SRP) as it emerges from the ribosome (Walter *et al.*, 1982; Walter and Blobel, 1982a, 1982b; Meyer *et al.*, 1982). Further translation of the mRNA is inhibited until the ribosome-SRP complex has docked at a site on the rough endoplasmic reticulum that allows the nascent protein to be co-translationally translocated through the membrane to the luminal side. As far as is known, there is no way by which a protein can re-cross this barrier and return to the cytoplasm or move to the nucleus. Lingappa and his co-workers (Lingappa *et al.*, 1984) have shown that a chimeric protein consisting of the signal peptide from β-lactamase and sequences from chimpanzee α-globin is co-translationally translocated into dog pancreas membrane vesicles in a wheat germ cell-free translation system. In intact cells, hydrophobic signal peptides should therefore act as *cis*-dominant markers for proteins that are destined for the exocytotic pathway. Consequently, attachment of a signal peptide should divert an intracellular protein from its normal location into the major (and perhaps sole) pathway that leads newly-synthesized proteins to the cell surface.

Here, we describe the biosynthesis, intracellular processing and properties of a chimeric molecule (HA-T antigen) that consists of the signal sequence of the hemagglutinin (HA) of the Japan strain of influenza virus joined to SV40 large T antigen. We show that newly-synthesized HA-T antigen efficiently and completely crosses the membrane of the endoplasmic reticulum into the lumen, where it undergoes signal cleavage and primary glycosylation of the N-linked type. However, transport of the molecule to the Golgi apparatus is extremely inefficient and very little or no HA-T antigen reaches the cell surface. Furthermore, the biochemical properties of HA-T antigen are very different from those of nuclear and surface T antigens. For these and other reasons, it is unlikely that T antigen normally travels to the cell surface along the conventional exocytotic pathway. An alternative mechanism, involving transport of T antigen from the nucleus to the cell surface, is proposed.

Results

Construction of a chimeric gene encoding HA-T antigen

A chimeric gene was constructed that encodes the amino acid sequence shown in Figure 1. A fragment of DNA encoding the 15 N-terminal amino acids of the HA from the Japan strain of influenza virus (Gething *et al.*, 1980) was joined in-frame to a segment of DNA coding for the C-terminal 694 amino acids of SV40 T antigen (i.e., the entire coding sequence apart from the 14 amino acids that form the N terminus of the molecule). The chimeric gene therefore codes for a precursor protein that is one amino acid longer than authentic T antigen and contains the cleavage site for signal peptidase. After processing, the mature form of HA-T would be 14 amino acids shorter than wild-type T antigen.

The isolation and properties of murine cell lines that synthesize HA-T antigen

A vector (BV1MT.HA-T, Figure 9) was constructed that contains: (i) the region of bovine papilloma virus (BPV) DNA (the 69% *Hind*III-*Bam*HI fragment) coding for functions responsible for morphological transformation of murine cells (Lowy *et al.*, 1980) and for maintenance of the viral DNA in an extrachromosomal state in murine cells (Lusky and Botchan, 1984); (ii) plasmid sequences that allow the vector to be propagated in *Escherichia coli*; (iii) a fragment of DNA derived originally from the human β-globin gene cluster. In some systems (DiMaio *et al.*, 1982; Zinn *et al.*, 1983; Sambrook *et al.*, 1985) but not all (DiMaio *et al.*, 1984), this fragment appears to carry an activity that enhances the ability of BVP vectors to replicate as episomes in mammalian cells; (iv) the sequences coding for HA-T antigen which were inserted immediately downstream of the murine metallothionein-I promoter (Hamer and Walling, 1982) and upstream of the poly(A) addition site that marks the end of the SV40 early region.

NIH-3T3 cells were transfected with a mixture of BV1MT.HA-T and a plasmid pON_3 which contains a composite gene consisting of the SV40 early promoter and the aminoglycoside transferase gene of Tn5. Expression of the *neo*^{r} gene renders mammalian cells resistant to the antibiotic G418 (Jimenez and Davies, 1980; Colbere-Garapin *et al.*, 1981; Southern and Berg, 1982). Colonies that grew in the presence of the antibiotic were expanded into cell lines and screened by a quantitative, solid-phase radioimmune assay and by immunofluorescence for the production of HA-T antigen. Approximately 40% of the cell lines tested expressed the antigen and several of these lines were re-

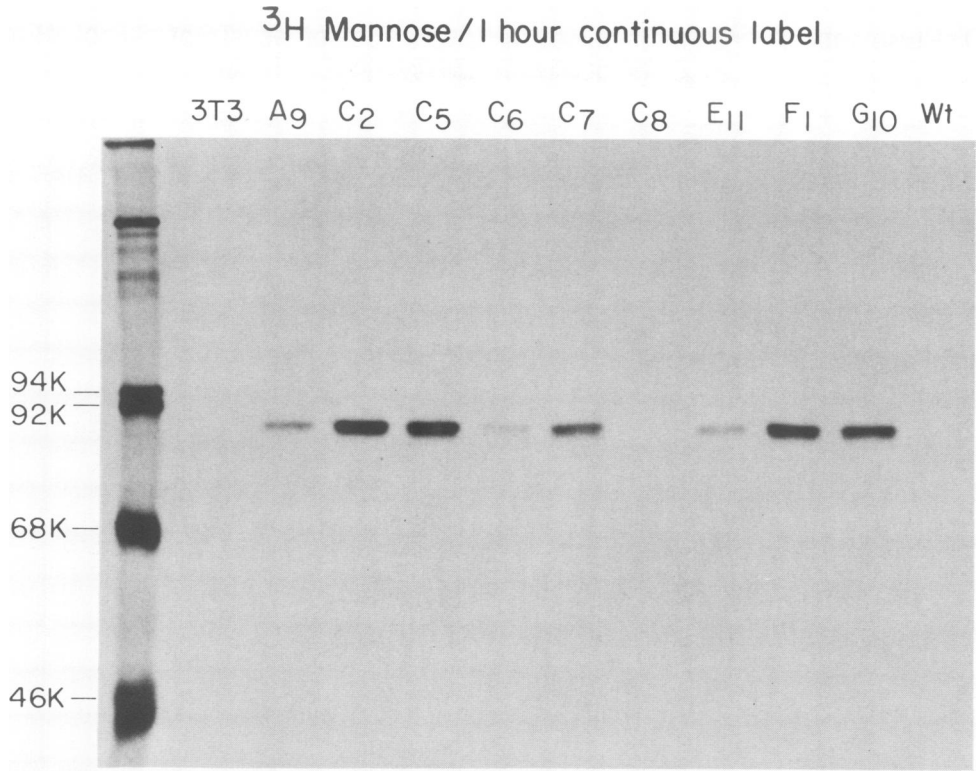


Fig. 2. Primary glycosylation of HA-T antigen. Monolayers (60 mm) of cells were radiolabelled for 1 h in 1 ml medium containing 100 μCi [^{3}H]D-mannose. After labeling, the cells were washed and lysed. Clarified extracts were prepared and molecules related to SV40 large T antigen were immunoprecipitated with a monoclonal antibody (pAb416) specific for large T antigen (Harlow *et al.*, 1981). Immune precipitates were collected on protein A-Sepharose and analyzed by electrophoresis through 7.5% polyacrylamide-SDS gels and fluorography. **Lanes A_9, C_2, C_5, C_6, C_7, E_{11}, F_1 and G_{10}** contain immunoprecipitates of extracts of individual cell lines that synthesize HA-T antigen. **Lane C_8** contains an immunoprecipitate of a cell line that exhibits resistance to G418, but does not synthesize HA-T antigen. The lane marked **3T3** contains an immunoprecipitate of non-transfected 3T3 cells. The lane marked **Wt** contains material immunoprecipitated from an extract of a cell line expressing wild-type SV40 large T antigen. This cell line, which was provided by Dr. R.Gerard, expresses equivalent amounts of the wild-type SV40 T antigen gene from the murine metallothionein promoter cloned in a BPV shuttle vector of the type shown in Figure 9.

cloned and used in the studies described below. All of them synthesize large quantities of HA-T antigen ($\sim 5 \times 10^6$ molecules/cell or 5 – 10 times the amount of wild-type T antigen expressed in COS-1 cells, as assayed by solid-phase radioimmune assay).

The transcriptional activity of metallothionein promoters is increased after treatment of cells with heavy metals (Karin *et al.*, 1981; Durnham and Palmiter, 1981; Hamer and Walling, 1982). However, the cell lines used in this study, which were selected for their ability to produce large quantities of HA-T antigen constitutively, showed at best a 2.5-fold increase in the expression of HA-T antigen when the cells were incubated for 8 – 24 h in media containing cadmium or zinc (1 – 5 μM). Several other hybrid genes, consisting of the promoter and control region of the murine metallothionein-I gene fused to protein-coding sequences, have been introduced into cultured cells in BPV-derived vectors. In some cases (e.g., human growth hormone, Pavlakis and Hamer, 1983) but not all (e.g., hepatitis surface antigen, Hsiung *et al.*, 1984; influenza virus hemagglutinin, Sambrook *et al.*, 1985), expression of these hybrid genes is increased by exposing the cells to heavy metals. Cells synthesizing HA-T antigen fall into the latter class.

NIH-3T3 cells that express large quantities of HA-T antigen closely resemble the parental cells in their morphology and growth rate: their saturation density is approximately twice that of NIH-3T3 cells. We conclude that the production of large amounts of HA-T antigen is insufficient to cause these cells to display a fully-transformed phenotype. By contrast, cell lines that synthesize approximately equivalent amounts of authentic, wild-

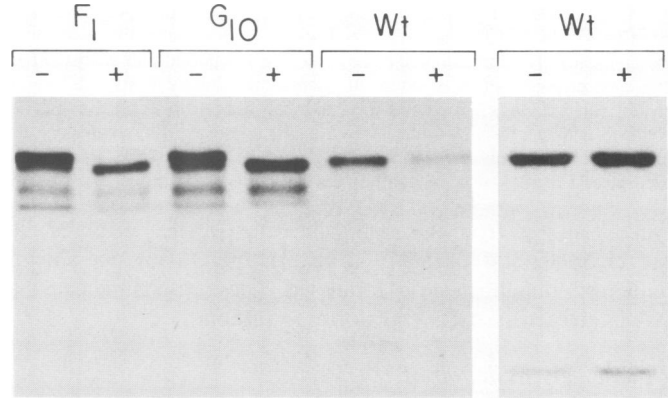


Fig. 3. Effect of tunicamycin on the glycosylation of HA-T antigen. Monolayers (60 mm) were incubated for 1 h in the presence (+) or absence (−) or tunicamycin (4 μg/ml). The cells were then washed, incubated for 30 min in medium lacking methionine and radiolabeled for 1 h in 1 ml medium containing 100 μCi [^{35}S]methionine. Tunicamycin was present throughout the starvation and labeling periods in all samples marked (+). The cells were then lysed and immunoprecipitates were prepared and analyzed as described in the legend to Figure 2. The Figure shows a comparison of three cell lines: F_1 and G_{10}, both of which synthesize HA-T antigen; and Wt, which expresses equivalent amounts of wild-type T antigen. The apparent inhibition of synthesis of wild-type T antigen by tunicamycin (left hand panel) is an artifact caused by unequal loading of samples onto the SDS-polyacrylamide gel. Usually, synthesis of wild-type T antigen was not detectably affected by tunicamycin (see experiment in right-hand panel).

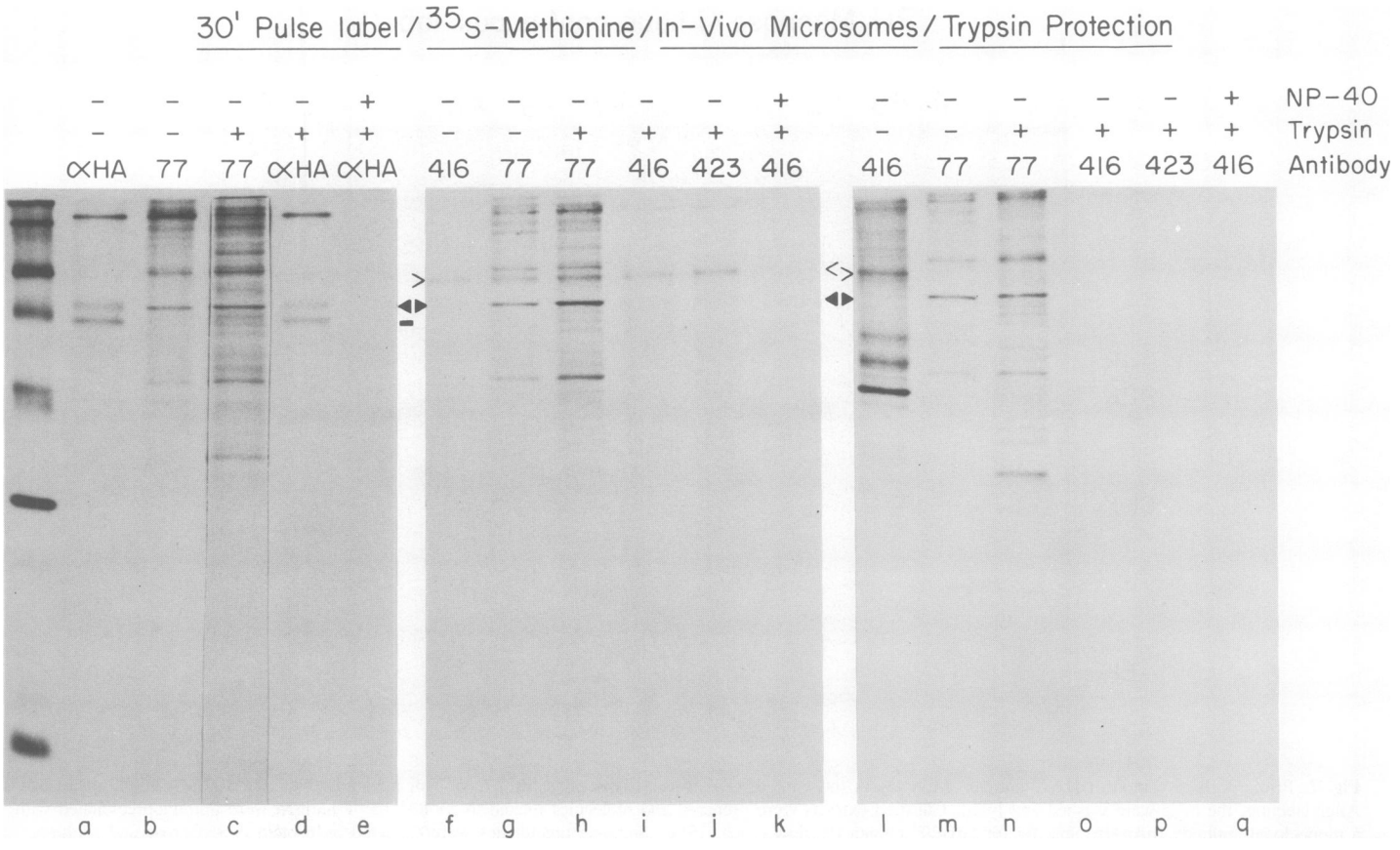


Fig. 4. HA-T antigen is completely translocated across the membrane of the rough endoplasmic reticulum. Monolayers (100 mm) of cells were labeled for 1 h with 300 μCi [^{35}S]methionine after starvation for 30 min in medium lacking methionine. Microsomes were then prepared essentially as described by Rose and Bergmann (1983) and divided into three aliquots, which were incubated either with no additions, or with trypsin (100 μg/ml), or with trypsin (100 μg/ml) and Nonidet P-40 (1%). After 1 h at 37°C, soybean and egg-white trypsin inhibitors were added to a concentration of 100 μg/ml, and the samples were then sedimented through a solution of 10% sucrose (Beckman SW41, 38 000 r.p.m. for 5 h at 4°C). The pellets were dissolved in a solution containing 1% Nonidet P-40, 0.4% sodium deoxycholate, 10 mM Tris-HCl pH 7.4 and 66 mM EDTA, and centrifuged briefly to remove debris. Molecules related to T antigen were then precipitated with monoclonal antibodies specific for the N-terminal (pAb416) or C-terminal (pAb423) regions of SV40 T antigen (Harlow *et al.*, 1981); molecules related to HA were precipitated with a polyclonal serum; 77-kd binding protein [originally called heavy chain binding protein (HBP) Haas and Wabl, 1983] was precipitated with a monoclonal antibody kindly provided by J.Kearney, D.Bole and L.Hendershot, University of Alabama, Birmingham). The immunoprecipitates were then collected and analyzed by SDS-PAGE using 15% gels. The left hand panel (**lanes a–e**) shows immunoprecipitates of the contents of microsomes isolated from a cell line expressing a secreted version of HA (HA^{sec}; Gething and Sambrook, 1982). The central panel (**lanes f–k**) shows the results obtained from a cell line (C_7) expressing HA-T antigen. The right-hand panel (**lanes l–q**) displays results obtained with microsomes isolated from a cell line expressing equivalent amounts of wild-type T antigen. The positions of HA-T antigen, HA, and the 77-K protein are marked by the symbols >, – and ◇, respectively. The conditions of digestion and the antibody used to precipitate the samples are shown above each lane. The mol. wts. of the markers (left-hand track) in decreasing order are 200, 92, 68, 46, 32 and 17 kd.

type T antigen from the identical vector, display features that are characteristic of SV40 transformation (data not shown; R.Gerard, unpublished observations). These results indicate that HA-T antigen is not a transforming protein.

Primary glycosylation of HA-T antigen

The amino acid sequence of large T antigen contains a canonical site for N-linked glycosylation (153-Asn-Arg-Thr). This site is normally unoccupied in wild-type T antigen, because the protein does not come into contact with glycosylating enzymes that reside in the lumen of the endoplasmic reticulum. All cell lines expressing HA-T antigen synthesized large quantities of a molecule that was labelled with radioactive mannose during a 1 h pulse and was precipitated with antisera specific to T antigen (Figure 2). By contrast, no label was incorporated into T antigen in a control cell line that synthesizes equivalent amounts of wild-type T antigen. The incorporation of radioactive mannose into HA-T antigen was abolished in the presence of tunicamycin, indicating that the glycosylation was of the N-linked type (data not shown). We conclude: (i) that the potential site for N-linked glycosylation remains unoccupied in wild-type T antigen, (ii) that the presence of the signal peptide causes HA-T antigen to cross the membrane of the rough endoplasmic reticulum and enter the lumen, where it undergoes primary glycosylation. Figure 3 shows the result of an experiment in which several cell lines synthesizing HA-T or wild-type T antigen were labelled for 1 h with [^{35}S]methionine in the presence or absence of tunicamycin. In the absence of the drug, the labelled HA-T antigen was slightly larger than authentic T antigen – presumably because of the addition of core oligosaccharides. HA-T antigen synthesized in the presence of tunicamycin is smaller than wild-type T antigen by an amount that is consistent with the absence of core oligosaccharide and cleavage of the signal peptide. The size of wild-type T antigen synthesized in the presence and absence of tunicamycin is identical. After processing in the lumen of the endoplasmic reticulum, the amino acid sequence at the N terminus of HA-T antigen should be NH_2Asp-Leu-Leu-Gly-Leu-Glu..... Automated microsequencing of the isolated protein into which [^{3}H]Leu had been incorporated during biosynthesis confirmed the presence of Leu at positions 2,

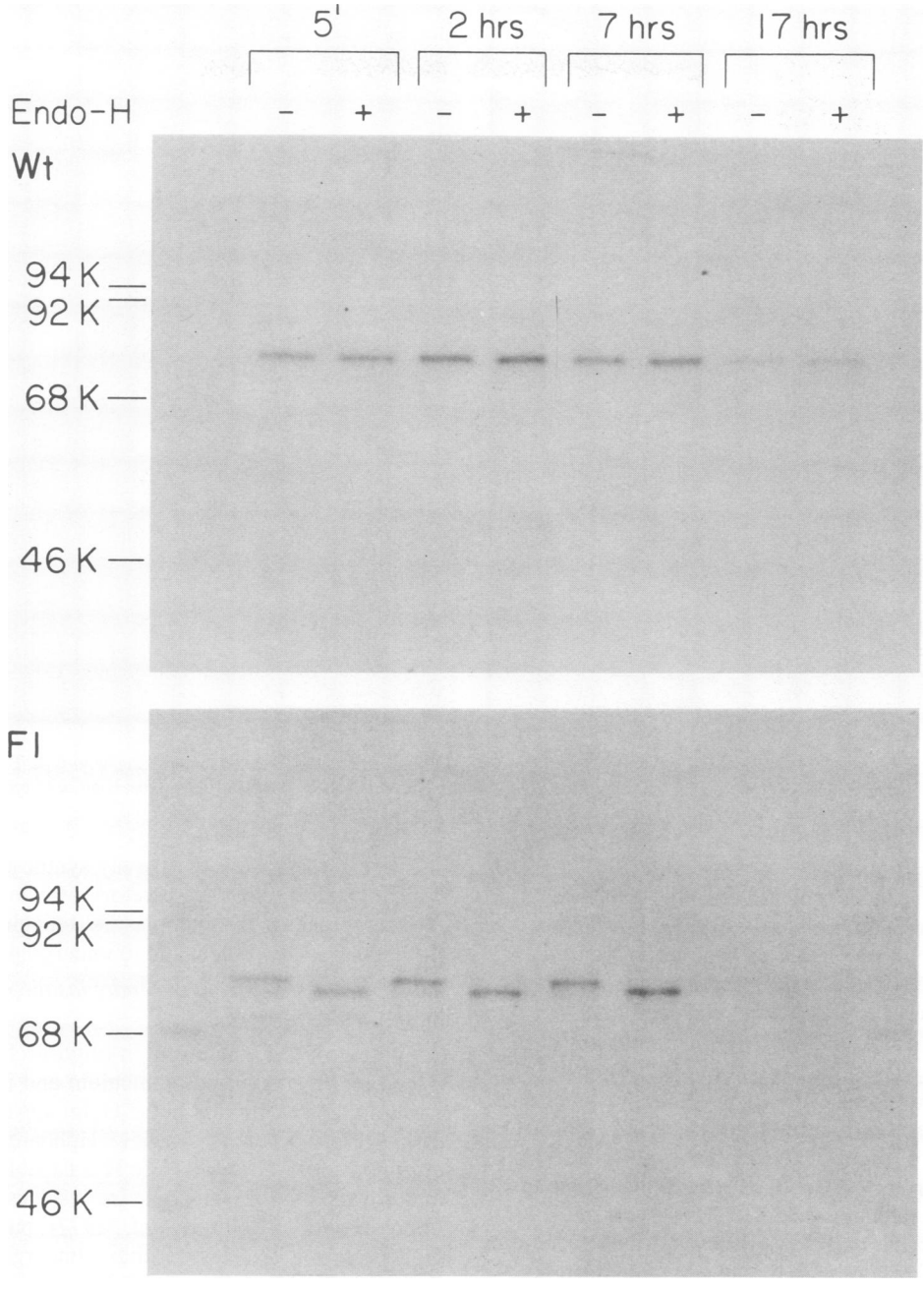


Fig. 5. Sensitivity of HA-T and T antigens to digestion with endoglycosidase H. Monolayers (60 mm) were incubated for 30 min in methionine-free medium and then pulse-labelled for 10 min with 100 μCi of [^{35}S]methionine in 1 ml medium. The cells were then washed and incubated for various periods of time (5 min, 2 h, 7 h or 17 h) in complete medium. At each time point, a monolayer of the cells was lysed and molecules related to T antigen were precipitated from the extracts as described in the legend to Figure 2. The immunoprecipitated material was divided into two parts, one of which (+) was treated with endoglycosidase H as described by Doyle *et al.* (1985). The material was then analyzed by SDS-polyacrylamide gel electrophoresis as described in the legend to Figure 2. **Upper panel,** extracts of cells synthesizing wild-type T antigen. **Lower panel,** extracts of cells (F_1) synthesizing HA-T antigen.

3 and 5 (data not shown). No radioactivity was detected at residues 4, 10 and 12 (the positions occupied by leucine in the signal peptide of HA). We conclude that HA-T antigen is synthesized as a precursor from which the signal peptide is later removed – presumably after the protein has been translocated into the lumen of the endoplasmic reticulum.

No SV40 small t antigen was detected in cells expressing either large T antigen or HA-T antigen. Why small t antigen should be synthesized so inefficiently in these cells is unknown. However a similar result has been obtained when the early region of SV40 has been expressed in other mammalian vector/host systems (Thummel *et al.*, 1983).

HA-T is completely translocated across the membrane of the rough endoplasmic reticulum

Since newly-synthesized HA-T antigen undergoes primary glycosylation, at least the N-terminal 154 amino acids of the molecule must cross the membrane of the rough endoplasmic reticulum. To determine whether the entire polypeptide was translocated, we measured the ability of microsomes, prepared by homogenizing cells, to protect HA-T antigen from digestion with externally-added proteases. Figure 4 shows the result of an experiment in which microsomes, prepared from cells that had been labelled for 20 min with [^{35}S]methionine, were treated with trypsin in the presence and absence of detergent (NP-40). As

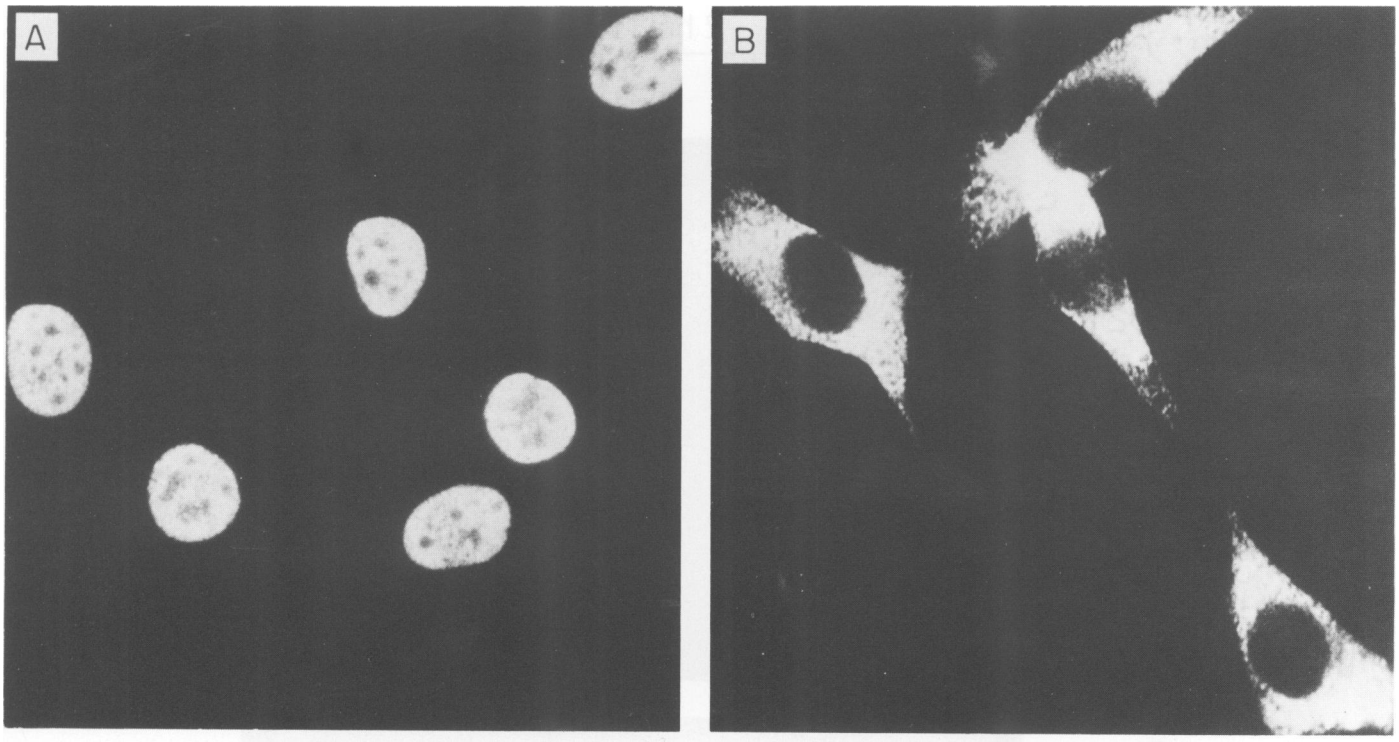


Fig. 6. Immunofluorescent staining of cells expressing wild-type T antigen or HA-T antigen. NIH-3T3 cells synthesizing wild-type T antigen or HA-T antigen from BPV vectors were fixed and stained by indirect immunofluorescence (Pope and Rowe, 1964). **Panel a**, cells expressing wild-type T antigen. **Panel b**, cells (C_7) expressing HA-T antigen.

a control, we used a cell line that expresses from a BPV-based vector, high levels of the anchor-minus version of influenza virus hemagglutinin (HA^{sec}) (Sambrook *et al.*, 1985). HA^{sec} behaves as a classical secretory molecule and is completely translocated across the membranes of the rough endoplasmic reticulum, transported through the Golgi apparatus and secreted from the cell surface (Gething and Sambrook, 1983; Sambrook *et al.*, 1985). Microsomes prepared from a cell line expressing HA^{sec} are fully competent in protecting the newly-synthesized molecule from digestion with protease (Figure 4, lanes b, d and e). Similarly, microsomes prepared from a cell line that synthesizes HA-T contain a completely protected T antigen species that is immunoprecipitable with monoclonal antibodies (Harlow *et al.*, 1981) directed against either the carboxy-terminal (PAb 423) or the amino-terminal (PAb 416) domains of authentic T antigen (lanes f, i, j and k). Cell lines producing wild-type T antigen yield microsomes which show only very small amounts of the protein (lane 1). This T antigen is not protected from digestion by trypsin (lanes o – q) and probably becomes associated with the outside of microsomes during preparation of the vesicles. Disruption of microsomes with detergent renders HA^{sec} and HA-T susceptible to digestion with trypsin (lanes e and k).

Lanes b and c, g and h, and m and n show the results of precipitation of microsomal-associated proteins with an antiserum against a 77-K cellular protein. The protein (HBP or BiP), originally found in association with nascent immunoglobulin heavy chains (Haas and Wabl, 1983), is localized on the luminal side of the rough endoplasmic reticulum (J.Kearney, D.Bole and L.Hendershot, personal communication), where it associates transiently with a variety of newly-synthesized secretory and membrane proteins (L.Hendershot and M.-J.Gething, unpublished results). As seen in Figure 4, both HA^{sec} and HA-T antigen associate efficiently with the 77-K protein: wild-type T antigen does not.

These results show that newly-synthesized HA-T antigen is indistinguishable from a secretory protein in its ability to cross the membrane of the endoplasmic reticulum, to associate with the cellular 77-K protein and to undergo primary glycosylation.

Transport of HA-T from the endoplasmic reticulum to the Golgi apparatus is blocked

Trimming of the mannose-rich oligosaccharide is normally initiated in the endoplasmic reticulum and is completed upon arrival of the glycoprotein in the Golgi apparatus, where terminal glycosylation occurs (see review, Hubbard and Ivatt, 1981). The enzyme endo-β-N-glycosidase H is capable of cleaving between the proximal N-acetylglucosamine residues of mannose-rich core oligosaccharide (Robbins *et al.*, 1977), but is ineffective against glycoprotein molecules that have undergone further modification in the Golgi apparatus. Hence resistance to the activity of endoglycosidase-H is a useful assay for the movement of glycoproteins from the endoplasmic reticulum to the Golgi apparatus. Figure 5 shows that HA-T antigen labelled with [^{35}S]methionine for 10 min remains sensitive to endoglycosidase-H for a period of 7 h (longer exposures of the autoradiograph show that this sensitivity persists for at least 17 h after the pulse). By contrast, a typical secretory protein such as HA^{sec} becomes completely resistant to endoglycosidase H within 2 or 3 h (Gething and Sambrook, 1983; Sambrook *et al.*, 1985). Thus the N-linked glycan of HA-T antigen does not undergo the series of modifications that mark the progress of normal secretory and membrane proteins to and through the Golgi apparatus. This result strongly implies that HA-T antigen is blocked in its movement from the endoplasmic reticulum to the Golgi apparatus.

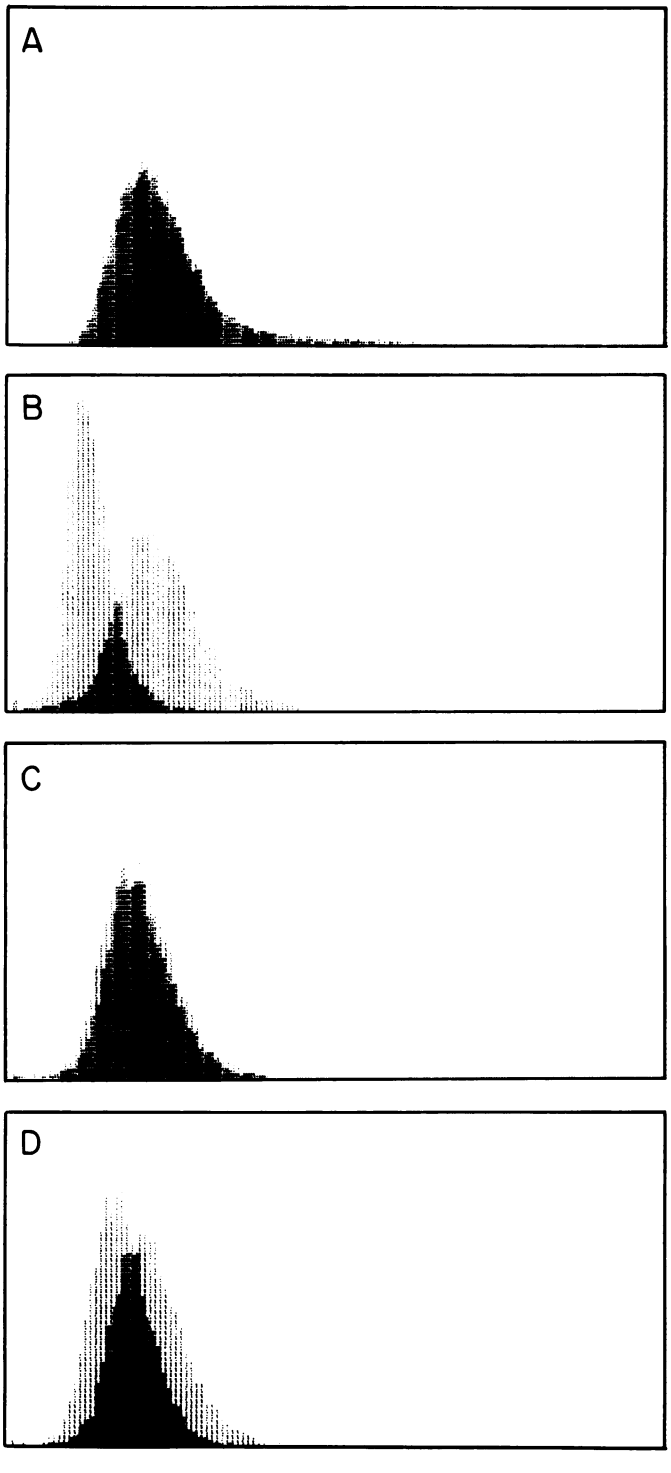
Intracellular localization of HA-T antigen

Cells producing wild-type T antigen display a characteristic pattern of nuclear immunofluorescence (Pope and Rowe, 1964). However, cells producing HA-T antigen show a dramatically dif-

ferent picture (Figure 6) with dark nuclei and bright cytoplasmic fluorescence. The failure to detect nuclear fluorescence is consistent with the biochemical data which indicate that all or virtually all of newly-synthesized HA-T antigen is translocated across the membrane of the rough endoplasmic reticulum into the lumen where it undergoes primary glycosylation. Furthermore, the lack of detectable staining of the Golgi apparatus once again suggests that HA-T antigen is blocked or is transported very slowly indeed from the endoplasmic reticulum.

HA-T antigen reaches the cell surface very inefficiently

To estimate the amount of T antigen on the cell surface, live cells synthesizing wild-type T antigen or HA-T antigen were stained with fluorescent anti-T sera and analyzed by flow cytometry (Figure 7). Panel A shows the results obtained when murine cells expressing wild-type SV40 T antigen from a BPV vector were compared with a line of SV40-transformed cells (mKSA) that displays a high level of T antigen-specific surface fluorescence (Henning *et al.*, 1981). Both cell lines yield co-incidental traces and therefore display the same amount of surface fluorescence. The intensity of the fluorescence is ~8-fold greater than that observed when the cells are stained with non-immune control sera (panel B). Control cells display the same low intensity of surface fluorescence with both antisera (data not shown). Ten out of the 12 cell lines producing HA-T antigen display levels of surface fluorescence with anti-T sera that are indistinguishable from background. An example of one of these cell lines is shown in panel C. Two out of the 12 cell lines examined display a very low level of T antigen-specific surface fluorescence (~2-fold greater than background – panel D). The reason for this low level of fluorescence is unknown. In no case however, did the intensity of fluorescence approach that displayed by cells synthesizing equivalent amounts of wild-type T antigen. We therefore conclude that HA-T antigen is transported to the cell surface very inefficiently if at all.



No HA-T antigen could be detected in the medium taken from cultures of cells synthesizing the molecule by either radioactive pulse-chase experiments or solid-phase radioimmune assay. These assays are sensitive enough to have detected in the supernatant ~0.25% of the intracellular HA-T antigen. We therefore conclude that HA-T antigen is not a secretory molecule.

HA-T antigen is not phosphorylated, acylated or bound to cellular p53

In SV40-transformed cells, both the nuclear (Tegtmeyer *et al.*, 1977) and surface forms (Soule and Butel, 1979) of wild-type T antigen are found as phosphoproteins in association with a cellular protein (p53) which also is phosphorylated (Lane and Crawford, 1979; Linzer and Levine, 1979). Cells synthesizing wild-type T antigen or HA-T antigen were labeled with [^{32}P]-orthophosphate and extracts were prepared. Extracts of cells synthesizing wild-type T antigen contain two phosphoproteins that were co-precipitated by anti-T sera and whose mol. wts. (94 000 and 53 000) correspond to T antigen and p53. These phosphorylated species were not detected in any of the cell lines producing HA-T antigen (data not shown). Precipitation of extracts of cells producing wild-type T antigen with anti-p53 sera yielded the same two bands (p53 and its associated T antigen).

Fig. 7. Analysis of T antigen and HA-T antigen on the cell surface by flow cytometry. The conditions used for cytofluorography of unfixed cells stained with anti-T or control sera are described in Materials and methods. Each panel shows the number of cells (vertical axis) that fluoresce with a given intensity (horizontal axis). The intensity of fluorescence is displayed in arbitrary units on a logarithmic scale stretching over three log units. Each panel contains two traces: the area of overlap between these traces is shown in darker hatching. **Panel A.** A comparison between NIH-3T3 cells synthesizing wild-type T antigen from a BPV vector and a conventional line of SV40-transformed murine cells (mKSA, Henning *et al.*, 1981). The two traces are coincident. **Panel B.** Surface fluorescence of NIH-3T3 cells expressing wild-type T antigen and stained with anti-T serum (right hand trace) or control serum (left hand trace). The distance between the medians of the two traces is equal to an 8.5-fold increase in fluorescent intensity. **Panel C.** Surface fluorescence of cells (C_7) expressing HA-T antigen and stained with anti-T serum or control, serum. The two traces are coincident. The results obtained with C_7 cells are typical of the majority of HA-T producing cell lines. **Panel D.** Surface fluorescence of cells (F_1) expressing HA-T antigen and stained with antiserum (right hand trace) or control serum (left hand trace). F_1 cells are atypical in that they display a small amount of specific surface fluorescence when stained with anti-T sera (see Results). The distance between the medians is equivalent to a 1.9-fold increase in fluorescent intensity.

However, no proteins were specifically precipitated by these sera from extracts of cell lines synthesizing HA-T antigen (results not shown). We conclude that HA-T antigen, by contrast to the nuclear and surface forms of wild-type T antigen, is not a phosphoprotein and is not complexed to cellular p53.

Finally, it has been reported that the surface form of wild-type T antigen is acylated and tightly bound to a substructure of the plasma membrane, the plasma membrane lamina (Klockmann and Deppert, 1983a, 1983b; Klockmann *et al.*, 1984). Where in the cell fatty acids become attached to T antigen is not known. However, in the case of authentic membrane proteins of both viral and cellular origin, in which this type of post-translational modification is widespread, acylation takes place (at least in part) on the cytoplasmic face of the membrane of the endoplasmic reticulum in the late endoplasmic reticulum-early Golgi stage of the exocytotic pathway (see review, Schmidt, 1982). We were unable to detect any incorporation of radiolabelled palmitate into HA-T antigen and we conclude that the molecule is not acylated *via* palmitate residues – a result that is consistent with the observation that HA-T antigen is completely translocated across the membrane of the endoplasmic reticulum to a site where it is no longer accessible to acylating enzymes present in the cytoplasm.

Table I. A comparison of the properties of nuclear T antigen, surface T antigen and HA-T antigen

	Nuclear T	Surface T	HA-T
1. Location	Nucleus	Surface	ER
2. Phosphorylation	+	+	–
3. Binding to p53	+	+	–
4. Palmitate addition	–	+	–
5. Properties associated with N-linked glycosylation			
a. Tunicamycin sensitivity	–	–	+
b. Mannose labeling	–	–	+
c. Endo-H sensitivity	–	–	+
6. Binding to 77 K	–	–	+

The properties of HA-T antigen are derived from the data presented in this paper. Wild-type T antigen expressed from a BPV vector is indistinguishable in its properties from nuclear T antigen synthesized in cells transformed or lytically infected with SV40. The properties of surface T antigen are summarized from data published by others (see references cited in Introduction).

Discussion

HA-T antigen differs from nuclear and surface T antigen both in its biochemical properties and intracellular location (see Table I). In part, the biochemical differences reflect events at the potential N-linked glycosylation site at Asn^{154}, which in HA-T antigen is an efficient acceptor of mannose-rich core oligosaccharides. By contrast, the same site in nuclear and surface T antigens remains unoccupied. Instead of entering the nucleus or moving to the cell surface, HA-T antigen is co-translationally translocated across the membrane of the rough endoplasmic reticulum but is unable to move further along the secretory pathway. Directed to the lumen of the rough endoplasmic reticulum, HA-T antigen does not become exposed to phosphorylating or fatty acid acylating enzymes in the cytoplasm (Scheidtmann *et al.*, 1982; van Roy *et al.*, 1983; Schmidt, 1982), nor does it have the opportunity to associate with cellular p53 in the nucleus. Instead it associates with a cellular protein (77 K) that appears to be involved in early events in the exocytotic pathway. From these results, the following points were concluded.

(i) The hydrophobic signal peptide acts as a *cis*-dominant marker that overrides other transport signals in SV40 T antigen – for example, the nuclear transport signals that map in the seven amino acids around Lys^{128} (Kalderon *et al.*, 1984a, 1984b). Almost certainly, this dominance stems from the location of the signal peptide at the N terminus of the nascent protein, where the interaction with signal recognition particles prevents further synthesis of the polypeptide until the ribosome is attached to a docking site on the membrane of the rough endoplasmic reticulum. When translation resumes, the polypeptide is co-translationally transported through the membrane in a manner that apparently affords no opportunity for downstream nuclear transport signals to function.

(ii) The translocation of a protein through the membrane of the rough endoplasmic reticulum is not sufficient to guarantee that it will move further along the secretory pathway. This conclusion is consistent with observations of the behavior of chimeric and mutant forms of authentic secretory protein, many of which

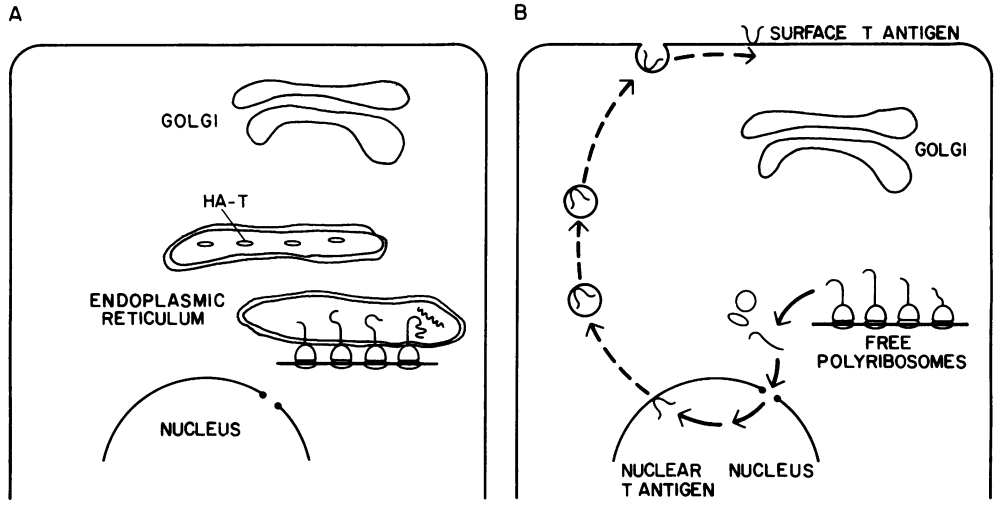


Fig. 8. A model to illustrate (**a**) the intracellular location of HA-T antigen, (**b**) a possible scheme for transport of wild-type T antigen through the nucleus to the cell surface. We realise that the topological requirements of this model place constraints on the mechanism of fragmentation of the nuclear envelope at mitosis.

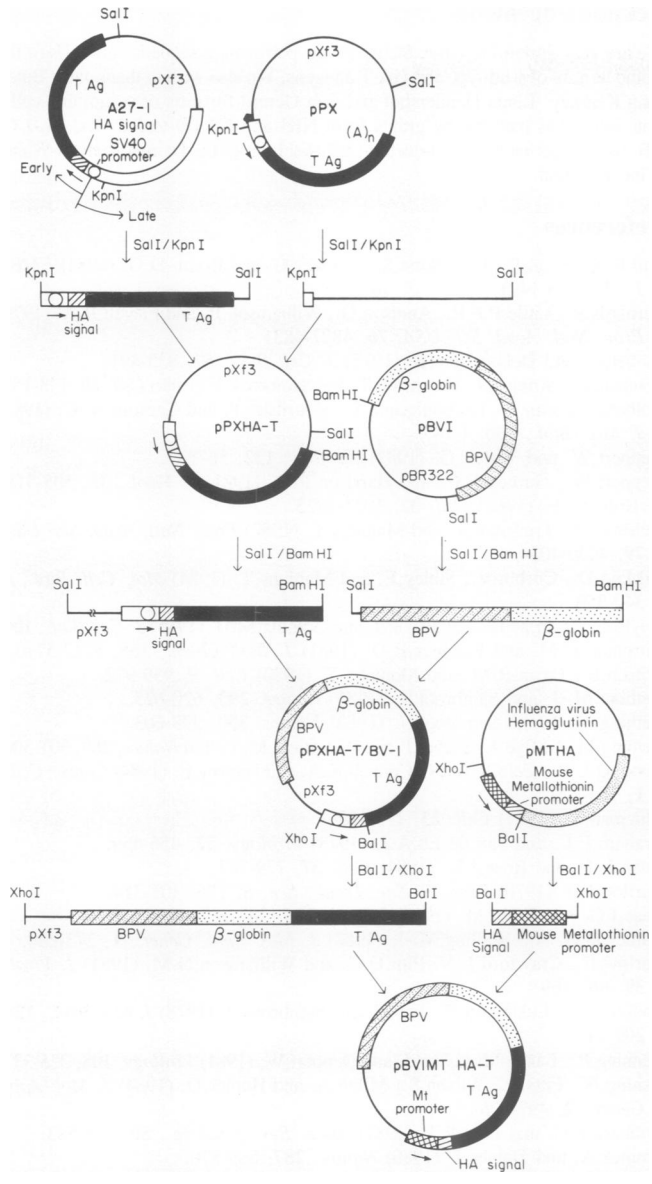


Fig. 9. Construction of a bovine papilloma virus shuttle vector expression HA-T antigen. The details of the construction are given in Materials and methods.

show defects in transport to the cell surface (see, for example, Rose and Bergmann, 1983; Guan and Rose, 1984; Wills *et al.*, 1984; Doyle *et al.*, 1985). The events that occur during movement of secretory and transmembrane proteins from the rough endoplasmic reticulum to the Golgi complex are not yet fully defined. However there is circumstantial evidence pointing to a correlation between the ability of a protein to fold correctly and its rate of movement through the early stages of the secretory pathway (Guan and Rose, 1984; Doyle *et al.*, 1985). In addition to folding correctly these proteins may need to interact with hypothetical cellular receptors which have been postulated to carry them from their site of synthesis in the endoplasmic reticulum to the Golgi apparatus. The failure of HA-T antigen to move from the endoplasmic reticulum can therefore be explained in two ways. First, HA-T antigen is a protein that has been removed from its normal milieu and placed in a foreign environment. There would be no evolutionary reason why cellular receptors, if they exist at all, should be equipped to deal with HA-T antigen. Second, the presence of a bulky glycan group may not be conducive to proper folding of the polypeptide or to efficient oligomerization of the molecule. In addition, it is possible that the temporary presence of the signal peptide in place of the 14 amino acids that normally form the N terminus of T antigen may inhibit or retard the folding of the molecule. However this seems unlikely, since forms of T antigen with far more extensive changes at their N termini (e.g., Ad2^{+}D2, Hassell *et al.*, 1978) retain the ability to fold into a functional form and to oligomerize (see Tjian, 1978; Tjian *et al.*, 1978).

(iii) Because HA-T antigen fails to move efficiently along the secretory pathway and because its biochemical properties are very different from those of surface T antigen, it seems unlikely to us that wild-type T antigen reaches the cell surface by travelling along the conventional exocytotic route. We are therefore left with a paradox. At some stage in its life, surface T antigen must cross a membrane in order to be present on the outside of the cell: yet it has none of the hallmarks of an integral or transmembrane protein. Under these circumstances there seem to be two alternative possibilities. First T antigen may have the ability spontaneously to insert itself into a membrane. Proteins with this property [e.g., bacteriophage M13 procoat (Silver *et al.*, 1981)] have been recognized, although they seem to be rather rare, especially in mammalian systems. However, T antigen seems to be ill-equipped for this task: it contains no strongly hydrophobic regions and is not synthesized from a larger precursor. This explanation therefore seems unlikely to us. An alternative possibility is shown in Figure 8. We postulate that in SV40-transformed cells, all T antigen molecules, no matter what their final destination, are synthesized in the same pool of 'free' polysomes in the cytoplasm and then travel to the nucleus using the nuclear transport signals described by Lanford and Butel (1984) and Kalderon *et al.* (1984a, 1984b). We hypothesize that at least a fraction of nuclear T antigen becomes associated either directly or *via* intermediate molecules (which perhaps include DNA) with the inner wall of the nuclear membrane. At cell division, this membrane is known to break down into small vesicles that become dispersed throughout the cytoplasm (see Maul, 1982). If, after cytokinesis, a minority of these vesicles fuses directly with the plasma membrane or with other vesicles that are bound for the surface, their contents would be everted onto the outer face of the plasma membrane. Fatty-acid acylation of T antigen could occur either during transport to the cell surface or at the surface itself. This explanation is consistent with the known properties of T antigen. This hypothesis might also apply to another nuclear protein (influenza virus nucleoprotein) that, like SV40 T antigen, lacks hydrophobic signal sequences and yet is expressed in small amounts on the surface of cells which express the NP gene (Townsend and Skehel, 1984; Townsend *et al.*, 1984). Like SV40 T antigen, NP is a nucleic acid-binding protein.

If this hypothesis stands up to experimental test, it will be interesting to determine whether normal cellular proteins travel along this novel pathway from nucleus to the cell surface.

Materials and methods

General methods

Preparation of DNAs, digestion with restriction enzymes, agarose gel electrophoresis, ligation, addition of synthetic linkers and bacterial transformation were done by standard methods (Maniatis *et al.*, 1982 and references therein).

Construction of a BPV shuttle vector expressing HA-T antigen

The gene coding for the HA from the Japan strain of influenza virus contains an *Ava*II site at nucleotide 88 (Gething *et al.*, 1980) immediately downstream

from the codon which specifies the last amino acid of the hydrophobic signal sequence. A fragment of DNA was prepared that carried the complete signal sequence and ended at this *Ava*II site. This fragment was joined to the *Ava*II site at nucleotide 5118 of SV40 DNA in such a way as to create a chimeric gene in which the first 14 codons of the SV40 early region were replaced by the first 15 codons of the HA gene.

Expression of the chimeric gene in the resulting plasmid (A27.1, see Figure 9) was controlled by the SV40 early promoter. The gene was placed under the control of the murine metallothionenin I promoter (Hamer and Walling, 1982) and inserted into a bovine papilloma virus shuttle vector according to the scheme shown in Figure 9. The plasmids used in this construction and their derivations are given below.

(i) pPX, which consists of the *Cla*I-*Bam*HI fragment of plasmid pXf3 (Maniatis *et al.*, 1982) joined to a 2.8-kb segment of SV40 extending counterclockwise from the *Hpa*II site at nucleotide 346 through the origin, promoter and early coding region to the *Bam*HI site at nucleotide 2533. The *Hpa*II/*Cla*I site was then altered to an *Xho*I site by addition of synthetic linkers.

(ii) pBPV-BV1 (Zinn *et al.*, 1983) which contains the 5.5-kb subgenomic transforming fragment of BPV DNA, a deleted version of a 7.6-kb fragment of DNA derived originally from the human β-globin gene cluster (Bernards *et al.*, 1979; Fritsch *et al.*, 1980) and the plasmid pBRd (DiMaio *et al.*, 1982).

(iii) pMTHA (Sambrook *et al.*, 1985), which consists of the 3.5-kb *Cla*I-*Bam*HI fragment of pPX, a fragment of 800 bp carrying the murine metallothionein I (Hamer and Walling, 1982), the coding region derived from a full length cDNA clone of the hemagglutinin gene of the A/Jap/305/57/H2N2 strain of human influenza virus (Gething *et al.*, 1980) and a fragment of 200 bp that carries the polyadenylation signal from the distal end of the SV40 early region.

Analysis of HA-T and wild-type T antigens

The methods for pulse-labelling of cells with [^{35}S]methionine and [^{3}H]mannose in the presence and absence of tunicamycin (4 μg/ml), immunoprecipitation of radiolabelled HA-T and T antigens, analysis of immunoprecipitates by SDS-polyacrylamide gel (7.5%) electrophoresis, digestion of immunoprecipitates with endoglycosidase H, and quantitative radioimmune assay of T antigens were done according to methods described by Gething and Sambrook (1981, 1982). Labelling of T antigens with [^{32}P]orthophosphate was performed essentially as described by Scheidtmann *et al.* (1982).

Growth and transfection of cells

Cells were maintained in Dulbecco's modification of Eagle's medium supplemented with 10% fetal bovine serum in a humidified incubator containing 5% CO_2 at 37°C. The cells were subcultured twice a week and were not allowed to grow to confluency. Transfected derivatives were selected initially for resistance to G418 but the cloned sublines were subsequently maintained in medium lacking the antibiotic.

BPV vectors carrying the gene for either wild-type T antigen or HA-T antigen were introduced into the cells as a co-precipitate with calcium phosphate (Graham and van der Eb, 1973), essentially as described by DiMaio *et al.* (1982). The transfection mixture contained 1 μg of the appropriate plasmid, 100 ng of plasmid pON_3 and 20 μg of sheared mouse DNA as carrier. Forty-eight hours later, the cultures were subdivided (split ratio 1:5) into medium containing G418 (0.3 mg/ml). Cell death usually began 3 days later and surviving colonies were picked after 10 – 14 days incubation, using glass cloning cylinders into 12-well multiplates (Linbro). These clones were screened for the expression of T antigens by indirect immunofluorescence (Pope and Rowe, 1964) and by quantitative radioimmune assay. Positive isolates were recloned and grown to mass culture for biochemical analysis.

Flow cytofluorometry

Cells were examined for expression of T antigen on the surface by quantitative cytofluorometry using an Epics C flow cytometer (Coulter). Cells were grown to subconfluency on standard tissue culture plates and were treated with 0.05% EDTA in phosphate buffered saline (lacking calcium and magnesium) for 15 min. The cells were then removed from the plates by aspiration, washed three times in medium containing 1% heat-inactivated fetal bovine serum and incubated for 30 min at 0°C either with a 1:1000 dilution of a FITC-conjugated mixture of 12 monoclonal antibodies specific for different epitopes on large T antigen (Harlow *et al.*, 1981), or with medium containing non-conjugated heat-inactivated fetal bovine serum, or with one of a variety of fluorescent non-immune sera or monoclonal antibodies directed against cell surface proteins (e.g., hemagglutinin) that are not expressed on the surface of the cell lines that are synthesizing SV40 T antigens. The cells were then washed twice with medium containing 1% heat-inactivated fetal bovine serum, resuspended at a concentration of 2 x 10^6 cells/ml and analyzed by flow cytofluorometry. Cells treated with the fluorescent control sera mentioned above showed no increase in fluorescence over the background level of autofluorescence.

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