CELL SURFACE PROTEIN CHANGES

ASSOCIATED WITH HSV-2 TRANSFORMATION

by

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ABSTRACT

In these studies the cell surface proteins of
Herpes simplex virus type 2 (HSV-2) transformed hamster
embryo fibroblasts (HaEF) were examined using a variety
of radioactive isotope labeling techniques with subsequent
analysis on sodium-dodecyl sulphate- polyacrylamide slab
gels. As well, a number of biological parameters of
these cell lines were investigated including cell morphology,
doubling time, saturation density, and tumourigenecity.

Cell surface iodination performed on the series
of HSV-2 transformed HaEF revealed at least three cell
surface alterations. One such protein was the major
iodinated species of 220,000 daltons commonly called
LETS. LETS was reduced in the transformed lines. All
transformed lines contained less than 50% of the level
of LETS found in normal secondary HaEF.

A variation was found for the HSV-2 transformed
cells in their ability to produce tumours. A relation-
ship was observed between tumour-forming ability and the
growth properties of cell doubling time and saturation
density. The more tumourigenic lines grew to higher
saturation densities and doubled their numbers in the
shortest time. In the HSV-2 transformed lines a correlation
was also found between the amounts of LETS protein on the
surface of the cells and tumourigenecity. The amount

of LETS present was considerably greater for the weakly tumourigenic lines than for the more tumourgenic lines.

There were few protein changes detected when whole cells labeled with ^{35}S-methionine were examined, however, numerous alterations were evident when isolated plasma membranes from normal, secondary HaEF and the various HSV-2 transformed lines labeled with ^{35}S-methionine or inorganic ^{32}P-phosphate were analyzed. These latter studies indicated that several plasma membrane protein changes are associated with HSV-2 transformation.

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INTRODUCTION

There is great interest today in the oncogenic
potential of the herpes viruses because of their suspected
role in specific human malignancies. This interest arose
from the fact that these viruses have been proven to
be oncogenic in several different vertebrate species.
Marek's disease, a general lymphomatosis in chickens, is
caused by a herpes virus (Purchase, 1972). Herpes saimiri,
isolated from squirrel monkeys, is highly oncogenic in
marmosets and owl monkeys causing a lethal malignant lymphoma
(Melendez et al., 1972). The target cell in each case
is the T-lymphocyte. Both these viral diseases satisfy
Koch's postulates and can be prevented by immunization
with viral vaccines (Witter et al., 1970; Laufs and
Fleckenstein, 1972). A herpes virus has also been shown
to be the causitive agent in Lucke's tumour, an adenocarcinoma
found in the kidney of the American leopard frog (Mizell
et al., 1969). Although animal systems have greatly increased
our understanding of the molecular biology and pathology
of herpes viruses, they do not serve as good models for
human malignancies since the incidence and variety of
tumours found in man are different than those found in
animal systems studied thus far (Priester and Mantel, 1971).

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For example, there are no animal model systems for the study of cervical carcinoma, a common form of cancer in man.

There are at least five different herpes viruses which infect humans: herpes simplex subtype 1 (HSV-1), herpes simplex subtype 2 (HSV-2), herpes zoster (HZV), Epstein-Barr (EBV), and cytomegalovirus (CMV). Three of the above viruses have been linked to malignancies in man. An association exists between EBV and both carcinoma of the nasopharynx (Henle and Henle, 1973) and African Burkitt's lymphoma (Henle and Henle, 1973). CMV has been linked to Kaposi's sarcoma (Giraldo et al., 1972). HSV-2 has been associated with carcinoma of the cervix by sero-epidemeological criteria (Rawls et al., 1969). In these latter studies a significantly higher incidence of neutralizing antibody to HSV-2 in patients with cervical carcinoma was found compared to control populations. As well, in some human cervical cancer cells HSV-2 antigens have been detected (Aurelian, 1973), HSV-2 virions have been recovered (Aurelian et al., 1971), and HSV-2 DNA fragments have been demonstrated to be present (Frenkel et al., 1972). DNA-DNA hybridization experiments between radiolobeled HSV-2 DNA and DNA from cervical cancer cells revealed the presence of a segment representing 39% of the HSV-2 genome at a concentration of 3-5 copies per diploid cell (Frenkel et al., 1972). The majority of studies however suggest that

most cervical cancer cells do not contain HSV-2 DNA but
it is likely that the methods currently used are not
sensitive enough to detect small fragments of the HSV-2
genome. It has been shown that transformation of mammalian
cells can be induced in vitro with as little as 1.2×10^6
daltons of adenovirus or simian virus 40 (Graham et al.;
1974; Abrahams et al., 1975). If the same amount of
HSV-2 DNA is sufficient to induce oncogenic transformation,
the sensitivity of the molecular probe would have to detect
1-2% of the viral DNA. The total molecular weight of
HSV-2 viral antigens associated with cervical cancer cells
is about 280,000 daltons representing 2-3% of the virus's
coding capacity. Current methods would not be able to
detect this amount.

Herpes viruses are the largest and have the most
complex structure of the oncogenic DNA viruses. The virion
consists of four major components, the centrally located
core surrounded by three concentric structures: the capsid,
the tegument, and the envelope. The core is believed
to be an electron-dense toroid containing DNA with a less-
dense plug filling the hole. The capsid consists of 162
subunits called capsomeres in an iscosadeltahedron arrange-
ment with an 2-, 3-, 5-fold symmetry. The tegument is a
layer of amorphous material located between the capsid
and envelope. The envelope consists of a tri-laminar

membrane with spikes projecting from its outer surface. The complete viral particle has a diameter of approximately 180nm. A naked nucleocapsid is not infectious whereas enveloped nucleocapsids are. The purified virus contains at least 33 species of polypeptides ranging in size from 25,000 to 280,000 daltons. The genome is a linear double-stranded DNA molecule of approximately 10^8 daltons.

It is not known how the virus enters the cell but electron microscopic studies indicate that entry of the virus into the cell may occur by two mechanisms: fusion with the plasma membrane (Morgan et al., 1968) or by phagocytosis (Dales and Silverberg, 1969). After entry into the cell the envelope and outer protein-capsid is removed, leaving a DNA-protein complex which then enters the nucleus. There is extensive early viral transcription which precedes viral DNA synthesis and proceeds in the absence of protein synthesis. 'Some of these transcripts are transported to the cytoplasm where they are translated into α-polypeptides. These viral proteins are translocated into the nucleus where they mediate a new round of transcription from viral DNA. The β-polypeptides are produced from these new transcripts. They function in the synthesis of viral DNA, the termination of synthesis of host and α-polypeptides, and bring about the third group of trans-

cripts from which the δ-polypeptides originate. Most of
the δ-proteins are structural while others terminate
β-protein synthesis. Viral assembly begins in the nucleus.
It is thought that the viral DNA may penetrate into an
assembled capsid. Herpes viruses acquire a lipid envelope
by budding, usually from the nuclear membrane and probably
leave the cell via the endoplasmic reticulum (Roizman et al.,
1977).

HSV can establish a latent or persistent infection
in some host cells. A persistent infection is one in
which infectious virus is continually released, even in
the presence of circulating antibody and in the absence
of symptoms of disease (Rapp and Jerkofsky, 1973). A
latent infection is one in which at least the genome of the
virus is present but infectious virions cannot be recovered
except during manifestations of the disease. In both
these cases the virus can become suddenly active, often
with resultant cell destruction and disease. This viral
activation can be brought about by a variety of stimuli
including UV-light irradiation.

Herpes replication, as is the case in most DNA
viruses is a highly inefficient process yielding a large
ratio of defective to infectious particles. These defective
particles could cause transformation since replication of
herpes virus in permissive cells induce such severe cyto-
pathology that the cell dies. To transform cells by

HSV-2 the replication machinery of the virus must be
inactivated. This can be done in a number of ways:
UV-light inactivation (Duff and Rapp, 1971: Boyd and Omre,
1975), by incubating cells infected with wild type HSV-2
at 42°C (Dorai and Munk, 1973), photodynamic inactivation
(Rapp et al., 1973), incubating cells infected by temperature-
sensitive mutants at the non-permissive temperature
(McNab, 1974; Takahashi and Yamanishi, 1974), and infecting
cells with sheared HSV-2 DNA (Wilkie et al., 1975).

Cancer cells and cells transformed in vitro by
oncogenic viruses and other agents, possess a phenotype
which differs markedly from that of their normal counter-
parts. A number of these differences are associated with
the plasma membrane. Immunological techniques have shown
differences in surface antigens between normal and trans-
formed cells (cf. Nicolson, 1975). Lectins have been used
to show that a surface architectural change occurs following
transformation. Lectins are proteins with an affinity
for carbohydrate groups that are found at the cell surface.
Several of these, including concanavalin A and wheat germ
agglutin, have the property of agglutinating transformed
cells at concentrations which do not affect normal cells
(Burger, 1973).

One phenotypic difference between normal and
transformed cells of great interest concerns the regulation
of growth. Normal cells exhibit density-dependent inhibition

of growth. At a certain cell density, characteristic of
individual cell lines, the cells stop dividing and become
quiescent, a phase of the growth cycle often called G_0.
This phenomenon was originally called contact-inhibition
of growth. In contrast, transformed cells do not become
contact-inhibited but rather continue to proliferate
to much higher densities. The fact that cell-cell contact
appeared to inhibit cell proliferation first suggested
that the cell surface may be involved in growth control.
There is now much evidence to support this idea. First,
lectins can stimulate the conversion of lymphocytes from
small resting cells into large blast-like cells which
then undergo mitotic division (Nowell, 1960), presumably
by the binding of the lectin to specific carbohydrate
groups on the cell surface. Second, transport systems
for glucose, amino acids, and other important metabolites
which are necessary for cell division are found at the
plasma membrane. An increase in the uptake of glucose
is one of the earliest indications for the onset of cell
proliferation and oncogenic transformation (Weber, 1971).
Third, it has been shown that cAMP levels are low in rapidly
dividing normal cells and transformed cells, whereas the
levels are higher in density-inhibited cells (Otten et
al., 1971; Sheppard, 1972; Pastan and Johnson, 1974;
Pastan et al., 1975). Thus it appears that cAMP is involved

in growth control and the enzymes for cyclic nucleotide regulation are found for the most part in the plasma membrane. Fourth, many hormones which affect cell growth do not enter the interior of the target cell but rather seem to act via the cell surface (Cuatrecasas, 1975). Fifth, proteases may also play an important role in growth control by their action on the cell surface. It has been shown that confluent, non-dividing cells will undergo at least one round of division when treated with proteases (Burger, 1970; Sefton and Rubin, 1970). These cells, so treated, transiently take on some of the phenotypic characteristics of transformed cells. They become agglutinable by lectins (Burger, 1969; Inbar and Sachs, 1969), new antigens are expressed (Hayry and Defendi, 1970; Burger, 1971), elevated 2-deoxyglucose uptake ensues (Sefton and Rubin, 1971), and reduced levels of cAMP are present (Otten et al., 1971; Pastan and Johnson, 1975). It has been shown that fibroblasts from different species transformed by various carcinogens have proteolytic activities that are much greater than normal cells (Bossman, 1972; Schnebli, 1972; Unkeless et al., 1973; Ossowski et al., 1973). Many transformed cells do release a factor, plasminogen activating factor, which converts the serum proenzyme plasminogen into the protease, plasmin (Unkeless et al., 1973; Ossowski et al., 1973). It has been shown that plasmin activity is required for a variety of changes characteristic of

transformed cells such as: growth in agar, migration into
a wound in the absence of serum, and a characteristic
series of morphological changes (Ossowski et al., 1973).
It has been suggested that part of the transformed phenotype
may be maintained by the action of proteases on the cell
surface (Hynes, 1974). It appears then, that many of the
properties of transformed cells involve alterations in
the plasma membrane.

A better understanding of the molecular composition
and architecture of the plasma membrane of transformed
or cancer cells could suggest possible mechanisms for the
altered affinities and growth properties of these cells.
The first proposal for a possible membrane structure came
from studies on the red blood cell. It was suggested that
the surface membrane of red cells was composed of a double
layer of lipid molecules, with the polar groups situated
on the outside of the layers (Gorter and Grendel, 1925).
This was later modified to include pleated sheets of membrane
protein associated with the charged phosphate groups of
the phospholipids (Danielli and Davson, 1935). Early
electron microscopy studies supported the lipid bilayer
hypothesis of membrane structure (Robertson, 1959).
These studies revealed two thin electron opaque lines,
each about 20A thick, separated by about a 35A space.
This 75A wide structure was called a unit membrane and was
interpreted as supporting the Danielli-Davson membrane

model. High resolution X-ray diffraction patterns of myelin membranes yielding Fourier diagrams showed unequivocally that lipids in membranes were arranged in a bi-layer structure (Caspar and Kirschner, 1971). The Danielli-Davson model representing cell membranes as a static lipid bilayer covered by proteins arranged in pleated sheets was not supported by thermodynamic considerations and recent membrane data. It was pointed out that this model did not maximize the hydrophobic interactions between the proteins and lipids and the hydrophilic interactions between the polar groups of the phospholipids and the aqueous environment, that is, the model did not represent the lowest possible free energy for a lipid-protein membrane. Based primarily on thermodynamic considerations, Singer and Nicolson (1972) proposed the Fluid Mosaic Model. In this model the proteins are globular and folded in such a way as to give them hydrophobic and hydrophilic portions. The hydrophobic part would be embedded in the interior of the lipid bilayer in contact with the hydrophobic lipid tails, while the hydrophilic portions would be exposed to the aqueous environment. These are called integral proteins. They could float freely about in the lipid in a lateral direction but could not freely pass from one side of the membrane to the other. Transport proteins are envisioned as spanning the entire width of the membrane and are most likely composed

of subunits with an aqueous pore, much like the haemoglobin
molecule, allowing select ions and molecules to pass in
and out of the cell. There are other proteins which
associate with the membrane largely through ionic, hydrogen
bonding, and other interaction and can often be dissociated
molecularly intact from the membrane by mild treatment
such as increasing the ionic strength of the medium.
These are called peripheral proteins, and proteoglycans
and LETS are examples of this type at the outer membrane,
while the red blood cell's spectrin is an example for
this class of protein at the inner side of the plasma
membrane. Cytoskeletal components are similar to peripheral
proteins with largely an operational difference, that is,
they have a transient nature (ability to change organization
and association rapidly), dependence on cell energy systems
for maintenance, sensitivity to drugs, e.g., colcemid,
cytochalasin B, and have structural linkage to cell
organelles. There are three main types: thin filament
or microfilaments composed of actin, thick filament composed
of myosin, and small tubules or microtubules made up
of tubulin. Much recent evidence has supported and extended
this model of plasma membranes (cf. Nicolson, 1975).

   If the plasma membrane does regulate cell prolif-
eration then it is possible that biochemical changes at
the cell surface following transformation may be responsible
for the altered growth properties of these cells. Many

studies have been carried out to identify differences in the plasma membrane of normal and transformed cells and a number of changes have been detected, including alterations in proteins.

Perhaps a universal change in a membrane protein associated with transformation concerns one which was first detected by external surface labeling. This protein has been called band 1 (Hynes, 1973; Hynes and Humphryes, 1974), 250K protein (Hynes, 1974), galactoprotein 'a' (Gahmberg and Hakamori, 1973; Gahmberg et al., 1974), SF antigen (Ruoslahti et al., 1973), and the more common acronym, LETS (large, external, transformation sensitive) (Hynes, 1976). LETS has been shown to have the following characteristics. It has a high molecular weight ranging from 210,000 to 270,000 daltons depending on the system used (Hynes, 1973; Hynes and Humphryes, 1974; Robbins et al., 1974; Hogg, 1974; Critchley, 1974; Yamada and Weston, 1974). It is the major labeled band in SDS-polyacrylamide slab gels when external labeling techniques are employed (Hynes et al., 1976). LETS is extremely sensitive to proteases, that is, proteolytic treatment which does not detach the cells from the plate completely removes LETS (Hynes, 1973; Hynes and Humphryes, 1974; Wickus et al., 1974; Robbins et al., 1974; Critchley, 1974; Yamada and Weston, 1974; Graham et al., 1975; Hynes and Wyke, 1975).

It is a glycoprotein (Hynes and Humphryes, 1974; Robbins
et al., 1974; Stone et al., 1974; Critchley, 1974; Yamada
and Weston, 1974). The synthesis of LETS is density-
dependent; cells in logarithmic growth have less LETS
than density-inhibited cells (Hynes, 1974; Gahmberg, 1974;
Critchley, 1974; Hynes and Bye, 1974).
The amount of LETS is cell cycle dependent. There are
high surface levels of this protein on cells in G_0 whereas
there are lower levels in other parts of the cycle, the
lowest level being in mitosis (Hynes, 1974; Hynes and
Bye, 1974). LETS has also been shown to be reduced or
absent from cells transformed by both viruses and chemicals
(Hynes, 1973; Hynes and Humphryes, 1974; Gahmberg and
Hakomori, 1973; Wickus et al., 1974; Robbins et al., 1974;
Hogg, 1974; Stone et al., 1974; Critchley, 1974; Pearlstein
and Waterfield, 1974; Vaheri and Ruoslahti, 1974; Pearlstein
et al., 1976; Hynes and Bye, 1974). The loss of LETS
in transformed cells may be transformation dependent.
It has been shown that there is a gradual loss of LETS
with time from chick embryo fibroblasts infected with a
Rous sarcoma virus temperature-sensitive for the transforming
src function as the cells are lowered from the non-permissive
to the permissive temperature (Robbins et al., 1974).

There was early speculation that the removal of
LETS played a direct role in the control of growth of cells

in culture. It was suggested that the removal of this protein initiated cell division. The fact that transformed cells secrete a higher quantity of proteases, particularly plasminogen activator factor, suggested that the uncontrolled growth and reduced LETS was due to the removal of this protein by autodigestion. However, studies with a variety of proteases have shown that such is not the case (Blumberg and Robbins, 1974; Chen et al., 1974). At certain concentrations thrombin can stimulate cells to undergo DNA synthesis and divide but not remove LETS (Chen et al., 1974). With low amounts of trypsin, subtilisin, or ficin, LETS could be removed but there would be no significant increase in DNA synthesis and cell division (Chen et al., 1974). Thus removal of LETS is neither sufficient nor necessary for the onset of cell division.

The isolation of this surface glycoprotein (Yamada and Weston, 1974) led to experiments in which purified LETS was added to cultures of transformed cells in order to determine its biological effect. Addition of LETS isolated from normal cells to transformed cultures restored certain morphological features and adhesive properties characteristic of normal cells (Yamada et al., 1975) and the formation of well-defined actin cables which are not present in untreated transformed cells. When LETS is added to normal or transformed cells it produces an increased

migration of the cells and thus may determine how and where

cells migrate in vivo (Ali and Hynes, 1978). LETS added

to transformed cells does not effect 2-deoxyglucose uptake,

cAMP levels, protease production, or growth properties

(Yamada et al., 1976; Yamada and Pastan, 1975; Ali et al.,

1977; Chen et al., 1978). It is now believed that LETS

glycoprotein may play a role in cell adhesion, morphology,

cytoskeleton structure, and motility.

It has been reported that there is a correlation

between the expression of LETS and the oncogenic capability

or tumourigenicity of a series of adenovirus 2 transformed

cells lines (Chen et al., 1976). Gallimore and Chen

(1977) found that the reduced level of LETS protein

was the only trait correlating with tumourigenicity of

cells transformed by adenovirus 2 including anchorage-

independence of growth. It had been reported earlier

that anchorage-independent growth was the only virus-induced

alteration that correlated with tumourigenicity (Freedman

and Shin, 1974; Shin et al., 1975). In another study using

a series of human cell hybrids no correlation was found

between the amount of LETS and tumourigenicity (Der and

Stanbridge, 1978).

Although a direct role for the loss of LETS in

regulating cell proliferation has been ruled out, it is

possible that other plasma membrane protein alterations

may play important roles in the altered pattern of cell
proliferation in transformed cells. A protein of 206,000
daltons called omega was reduced or absent after RSV
transformation of chicken embryo fibroblasts (Robbins et
al., 1974). The location of omega in the cell is not known
but it is insensitive to externally added proteases.
A glycoprotein of molecular weight 142,000 daltons was
absent in membranes from RSV-CEF (Bussell and Robinson,
1973). This protein was found to be insensitive to trypsin
therefore an external location is unlikely. Another
glycoprotein of 47,000 daltons called delta was reduced
in RSV transformed CEF (Wickus et al., 1974; Robbins et al.,
1974; Isaka et al., 1975). although in a similar study
no decrease was observed (Stone et al., 1974). (This difference
may reflect the different methods of membrane preparation.)
The amount of plasma membrane-bound actin has been reported
to be reduced by as much as 30-50% following RSV trans-
formation of CEF (Hubbard and Cohn , 1975; Robbins et al.,
1974), but the total amount of actin per cell was unchanged.
Furthermore the actin in transformed cells is largely
de-polymerized (Pollack et al., 1975). There were two
proteins of molecular weights 73,000 and 95,000 , detected
in membrane preparations from normal cells which increased
in amount after transformation in both chicken and rat
cells transformed by several different avian sarcoma viruses

(Stone et al., 1974). These proteins have recently been shown to be involved in glucose transport (Banjo and Perdue, 1976; Shin et al., 1977).

The activity of plasma membrane proteins can be modified in another way besides quantitative increases or decreases. Phosphorylation or de-phosphorylation can alter the biological activity of proteins (Rubin and Rosen, 1975). The phosphorylation-de-phosphorylation systems at the cell surface may provide a mechanism for the modification of structural proteins, enzymes, or mitogen receptors with significant effects on the control of cell proliferation (Branton and Landry-Magnan,1978).

Because HSV-2 transforms cells at low frequency, as do all oncogenic DNA viruses, transformed cells have to be cloned and grown extensively. This extensive growth could lead to karyotype drift and clonal variation. Thus it is difficult to determine whether any phenotypic differences between normal and HSV-2 transformed cells are due to clonal variations or transformation. Whenever transformed cells are cloned there is the risk of selecting genetic variants which may show biochemical differences from each other and from the normal parent cell which are not due to transformation. A way around this is to examine a number of clones. It is hoped that any transformation-specific differences will be shown by all clones so

that the change can be demonstrated as transformation-dependent rather than a clonal variation. The present studies employed two clones of hamster embryo fibroblasts transformed by temperature-sensitive mutants of HSV-2 (Takahashi and Yamanishi, 1974) designated as ts4 and ts35. As well, two tumour-derived cell lines were utilized. The first tumour line, 333-8-9T, originated from a hamster tumour induced by a clone (333-8-9) of hamster embryo fibroblasts transformed by UV light-inactivated HSV-2 (Duff and Rapp, 1971). The second tumour line, ts35T, originated from a tumour excised and cultured in this laboratory produced by injecting ts35 into a hamster.

No infectious HSV-2 virions have been recovered from ts4 and ts35 as shown by plating these cells on HEL cells or by inoculation of cells, disrupted by sonication or freezing and thawing, onto HEL cells followed by incubation at the permissive temperature of 32°C (Takahashi and Yamanishi, 1974). The presence of HSV-2 genome in these lines was shown by the addition of rabbit antiserum HSV-2 to the transformed clones followed by the application of fluorescein-conjugated anti-rabbit goat serum. Distinct fluorescence was detected in the cytoplasm of a small proportion (5-10%) of the cells (Takahashi and Yamanishi, 1974). Injection of these cells into animals gave rise to tumours with ts35 being more tumourigenic than ts4

(Takahashi and Yamanishi, 1974).

There is only limited biological and biochemical data on tumour lines from HSV-2 transformed hamster embryo fibroblasts and none on the particular tumour lines examined in this report. Previous studies have shown that tumour lines from ts4, ts35 (Takahashi and Yamanishi, 1974) and 333-8-9 (Duff and Rapp, 1971) produce tumours in a much shorter time period. Five tumour lines from 333-8-9 contained HSV-2 antigens visible as a diffuse fluorescence throughout the cytoplasm when hamster antiserum to HSV-2 followed by anti-hamster gamma globulin conjugated to fluorescein isothiocyanate was used (Duff and Rapp, 1971). Tumour cells from a hamster injected with 333-8-9 cells contained less detectable HSV-2 DNA than that present in the parent line (Frenkel et al., 1976). No infectious HSV-2 virus was recovered from the parent 333-8-9 line (Duff and Rapp, 1971) and thus it is highly unlikely that the tumour line studied in this report contain infectious HSV-2 virions.

In the studies reported here a number of biological parameters of HSV-2 transformed hamster embryo fibroblasts were investigated. The growth properties of these cells were determined including saturation density and doubling time. Proteins on the cell surface were examined by cell surface iodination. Metabolic labeling with radioactive

amino acids and inorganic phosphate was used to study

the polypeptides in isolated, purified membranes. As well,

labeled whole cell patterns were also examined. All proteins

were analyzed using SDS-polyacrylamide slab gel electrophoresis

followed by autoradiography or fluorography. The oncogenic

potential of the HSV-2 transformed hamster lines was

assessed in vivo by injecting three concentrations of the

various cell lines into the backs of seven day-old and

seven week-old hamsters. These studies represent the

first experiments designed to examine the effects

of HSV-2 transformation on the cell surface, and to

correlate such with tumourigenicity.

MATERIALS AND METHODS

A. Tissue Culture

1. Hamster Embryo Fibroblasts (HaEF)

Primary, normal hamster fibroblasts were prepared using 13 to 14 day-old embryos from pregnant Golden Syrian hamsters (High Oak Ranch, Goodridge, Ontario). The animal was etherised until dead and then washed in a dettol solution. The embryos were removed, decapitated and minced with scissors. The tissue was washed twice with phosphate buffered saline (PBS) to reduce red blood cell contamination and then it was incubated in 0.25% trypsin for 20 minutes at room temperature. Cells were pelleted by centrifugation for 5 minutes at 1500 rpm in a 50ml plastic centrifuge tube (Falcon). The pellet was resuspended in α-MEM supplemented with 10% fetal calf serum, 100ug/ml streptomycin, 100 units/ml penicillin, 0.03% w/v glutamine, 0.75g/L $NaCO_2$ (all reagents from Gibco) and plated in 150cm² culture plastic flasks (Corning). The cells were grown overnight in a 37°C incubator with a 5% CO_2/95% air atmosphere. Medium was changed after 24 hours. After 3-4 days, these primary cultures were trypsinized and replated as secondary cultures which were used for all experiments.

2. Herpes simplex virus, subtype 2 (HSV-2) transformed lines

In these studies four hamster fibroblast lines transformed by HSV-2 were employed. Two of these lines, .ts4 and ts35, were clones derived from secondary hamster embryo fibroblasts infected with temperature sensitive mutants of HSV-2 (Takahashi and Yamanishi, 1974). A third line; 333-8-9T was derived from hamster tumours which formed after the injection of 333-8-9 cells. This cell line was originally cloned from secondary hamster embryo fibroblasts infected by UV light-inactivated HSV-2 (Duff and Rapp, 1971). The fourth line, ts35T, was established in this laboratory from a tumour excised from a hamster injected intradermally with the parent line, ts35. The transformed lines were cultured under the same conditions as the HaEF and they were passaged twice a week at a 1/20 dilution. The tumour-derived lines, ts35T and 333-8-9T, were used for experiments between the 5th and 20th passage while the two cloned lines, ts4 and ts35, were used between the 60th and 80th passage. The cell lines were screened for microplasm using standard micro-biology techniques and were found to be negative by these methods.

B. Photography of cells in culture

Cell morphology was assessed using a Leitz reverse-

objective, phase-contrast microscope with camera attachment
and photographed with Kodak Tri-X black and white film.
Cells were photographed just prior to confluence.

C. In vitro studies on growth kinetics

Comparisons of cell doubling times and saturation
densities of the various cell lines and HaEF were assessed
in culture. To 60mm Petri dishes (Corning) were added
1×10^5 viable cells in 5ml of α-MEM. The medium was left
unchanged during the course of the experiment. Duplicate
cell counts were taken twice daily by haemocytometer and
the total number of cells per Petri dish was calculated
and converted to number of cells per square centimeter
of Petri dish growth surface.

D. Tumourigenicity studies

To measure the tumourigenicity of the various cell
lines, three concentrations of cells, 10^6, 10^5, and 10^4,
in 1ml of PBS were injected intradermally into the backs
of 7 day and 7 week-old Golden Syrian hamsters. Five
animals were used for each concentration. The hamsters
were checked weekly for the appearance of tumours. Tumour
size was also measured periodically using calipers.
As a control a group of hamsters was injected with
secondary hamster embryo fibroblasts.

E. Studies on plasma membrane and whole cell proteins

1. Lactoperoxidase iodination

Cell surface proteins were examined using the
surface iodination technique as described by Wickus
et al., (1974), with some modifications. Cells were
plated in α-MEM containing 5% FCS at a concentration
of 4.2×10^5 viable cells per 100mm Petri dish (Corning).
On the third day while the cells were in logarithmic
phase growth, surface iodination was performed. Cells
grown on Petri dishes were washed twice with PBS and
then 4ml of PBS were added. To this solution the
following reagents were added: 20ul of 5×10^4 M NaI
as carrier, 70ul of lactoperoxidase (1.84ug/ml, 47.8
units/mg at 30°C, Calbiochem), 25ul of 0.5M glucose,
70ul of carrier-free sodium I^{125} (2mCi/ml in PBS, NEN),
and 20ul of a 1/100 diluted glucose oxidase stock solution
(3.8mg/ml, 268 units/mg, Miles Laboratories). The reaction
was allowed to proceed for 15 minutes at room temperature
and it was terminated by two washes of PBS and then
0.5ml of hot dissolving buffer (see below) was added.
The contents of the dish were then scraped into a capped,
plastic test tube by means of a rubber policeman.
The sample was immediately placed in a boiling water

bath for 2 minutes then either used immediately for
SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE)
or frozen and stored at -20°C.

2. ^{35}S-methionine labeled whole cells and plasma membranes

Cells were metabolically labeled with ^{35}S-methionine
in order to examine the whole cell and plasma membrane
protein patterns. Cells were plated at 1x10^5 cells per
60mm Petri dishes. On the third day the medium was changed
to methionine-minus medium with the usual supplements
plus 10% (v/v) regular α-MEM containing 5% FCS. To
each plate containing 1ml of medium was added 50uCi
of ^{35}S-methionine (618.75Ci/mmole, NEN). The cells
were labeled overnight then washed twice with PBS.
Hot dissolving buffer (see below) was added and the contents
of the Petri dish was scraped into capped plastic test-
tubes and placed in a boiling water bath for 2 minutes.
The samples were either used immediately for SDS-PAGE
or frozen and stored at -20°C.

For ^{35}S-methionine labeled plasma membranes,
8x10^5 viable cells were plated in 150cm^2 plastic tissue
culture flasks and they were grown for 2-3 days. At
a point prior to confluence methionine-free medium was
added as described above except that 100uCi of ^{35}S-meth-
ionine was present (10uCi/ml). The cells were labeled
overnight.

3. ^{32}P inorganic phosphate labeled membranes

Cells were metabolically labeled with ^{32}P in-
organic phosphate to examine the protein phosphorylation
pattern in plasma membranes. Cells to be labeled were
prepared as described above for ^{35}S-methionine labeling
except that to each 150cm^2 tissue flask containing
phosphate-free medium was added 500uCi of carrier-free
^{32}P inorganic phosphate (NEN) and incubated for 2 hours.

F.   Membrane isolation

Plasma membranes were prepared by the method
of Carlsen and Till (1975). Cells were washed twice
with PBS and then 15ml of ice-cold swelling solution
was added (4 parts 0,001M ZnCl$_2$ to 1 part DMSO) and
they were left in the cold for 45 minutes. This swelling
solution was poured off and 15ml of freshly prepared
ice-cold saturated solution of fluorescein mercuric
acetate in 0.02M tris (pH 8.1) was added. The flasks
were then placed on a horizontal shaking platform set
at 120 cycles per minute. When large sheets of membranes
were seen floating (20-40 minutes), the shaking was
stopped and the supernatant was poured into 30ml glass
centrifuge tubes (Corning). These were spun at 8000
rpm in a Sorvall centrifuge using the Sorvall HB$_4$ rotor.
Plasma membranes in the pellet were further purified

using the aqueous two-phase system of Brunette and

Till (1971), as modified by Gaffney et al., (1974).

The pellet was resuspended in 10ml of ice-cold top phase

then 10ml of bottom phase was added. The two phases

were thoroughly mixed then centrifuged at 3000 rpm in

the HB Sorvall rotor for 10 minutes. The purified

membrane, found at the interphase, was pipetted off into

a 30ml glass centrifuge tube containing 20ml of ice-

cold distilled water. It was centrifuged for 15 minutes

at 8000 rpm in the Sorvall HB rotor. The pellet was

routinely examined for the presence of nuclei and whole

cell contaminants. If these were present the above

purification procedure was repeated until these

contaminants were removed. The final purified membrane

pellet was dissolved in hot dissolving buffer (see below)

and placed in a boiling water bath for 2 minutes.

The sample was either used immediately for SDS-PAGE

or frozen and stored at $-20^{\circ}C$.

G. Sodium dodecyl sulphate-polyacrylamide slab gel
    electrophoresis (SDS-PAGE)

Labeled proteins were analyzed by SDS-polyacrylamide

slab gel electrophoresis using a system based on that of

Laemmli (1970). All separating gels were gradients

of polyacrylamide from 7.5% to 15% in 1% glycerol

sandwiched between glass plates measuring 15cm x 24cm.

The stacking gel consisted of 5% polyacrylamide. All

polyacrylamide gels contained 0.8% bis as cross-linker
unless otherwise stated. The stock electrode buffer
was composed of 0.2M glycine dissolved in a 0.025M
tris buffer with a final concentration of 0.1% SDS.
The samples were dissolved in a buffer consisting of
0.05M tris (pH 6.8) containing 1% SDS, 1% 2-mercapto-
ethanol and 10% glycerol. Acid-precipitable radioactivity
was determined by precipitation with 10% trichloracetic
acid and filtration using glass fibre filters. These
were dried and then counted in a Bechman liquid scintillation
counter. Samples containing equal amounts of radio-
activity were subjected to electrophoresis at a constant
current of 35mA. Gels were stained with Coomassie
Blue, destained with isopropanol-acetic acid and then
dried under vacuum. The labeled protein pattern was
visualized by autoradiography using Kodak RP X-Omat
film which was pre-sensitized with red light (cf. Laskey
and Mills, 1975). In some cases the gels were impregnated
with PPO following the method of Bonner and Laskey
(1974). Quantitative data was obtained by scanning the
autoradiograms with a MKIII CS Joyce-Loebl double beam
recording densitometer. Peaks traced on the graph paper
corresponding to protein bands of interest were cut out
and weighed. Nominal molecular weights of protein bands
were determined by comparing the migration distance with
that of known standards such as gamma-globulin (160,000

daltons), albumin (67,000 daltons), ovalbumin (45,000 daltons),myoglobin (17,200 daltons), and cytochrome c (12,700 daltons).

RESULTS

A. Morphology

The morphological characteristics of the various cell lines used in the present studies were examined by light microscopy. Both the HaEF (fig. 1) and the ts4 line (fig. 2) were similar in size. They also exhibited long, thin, pointed processes typical of fibroblasts. The ts35 line (fig. 3 and 4) was comprised of two populations of cells that could be discernible at the light microscope level. The more numerous cells were larger than ts4 and HaEF, and tended to be flatter. The other population of cells in the ts35 line were ten times or more the size of the smaller cells. These giant cells, present at less than 10% of the total cell population, were often multinucleate with many long, thin, cellular processes. The smaller cells tended to form a roughly circular border around these larger cells. The 333-8-9T line (fig. 6) has a morphology somewhat intermediate to that of ts4 and the smaller cells of the ts35 line. The ts35T line (fig. 5) bore a greater resemblance to the smaller cells of the parent ts35 line and thus the giant cells found in ts35 appeared to be lost by passage through the hamster. Both the ts35T 333-8-9T lines had a high percentage of very refractile,

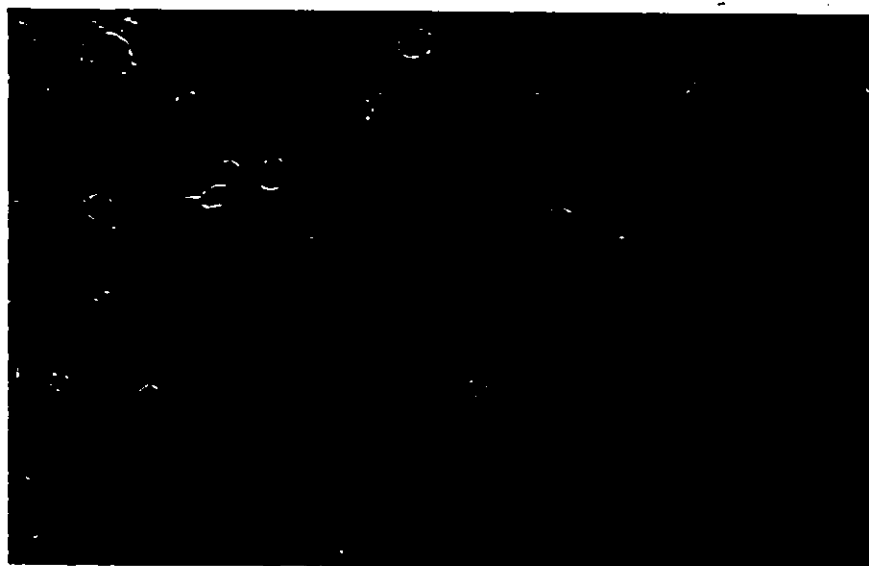FIGURE 1.   HaEF   X100

FIGURE 2.   ts4 X100
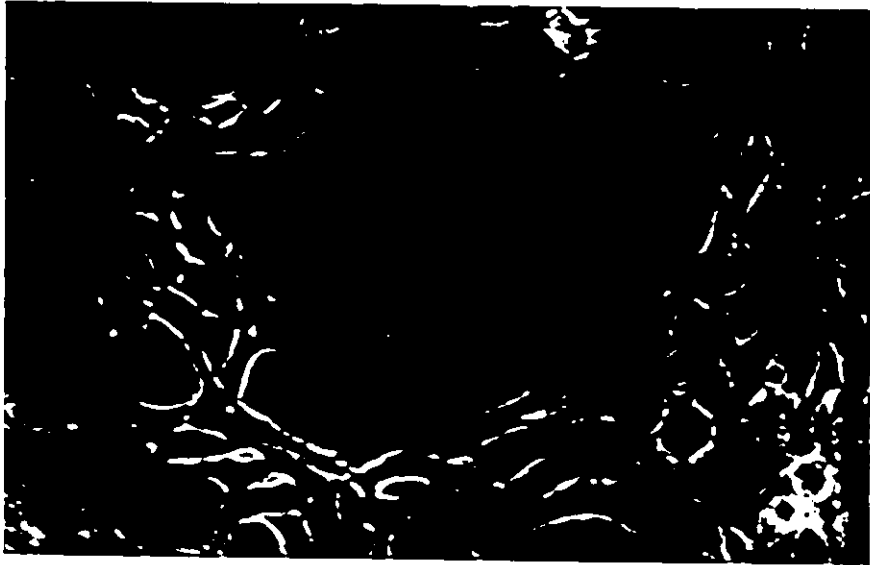
FIGURE 3. ts35 X100

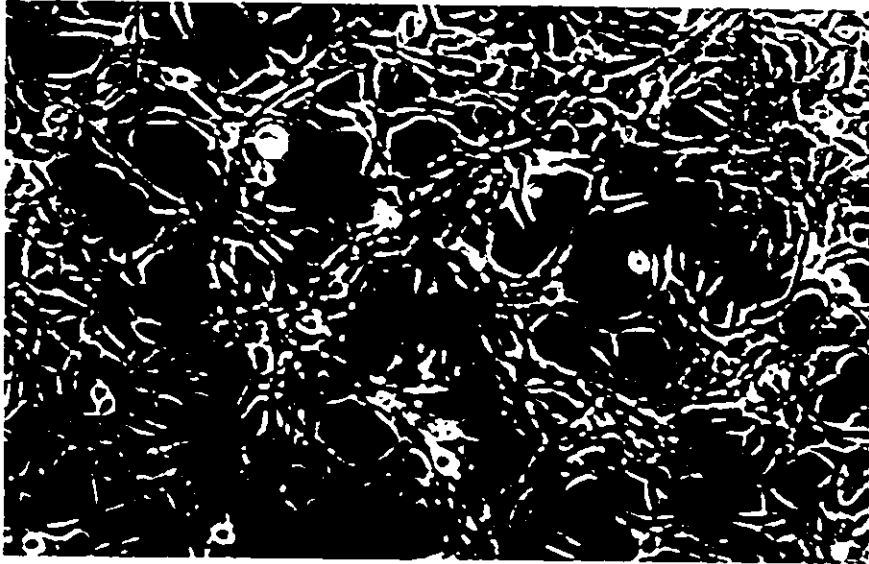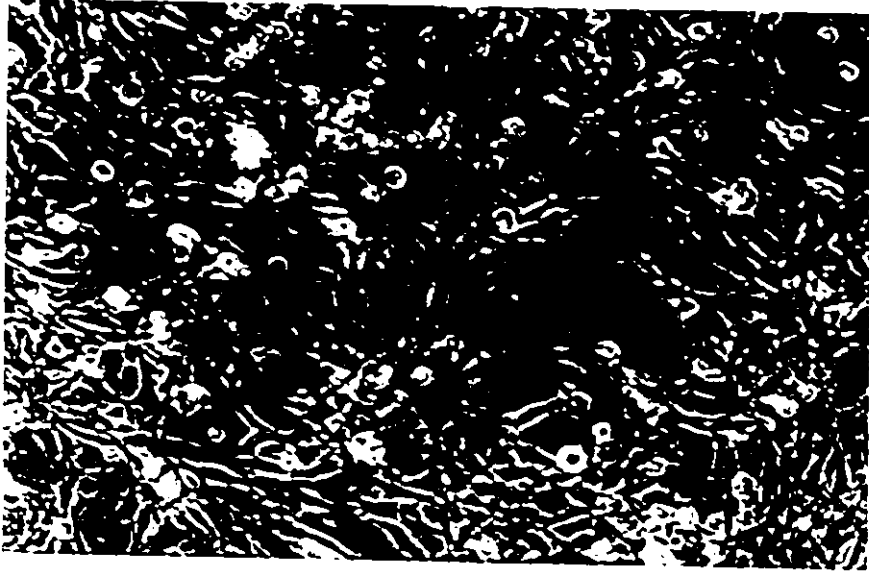FIGURE 4. ts35T X150

FIGURE 5. ts35T X100

FIGURE 6.   333-8-9T   X100

rounded cells.

B. Tumourigenicity studies

The oncogenic potential of the in vitro trans-
formed lines was assessed in vivo. Cells were injected
intradermally into the backs of seven day and seven
week old (weanlings) Golden Syrian hamsters at a concentration
of 10^6, 10^5, or 10^4 cells. As shown in table 1, all the
transformed lines produced tumours, however the time
required for the appearance of these tumours differed
dramatically among the cell lines tested.

Weanlings that were injected with the 333-8-9T
line formed tumours rapidly at all doses of cells. In
2 weeks there were tumours in all five animals at the
highest dosage of 10^6 cells. It was not until the 3rd
and 5th week that tumours arose in 100% of the weanlings
injected with 10^5 and 10^4 cells of the 333-8-9T line,
respectively.

The ts35 line produced tumours in 100% of the
weanlings in 4 and 6 weeks after injecting 10^6 and 10^5
cells respectively. Only 60% of the animals had tumours
using 10^4 cells at the end of 8 weeks.

The ts4 line produced tumours in 80% of the
weanlings injected with 10^6 cells at24 weeks. Tumours
appeared in 60% and 80% of the animals at 26 and 40 weeks
after injecting 10^5 and 10^4 cells respectively. Also

TABLE 1

TUMOURIGENICITY OF HSV-2 TRANSFORMED HAMSTER FIBROBLASTS

A. WEANLINGS[1]

Tumour Formation (weeks) with input of:	HaEF	ts4	ts35	ts35T	333-8-9T
10^6 cells	>40	24	4	nd	<2
10^5 cells	>40	26	6	nd	3
10^4 cells	>40	40	8	nd	5

B. NEWBORN[2]

10^6 cells	>5	>5	4	nd	<2
10^5 cells	>5	>5	5	nd	<4
10^4 cells	—	—	—	nd	—

1. Hamsters were inoculated with various numbers of cells and the data presented represents the time at which at least 50% of the animals (i.e. 3 out of 5) developed tumours.

2. Hamsters were inoculated with various numbers of cells and the data presented represents the time at which at least 50% of the animals (i.e. 3 out of 5) developed tumours. This experiment was terminated between the 4th and 5th week.

shown in table 1 is the newborn tumourigenicity results
which are similar to the above data with weanlings.

No tumours resulted in animals injected with the
control secondary hamster embryo fibroblasts at any cell
concentration.

As shown in fig. 7, once the tumour became
established, growth was rapid in weanlings injected with
the 333-8-9T and ts35 lines. Tumours resulting from the
injection of 333-8-9T cells grew faster than those from
ts35 line. No quantitative measurements were taken to
assess tumour growth rate using ts4 cells but growth
also appeared to be quite fast, but not as rapid as with
333-8-9T and ts35. All tumours were hard and solid.
The 333-8-9T line was the only cell line which produced
necrotic tumours. The necrosis started very early in
tumour growth. Most animals had 1 large tumour at the
site of injection. A few animals had multiple tumours
but these were clustered close to the site of injection.

C. Doubling time and saturation density of cell lines
   in culture

The first quantitative parameters investigated
concerning the growth properties of HSV-2 lines were
cell doubling time and saturation density of the various
HSV-2 transformed lines in culture. The doubling time
was the time required for a population of cells to increase
their numbers by a factor of two. The saturation density

TUMOUR SIZE (cm)

TIME AFTER INJECTION (weeks)
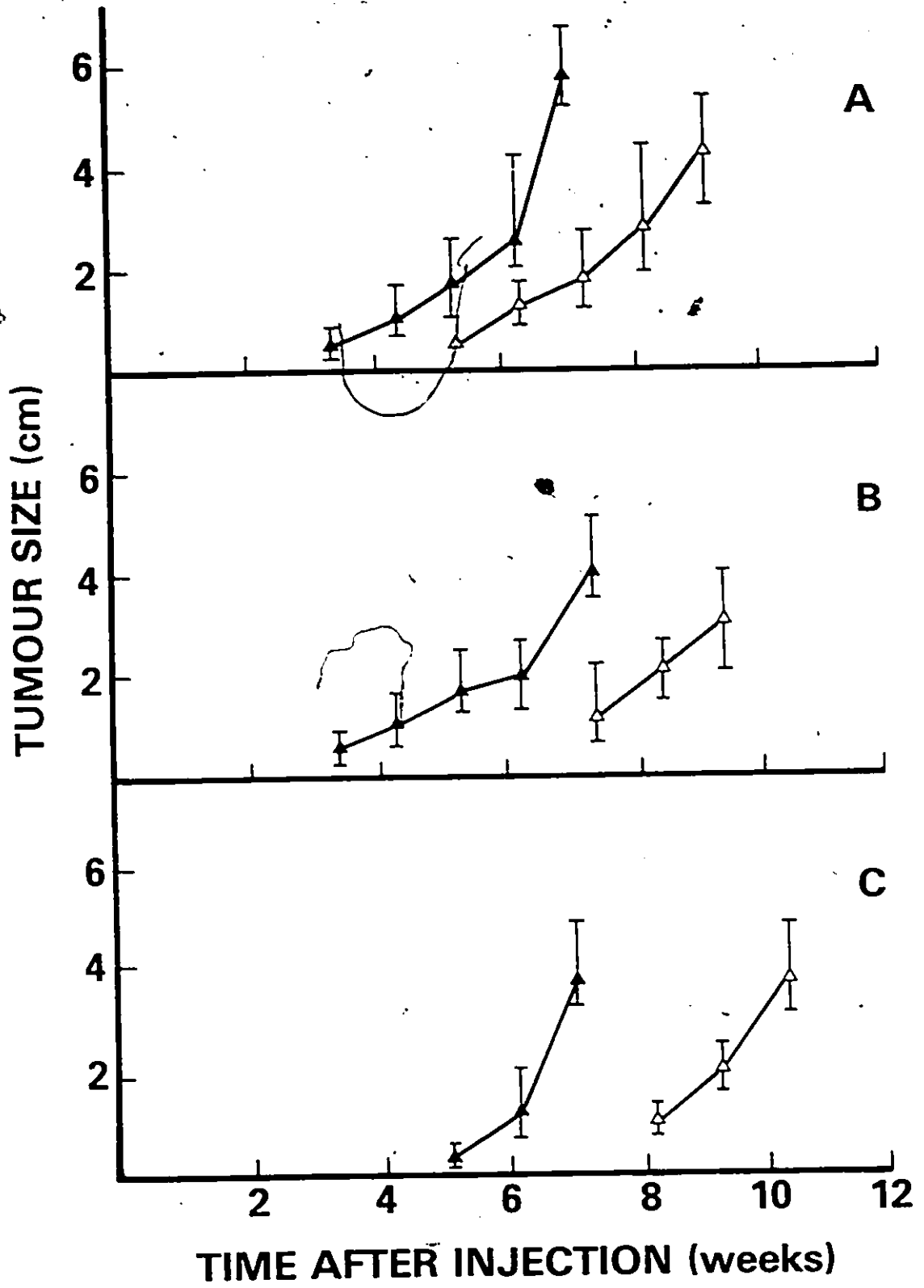
was that density at which cells stopped growing and

entered quiescence or G_0. Previous studies with a

variety of transformed cells showed transformed cultures

typically grew more rapidly and to higher saturation

densities than their normal cell counterparts (cf. Tooze,

1973). Saturation density had often been related to

tumourigenicity that is, the higher the saturation density

the more tumourigenic the cell (Aaronson ahd Todaro, 1968).

This higher cell density of transformed lines was believed

to be a reflection both of a loss of contact inhibition

and an increase in the efficiency with which serum was

used by the cell (Tooze, 1973).

The HSV-2 transformed lines and normal HaEF were grown

at three concentrations of fetal calf serum: 10% (fig. 8),

5% (fig. 9), and 1% serum (fig. 10). At all serum

concentrations, the transformed lines either had equal to

or decreased doubling·times compared to normal HaEF (Table 2)

At 10% serum the doubling time for 333-8-9T was 8 hours

while that for ts35, ts4, and HaEF was approximately 12

hours. At 5% serum the doubling time for 333-8-9T was

16 hours, for ts35T 18 hours, ts35 16 hours, and HaEF

50 hours. At 1% serum 333-8-9T, ts35T and ts35 took

approximately 22 hours to double in cell number while for

ts4 and HaEF the time required was 50 and 75 hours,

respectively.

Saturation densities for the various cell lines

FIGURE 8

The various cell lines were plated at 1.5×10^5
cells per 60mm Petri dish in α-MEM supplemented with 10%
fetal calf serum. Duplicate cell counts were taken twice
daily by means of a haemacytometer and converted to
number of cells per cm^2. The medium was left unchanged
during the course of the experiment.

HaEF   —()—O:

ts4   —o—θ·

ts35   —Δ~Δ—

ts35T   nd

333-8-9T   —Δ~Δ—

FIGURE 9

The various cell lines were plated at 1.5×10^5
cells per 60mm Petri dish in α-MEM supplemented with
5% fetal calf serum. Duplicate cell counts were taken
twice daily by means of a haemacytometer and converted
to number of cells per cm^2. The medium was left unchanged
during the course of the experiment.

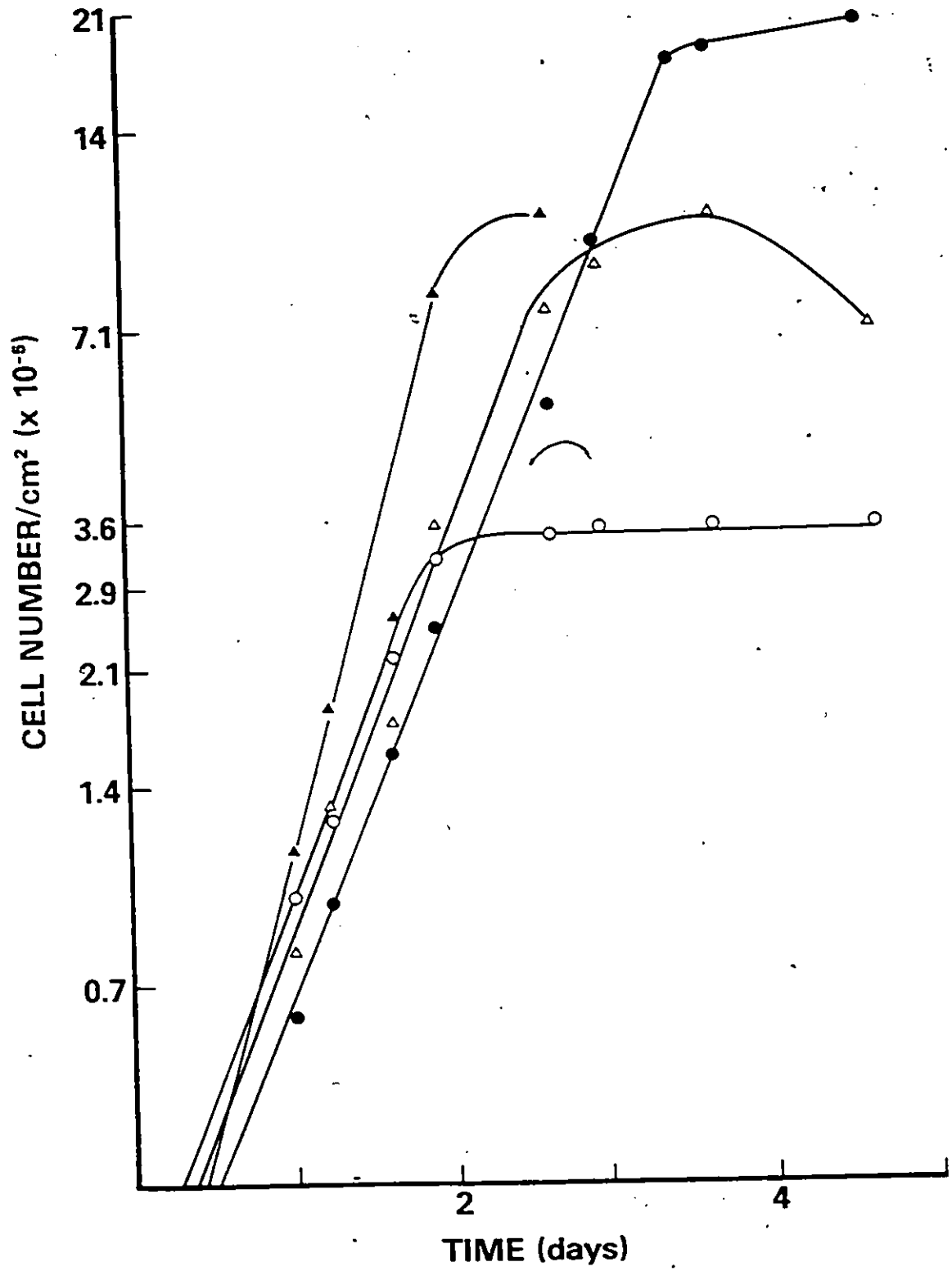HaEF —o—o—

ts4 —●—●—

ts35 —△—△—

ts35T —▽—▽—

333-8-9T —▲—▲—

FIGURE 10

The various cell lines were plated at 1.5×10^5 cells per 60mm Petri dish in α-MEM supplemented with 1% fetal calf serum. Duplicate cell counts were taken twice daily by means of a haemacytometer and converted to number of cells per cm^2. The medium was left unchanged during the course of the experiment.

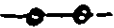HaEF —○—○—

ts4 —●—●—

ts35 —◁—△—

ts35T —□—◁—
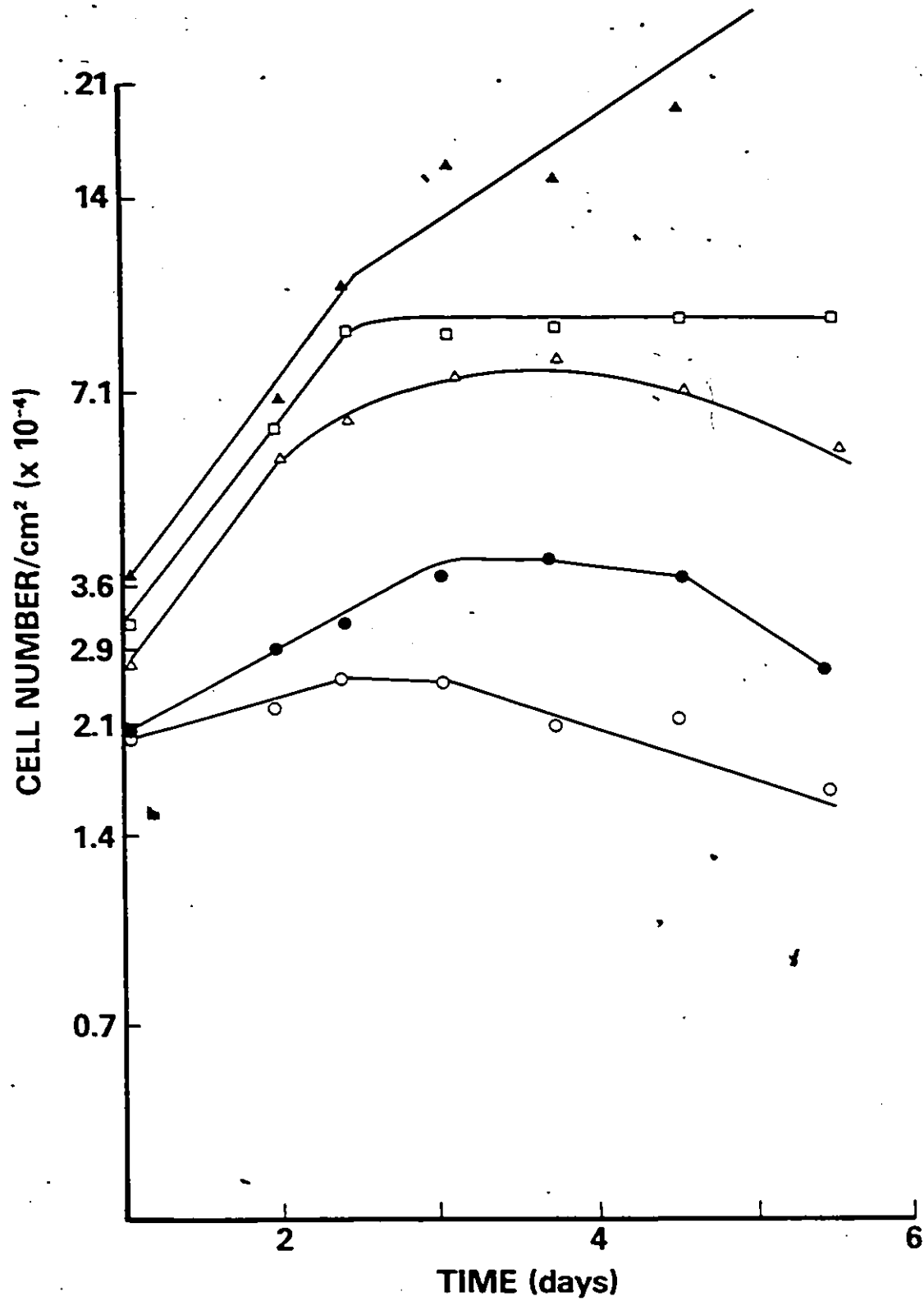
333-8-9T —▲—▲—

TABLE 2

CELL DOUBLING TIME OF HSV-2 TRANSFORMED HaEF (hours)

CELL LINE	SERUM CONCENTRATION		
	10%	5%	1%
HaEF	12	50	>75
ts4	12	14	50
ts35	12	16	22
ts35T	nd	18	22
333-8-9T	8	16	22

The various cell lines were plated at 1.5×10^5
cells per 60mm Petri dish in α-MEM supplemented with
three different concentrations of fetal calf serum.

Duplicate cell counts were taken twice daily by means of a
haemacytometer and converted to number of cells per cm^2.
Doubling time for the various cell lines was taken
as the slope of the curve from figures 8, 9, and 10.

TABLE 3

SATURATION DENSITIES OF HSV-2 TRANSFORMED HaEF (cells per cm^2)

CELL LINE	SERUM CONCENTRATION		
	10%	5%	1%
HaEF	$3.6x10^5$	$2.1x10^4$	$2.5x10^4$
ts4	$2.1x10^6$	$1.1x10^5$	$3.9x10^5$
ts35	$1.1x10^6$	$4.6x10^4$	$7.5x10^5$
ts35T	nd	$*4.3x10^4$	$9.3x10^5$
333-8-9T	$*1.1x10^6$	$*7.1x10^4$	$3.6x10^6$

The various cell lines were plated at $1.5x10^5$ cells per 60mm Petri dish in α-MEM supplemented with three different concentrations of fetal calf serum. Duplicate cell counts were taken twice daily by means of a haemeacytometer and converted to number of cells per cm^2. The saturation densities for the various cell lines was determined from figures 8, 9, and 10.

*Adequate values could not be obtained as the cells detached from the plates.

were also determined from cell growth curves (fig.'s 8-10).
Using 5% and 10% serum, suitable values could not be
obtained as the ts35T and 333-8-9T cells detached from
the plate. Such detachment occurred after 2 days in culture
at 10% serum and after 3 days in culture at 5% serum.
The majority of cells floating in the supernatent were
dead as indicated by trypan blue exclusion. The 333-8-9T
line was the most susceptible to this problem at high serum
concentration. All cell lines remained attached to the
plates at 1% serum however , and thus this concentration
was used to measure saturation densities. As shown in
Table 3, the saturation densities at 1% serum for 333-8-9T
were greater than 3.6×10^5 cells/cm^2, ts35T 9.3×10^4 cells/cm^2,
ts35 7.5×10^4 cells/cm^2, ts4 3.9×10^4 cells/cm^2, and HaEF
2.5×10^4 cells/cm^2. Thus the order of decreasing saturation
density was 333-8-9T, ts35T, ts35, ts4, and HaEF. · Comparing
these data with those presented in table 1, it was found
that saturation density correlated directly with tumouri-
genicity for the various cell lines tested.

C. Analysis of cell surface proteins by surface iodination

Cell surface proteins may be directly involved
in growth control and phenotypic alterations associated
with transformation. Thus experiments were carried out
to compare the cell surface proteins of secondary hamster
embryo fibroblasts and the various HSV-2 transformed hamster
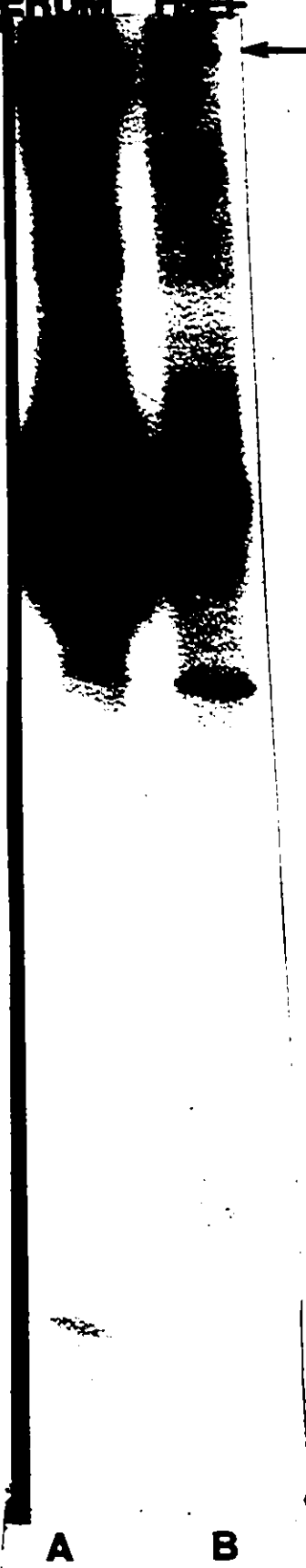
FIGURE 11

An autoradiogram of a 7.5% to 15% SDS-poly-
acrylamide slab gel electropherogram of fetal calf
serum (A) and HaEF (B) that were iodinated. Primary
HaEF were replated in α-MEM containing 5% fetal calf serum
at 4.2×10^5 cells per 100mm Petri dish. On the third
day the cells were iodinated and analyzed as in
Material and Methods.

48.

SERUM H.EF

← LETS

A   B

lines. The technique of cell surface iodination was employed for this study. It has been shown that this technique labeled only those proteins at the external surface of the cells (cf. Hynes, 1973; Phillips and Morrison, 1971; Hubbard and Cohn, 1972).

Cells were iodinated using ^{125}I and analyzed on SDS-polyacrylamide slabs gels. The autoradiograms from these gels revealed a number of labeled species (fig. 10-a). Some of these labeled proteins could be serum-derived since this was not a metabolic label. A comparison of ^{125}I labeled serum (fig. 11-a) and normal HaEF (fig. 11-b) revealed that many of the labeled proteins present on the cell surface were serum derived. However, several other proteins were labeled which did not correspond to serum proteins and these were presumably cell-derived constituents. One such protein was the major iodinated species having a nominal molecular weight of 220,000 daltons. This protein was also found to be trypsin-sensitive, that is, at trypsin concentrations that would not detach cells from the plates would completely remove this protein from the surface of the cell (data not shown). It is also a major cell glycoprotein as shown when cells are labeled with ^{3}H-glucosamine (data not shown). These characteristics strongly suggest that this polypeptide is probably the LETS protein. Comparison of the cell surface iodination patterns of normal and HSV-2 transformed hamster

FIGURE 12

An autoradiogram of a 7.5% to 15% gradient
SDS-polyacrylamide slab gel electropherogram of HaEF (A),
ts4 (B), ts35 (C), ts35T (D), and 333-8-9T (E), that
were iodinated.

Primary HaEF and the various cell lines were
replated at 4.2×10^5 cells per 100mm Petri dish. On the
third day the cells were iodinated and analyzed as
in Materials and Methods.

HEF ts4 ts35 35T 333-8-9T

← LETS
←180K
←150K

←S1
←S2

A   B   C   D   E

cells (fig. 12) revealed that this large protein was present in lower amounts in the HSV-2 transformed cells lines (fig. 12-b, c, d, e) compared to the HaEF (fig. 12-a). Quantitative data (table 4) from densitometer scanning of autoradiograms showed ts35T had 14.5%, 333-8-9T 18%, ts35 29.3%, and ts4 49.4%, of the HaEF value. These values were an average of four different experiments using autoradiograms from gels with iodinated cell samples containing equal radioactivity. Similar results were observed with iodinated cell samples containing equal quantities of cell protein (data not shown). Although the absolute values varied as much as 30%, the relative amounts of LETS for each HSV-2 transformed hamster cell line was the same in each experiment. Comparing these data with those presented in table 1, it was found that the relative amounts of this protein from the HSV-2 transformed hamster cell lines correlated inversely with tumourigenicity.

Two other proteins which appeared to be cell-derived with molecular weights of 180,000 and 150,000 daltons (fig. 12-a, b, c) were present in HaEF , ts4, and ts35, but not in the tumour-derived cell lines, ts35T and 333-8-9T. As well, the proteins labeled S_1 and S_2 were determined to be serum-derived as shown in fig. 11.

F. Studies on whole cells and membrane proteins labeled with ^{35}S-methionine.

The studies described above indicated that the

amount of one cell surface protein, LETS, was altered by
HSV-2 transformation. To determine if other cell surface
or whole cell proteins were modified by HSV-2 transformation,
studies were carried out using ^{35}S-methionine labeled
preparations and analyzed on SDS-polyacrylamide slab gels.

As shown in fig. 13 only a few protein changes
were observed in whole cell protein patterns. A
polypeptide with a molecular weight of 225,000 daltons
was present in the HaEF but absent in all transformed
lines. Another (perhaps a doublet) of approximately
47,000 daltons was reduced in the transformed lines compared
to the HaEF. Thus HSV-2 transformation of hamster embryo
fibroblasts results in only a few alterations in proteins
detectable at the whole cell level.

With the technique of surface iodination only those
few proteins which were on the outer surface of the cell
and accessible to the iodinating enzyme were labeled.
In order to analyze the other cell surface proteins in
in normal and HSV-2 transformed hamster cells, cultures
were labeled with ^{35}S-methionine and purified plasma
membranes were isolated and analyzed on SDS-polyacrylamide
slab gels. The autoradiogram of fig. 14 revealed at least
8 major differences among the various cells examined.
A 25,000 dalton protein was present in the HaEF but greatly
reduced in all transformed lines. A 29,000 dalton protein

TABLE 4

Quantitation by densitometer scanning of cell surface LETS and membrane bound actin.

cell line	LETS	ACTIN
HaEF	100%	100%
ts4	49.4%	119%
ts35	29.3%	115%
ts35T	14.5%	88.8%
333-8-9T	18%	88.1%

Quantitative data was obtained by scanning the autoradiograms with a MK111 CS Joyce-Loebl double beam recording densitometer. Peaks traced on the graph paper corresponding protein bands of interest were cut out and weighed. The values were taken as a per cent of the HaEF value.

FIGURE 13

An autoradiogram of a 7.5% to 15% SDS-poly-
-acrylamide slab gel with 0.4% bis as cross-linker
showing HaEF (A), ts4 (B), ts35 (C), ts35T (D), and
333-8-9T (E), labeled with ^{35}S-methionine.

Primary HaEF and the various cell lines were replated
in α-MEM containing 5% FCS at 1.5×10^5 cells per 60mm
Petri dish. On the third day the medium was changed
to methionine-minus medium with the usual supplements
plus 10% (v/v) regular α-MEM containing 5% FCS.
To each plate containing 1ml of medium was added
50uCi of ^{35}S-methionine (618.75Ci/mmole). The cells
were labeled overnight.

536
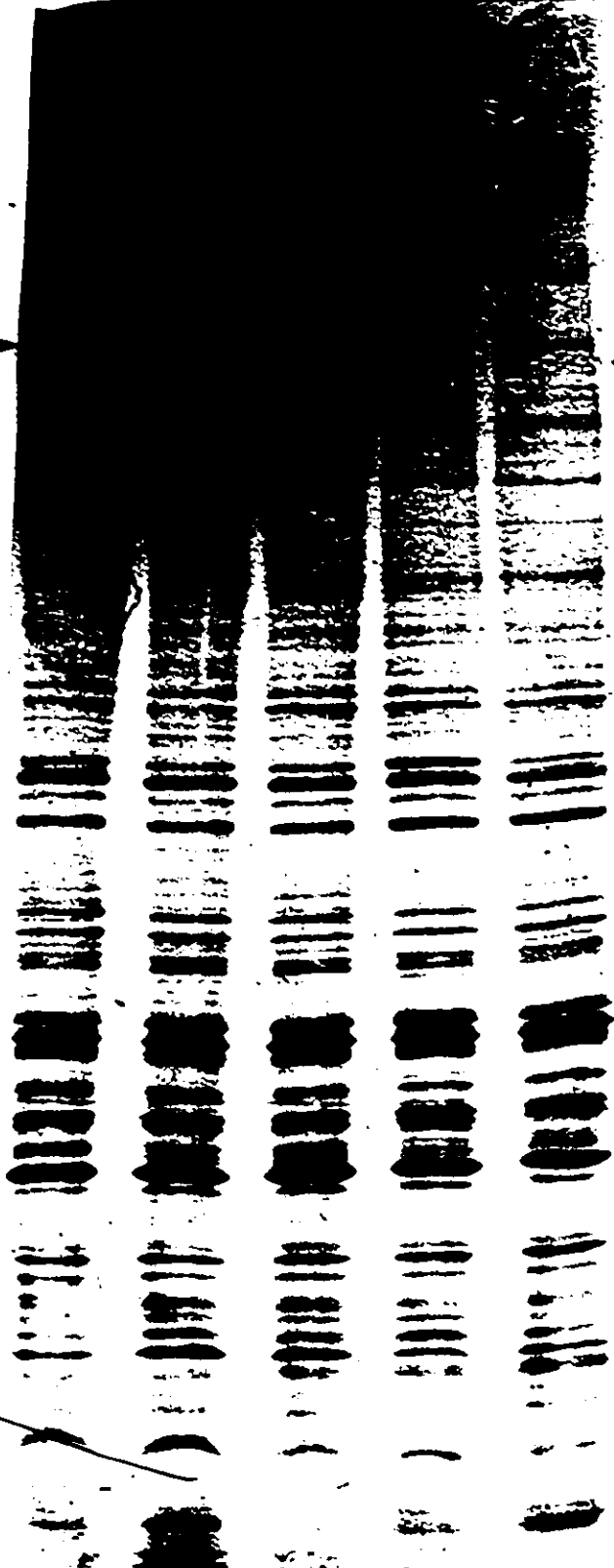
FIGURE 14

An autoradiogram of a 7.5% to 15% gradient
SDS-polyacrylamide slab gel showing purified membranes
of HaEF (A), ts4 (B), ts35 (C), ts35T (D), and 333-8-9T
(E), labeled with ^{35}S-methionine.

Primary HaEF and the various cell lines were
replated in α-MEM containing 5% FCS at 8×10^5 cells
per 150cm^2 tissue culture flasks. At a point prior
to confluence methionine-free medium was added containing
100uCi of ^{35}S-methionine in 10ml of medium. The cells
were labeled overnight.

FIGURE 14A (inset)

Samples from the above experiment were re-run under
the same conditions as outlines above. The inset is a portion
of the autoradiogram showing 45K actin present in all cell
lines but the 47K polypeptide present only in the HaEF.

HaEF   ts4   ts35   35T 333—8—9T

220K→

145K→

110K

63K

47K→

49K

43K→

45K
(actin)

34K

32K
31K

29K→

25K→

A   B   C   D   E
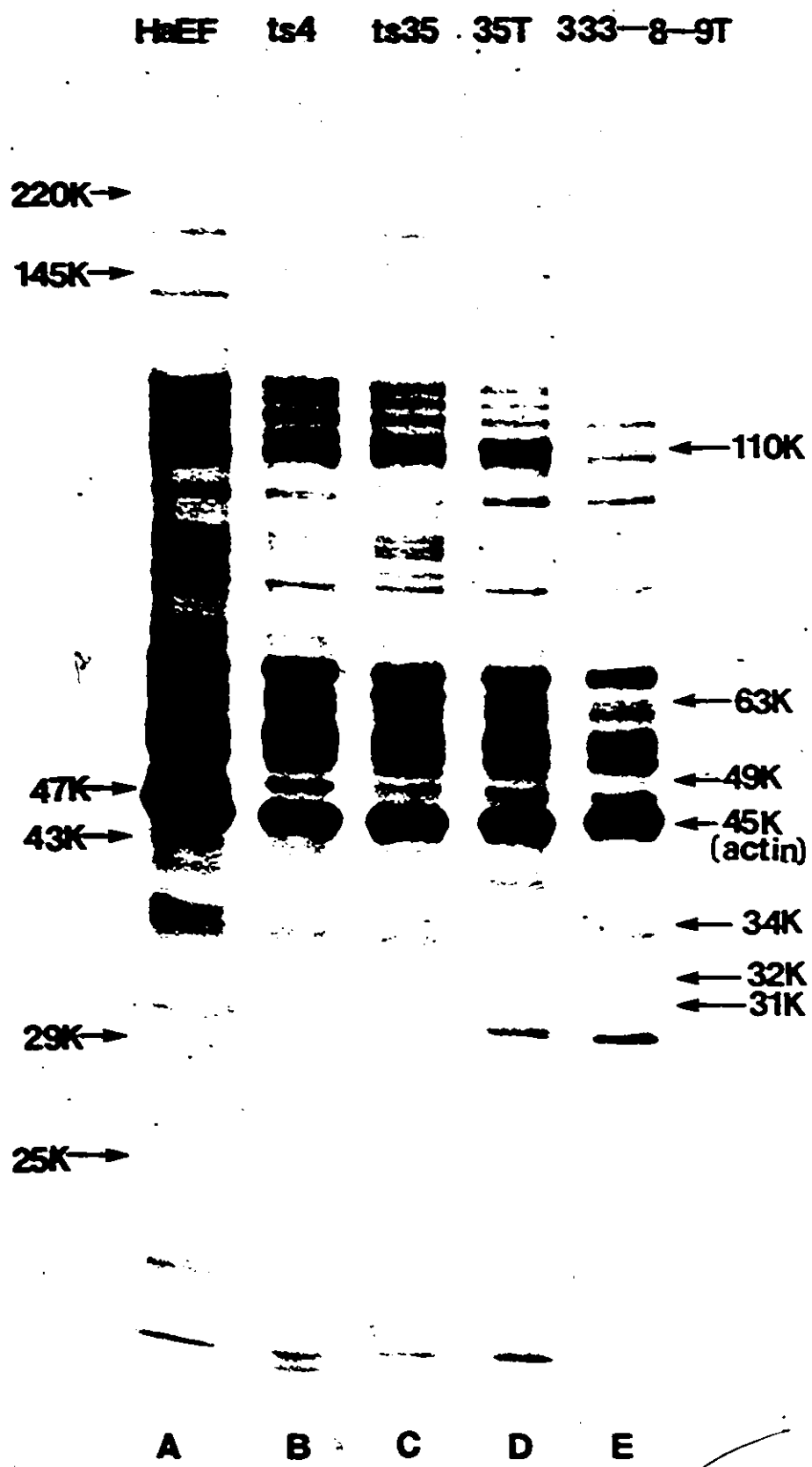
was reduced in all lines compared with the HaEF. There
were two proteins with molecular weights of 31,000 and
32,000 daltons, which were present in 333-8-9T and ts35T
but greatly reduced or absent in ts35, ts4, and HaEF.
A species of molecular weight 43,000 daltons was present
in higher amounts in HaEF, reduced in ts4, further reduced
in ts35, and absent in ts35T and 333-8-9T. There was no
significant difference in the amount of the actin protein
(45,000 daltons) from HaEF compared with the transformed
lines (table 4.). In the HaEF there were two closely
resolved proteins with a molecular weight of 47,000 daltons.
The larger of these two polypeptides was absent from the
transformed lines. Samples from this experiment were
analyzed a second time on SDS-polyacrylamide gels in an
effort to improve the resolution of the 47,000 dalton
polypeptide. Shown in fig. 14A is a section from an
autoradiogram of such a gel which showed an improved
resolution of the 47,000 dalton species. This revealed
with greater clarity that this 47,000 dalton protein was
present only in the HaEF. A protein of 49,000 daltons
present only in ts4, ts35, and ts35T as well as increases
of the following proteins; a 34,000 and 63,000 dalton
species found in ts35, a 110,000 dalton protein present
in ts35T, and a 145,000 dalton polypeptide observed in
HaEF and ts35, all represent clonal variations. A large

protein of 220,000 daltons was present in higher amounts
in HaEF, reduced in ts4 and ts35, and absent in ts35T
and 333-8-9T. Thus isolated plasma membrane from hamster
embryo fibroblasts infected with HSV-2 revealed many
differences compared with normal HaEF.

G. Phosphorylation studies on plasma membranes of HaEF
and HSV-2 transformed lines.

Changes in cell surface or intracellular phospho-
proteins could also play a role in HSV-2 transformation.
As a preliminary study, cells were therefore labeled with
^{32}P inorganic phosphate and analyzed on SDS-polyacrylamide
slab gels. The autoradiogram of fig. 15 revealed 6 major
differences among the cells examined. Proteins of 60,000
and 39,000 daltons were absent in ts35T and 333-8-9T but
present in ts35, ts4, and HaEF. There was a 19,000
dalton species which was phosphorylated more in the HaEF
than in the HSV-2 transformed lines whereas a 32,000
dalton polypeptide was phoshorylated more in the HSV-2
transformed lines than in the HaEF. As was the case for
plasma membranes labeled with ^{35}S-methionine, numerous
clonal variations could be detected among the various
cell lines labeled with ^{32}P inorganic phosphate. Thus
there does appear to be a difference in the phosphoprotein
pattern between normal HaEF and HSV-2 transformed hamster
fibroblasts at the membrane level.

FIGURE 15

An autoradiogram of a 7.5% to 15% gradient
SDS-polyacrylamide slab gel showing purified
membranes of HaEF (A), ts4 (B), ts35 (C), ts35T (D),
and 333-8-9T (E), labeled with inorganic ^{32}P.

Primary HaEF and the various cell lines were
replated in α-MEM containing 5% FCS at 8×10^5 cells
per 150cm^2 tissue culture flasks. At a point prior
to confluence phosphate-free medium was added containing
500uCi of carrier-free ^{32}P inorganic phosphate in 10ml
of medium and incubated for 2 hours.

HaEF   ts4   ts35   35T   333 8 9T

60K→

39K→

←32K

19K →

A   B   C   D   E
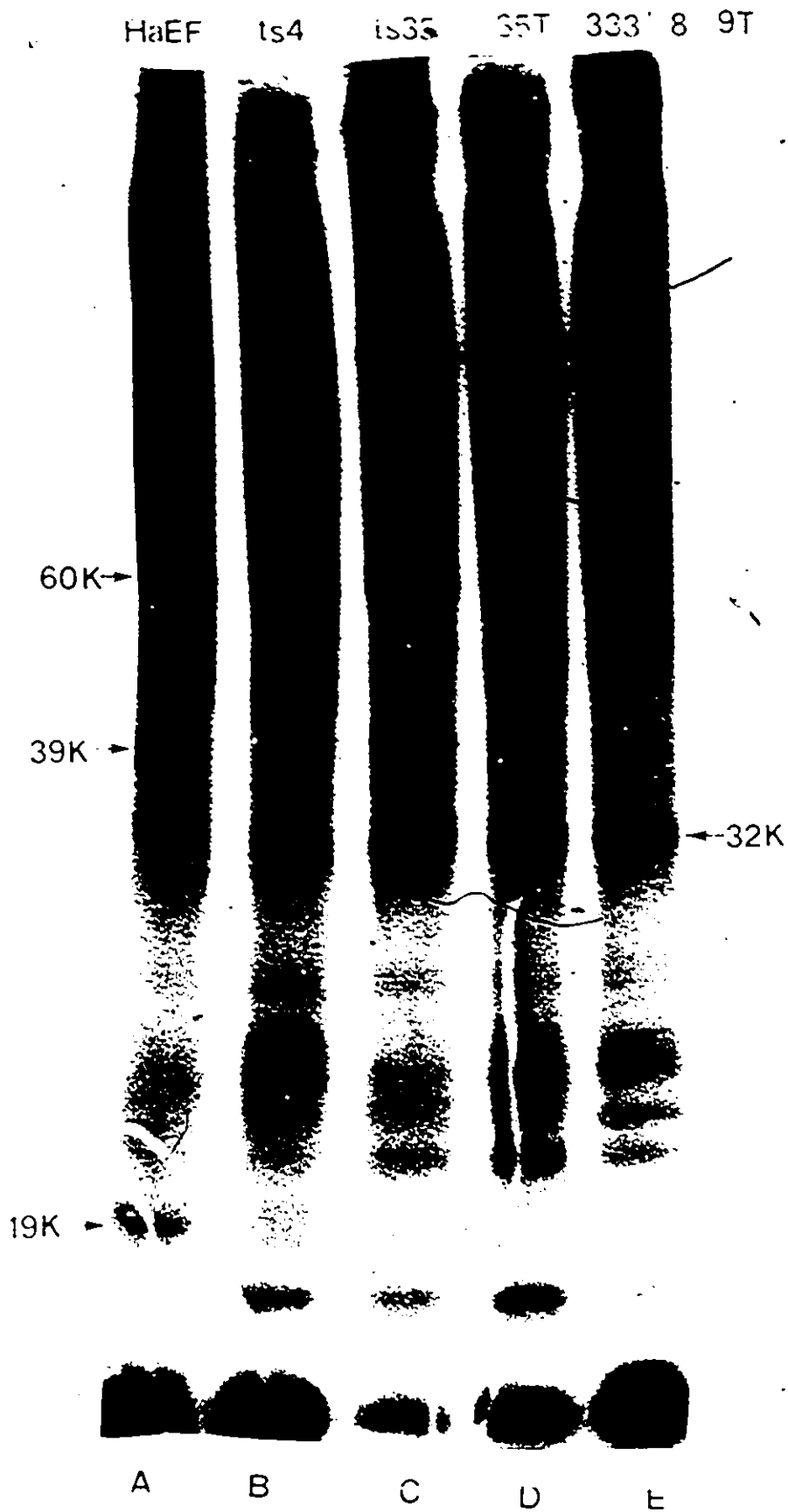
DISCUSSION

The data presented in this report represent the first biochemical studies designed to investigate the cell surface and plasma membrane proteins of cells transformed by HSV-2.

Cell surface iodination performed on a series of HSV-2 transformed hamster embryo fibroblasts revealed at least three cell surface protein alterations. Two of these proteins, 180,000 and 150,000 daltons, were absent in ts35T and 333-8-9T. Both of these lines were derived from tumours and it is likely that passage through the animal selected for cells lacking these proteins. The third protein alteration was the major iodinated species of 220,000 daltons commonly called LETS. Lets was reduced in the transformed lines. All transformed lines contained less than 50% of the level of LETS found in normal secondary HaEF.

A variation was found between the HSV-2 transformed cells in their ability to produce tumours. A relation-ship was observed between tumour-forming ability and the growth properties of cell doubling time and saturation density. The 333-8-9T cells were highly tumourigenic, doubled their numbers every 22 hours and grew to high saturation density at 1% serum. On the other hand ts4

cells were weakly tumourigenic, had a doubling time of
50 hours and grew to a saturation density about one tenth
of 333-8-9T cells at 1% serum. This correlation between
saturation density and doubling time in vitro and initiation
of tumour formation in vivo is an observation which is
typical of transformed cells (Aaronson and Todaro, 1968;
Gallimore, 1974; Risser and Pollack, 1974). In the HSV-2
transformed lines a correlation was also found between
the amount of LETS protein on the surface of the cells
and tumourigenicity. The amount of LETS present was
considerably greater for the weakly tumourigenic ts4 cells
than for the more tumourigenic ts35 and 333-8-9T.

Reduced amounts of LETS in relation to tumourigenicity
has also been observed with a series of adenovirus 2
transformed rat cells (Chen et al., 1976; Gallimore et
al., 1977). These authors suggested that LETS protein
may function as an extracellular 'glue' and that its
presence at the cell surface may inhibit tumour formation.
They propose that tumours are eventually produced by
selection of cells in the inoculum that contain lower
quantities of LETS. The data presented here support this
hypothesis. The two lines which contain the lowest
levels of LETS, ts35T and 333-8-9T, were both tumour-
derived. Also comparing ts35T tumour cells with their
parent transformed line ts35, passage through hamsters
resulted in a population of cells containing one half the
amount of LETS protein. That some type of selection was

occuring in vivo was also shown by the fact that the
ts35T line did not contain the population of giant cells
present in the parent ts35 line.

Two proteins were found to be altered in both
membranes and whole cells labeled with ^{35}S-methionine.
The first was a high molecular weight protein, 225,000·
dalton present in normal HaEF but absent in all trans-
formed whole cell preparations. With isolated plasma
membranes a protein of similar size was absent only in
the tumour-derived lines, 333-8-9T and ts35T. A 206,000
dalton proteins was described by Robbins et al., which
was reduced or absent after RSV-transformation of CEF.
This protein, called omega, was insensitive to externally
added protease and was absent, or present only in small
amounts, in isolated plasma membrane preparations. It.
is possible that the 225,000 dalton species reported
here is analogous to omega. The second protien of 47,000
daltons was reduced or absent in all transformed whole
cell and plasma membrane preparations. A CEF protein
of about 47,000 daltons, termed delta, was found in reduced
amounts in whole cells and membranes following RSV-
transformation (Wickus et al., 1974; Robbins et al.,
1974; Isaka et al., 1975). Delta was not accessible to
lactoperoxidase iodination or added protease (Branton,
unpublished data).

The numerous other plasma membrane protein alterations

can be grouped into four classes. The first class are
proteins which are present in all the HSV-2 transformed
lines but reduced compared to the HaEF. Examples of
these are the 25,000 and 29,000 dalton species. The second
class of protein alteration is represented by the 31,000
and 32,000 dalton polypeptides which are present in the
tumour lines ts35T and 333-8-9T lines, but greatly reduced
or absent in the other HSV-2 transformed lines and the
HaEF. The presence of these proteins may have been related
to the fact that ts35T and 333-8-9T were derived from
tumours. Passage of transformed cells through the hamster
may have selected for cells expressing these proteins.
The third class of proteins is represented by the 43,000
dalton species which is absent from the tumour-derived
lines. Selection for cells lacking these proteins may
have occured as a result of passage through the hamster.
The final class of protein alteration is a large group.
Polypeptides of this class may be found in one line such
as the 34,000 dalton species in ts35 or more than one line
such as the 49,000 dalton protein found in ts4, ts35,
and ts35T yet they are greatly reduced or absent in the
other lines. This group most likely arises from clonal
variation and demonstrates the importance of utilising
a number of transformed clones in this type of study.

There is very little data available on proteins
which increase upon transformation by DNA viruses.

Observations of enhanced; metabolic labeling (Stone et al.,
1974; Isaka et al., 1975: Vaheri and Ruoslahti, 1974),
surface labeling using the galactose oxidase method
(Gahmberg and Hakomore, 1973; Critchely, 1974), and de
nova synthesis (Isaka et al., 1974) of proteins in cells
transformed by avian sarcoma viruses have been reported. In the
present studies it was of interest to determine if HSV-2
virus specific proteins could be detected in the various
transformed lines. No evidence for such viral polypeptides
was found using either labeled plasma membranes or whole
cell preparations or surface iodinated material. It
is possible that virus-specific antigens, if present
are in such small amounts that the methods employed here
are not sensitive to detect them.

The reduction of surface or plasma membrane proteins.
may be attributable to a number of factors. A protein
may be prevented from engaging in a proper association
with other surface or membrane components without any
direct effect on synthesis or absolute amount. On the
other hand there could be complete cessation of synthesis,
reduction in synthesis, or incomplete synthesis such as
alterations in glycosylation or in cleavage of precursors.
It also is possible that there may be an increase in the
rate of loss from the surface due to a passive loss or
active removal or degradation. Combinations of the above
may also occur. The experimental design of this report

cannot discriminate between any of these possibilities.
However, there is data available elsewhere on the LETS
protein which allows a discussion as to perhaps why this
protein decreases upon transformation. LETS protein has
been shown to be reduced on the surface of transformed
cells and on purified plasma membrane preparations.
In some systems the absolute amount per cell is reduced
upon transformation (Robbins et al., 1974). LETS
continues to be synthesized in transformed cells. An
hypothesis which has received much support is that LETS,
which is extremely sensitive to proteolysis, is removed
from transformed cells by proteases secreted by the cells
themselves. If transformed cells are mixed with labeled
normal cells there is an accelerated loss of LETS from the
normal cells (Hynes, 1974). Addition of inhibitors of
proteolysis or removal of plasminogen from the serum
by affinity chromatography (Hynes and Pearlstein, 1976)
gave inconclusive results. Using fibrinolysis as an assay
for plasmin and probably other protease activity, studies
have been carried out to investigate the correlation between
proteolytic activity and presence or absence of LETS
protein. There was good correlation for virally transformed
lines but many spontaneous tumour lines were positive for
LETS yet were also active in fibrinolysis. The role,
if any, of plasmin plus plasminogen activator in the
removal of LETS on transformed cells is therefore incon-

clusive, however other proteases undetected by the fibrinolysis assay and working independent of plasmin could be involved.

A preliminary analysis of membranes isolated from the various HSV-2 transformed hamster lines that were labeled with inorganic ^{32}P revealed a number of alterations in phosphoproteins. There were reductions (19,000 daltons) and increases (32,000 daltons) in phosphorylation of proteins compared with the HaEF, as well as phosphoproteins which were absent (60,000 and 39,000 daltons) or present (53,000 and 23,000 daltons) only in the tumour lines.

The transformation of hamster embryo fibroblasts by HSV-2 results in the reduction of the cell surface LETS glycoprotein. This has been observed in cells transformed by other DNA viruses such as polyoma, simian virus 40, and adenovirus 2, as well as numerous RNA tumour viruses. There appears to be a selection for HSV-2 transformed hamster fibroblasts in vivo that contain reduced LETS and it is therefore possible that LETS may play a role in inhibiting tumour formation. This has been observed in rat-cells transformed by adenovirus 2. That transformation of hamster fibroblasts by HSV-2 results in an altered plasma membrane is clear from the number of other protein changes reported in these present studies. Although little can be said at this time of their specific function in the plasma membrane it is possible that these changes may effect cell function and growth control.

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