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DETECTION OF AN EARLY SURFACE CHANGE DURING THE PROCESS OF ONCOGENIC TRANSFORMATION

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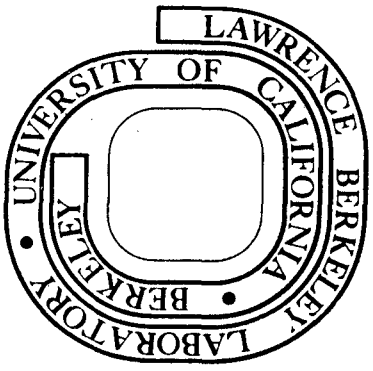

LBL-7517

DISCLAIMER

DETECTION OF AN EARLY SURFACE CHANGE DURING THE PROCESS OF ONCOGENIC TRANSFORMATION

(Chick embryo fibroblasts/Rous sarcoma virus/temperature sensitive mutant/fluorescamine/fluoresence)

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Abstract

The reagent fluorescamine, that can specifically label surface components of cells grown as monolayers in culture, has been used to probe alterations in chick embryo fibroblasts infected with a temperature sensitive mutant of Rous sarcoma virus, Prague A, LA24.

The fluoresence of bound fluorescamine on cells at the permissive temperature (35°) was found to be about 1/3 that of cells cultured at the non-permissive temperature (41°). During the development of the transformed phenotype, that is after transfer of the cells from 41° to 35°, the decrease in surface fluorescence was observed to be an early event, occurring within the first 4 to 8 hours after temperature shift. This alteration was found to take place on a similar time scale to changes in 2-deoxyglucose transport, and to an increased rate of DNA synthesis, but prior to any major morphological changes.

The change was found to be related to cell transformation rather than to growth differences of the cells at the two temperatures. Further, it was found that fluorescamine was not monitoring the loss of LETS glycoprotein from the surface, nor the loss of any other surface components that could be detected by lactoperoxidase catalysed iodination of surface proteins.

It is suggested that fluorescamine may be monitoring some previously unreported alteration.

Introduction

Transformation of cells in culture by oncogenic agents leads to a wide range of changes in cellular properties including loss of growth control, altered morphology, increased mobility of surface lectin receptors, and changes in cytoskeletal organization. Considerable evidence obtained over the last few years has suggested that alterations in the cell surface may be responsible for many of these observations (1,2,3). This has encouraged numerous investigations into the molecular orgnaization of the cell surface, and the characterization of changes that occur upon transformation. Previous work in this laboratory has involved the use of fluorescamine to probe the surfaces of normal and transformed cells.(4,5,6). This reagent reacts with primary amines to give fluorescent adducts, and under controlled conditions can be used to label primarily surface components of intact cells growing as monolayers in culture. Fluorescence measurements of total bound fluorescamine on a variety of cells transformed by RNA viruses, a DNA virus or a chemical carcinogen have demonstrated that transformed cells exhibit considerably lower fluorescence intensities than their normal counterparts (6).

In this study we have examined this particular surface alteration in chick embryo fibroblasts infected with a temperature sensitive mutant of Rous sarcoma virus, LA24, a mutant of Prague A, strain. The use of a temperature sensitive virus has allowed the actual development of the transformed state to be monitored, after transfer of infected cells from the non-permissive temperature (41°) to the permissive temperature (35°). We have correlated the fluorescamine monitored change with several parameters of the transformed state, with particular emphasis on its relationship to surface protein and glycoprotein changes which have been reported previously. Our approach has been to monitor the surfaces of infected cells with fluorescamine after transfer to the permissive temperature, and to examine alterations

in morphology, thymidine incorporation and 2-deoxyglucose uptake (7) as a measure of the development of the transformed state. We have also examined the relationship of the change monitored by fluorescamine to alterations in cell growth.

Materials and Methods

Cell Culture

Chick embryo fibroblasts were prepared and cultured as previously described (8). Primary cultures were infected with virus (LA 24) by the addition of 3.5×10^6 FFU to approximately 5×10^5 cells in each 100 mm culture dish. The cells were maintained at 39° until secondary seeding (2×10^6 cells per 100 mm dish) when they were transferred to 41°. Tertiary cells were seeded at $5\text{-}7 \times 10^5$ cells per 60 mm dish and maintained at 41° until the beginning of the experiment. Uninfected cells were treated in a similar manner. The cells were usually cultured in medium 199 supplemented with 2% tryptose phosphate broth, 2% calf serum, 1% heated chicken serum, and 0.1% glucose. For experiments using slow growing cells, tertiary cultures were seeded in medium 199 supplemented with 0.5% heated chicken serum and 0.1% glucose. Infected cells were fed daily by the addition of 0.5 ml supplement (70% tryptose phosphate broth, 2% glucose, 0.75% sodium bicarbonate) to 5 ml medium. Four hours prior to temperature shift, all cultures (normal and infected) were fed with 0.5 ml supplement/5 ml medium.

Virus Cloning

A cloned stock of virus was obtained by selecting a single colony of transformed cells in agar. Virus was collected from the cells by replacing the medium every 2 hours with 2 ml fresh medium.

Labelling of Cells with Fluorescamine

This was carried out as previously described (4). Labelling mixture (2 ml) containing 500 µg/ml fluorescamine, 0.5% acetone, 0.2 M sodium borate pH 9.0, was added to cells in 60 mm plates which were shaken gently for 30 seconds. The label was removed rapidly and the monolayers were washed and solubilized in 0.067 M sodium borate, pH 9.0, containing 2% SDS.

Samples were diluted 1/10 with 0.067 M sodium borate, pH 9.0, and fluorescence emission at 470 nm was monitored with a Perkin Elmer Fluorescence Spectrometer with the excitation wavelength set at 390 nm. Labelling was performed on triplicate plates and fluorescence was expressed in arbitrary units per µg protein which was determined by the method of Lowry (9).

Measurement of 2-deoxy-D-glucose Uptake and Thymidine Incorporation

These were measured by the methods of Martin et al. (7).

Lactoperoxidase Catalyzed Iodination

This was carried out using the procedure of Hynes (11), except that labelled cells were solubilized directly on the plate by the addition of 150 µl of a solution containing 2% SDS, 50 mM Tris Cl pH 6.7, 2 mM phenylmethylsulphonyl fluoride (PMSF). Samples were solubilized by heating to 100° for 15 mins.

Gel Electrophoresis

This was carried out using the procedure of Laemmli (10). When iodinated components were separated by this technique, gels were dried immediately after electrophoresis and band positions identified by autoradiography on Kodak SB 54 Xray film.

Isolation of LETS Glycoprotein and Preparation of Anti-LETS Antiserum

This was carried out using the method of Yamada et al. (12) with the following modifications to minimize proteolytic degradation. Monolayers were washed initially with Hanks buffer containing freshly added PMSF (3 mM), and then extracted for 1 hr with medium 199 containing PMSF (3 mM), tosyllysylchloromethyl ketone (TLCK, 50 µg/ml), tosylphenylalanyl chloromethyl ketone (TPCK, 50 g/ml), and ε amino caproic acid (1 mg/ml). Monolayers were subsequently extracted twice for 1 hr periods with fresh medium containing inhibitors and also urea (1M). The proteolytic inhibitors were added just prior to use. The two 1 hr urea extractions were pooled and treated as

reported by Yamada et al. (11). LETS glycoprotein was finally purified by preparative SDS polyacrylamide gel electrophoresis. This protein was extracted after freezing the gel band containing LETS in liquid nitrogen, grinding it to a fine powder and then shaking the particles in Tris-glycine buffer (25mM) pH 8.3, containing 0.1% SDS. The eluted protein was dialysed against ammonium bicarbonate (0.2M) and concentrated by lyophilsation. In order to raise an antiserum against LETS the lyophilised material was dissolved in a small volume of water and then treated with $AlCl_3$ to precipitate the SDS-protein complex (13). The complex containing 100 µg protein was mixed with an equal volume of Freund's Complete Adjuvant and then injected into a rabbit at multiple sites. The animal's response was boosted after 4 weeks with another injection of 100 µg protein. Blood was collected 10 days after the final injection, and serum prepared.

Visualization of LETS by Immunofluorescence Microscopy

Infected chick embryo fibroblasts cultured on glass coverslips were fixed with 3.5% formaldehyde in phosphate buffered saline (PBS) at room temperature. After washing 4 times with PBS, coverslips were incubated at 37° for 1 hr with appropriate dilutions of rabbit antiserum against LETS glycoprotein. Coverslips were washed 4 times for 10 minute periods with PBS and then incubated with fluorescein conjugated goat antirabbit antiserum (Miles-Yeda Ltd.) at 37° for 1 hr. Coverslips were washed a further 4 times as above and then examined under the fluorescence microscope.

Growth Curves

Cell growth was estimated by counting the number of cells in a marked area of the culture plate. Routinely this was done by photographing the same area on the plates at various times and then counting the number of cells shown in the photographs.

Results

Surface Alterations Detected in LA24 Infected Cells by Fluorescamine Labelling

Tertiary passage cells were plated at 41° (non-permissive) and 35° (permissive), and labelled with fluorescamine at various times after plating. While significant changes in the surface fluorescence were detected during growth of cells at each temperature, cultures at 41° were generally 3 to 5 fold more fluorescent than those grown at 35° (Fig. 1). The variation in fluorescence values at each temperature was probably a consequence of density dependent alterations in the surface components. The exact value of the fluorescence varied from one experiment to the next but qualitatively similar results were obtained in all experiments.

Cells were cultured at 41° for 16 hr, shifted to 35° and labelled with with fluorescamine at various times thereafter. Alterations in surface fluoresence were detected within the first 4 hrs after shift and by 8 to 12 hrs the fluorescence values were comparable to those of cells maintained at 35° from the time of plating. Cultures grown at 35°, shifted to 41° and subsequently labelled with fluorescamine exhibited the reverse effect, that is within 12 hrs levels of fluorescence had increased to those of cells maintained at 41° from the time of plating. These changes were found to take place with similar kinetics and occurred independent of the time after plating when the temperature was shifted (Fig. (1), (ii),(iii)). Normal uninfected cells did not demonstrate any of these alterations upon temperature shift, (insert in Fig. 1). In this experiment the fluorescence values of labelled normal cells differed from those of the virally infected cells. This may be a consequence of the infection process itself or the experimental variability discussed above.

Changes in Cell Morphology and 2-deoxyglucose Uptake Following Temperature Shift

In order to assess the development of the transformed state cell morphology was monitored at various times after shift to 35°, (Fig. 2). No major changes in morphology took place within the first 8 hrs but by 16-24 hrs the 35° cells had become rounded and refractile. By 49 hrs all the cells kept at 35° had become fully rounded and foci had developed in some regions of the plate. Thus major morphological changes took place much later than the changes measured with fluorescamine.

In addition to morphological changes another characteristic feature of transformed chick embryo fibroblasts is that they exhibit a higher rate of 2-deoxyglucose uptake than normal cells. The rate of uptake by cells grown at 41° was monitored at various times after shift to 35° and within 8 hrs was observed to increase to the level of cultures maintained at 35° from the time of plating. This change was reversible and the rate of uptake in cells initially plated at 35° fell rapidly when cells were transferred to 41°, as reported previously (7), (Fig. 3).

Thus the changes that were detected by the use of fluorescamine took place on a similar time scale to changes in 2-deoxyglucose uptake but prior to major morphological changes.

Changes in Thymidine Incorporation After Shift to the Permissive Temperature

Cells were maintained at low serum concentrations to decrease their growth rate. On transfer from 41° to 35° these cells exhibited an increased rate of DNA synthesis as shown by thymidine incorporation into the acid precipitable fraction (Fig. 4, (i)). This change was found to take place within the first 8 hrs after temperature shift. The early peak

in the rate of DNA synthesis in the control cultures which were kept at 41° throughout the experiment, was probably a consequence of feeding the cultures just prior to temperature shift.

When these slow growing cells were labelled with fluorescamine at various times after downward temperature shift a decrease in fluoresence was observed which was similar to that found in rapidly growing cultures. By 4 to 8 hrs after shift the fluorescence of labeled cells at 35° was about 1/3 that of the 41° control cultures (Fig. 4, (ii)). Thus the change monitored by fluorescamine took place on the same time scale as the increased rate of DNA synthesis.

The Relationship of Fluorescamine Detected Changes to Growth Rates

Growth rates were estimated for both uninfected cultures and cells infected with LA24 at 41° and at 35°. At 35° both infected and uninfected cells increased in number at a slower rate than at 41°, but at neither temperature did the infection process affect the growth rate (Fig. 5). Since the change detected with fluorescamine occurred only in the infected cultures, it can be concluded that the alterations detected with fluorescamine are not the result of differences in growth rates but relate directly to the transformation process.

The Relationship of Changes Detected Using Fluorescamine to Surface Changes Monitored by Iodination

Surfaces of normal and transformed cells have been examined previously using a number of techniques and several significant changes noted upon cell transformation (reviewed by Hynes (3)). The iodination of surface components by the enzyme lactoperoxidase has been used to demonstrate the the loss of LETS glycoprotein from the surface of some transformed cells as well as changes in other iodinated components. In order to assess how such alterations relate to the change monitored with fluorescamine, cells

were labelled with ^{125}I at various times after shift from 41° to 35°, and total cell protein was resolved by SDS polyacrylamide gel electrophoresis. The labelled components were identified by autoradiography (Fig. 6). No changes in the iodination profiles were detected within 24 hours of the shift to the permissive temperature. Differences in the intensities of adjacent tracks seen in Fig. 6 are due to variations in the quantity of material loaded on the gel. Densitometer scans of the autoradiograms demonstrated that there were no changes in the relative quantities of labelled components at any time after temperature shift. The quantity of radioactive label in LETS glycoprotein was measured directly by cutting out the band from the gel and estimating the bound ^{125}I with a -counter. At 8 and 15 hours after temperature shift the proportion of total counts in LETS glycoprotein was the same for cells grown at 41° and 35°. By 24 hours post shift cells at 41° demonstrated an increased proportion of ^{125}I in LETS compared to cells at 35°. However, even at this time cells at 35° had very significant levels of the glycoprotein (data not shown). Thus it appears that changes detected by fluorescamine did not correlate with any changes that could be monitored by iodination procedures.

The presence of LETS glycoprotein on these cells after temperature shift was further demonstrated using fluorescent antiserum against LETS glycoprotein (Fig. 7). Fluoresent staining was clearly visible on cells kept at 35° for 24 hrs, by which time many cells had developed a rounded morphology.

Discussion

The studies reported here demonstrate that a surface alteration that takes place early in the process of transformation can be monitored using the probe, fluorescamine. This is a reversible change in LA24 infected

chick fibroblasts and moreover, is transformation-related rather than growth-related.

Experiments carried out to examine the relationship of this change to alterations in surface components detected by iodination demonstrated that fluorescamine was not monitoring the release of iodinatable surface components. The demonstration of the presence of LETS glycoprotein on cells infected with the virus at its permissive temperature, is in conflict with the report of Hynes and Wyke (14). Using the same mutant, LA24, they reported the loss of LETS from the cell surface 8 to 24 hrs after temperature shift. While even in their system this change occurred later than that detected using fluorescamine, it seemed important to reexamine our results using a different procedure. We thus raised an antiserum against LETS glycoprotein and confirmed our iodination data by immunofluoresence microscopy. Two factors may be important in considering the discrepancy. Firstly, our studies were carried out on a cloned LA24 mutant. In selecting this clone we may have isolated a mutant that was distinct from that used by Hynes and Wyke. Secondly, our iodination techniques differ slightly from those used by Hynes and Wyke. As described in the "Materials and Methods" section, our labelled cells were solubilized directly from the plate while Hynes and Wyke scraped cells from the plate using a rubber policeman and collected them by centrifugation prior to solubilization. In our hands this latter method has been more prone to problems of proteolytic degradation (for both 41° and 35° cultures) than that which we followed.

Since the procedure used for labelling cells with fluorescamine differs significantly from that used for iodinating surface components, it is possible that some alterations may actually be induced during the labelling procedure and hence would not be monitored by other labeling techniques. We have

thus labelled cells at 35° and 41° with ^{125}I, after labelling with fluorescamine. Differences in iodination profiles as a consequence of the fluorescamine labelling procedure were not observed.

Apart from changes in LETS the loss of 210,000 MW and 40,000 MW components has also been reported for transformed cells (3). These alterations were not observed in our system. Other surface changes that have been found upon transformation have included increases in components of MW 90,000 and 75,000 (15). These have recently been shown to relate to glucose starvation rather than transformation (16), and are unlikely to have accumulated under our culture conditions as the medium was supplemented with glucose. Fluorescamine therefore appears to be monitoring a novel alteration at the surface, that has not previously been described. It is significant that it has been found for all normal/transformed pairs that have been examined so far (6).

Currently, we are examining the molecular basis of this change. Clearly as the alteration takes place very early in the development of the transformed state it will be important to investigate its relationship to the onset of DNA synthesis in these cells. Furthermore, this feature may prove to be a useful indication that a cell is in process of becoming transformed. A detection system for transformation based on such an early event undoubtedly will have many applications (6).

Acknowledgments

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Figure Legends

Fig. 1. Fluorescence of exponentially growing cells labelled at various times after temperature shift. Blocks A, B, C, show data obtained for Prague A LA24 infected cells. Monolayers were cultivated either at 41° or 35° and at 16 hrs (A), 22 hrs (8) and 28 hrs (C), after plating some plates were transferred from 41° to 35°, and others from 35° to 41°. Monolayers were labelled with fluorescamine at various times thereafter. Block D demonstrates fluorescence values of normal (uninfected) cells maintained at 41° or shifted from 41° to 35° at 16 hrs after plating.

●———● 41° throughout experiment.

●------● cells plated at 41° and shifted to 35°.

o———o 35° throughout experiment.

o------o cells plated at 35° and shifted to 41°.

Fig. 2 Phase micrographs of Prague A LA24 infected cells. Cells were maintained at 41° throughout the experiment or transferred to 35° 16 hrs after plating. Time (hrs) refer to hours after temperature shift.

Fig. 3. The rates of uptake of ^{3}H 2-deoxyglucose monitored in exponentially growing cells infected with Prague A, LA24.

●———● cells kept at 41° throughout experiment.

●------● cells plated at 41° and shifted to 35° at 16 hrs after plating.

o———o cells plated and kept at 35° throughout experiment.

o------o cells plated at 35° and shifted to 41° at 16 hrs after plating.

Fig. 4.

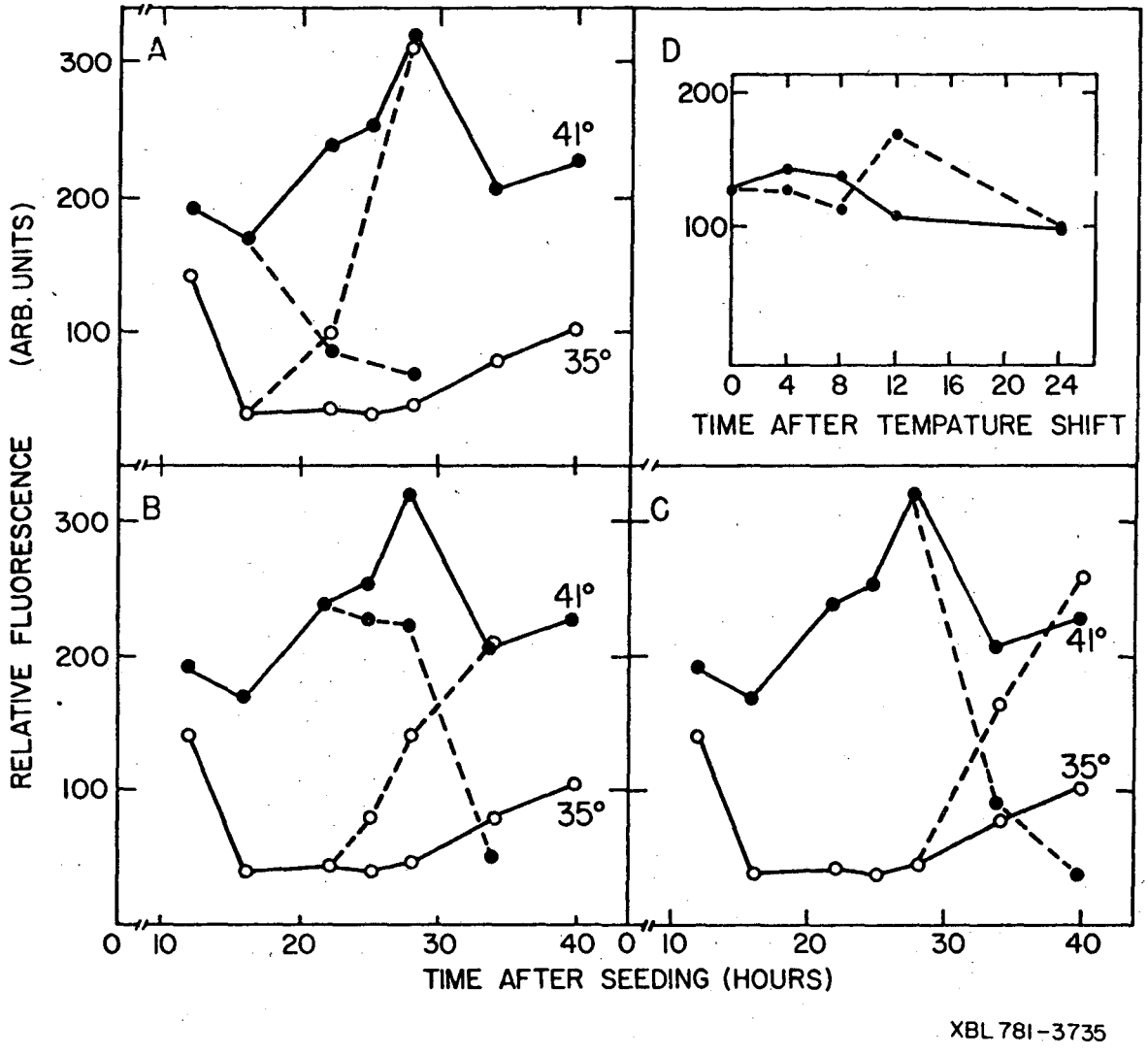
(i) Rates of incorporation of ^{3}H thymidine into trichloro acetic acid precipitable material in Prague A, LA 24 infected cells. Cells were grown in medium containing low serum (0.5% chick serum). ●———● cells kept at 41° throughout the experiment; ●------● cells shifted from 41° to 35° at 16 hrs after plating.

(ii) Fluorescence of fluorescamine labelled Prague A, LA 24 infected cells. Cells were grown as described in (i) and at 16 hrs after plating some were transferred to 35°. These were subsequently labelled at various times with fluorescamine. Values measured at 35° were compared with those measured at 41°.

Fig. 5. Growth of normal uninfected, and Prague A, LA 24 infected cell at 41° and 35°. Cell numbers were estimated as described in the Methods section. ●———●, Prague A, LA24 infected cells cultured at 41°.▲———▲, normal cells cultured at 41° o———o, Prague A, LA 24 infected cells cultured at 35°,△——△ Normal cells cultured at 35°.

Fig. 6. Autoradiogram of ^{125}I labelled surface components resolved by SDS polyacrylamide gel electrophoresis. Prague A, LA24 infected cells were cultivated at 41° and at 16 hrs after plating some plates were shifted to 35°. Cell surfaces were labelled with ^{125}I at various times after shift as denoted by hours. Arrow indicates the position of LETS gylcoprotein.

Fig. 7. Phase and fluorescence micrographs of Prague A, LA24 infected cells labelled with fluorescein conjugaged antiserum against LETS glycoprotein. A and B are phase and fluoresence micrographs of cells kept at 41° throughout the experiment. The fluorescent fibres are cables of LETS glycoprotein. C and D are phase and fluoresence micrographs of cells shifted to 35° for 24 hrs after initial plating at 41°. Intensely staining LETS fibres are seen in these cells also.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B
C
D
41°
35°
300
200
100
0
10
20
30
40
4
8
12
16
20
24
RELATIVE FLUORESCENCE (ARB. UNITS)
TIME AFTER SEEDING (HOURS)
TIME AFTER TEMPATURE SHIFT
XBL 781-3735

Fig. 1

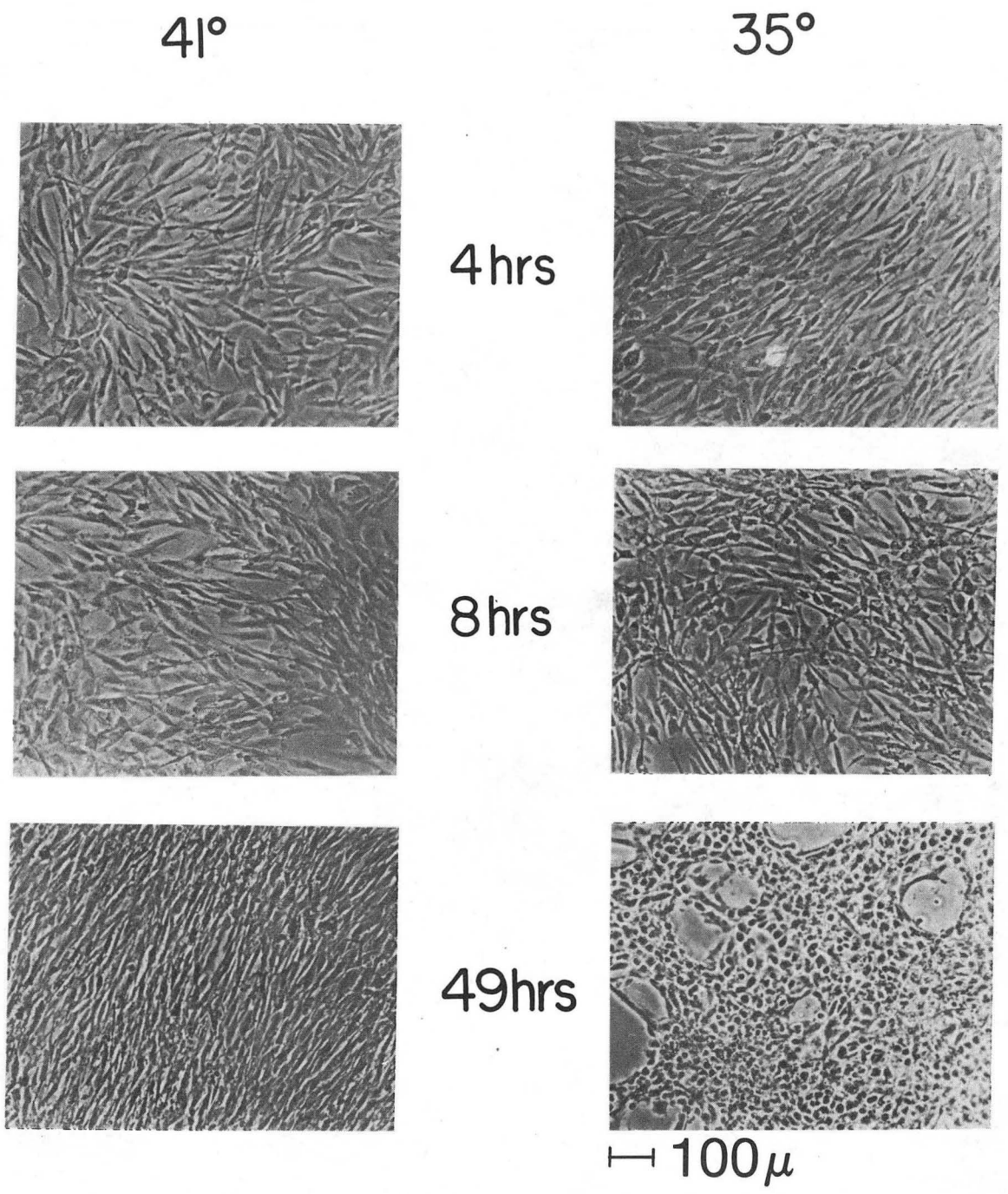

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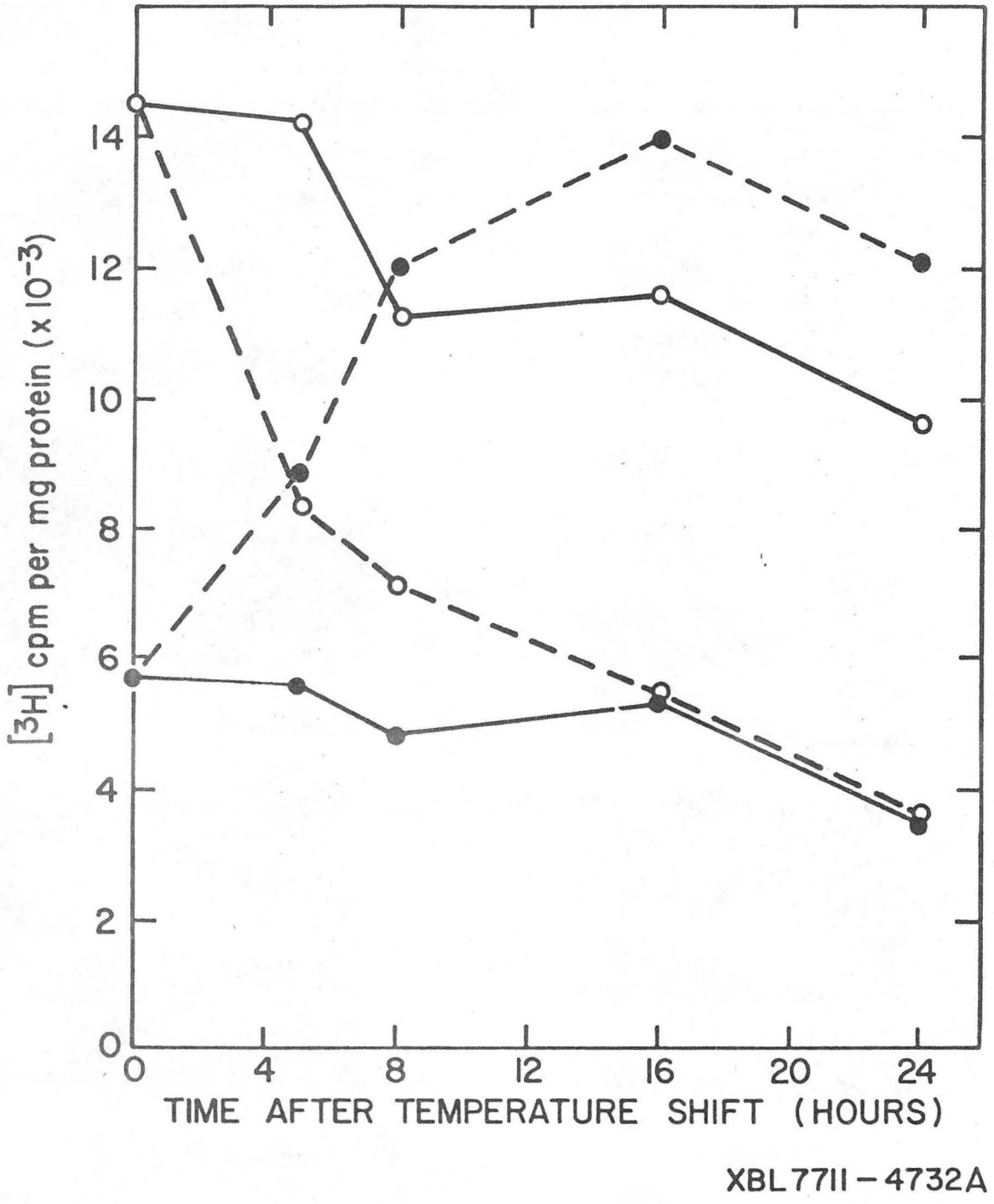
Fig. 2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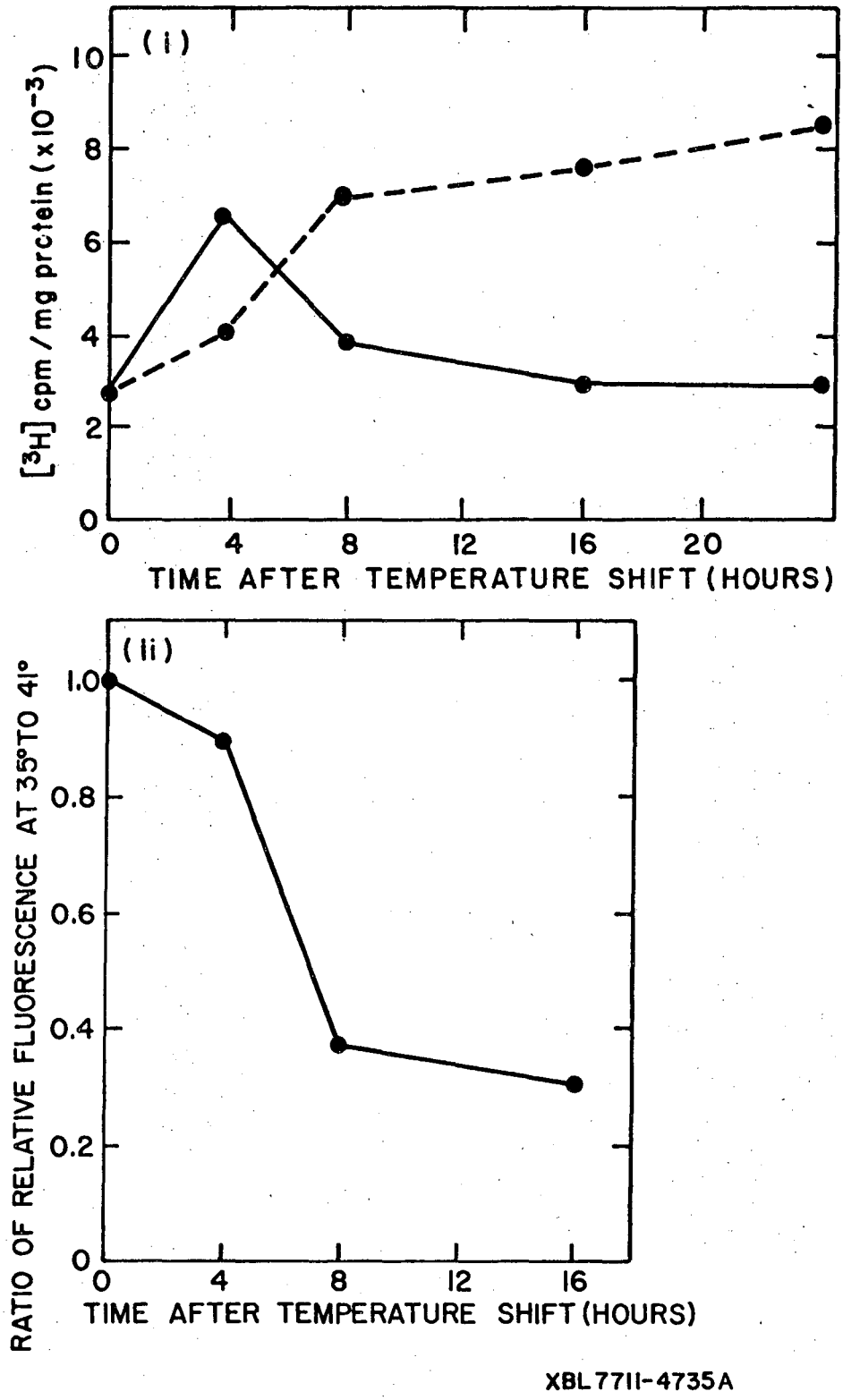
2
4
6
8
10
12
14
0
4
8
12
16
20
24
TIME AFTER TEMPERATURE SHIFT (HOURS)
XBL7711-4732A

Fig. 3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[3H] cpm / mg prctein (x10-3)
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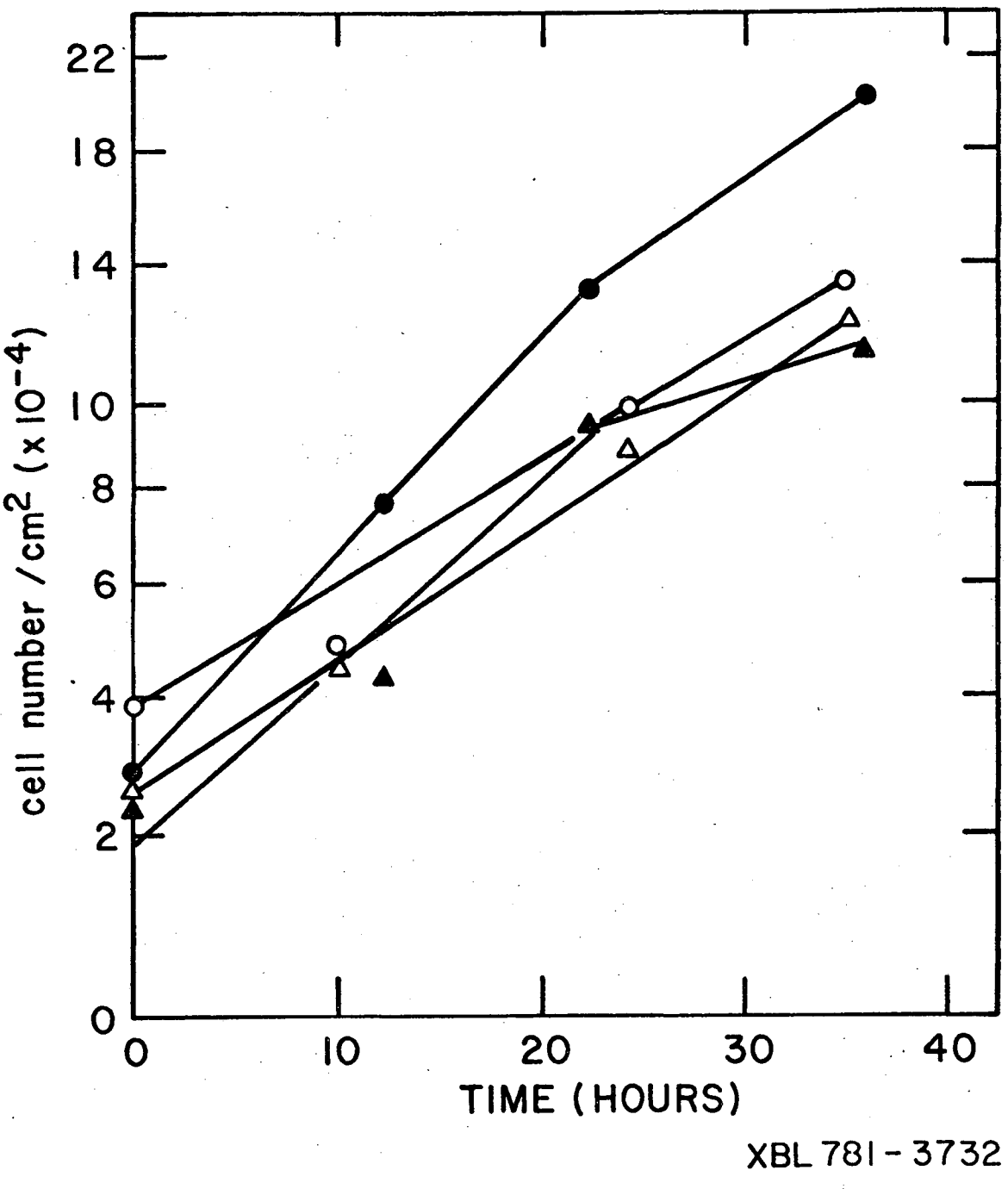
2
4
6
8
10
0
4
8
12
16
20
TIME AFTER TEMPERATURE SHIFT (HOURS)
(ii)
RATIO OF RELATIVE FLUORESCENCE AT 35°TO 41°
0
0.2
0.4
0.6
0.8
1.0
0
4
8
12
16
TIME AFTER TEMPERATURE SHIFT (HOURS)
XBL7711-4735A

Fig. 4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18
14
10
8
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10
20
30
40
TIME (HOURS)
XBL 781-3732

Fig. 5

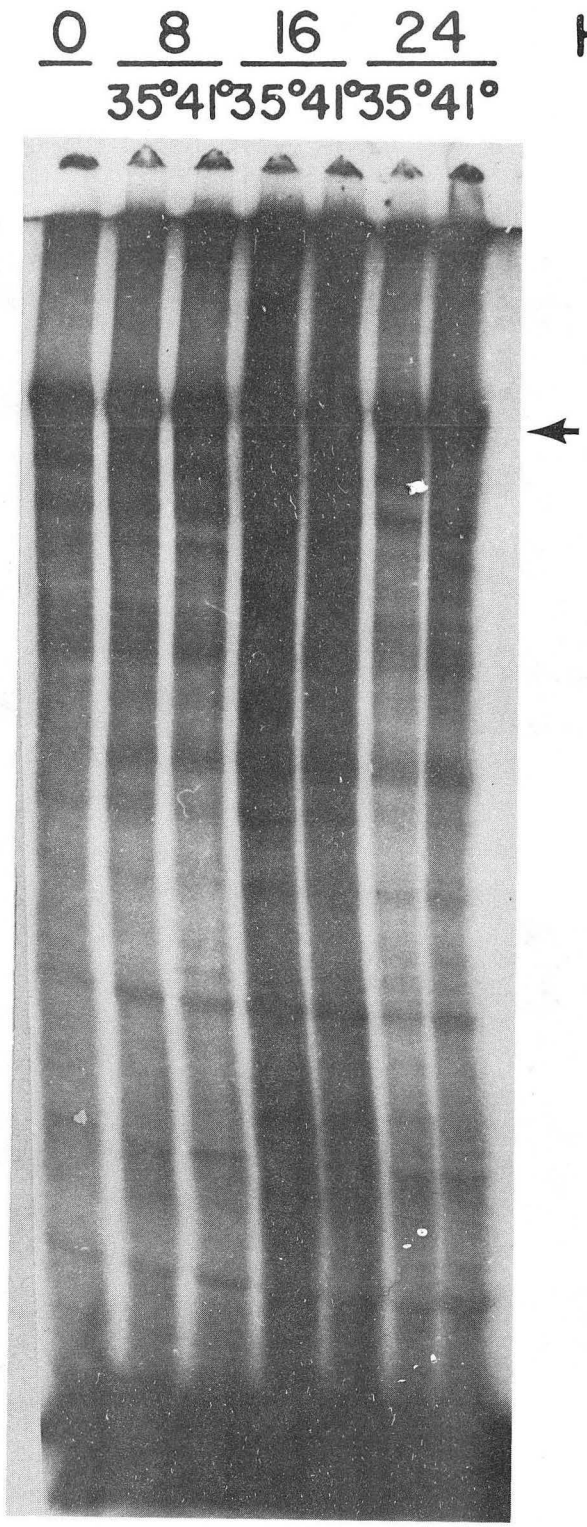

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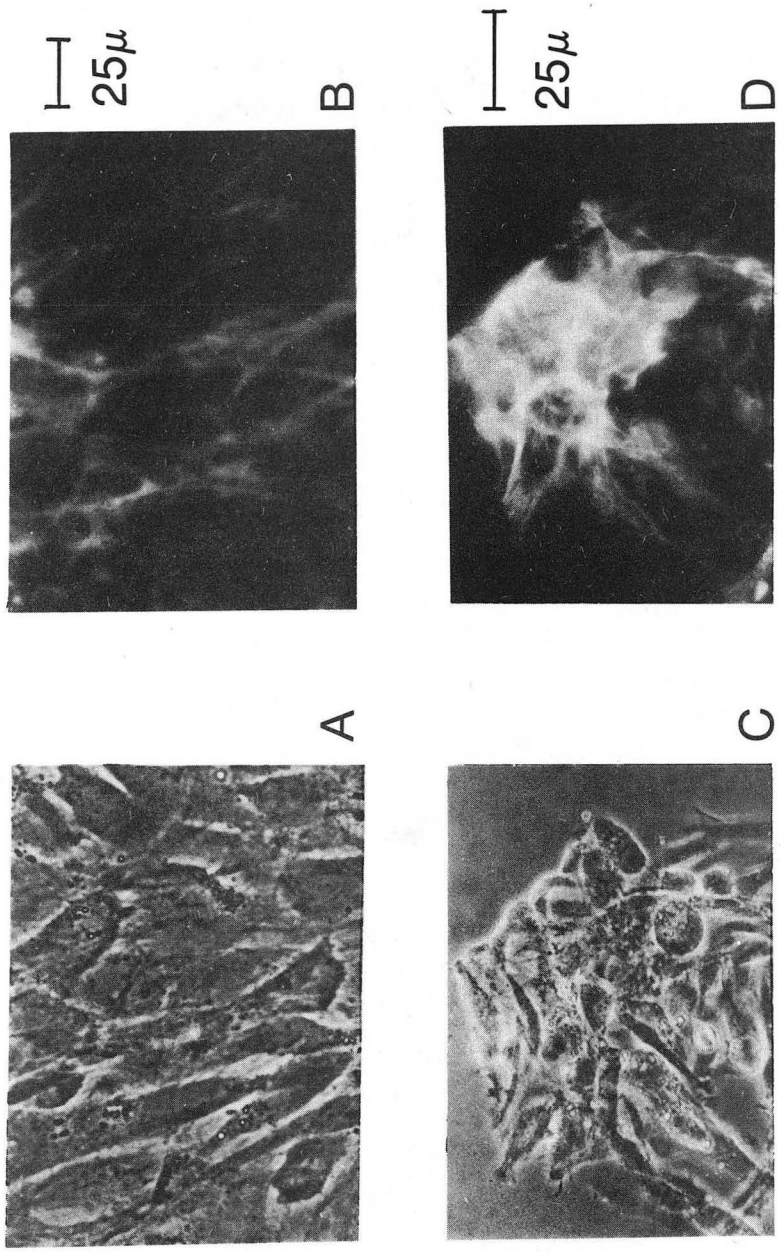
Fig. 6



XBB 779-8603A

Fig. 7

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