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ISOLATION AND PROPERTIES OF A GLUCOSAMINE-REQUIRING MUTANT OF CHINESE HAMSTER LUNG CELLS

TETSUO ONODA[1] and AIZO MATSUSHIRO

Department of Microbial Genetics, Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita, Osaka

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SUMMARY A glucosamine-requiring mutant was isolated from Chinese hamster lung cells (CHL) after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. It required either D-glucosamine or D-galactosamine for growth, 100 μg/ml of glucosamine being required for its maximal growth rate. When these compounds were not present in the medium the mutant became round or spindle-shape with many vacuoles in the cytoplasm. The parental cells (CHL) did not change morphologically throughout incubation, irrespective of the presence or absence of glucosamine.

Measurement of incorporation of ^{14}C-thymidine into DNA gave further evidence that the mutant cells could grow in the presence of glucosamine.

INTRODUCTION

There is now much evidence that the cell surface may be important in regulating cell proliferation and DNA replication in both bacterial cells (Ryter, 1968; Marvin, 1968) and animal cells (Abercrombie and Ambrose, 1962). The possibility that the cell surface participates directly or indirectly in these processes is supported by the demonstration that when cells are transformed by viruses or other carcinogens they acquire new growth patterns and their surface components change (Burger, 1970). However, little is known about the regulatory mechanism or the role of the surface membrane in the cycle of cell duplication. Glucosamine is a component of many macromolecules in the cell envelopes of both bacteria (Osborn, 1969) and animal cells (Ginsburg and Neufeld, 1969). Bacterial cell envelopes, consist of peptidoglycan, lipopolysaccharide, and teichoic acid while animal cell membranes consist of glycolipids and glycoproteins. Studies on the biosynthesis and functions of macromolecules containing glucosamine should be facilitated by isolation of mutants which require exogenous glucosamine for growth. Recently, a bacterial mutant requiring glucosamine for growth was isolated (Freese et al., 1970; Wu and Wu, 1971; Sarvas, 1971), but no such mutant has

1 Present address: Department of Bacteriology, Hiroshima University, School of Medicine, Hiroshima 734.

been obtained from mammalian cells.

This paper describes the isolation and biological properties of a glucosamine-requiring mutant from Chinese hamster lung cells (CHL).

MATERIALS AND METHODS

1. *Cell line and culture method*

The parental cells used were Chinese hamster lung cells (CHL). The basal medium used was Ham's F12 (F12) or sometimes Eagle's MEM (MEM) supplemented with calf serum (10%), streptomycin (100 μg/ml) and penicillin (100 IU/ml). Experiments were carried out in 60 mm plastic dishes unless otherwise stated. The routine technique was employed for Giemsa staining. Cell counts were performed in duplicate in a hemocytometer.

2. *Selection technique*

Selection of the mutant was done essentially by the method of Kao and Puck (1968). Sparse cell monolayers were incubated in F12 medium in the presence of 1.0 μg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) for 24 hr. Cells were then washed free of the mutagen and reincubated for 5 days in fresh F12 medium with glucosamine (200 μg/ml). The surviving cells were trypsinized, transferred at approx 5×10^5 cells per plate to petri dishes (120×15 mm) and then starved in F12 without glucosamine. After 24 hr, the medium was replaced by F12 without glucosamine and thymidine but supplemented with 10% calf serum and 5×10^{-5}M 5-bromodeoxyuridine (BUdR). Cells were further incubated for 24 hr, washed with saline, and placed under visible light (40 W standard fluorescent lamp) for 2 hr. Then the culture medium was replaced by fresh medium containing glucosamine (200 μg/ml) and cultures were incubated for 6 days. Treatment with NG was then repeated once more and the BUdR selection cycle was repeated three times to increase the number of mutant cells. After a total of four selections with BUdR, single cells were sparsely plated and allowed to form small colonies. Surviving clones were picked up, transferred, and tested for growth on F12 with or without glucosamine. Cells from clones that did not grow in F12 medium without glucosamine were further examined.

3. *Serum*

The response of mutant cells in glucosamine-free medium varied depending on the batch of serum used. Of six batches, the batch on which the mutant cells showed the least growth response in the absence of glucosamine was selected, for use throughout this work.

4. *Assay of DNA synthesis*

Synthesis of DNA was measured as incorporation of ^{14}C-thymidine into trichloroacetic acid (TCA)-insoluble material. Cells were labelled for 60 min with ^{14}C-thymidine (2 μCi per plate). Then the cultures were washed three times with ice-cold phosphate buffered saline (PBS). The cells were scraped off with a rubber policeman, precipitated with TCA, filtered on a millipore filter, dried, and counted in a scintillation counter.

RESULTS

1. *Isolation of glucosamine-requiring mutant cells*

Of about 1,200 clones isolated as described in Materials and Methods, two were found not to grow without glucosamine. Experiments were made on one of these two mutant clones.

2. *Effect of glucosamine concn on cell growth*

Mutant cells were seeded on 60×15 mm plastic plates at a density of 1.5×10^5 cells per plate. Various amounts of glucosamine were then added to the plates and cell counts were made after incubation for 4 days. Fig. 1 shows growth of the mutant in media supplemented with different amounts of glucosamine. For the maximal growth rate, 100 μg/ml of glucosamine were required. These experiments were repeated with the parental cells, CHL. The parental cells had the same growth rate in the presence and absence of glucosamine, i.e. their growth was independent of the glucosamine concn. High concn of glucosamine were inhibitory and had a cytotoxic effect on both the parental cells and mutant cells.

The effects of other amino sugars on cell growth were examined by repeating the experiments with galactosamine and mannosamine in

place of glucosamine. The results obtained are shown in Table 1. Galactosamine could replace glucosamine, but 100 μg/ml of mannosamine could not.

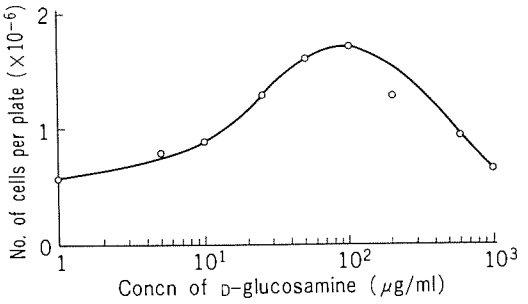

FIGURE 1. *Effect of glucosamine concn on growth of mutant cells. Cells were inoculated into F12 medium at a density of 1.5×10⁵ cells per plate, and incubated for 4 days at 37 C. Cell counts were made 4 days after inoculation.*

TABLE 1. *Effects of* D-*glucosamine,* D-*galactosamine and* D-*mannosamine on cell growth*[a]

Substrate	No. of cells (×10⁴)	
	Mutant	Parent
None	40	515
D-Glucosamine	180	529
D-Galactosamine	192	496
D-Mannosamine	12	290

[a] Mutant and parental cells were each inoculated into MEM medium at a density of 1.5×10⁵ cells per plate and incubated with 20% calf serum at 37 C. D-Hexosamine (100 μg/ml) were used in this experiment. Cell counts per plate of a parental cells were determined after 5 days and those of mutant cells after 6 days.

3. *Effect of the serum concn on cell growth*

The mutant and parental cells were grown in MEM medium with and without glucosamine (100 μg/ml) with various serum concn, and the average number of cells per plate was determined 4 days after inoculation. Table 2 shows the cell numbers of the parental and mutant cells per plate in media containing 5, 10, 20, and 30% calf serum. The final cell count varied directly with the amount of serum added. As shown in Fig. 2A, sparse mutant cells grew very slowly unless glucosamine was added, while in the presence of glucosamine (100 μg/ml) the growth rate of the cells increased with the serum concn of the medium. The growth rate of the parental cells increased with the amount of serum added, regardless of the presence or absence of glucosamine.

TABLE 2. *Effect of serum concn on cell growth with or without* D-*glucosamine (GlcN)*[a]

Concn of serum (%)	No. of cells (×10⁴)			
	Mutant		Parent	
	−GlcN	+GlcN	−GlcN	+GlcN
30	46	172	316	320
20	46	152	224	240
10	34	68	156	148
5	30	57	112	116

[a] Mutant and parental cells were each inoculated at a density of 2.0×10⁵ cells per plate and incubated for 4 days at 37 C. Then cell counts per plate were determined.

4. *Effect of the medium on cell growth*

The mutant and parental cells were grown in F12 or MEM medium with 20% serum and with or without glucosamine (100 μg/ml). The average number of cells per plate was determined at intervals for 10 days after inoculation and medium was renewed every four days. The results obtained are shown in Fig. 2. The mutant cells grow faster in F12 medium than in MEM medium. In the absence of glucosamine, the cells showed little growth in either F12 or MEM medium. On the other hand, growth of the parental cells was similar in F12 and MEM media: namely when the parental cells were inoculated into MEM and F12 media at a density of 2.0×10⁵ cells per plate, after 4 days, the cell densities per plate were 50.8×10⁵ and 52.6×10⁵ respectively (Fig. 2B). Therefore, growth of the parental cells was independent of the presence or absence of glucosamine.

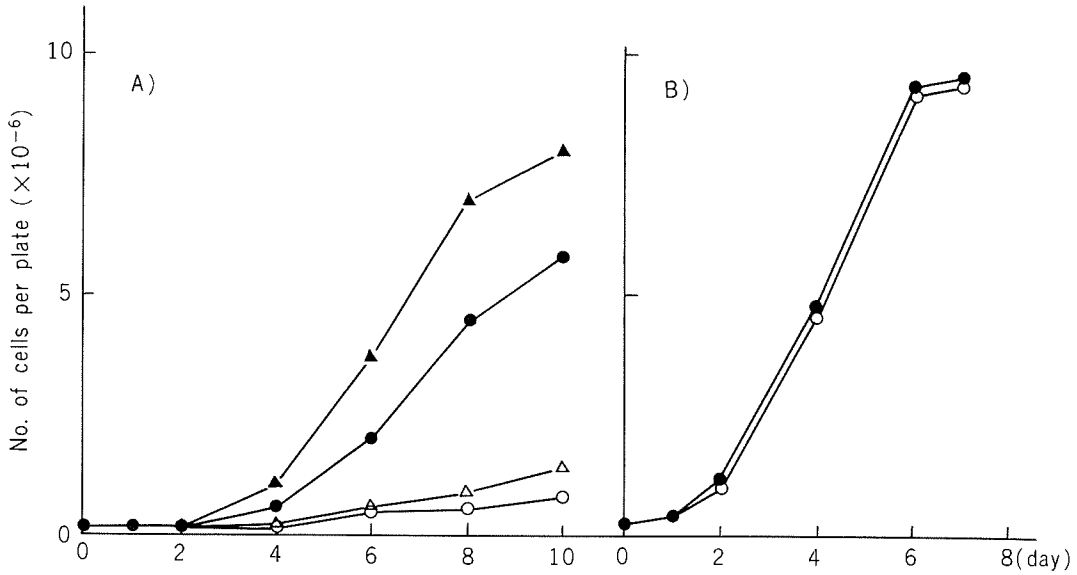

FIGURE 2. *Effect of media with and without glucosamine on cell growth.*
●, *MEM medium with glucosamine (100 μg/ml);* ○, *MEM medium without glucosamine;* ▲, *F12 medium with glucosamine (100 μg/ml);* △, *F12 medium without glucosamine.*
A) Mutant cells were inoculated into MEM and F12 media at a density of 1.2×10^5 *cells per plate and incubated for 8 days at 37 C.*
B) Parental cells were inoculated into MEM medium at a density of 1.5×10^5 *cells per plate, and incubated for 7 days at 37 C.*

5. *Effect of glucosamine on cell morphology*

In medium without glucosamine the mutant cells became markedly round or spindle-shaped with many vacuoles in their cytoplasm after 2 days (Fig. 3). The mutant cells showed more striking alteration in the absence of glucosamine in MEM medium than in F12 medium. However, in the presence of glucosamine the morphology of mutant cells was normal throughout incubation and vacuoles were rarely found in the cytoplasm. On addition of glucosamine the morphology of mutant cells which had be cultured in glucosamine-free medium slowly reverted to normal. The parental cells did not change morphologically in the absence of glucosamine.

6. *Recovery of cells maintained in glucosamine-free medium*

Mutant cells were seeded at 1.5×10^5 cells per plate into glucosamine-free medium. After 2, 4 and 6 days the medium was replaced by medium containing glucosamine and the cell density was determined at 2 day intervals for 8 days. Fig. 4A shows that the inhibition of cell division by glucosamine-free medium was reversible and the ability of mutant cells to divide was retained during culture for 6 days in glucosamine-free medium. To see whether cell growth was suppressed when glucosamine was removed from the medium, after 2 days inoculation the medium was replaced by media with and without glucosamine. As shown in Fig. 4B cells in the culture with glucosamine continued to increase in number, but those without glucosamine showed little increase in number.

7. *Incorporation of* ^{14}C*-thymidine*

Mutant and parental cells were seeded into MEM medium without glucosamine at a density of 2.0×10^5 cells per plate (35×15 mm

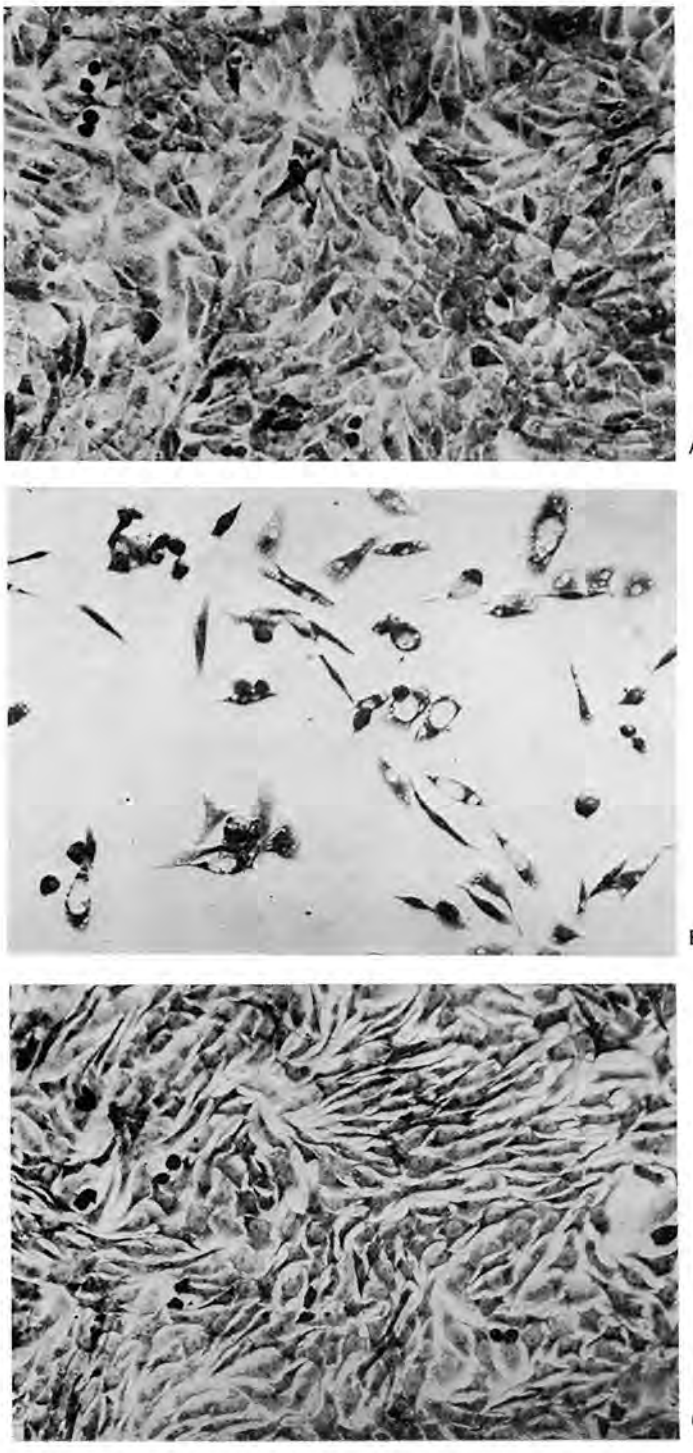

FIGURE 3. *Effect of glucosamine on cell morphology. Mutant and parental cells were each seeded at* 1.5×10^5 *cells per plate and incubated in MEM medium with or without glucosamine (100 μg/ml) with 20% calf serum. Mutant cells were stained with Giemsa solution at 6 days after inoculation and parental cells 4 days after inoculation. A) Mutant cells with glucosamine (100 μg/ml). B) Mutant cells without glucosamine. C) Parental cells without glucosamine.* × 100.

plastic petri dishes). After 24 hr, 100 μg/ml of glucosamine were added to half the cultures. At intervals, cultures were labeled for 60 min with 2 μCi of ^{14}C-thymidine. The results obtained are shown in Figs. 5A and B. The mutant cells showed an increased rate of thymidine incorporation, on addition of glucosamine but not in its absence. On the other hand, there was no significant difference in incorporation of ^{14}C-thymidine by parental cells with and without glucosamine. Thus this experiment on ^{14}C-thymidine incorporation into DNA confirmed the finding that the mutant cells can grow only in the presence of glucosamine.

DISCUSSION

Until recently a major limitation in studies on the genetics of mammalian cells at the molecular level has been the paucity of well characterized, stable gene mutations. A number of different mutants of mammalian cells have been reported (Chu and Maling, 1968; Taylor, Souhrada and

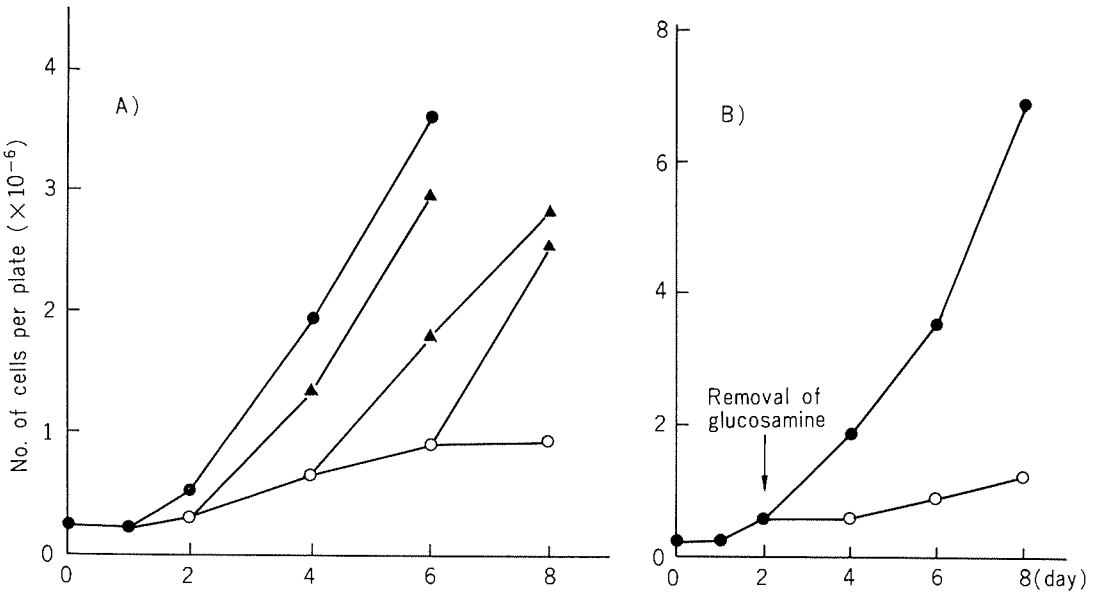

FIGURE 4. *A) Recovery of growth of mutant cells in glucosamine-free medium on adding glucosamine. Cells were incubated in the medium with 20% calf serum at 37 C. Glucosamine was added from the start of the experiment* (●). *Culture was incubated in glucosamine-free medium* (○). *Glucosamine (100 μg/ml) was added to cultures at 2 day interval.*
B) Arrest of growth of mutant cells on removing glucosamine. Glucosamine was present from the start of experiment (●). *Glucosamine was removed at the time (2 day from the start) indicated by an arrow* (○).

McCall, 1971; Kao and Puck, 1969; 1971). Kao and Puck (1968) reported isolation of auxotrophic mutants deficient in glycine, glycine+thymidine+hypoxanthine, and inositol by destruction of prototrophs by exposure to BUdR followed by illumination with near-visible light.

This paper describes the isolation and biological properties of a glucosamine-requiring mutant of Chinese hamster lung cells. The appearance of the requirement for glucosamine in auxotrophic cells isolated by treatment with NG is noteworthy. Glucosamine is the major source of the polysaccharide in macromolecules of the cell membranes of both bacteria (Osborn, 1969) and animal cells (Ginsburg and Neufeld, 1969). Wu and Wu (1971) recently reported isolation of a mutant of *Escherichia coli* K-12 which requires the glucosamine, *N*-Acetylglucosamine for growth. This mutant is deficient in the activity of L-glutamine-D-fructose-6-phosphate amino transferase. Similar mutants have recently been isolated from *Bacillus subtilis* by Freese et al. (1970) and from *Escherichia coli* by Sarvas (1971). A simiar mutant has not been isolated from animal cells.

The precise physiological role of the surface components containing glucosamine of animal cells was not elucidated. The mutant cells isolated in this work should be very useful in studies on the surface component containing glucosamine of Chinese hamster lung cells. Contact inhibition of growth of these cells can be inhibited by treatment with proteolytic enzymes and other substrates acting on the cell surface (Todaro, Lazar and Green, 1965; Vasiliev et al., 1970; Jainchill and Todaro, 1970; Vaheri, Ruoclahati and Nordling, 1972; Burger, 1970; Sefton and Rubin, 1970), indicating that the cell surface may be important in regulating cell proliferation. Burger and Noonan (1970) reported that by covering sites on transformed cells with monovalent concana-

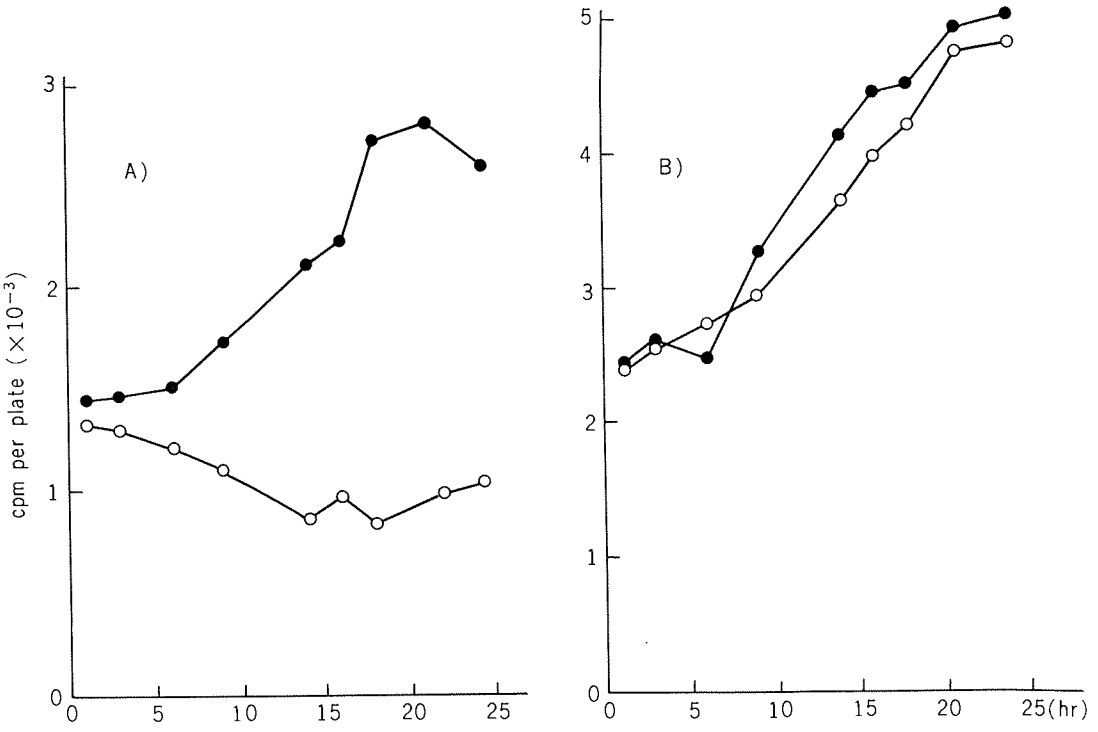

FIGURE 5. *Incorporation of ^{14}C-thymidine in the presence or absence of glucosamine. Mutant and parental cells were each seeded at a density of 2.0×10^5 cells per plate and incubated in MEM medium without glucosamine for 24 hr. Then glucosamine (100 μg/ml) was added to half of the cultures and at intervals the cultures were labelled for 60 min with 2 μCi of ^{14}C-thymidine. Trichloroacetic acid-insoluble radioactivity was measured at intervals after addition of glucosamine.*
A) Mutant cells. B) Parental cells. ●, *Cultures with glucosamine;* ○, *Cultures without glucosamine.*

valin A, the growth pattern of transformed fibroblastes is restored to that of normal cells. Onodera and Sheinin (1970) reported that not only are the surface components, including glucosamine, resynthesized after cells have been subcultured with trypsin but its synthesis is significantly enhanced after mitosis when cells enter a second G1 period. Furthermore, Gerner, Glick and Warren (1970) reported that there is a marked increase in the rate of incorporation of ^{14}C-glucosamine into the surface membrane just after division. Glucosamine is a component of many macromolecules of the cell membranes of both bacterial and animal cells, but its role in the cycle of cell proliferation is unknown. In this work we found that the growth rate of mutant cells increased on addition of glucosamine.

Immediately after subculture without glucosamine the cells are round. Then they spread over the glass surface and acquire the spindle shape of fibroblasts in culture. In this work we found that in medium without glucosamine the mutant cells developed many large vacuoles in the cytoplasm became markedly round or spindle-shaped. However, in the presence of glucosamine the cells had a normal morphology. Mutant cells with altered morphology after culture in medium without glucosamine slowly reverted to the normal morphology when this compound was added. The parental cells showed no morphological alteration irrespec-

tive of the presence or absence of glucosamine. These morphological alterations in mutant cells may have some relation with the deficiency of a metabolic pathway of glucosamine synthesis.

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