Characterization of a Cell Cycle Mutant derived from Hamster Fibroblast: Reversion Analysis

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ABSTRACT

K12 is a temperature-sensitive (ts) mutant cell line derived from Chinese hamster fibroblasts. When incubated at the nonpermissive temperature, K12 cells exhibit the following properties: (a) the cells cannot initiate DNA synthesis; (b) the synthesis of cytosol thymidine kinase is suppressed; and (c) the synthesis of three cellular proteins of molecular weights 94, 78, and 58 kdaltons is greatly enhanced. Here we characterize a spontaneous revertant clone, R12, derived from the K12 cells. We selected the revertant clone for its ability to grow at the nonpermissive temperature. Our results indicate that all the traits which constitute the K12 mutant phenotype are simultaneously reverted to the wild type in the revertant cell line, suggesting that the is mutation of the K12 cells is of regulatory nature and exerts multiple effects on the expressed phenotypes.

Somatic cell mutants can be useful tools to study the control of gene expression in higher animal cells (1). In particular, regulatory mutants exhibiting pleiotropic changes in the cellular phenotype may provide model systems whereby the mechanisms of coordinated gene expression can be examined at the molecular level.

K12 is a temperature-sensitive (ts) mutant derived from Chinese hamster fibroblasts. The K12 cells were among several mutant clones isolated after treatment of Wg1A parent cells with ethyl methane sulfonate (2). These clones were selected by virtue of their ability to proliferate at 35°C but not at 40.5°C.

K12 has been identified as a cell cycle mutant blocked at a step in G1 required for the initiation of DNA synthesis (2-5). When incubated at the nonpermissive temperature (40.5°C), K12 cells exhibit several interesting properties. While the cells remain attached to the dish and maintain normal levels of RNA and protein synthesis for at least 20 h, the levels of cytosol thymidine kinase and deoxycytidylylate deaminase activities, which normally increase with DNA synthesis, are suppressed (6). At the same time, three cellular proteins of molecular weights 94, 78, and 58 kdaltons are being accumulated (5, 7). Since the same set of proteins are overproduced in these hamster cells when the cells are starved of glucose, these proteins have been identified as “glucose-regulated proteins” (7-9). Investigation of the effect of actinomycin D on the enzyme levels and proteins synthesized by K12 cells at 40.5°C and 35°C suggests that the ts lesion is affecting new synthesis of these enzymes and proteins (6, 10). Moreover, results obtained by in vitro translation of the mRNA isolated from K12 cells further imply that the synthesis of these proteins is regulated at either the transcriptional or post-transcriptional level (10).

One explanation for the pleiotropic nature of the K12 mutation is that the ts lesion affects some regulatory function involved in (a) the initiation of DNA synthesis, (b) the activation of thymidine kinase and deoxycytidylylate deaminase synthesis, and (c) the suppression of synthesis of the three “glucose-regulated proteins.”

To determine whether the pleiotropic phenotype of K12 cells is the consequence of a single mutational lesion, we set out to isolate spontaneous revertants which are able to grow at the nonpermissive temperature. In this report, we describe the isolation and characterization of one such revertant clone, R12. Our results indicate that all of the traits which constitute the K12 mutant phenotype are simultaneously reverted to wild type in the revertant cell line.

MATERIALS AND METHODS

Materials

Dulbecco’s modified Eagle medium (DMEM) was purchased from Gibco Laboratories, Grand Island Biological Co. (Grand Island, NY), and calf serum was purchased from Bicocell Laboratories (Carson, CA). Methyl-[3H]thymidine and [3H]leucine were purchased from Amersham Corp. (Arlington Heights, IL). Polyacrylamide gel reagents were obtained from Bio-Rad Laboratories (Richmond, CA). Colcemid and all other chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Methods

Selection of Spontaneous Revertants: The source of the hamster cell lines (Wg1A and K12) and conditions for routine cell maintenance were previously described (7). For the selection of revertants, K12 cells were seeded at

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thymidine monophosphate (dTMP) per microgram of protein in 10 min. The extracts is defined as the number of picomoles of thymidine (dThd) converted to liquid scintillation counter. The thymidine kinase activity contained in the radioactivity eluted was determined by counting aliquots of the hydrolysate in a

The disks were then fixed with methanol:acetic acid (3:1), spread over slides, and stained with Giemsa.

**CELL SYNCHRONIZATION:** Cells were seeded at 10^5 cells/cm² in 60-mm culture dishes and incubated at 35°C in 5 ml of DMEM medium supplemented with 10% calf serum, without a change of medium for 4-5 d. At that time, most cells were synchronized by serum deprivation. Upon addition of fresh medium, the arrested cells were induced to proliferate. DNA synthesis at various times after addition of fresh medium was monitored by labeling the cultures with 0.25 µCi/ml methyl-[3H]thymidine for 30 min before extraction, and measuring the incorporation of labeled thymidine into TCA-precipitable cpm as described (5).

**ASSAY OF CYTOSOL THYMIDINE KINASE ACTIVITY:** Cell extracts were prepared at 4°C as follows: before extraction, the cells were washed once with phosphate-buffered saline (PBS), and the cell pellet was resuspended in 0.5 ml of sonication buffer (0.01 M TrisHCl, pH 7.5, 0.15 M NaCl, 20 µM thymidine, 2 mM dithiothreitol, and 10% glycerol) and sonicated for three 15-s intervals. The suspension was then spun in a Brinkmann Instruments, Inc. centrifuge (Westbury, NY) (12,000 g) for 10 min. The supernatant was removed and centrifuged at 39,000 rpm for 1 h in a type 50 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA). Enzyme activities were determined using this final supernatant. The concentration of protein in the supernatant was determined with the Bio-Rad protein assay (Bio-Rad Laboratories) using bovine serum albumin (BSA) as the standard. The thymidine kinase activity present in the enzyme extracts was assayed by modification of a published procedure (11). Briefly, various concentrations of protein from each extract were added to a standard reaction mixture containing 0.19 M TrisHCl, pH 7.5, 1.9 mM MgCl₂, 1.9 mM ATP, 10 mM DTT, 10 mM NaF, 0.19 mM thymidine, 1% BSA, and 25 µCi methyl-[3H]thymidine (specific activity 50 Ci/mmol), in a final volume of 0.1 ml. The reaction mixture was incubated at 37°C for 30 min. At the end of the incubation period, 0.05 ml of each reaction mixture was applied onto a Whatman DE81 paper disk (Whatman Inc., Paper Div., Clifton, NJ) and immediately dropped into 100% methanol (10 ml/disk). The disks were stirred gently for 15 min at room temperature, after which they were transferred to distilled water (10 ml/disk) and stirred gently for an additional 15 min at room temperature. The disks were allowed to air-dry overnight or by incubation at 60°C for 1 h. The radiolabeled dTMP was eluted from the disks by immersing the disks in a 2-ml solution containing 0.03 N HCl and 0.1 N KCl inside a capped scintillation vial overnight. The amount of radioactivity eluted was determined by counting aliquots of the hydrolysate in a liquid scintillation counter. The thymidine kinase activity contained in the extracts is defined as the number of pico moles of thymidine (dThd) converted to thymidine monophosphate (dTMP) per microgram of protein in 10 min.

**RESULTS**

**Isolation of Revertant Clones**

Over a period of 1 yr, approximately 50 frozen aliquots of K12 cells (4 x 10^6 cells each) were tested for the presence of revertant cells which were able to proliferate at the nonpermissive temperature. Of these, only one aliquot of cells gave rise to ~100 clones which survived incubation at 40.5°C. Such spontaneous revertants, present in this particular population at a frequency of ~10⁻⁴, were not observed in any of the other cultures tested. Therefore, it is most likely that before selection a single mutated cell gave rise to the revertant clones observed in this batch of cells. Thus, although the spontaneous reversion frequency of the K12 mutation was extremely low, we were able to isolate revertant clones from a single population of cells in which reversion had occurred. The subclones surviving selection at 40.5°C were grown to mass culture and designated R12, a revertant derived from K12.

**Cell Morphology of Wg1A, K12, and R12**

The parent cell line, Wg1A, its ts mutant K12, and the revertant R12 all grew with an average generation time of ~25 h at 35°C in DMEM medium supplemented with 10% calf serum. All three cell lines exhibited spindle-cell morphologies characteristic of fibroblasts but both K12 and R12 appeared larger in cross section than Wg1A cells (Fig. 1). When the cells were shifted to 40.5°C, Wg1A and R12 cells continued to grow to confluence, whereas K12 cells stopped growing and assumed an elongated cell shape as distinct from the rectangular shape exhibited by Wg1A and R12 cells at 40.5°C.

**Chromosome Numbers**

It has been previously determined that Wg1A cells have an average chromosome number of 21 (2, 5). The K12 mutant, when isolated, was reported to have 20-21 chromosomes (4,
pressed after the first day, and, by the end of the second day, the plates were incubated at 35°C and the other half at 40.5°C. Cells, R12 cells divide at about the same rate as the parent cell WgIA cells at both incubation temperatures. Except for showing a slightly lower seeding efficiency as compared to WgIA cells, R12 cells divide at about the same rate as the parent cell line.

Cell Cycle Analysis

Since K12 has been shown to be a cell cycle mutant arrested at the midpoint of G1 when incubated at 40.5°C (5), we compared the progress of the hamster cells through the cell cycle. The cells were first synchronized by serum deprivation, then proliferation was stimulated by addition of fresh culture medium. One set of plates was incubated at 35°C, while duplicate dishes were incubated at 40.5°C. At different times, cultures were pulse-labeled with methyl-[3H]thymidine for 30 min with methyl-[3H] thymidine and processed for determination of radioactivity incorporated into 3% TCA-insoluble material. Concomitantly, duplicate dishes of synchronized cells were harvested for the preparation of enzyme extracts. The activity of thymidine kinase in each of the extracts was determined with a hemocytometer. (A) Cell number incubated at 35°C, (O---O) cultures incubated at 40.5°C. (B) Thymidine kinase activities of the enzyme may be more labile at 40.5°C than at 35°C, implying that the peak of DNA synthesis was always about twofold. We also observed that there was a direct correlation between the enzyme activity and the rate of DNA synthesis in all cell lines at 35°C. It was evident that for K12 cells both DNA synthesis and thymidine kinase activity were completely inhibited by incubation at 40.5°C. In contrast, the revertant R12 cells exhibited properties similar to those of the wild-type parental cells at both incubation temperatures.

Cytosol Thymidine Kinase Activities

Previous studies indicated that the cytosol thymidine kinase activity in K12 cells was drastically reduced when the cells were incubated at 40.5°C, and the step at which the activities were affected was likely to be at the transcriptional or post-transcriptional level (6). To determine whether the reduced synthesis of thymidine kinase was a consequence of the K12 mutation, we assayed the thymidine kinase activities of the WgIA, K12, and R12 cells at both 35°C and 40.5°C using synchronized cultures. The results are shown in Fig. 4b. We observed that there was a direct correlation between the enzyme activity and the rate of DNA synthesis in all cell lines at 35°C. It was evident that for K12 cells both DNA synthesis and thymidine kinase activity were completely inhibited by incubation at 40.5°C. In contrast, the revertant R12 cells exhibited properties similar to those of the wild-type parental cells at both incubation temperatures.

In all our assays, we consistently observed that the ratio of enzyme activities per microgram of protein extract for the polyploid cells (K12, R12) and pseudodiploid cells (WgIA) at the peak of DNA synthesis was always about twofold. We also observed that the specific activity of the enzyme was always lower by a factor of two at 40.5°C than at 35°C, implying that the enzyme may be more labile at 40.5°C.

Enhanced Synthesis of Three Proteins

A unique characteristic of the K12 cell line is that upon incubation at 40.5°C three cellular proteins of molecular weights 95, 78, and 58 kdaltons are overproduced (5, 7). This set of proteins has been identified as "glucose-regulated proteins" observed to accumulate in animal cells when the cells are starved of glucose (7-9). The isolation of the revertant R12
associated with DNA replication was specifically affected at RNAsynthesis (6). Nonetheless, the synthesis of two enzymes mutation does not lead to an overall cessation ofprotein or cell cycle, as distinguished from the initiation of replication of (3-5). This event apparently takes place in the G1 phase of the tion essential for "commitment" of the cells to DNAsynthesis although previous studies suggested that it affects a cell func-

Overall, these findings suggest that at the nonpermissive temperature a regulatory molecule defined by the K12 mutation is nonfunctional. The functional deficiency of this component exerts a pleiotropic effect on the formation of various enzymes and proteins whose synthesis or repression signals a commitment to DNA replication.

In conclusion, the properties of K12 and its revertant R12 suggest that the ts mutation of the K12 cells is of regulatory nature and exerts multiple effects on the expressed phenotypes. With technologies now available for the isolation of functional DNA sequences (14), it will be most interesting to use the K12 system for the isolation and characterization of the mutated regulatory sequence.

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REFERENCES

1. Eiminovich, L., and L. H. Thompson. 1978. The nature of conditionally lethal tempera-
ture-sensitive mutations in somatic cells. J. Cell Physiol. 95:361-364.

2. Roscoe, D. H., M. Read, and H. Robinson. 1973. Isolation of temperature-sensitive mammalian cells by selective detachment. J. Cell Physiol. 82:325-332.

3. Roscoe, D. H., H. A. Robinson, and A. W. Carbonell. 1973. DNA synthesis and mitosis in a temperature-sensitive Chinese hamster cell line. J. Cell Physiol. 82:333-338.

4. Smith, B. J., and N. W. Wigglesworth. 1973. A temperature-sensitive mutation in a Chinese hamster cell line affecting DNA synthesis. J. Cell Physiol. 82:339-348.

5. Medoro, J. A., and V. Findham. 1978. Enhancement of the synthesis of specific cellular polypeptides in a temperature-sensitive Chinese hamster cell line (K12) defective for entry into S phase. J. Cell Physiol. 95:295-306.

6. Kim, S., and N. N. Jorgensen. 1976. Formation of thymidine kinase and deoxyuridylate deaminase in synchronous cultures of Chinese hamster cells temperature-sensitive for DNA synthesis. J. Cell Physiol. 88:57-64.

7. Lee, A. S. 1981. The accumulation of three specific proteins related to glucose-regulated proteins in a temperature-sensitive mutant cell line K12. J. Cell Physiol. 106:119-125.

8. Shiu, R. F. C., J. Pouyssegur, and I. Pastan. 1977. Glucose depletion accounts for the induction of two transformation-sensitive membrane proteins in Rous sarcoma virus-transformed chick embryo fibroblasts. Proc. Natl. Acad. Sci. U. S. A. 74:3840-3844.
9. Lee, A. S., A. Delegeane, and D. Scharff. 1981. A highly conserved glucose-regulated protein found in hamster and chick cells and preliminary characterization of its cDNA clone. *Proc. Natl. Acad. Sci. U. S. A.* 78:4922-4925.

10. Melero, J. A., and A. E. Smith. 1978. Possible transcriptional control of three polypeptides which accumulate in a temperature-sensitive mammalian cell line. *Nature (Land.).* 272:722-727.

11. Lee, L. S., and Y. C. Cheng. 1976. Human deoxythymidine kinase. *J. Biol. Chem.* 251:2600-2604.

12. Marin, G., and T. Labella. 1977. Chromosome replication in somatic hybrids of mouse and temperature-sensitive Chinese hamster cells. *J. Cell Physiol.* 90:71-78.

13. Melero, J. A. 1979. Isolation and cell cycle analysis of temperature-sensitive mutants from Chinese hamster cells. *J. Cell Physiol.* 98:17-30.

14. Lowy, I., A. Pellicer, J. F. Jackson, G.-K. Sim, S. Silverstein, and R. Axel. 1980. Isolation of transforming DNA: cloning the Hamster aprl Gene. *Cell.* 22:817-823.