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

DEBRA J. SCHARFF, ANGELO M. DELEGEANE, and AMY S. LEE
Department of Biochemistry, University of Southern California, School of Medicine, Los Angeles, California 90033

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 *ts* 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 deoxycytidylate 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 deoxycytidylate 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 Biocell Laboratories (Carson, CA). Methyl-[^{3}H]thymidine and [^{3}H]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

THE JOURNAL OF CELL BIOLOGY · VOLUME 92 MARCH 1982 629-633

~4 × 10^6 cells/75 cm^2 flask and incubated at 35°C for several days. After confluence had been attained, fresh medium was added, and the cells were shifted to 40.5°C. The K12 cells started to detach from the culture surface after 48 h. Dead cells were removed by pouring off the spent medium, and cells which were able to grow at 40.5°C were selected. Revertant clones were obtained after incubation at 40.5°C for 10–15 d. Subsequently, the cells were grown into mass culture and expanded for analysis.

CHROMOSOME ANALYSIS: Subconfluent, exponential cells were incubated at 35°C with 0.1 μg/ml colcemid for 1 h and mitotic cells were detached by gently shaking in a buffer containing 25 mM TrisHCl, 140 mM KCl, 0.6 mM EDTA, pH 7.4. After a low-speed spin (800 rpm for 10 min), the pellet containing the mitotic cells was resuspended in 75 mM KCl for 30 min at room temperature. The cells were then fixed with methanol:acetic acid (3:1), spread over slides, and stained with Giemsa.

CELL SYNCHRONIZATION: Cells were seeded at 10^4 cells/cm^2 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-[^{3}H]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) (12,800 *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 $MgCl_2$, 1.9 mM ATP, 10 mM DTT, 10 mM NaF, 0.19 mM thymidine, 1% BSA, and 25 μCi methyl-[^{3}H]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 0.05 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 picomoles of thymidine (dThd) converted to thymidine monophosphate (dTMP) per microgram of protein in 10 min.

LABELING PROTEIN WITH [^{3}H]LEUCINE AND PROCESSING OF CELL EXTRACTS: Cells were labeled with 10 μCi/ml of [^{3}H]leucine and cytoplasmic protein was prepared as described (7). For the preparation of total cell lysate, the cell pellet was immediately resuspended in lysis buffer containing 8 M ultra pure urea (Schwartz), 2% (wt/vol) Nonidet P-40 and 0.5% β-mercaptoethanol. The labeled proteins were analyzed by PAGE. Conditions for running the gels and fluorography were described (7).

RESULTS

Isolation of Revertant Clones

Over a period of 1 yr, approximately 50 frozen aliquots of K12 cells (4 × 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,

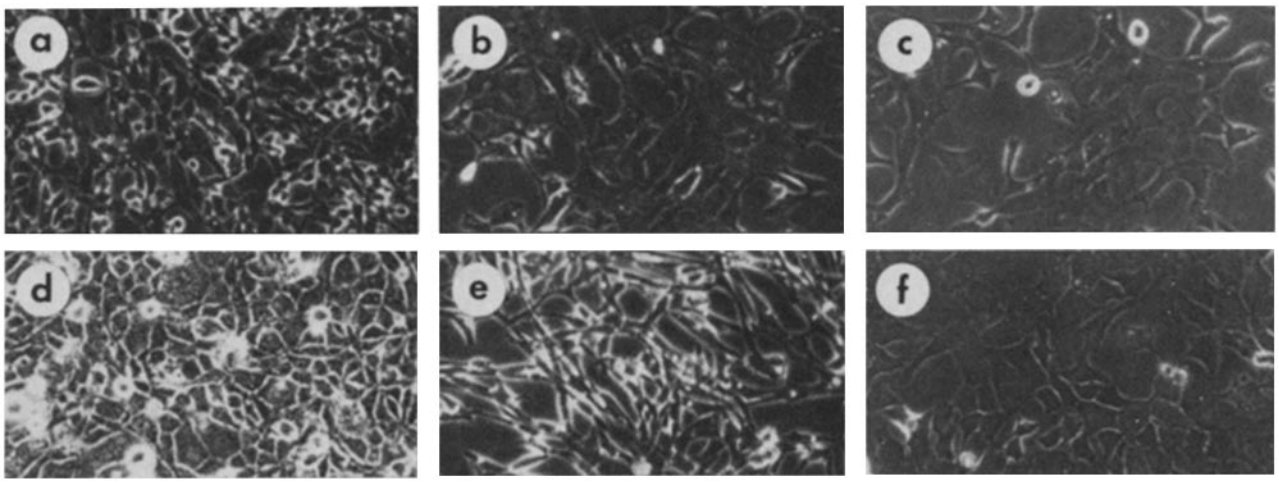

FIGURE 1 Morphologies of Chinese hamster fibroblast cells. Cells were seeded on plastic dishes and maintained in DMEM at the respective temperatures for 16 h. (*a*) Wg1A, 35°C; (*b*) K12, 35°C; (*c*) R12, 35°C; (*d*) Wg1A, 40.5°C; (*e*) K12, 40.5°C; and (*f*) R12, 40.5°C. The photographs were taken under phase contrast. × 100.

12) but subsequently became polyploid (5). The polyploid K12 cells exhibited the same mutant phenotype as pseudodiploid K12 cells. We have analyzed the chromosome distributions in these hamster cells: 90% of Wg1A cells have chromosome numbers in the range of 20–21, 75% of K12 cells have 35–37 chromosomes, and 82% of the R12 cells have 34–36 chromosomes (data not shown). These results eliminate the remote possibility that the R12 cells were derived from parental Wg1A cells contaminating the K12 cell culture.

Growth Properties

To test the growth properties of Wg1A, K12, and R12, sets of 60-mm dishes were seeded with the hamster cells. Half of the plates were incubated at 35°C and the other half at 40.5°C. On successive days, the number of cells on each dish was determined. The results obtained for the three cell lines are shown in Fig. 2. In the case of Wg1A cells, the increase in cell numbers was similar at either temperature. K12 cells divide at a rate similar to that of Wg1A cells at 35°C; however, at 40.5°C, the growth of the mutant cells was completely suppressed after the first day, and, by the end of the second day, <0.01% of the cells was still attached to the dish. By the third or fourth day, no viable cells remained. The revertant, R12, on the other hand, exbibited cell division rates similar to those of Wg1A cells at both incubation temperatures. Except for showing a slightly lower seeding efficiency as compared to Wg1A 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-[^{3}H]thymidine for 30 min to measure the rate of DNA synthesis. The results of such an analysis are shown in Fig. 3 *a*. When the cells were incubated continuously at 35°C, Wg1A, K12, and R12 cells all demonstrated an 8-h lag, followed by a wave of DNA synthesis which ended ~26 h after the stimulation of growth. In contrast to the K12 cells where DNA synthesis was completely suppressed at

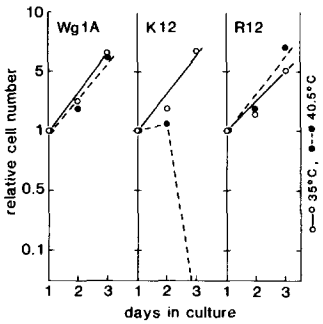

FIGURE 2 Growth curves of hamster cells. About 5 × 10^6 cells were seeded in each 60-mm culture petri dish and allowed to grow at 35°C for 1 d. Half of the cultures were then shifted to 40.5°C. On subsequent days, the cells were trypsinized and the number of cells was determined with a haemocytometer. (○——○) Cell number in cultures incubated at 35°C. (●——●) Cell number incubated at 40.5°C.

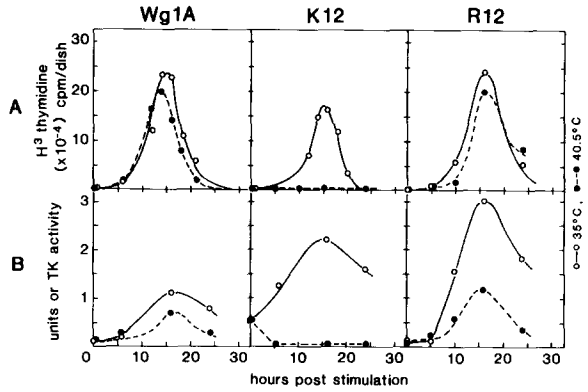

FIGURE 3 Measurement of rate of DNA synthesis and cytosol thymidine kinase activity during a cell cycle. Cells were synchronized in 60-mm petri dishes. At different times after addition of fresh medium, cultures were pulse-labeled for 30 min with methyl-[^{3}H]-thymidine and processed for determination of radioactivity incorporated into 5% 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 as described in Materials and Methods. (*A*) rate of DNA synthesis, as measured by the amount of methyl-[^{3}H]thymidine incorporated into the DNA. (*B*) Thymidine kinase activities. The activities were expressed as pmoles of dThd converted to TMP per microgram protein extract in 10 min. (○——○) cultures incubated at 35°C; (●——●) cultures incubated at 40.5°C.

40.5°C, R12 cells progressed through the cell cycle 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 Wg1A, K12, and R12 cells at both 35°C and 40.5°C using synchronized cultures. The results are shown in Fig. 4 *b*. We observed that there was a direct correlation between the enzyme activitiy 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 (Wg1A) at the peak of DNA synthesis was always about twofold. We also observed that the specific activitiy 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

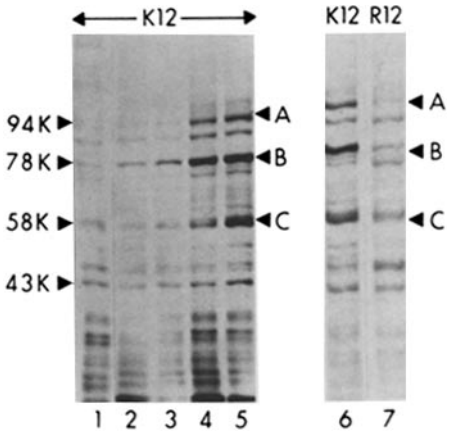

FIGURE 4 PAGE of proteins synthesized by K12 and R12 cells. Subconfluent cells were grown in DMEM and the proteins labeled with 10 μCi/ml [^{3}H]leucine. Cytoplasmic protein was prepared at the end of the labeling period as described (7). (Lane *1*) K12 labeled for 18 h at 35°C; (Lane *2*) K12 labeled for 5 h at 40.5°C; (Lane *3*) K12 labeled for 8 h at 40.5°C; (Lane *4*) K12 labeled for 16 h at 40.5°C; and (Lane *5*) K12 labeled for 24 h at 40.5°C. In a separate experiment, K12 and R12 cells were simultaneously labeled at 40.5°C for 16 h. Total cell lysate was prepared from these cultures: (Lane *6*) K12 cells at 40.5°C; and (Lane *7*) R12 cells at 40.5°C. About 3×10^4 cpm were applied to each gel lane. The proteins were separated by an 8.5% polyacrylamide gel. The slab gel was processed for fluorography as described (7). The molecular weights and positions of proteins *A*, *B*, and *C*, are as indicated.

provides an opportunity to test whether the overproduction of three proteins is a direct consequence of the K12 mutation, and whether the synthesis of the three proteins at 40.5°C is coordinately regulated. Therefore, K12 and R12 cells were labeled with [^{3}H]leucine at 35°C and 40.5°C, and the labeled proteins from cell extracts were analyzed on polyacrylamide gels. The results are shown in Fig. 4. When K12 cells were incubated at 40.5°C, three cellular proteins labeled A, B, and C were preferentially labeled and accumulated in K12 cells after several hours. In contrast, when R12 cells were similarly labeled at 40.5°C, specific labeling of all three proteins was reduced to the levels observed in Wg1A cells (7).

DISCUSSION

It is clear that although we were able to isolate spontaneous revertant clones from the K12 cells, the reversion frequency of the K12 mutation must be exceedingly low. Over a period of 1.5 yr during which the K12 cells were continuously grown and amplified in our laboratory, no cells able to survive incubation at 40.5°C for more than 3 d were observed except for the R12 isolate described here. Our observation is consistent with the low rate of spontaneous reversion ($<1.6 \times 10^{-7}$) reported earlier (2).

The precise nature of the K12 mutation is not known, although previous studies suggested that it affects a cell function essential for "commitment" of the cells to DNA synthesis (3–5). This event apparently takes place in the G1 phase of the cell cycle, as distinguished from the initiation of replication of individual replicons through the S phase.

Further work on the K12 system has demonstrated that the *ts* mutation does not lead to an overall cessation of protein or RNA synthesis (6). Nonetheless, the synthesis of two enzymes associated with DNA replication was specifically affected at either the transcriptional or post-transcriptional level. Since drugs which inhibit DNA synthesis, per se, do not prevent the formation of these enzymes, it is implied that the suppression of enzyme synthesis is a direct result of the *ts* mutation.

The enhanced synthesis of three cellular proteins of molecular weights 94, 78, and 58 kdaltons by K12 cells at 40.5°C is also a consequence of the *ts* mutation rather than an indirect effect due to the inhibition of DNA synthesis or incubation at the elevated temperature (5, 7, 9). Thus, in both wild-type cells and other cell cycle mutants derived from Wg1A in which DNA synthesis is also blocked, these three proteins were not overproduced at either incubation temperature (13). Furthermore, the K12 mutation is a genetic recessive and can be complemented by fusion with other cell cycle mutants (5).

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.

Our results obtained with the revertant R12 support the above hypothesis and provide new clues as to the nature of the *ts* lesion. The fact that we were able to isolate a spontaneous revertant suggests that the *ts* mutation is not due to a large deletion in the genomic nucleotide sequence. Secondly, the simultaneous reversion of all the phenotypes strongly suggests that a single mutational lesion underlies the pleiotropic mutant phenotype. Thirdly, the overproduction of the set of three cellular proteins may well be regulated by the same molecular mechanism. The coordinate nature of the induction is also exemplified by the simultaneous synthesis of the same set of proteins under other conditions such as glucose starvation.

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.

We thank Ms. V. Baker for excellent technical assistance, Drs. R. Mosteller for the suggestion of revertant isolation, R. H. Stellwagen for help in cell photography, and R. E. K. Fournier for many helpful discussions and critical review of the manuscript.

Our research was supported by a grant from the National Cancer Institute CA 27607. A. S. Lee is the recipient of a Junior Faculty Research Award from the American Cancer Society, JFRA24.

Received for publication 13 July 1981, and in revised form 23 October 1981.

REFERENCES

1. Siminovitch, L., and L. H. Thompson. 1978. The nature of conditionally lethal temperature–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 function in a Chinese hamster line affecting DNA synthesis. *J. Cell Physiol.* 82:339–348.
5. Melero, J. A., and V. Fincham. 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. Kit, S., and G. N. Jorgensen. 1976. Formation of thymidine kinase and deoxycytidylate deaminase in synchronized 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. P. 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 (Lond.).* 272: 725–727.
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 aprt Gene. *Cell.* 22:817–823.