A Temperature-sensitive Cell-cycle Mutant of Mammalian Cells, *tsJT16*, is Defective in a Function Operating Soon after Growth Stimulation

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Key words: cell cycle/*ts* mutant/ G_0 -S transition early events/signal transduction

ABSTRACT. A temperature-sensitive cell-cycle mutant, *tsJT16*, which has been isolated from Fischer rat fibroblasts, was defective in the function(s) that operated soon after growth stimulation. When G_0 -arrested *tsJT16* was stimulated to proliferate, it entered the S phase after 12–15 h at 34°C but failed to do so at 40°C. The function mutated in *tsJT16* was required to be normal for the first 4 h or less for cells to transit from the G_0 to S phase. The induction of cell-cycle-dependent genes such as *c-fos*, *c-myc* and ornithine decarboxylase was observed at both temperatures after growth stimulation. Although an increase in total protein synthesis occurred at both temperatures after growth stimulation, synthesis of one protein (p70) (pI 7.8 and Mr 70,000) was inhibited at 40°C. Synthesis of p70 was negligible in G_0 -arrested cells and blocked by actinomycin D in serum-stimulated cells at 34°C. These results suggest that *tsJT16* has a *ts* defect in one of the signal transduction processes to induce gene activation. *tsJT16* was also defective in progression of the G_1 phase of growing cells, consistent with the previous results in which growth stimuli were required at G_1 for continuation of proliferation.

A large body of evidence has been accumulating that different growth factors activate signal transduction pathways via different second messenger molecules and result in an induction of several cell-cycle-dependent genes whose products drive G_0 -arrested cells toward the S phase. Although it goes without saying that such a biochemical approach as stepwise analysis of a signal transduction process is fruitful in elucidating the mechanisms for initiation of cell proliferation, this approach has two major limitations: 1) it is less suitable for finding a new or unexpected sequence in signal transduction processes, and 2) it is not always easy to discriminate an essential or prerequisite event for cells to initiate proliferation from nonessential or accompanied events occurring in growth-stimulated cells.

The use of *ts* mutant cells theoretically complements these limitations, although it has its own limitations. A library of cell-cycle *ts* mutants should include clones defective in genes which are concerned with a wide variety of known and unknown steps of signal transduction processes for growth induction. In a *ts* mutant that fails to initiate proliferation at the nonpermissive temperature, the mutated function should be concerned with an

essential event for cell proliferation, leaving aside whether it acts directly or indirectly and specifically or nonspecifically. To date, no *ts* mutant of mammalian origin has been reported which had a *ts* mutation in early events after growth stimulation, in spite of the isolation of a large number of cell-cycle *ts* mutant clones (1).

In the present paper, we describe the first cell-cycle *ts* mutant of mammalian origin which has a defect in a function operating in the early stage, within 4 h or less, after growth stimulation. Although the precise biochemical step at which the mutated function operates is still unidentified, it is likely to be in one of the signal transduction processes from the cell surface to nuclei.

MATERIALS AND METHODS

Cells and cultures. *tsJT16* was isolated as previously reported (7) from Fischer rat embryo fibroblast line 3Y1 (9) and frozen-stored without further characterization. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Nissui, Tokyo) supplemented with 10% fetal bovine serum (M.A. Bioproducts, Md), penicillin G (100 units/ml), and streptomycin sulfate (100 µg/ml). 3Y1 was maintained at 37°C, and *tsJT16* was at the permissive temperature, 34°C. The nonpermissive temperature for *tsJT16* was 40°C. Routine

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checking by autoradiography (14) and DAPI staining (16) revealed no contamination with mycoplasmas.

Kinetic analysis. The cells were G₀-arrested by culturing a confluent monolayer in serum-free DMEM for 2–3 days. Arrested cells were stimulated for an induction of the G₀-S transition by replacing the culture fluid with fresh medium containing 10% fetal bovine serum. The progression toward the S phase was monitored by either autoradiography or by measuring radioactivity in acid-insoluble materials after labeling cells with [³H]thymidine (37 KBq/ml, 74 GBq/m mol, Amersham) as reported previously (11). Frequency of mitotic cells was measured under a microscope after fixation and Giemsa staining.

Northern blot analysis. Northern blot analysis was carried out, as described previously (8), by isolating total cytoplasmic RNA, size fractionating on agarose gel, transferring to nitrocellulose filter, hybridizing to nick-translated probes ($2\text{--}4 \times 10^8$ cpm/ μ g DNA), and exposing to Kodak X-Omat AR film. The plasmids used were pc3sp6vfos (carrying v-fos gene in a Riboprobe vector, a kind gift of Dr. S. Rittling), c-myc54 (carrying mouse c-myc gene, a kind gift of Dr. L. Kaszmarek) and pODC934 (carrying a mouse ornithine decarboxylase cDNA sequence (2), a kind gift of Dr. R. Baserga).

Protein synthesis and two-dimensional polyacrylamide gel electrophoresis. Cells in 35-mm plastic dishes were labeled for 1 h with [³H]leucine (111 KBq/ml, 185 GBq/m mol, New England Nuclear) in medium deficient in leucine with or without 10% dialyzed serum. After labeling, the cells were washed with phosphate-buffered saline and lysed in 0.5 N KOH. The lysate was kept overnight at room temperature and then precipitated with trichloroacetic acid. Radioactivities in acid-precipitable materials collected on a GF/c filter were measured in a liquid scintillation spectrometer.

For two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) of cellular protein, the cells plated in 60-mm dishes were labeled for 2 h with [³⁵S]methionine (TRAN³⁵S-LABEL, 1.1 MBq/ml, ICN) in DMEM deficient in methionine with or without 10% dialyzed serum. The cells were then washed with cold phosphate-buffered saline and solubilized in a solution containing 0.3% sodium laurylsulfate and 10% 2-mercaptoethanol. DNA was digested by incubating with DNase I (80 μ g/ml) for 5 min on ice. The lysate was mixed with solid urea (final concentration; 8.5 M) and an equal volume of a solution containing 8.5 M urea, 5% 2-mercaptoethanol, and 4% Nonidet P-40, and then applied on the first-dimensional gel. pH gradient was formed in the gel by Bio Lytes (Bio-Rad): 0.4% pH 3–7, 0.8% pH 5–7, and 0.8% pH 6–8. Two-dimensional gel electrophoresis was performed in 10% polyacrylamide gel according to the method of O'Farrell (10). After the run, the gels were fixed, stained with Coomassie brilliant blue, destained, impregnated with EN³HANCE (New England Nuclear), dried and exposed to Kodak X-Omat AR-5 film.

Fig. 1. Resumption of proliferation of *tsJT16* after serum stimulation. Confluent monolayers of *tsJT16* cells were growth-arrested in serum-free medium for 2 days and were stimulated to proliferate with serum. (a) Entrance into the S phase after serum stimulation of G₀-arrested *tsJT16* cells. The stimulated cells were cultured at 34°C (○) or 40°C (●) in the presence of [³H]thymidine. At the indicated times, the cells were fixed and the radioactivity was measured. (b) Changes in cell number following serum stimulation. The cells were stimulated at day 1 (arrow) and cultured at 34°C (○) or 40°C (●).

RESULTS

Kinetic analysis. *tsJT16* cells began to enter the S phase at 12–15 h poststimulation at 34°C, but at 40°C, neither entered the S phase (Fig. 1a) nor resumed proliferation (Fig. 1b). To determine the stage (execution point) at which progression of the cell cycle was blocked at 40°C, [³H]thymidine was added at the time of serum stimulation, and the cells were cultured at 34°C for various periods of time. Then the culture temperature was shifted up to 40°C until the end of the experiments at 34 h after stimulation, and the cultures were processed for autoradiography. Only the cells whose culture temperature was increased earlier than 4 h, or earlier than 2 h in another experiment, after serum stimulation were blocked against the G₀-S transition (Fig. 2). These results indicated that the gene mutated in *tsJT16* was required to operate normally only at a relatively early stage (at most, 4 h after serum stimulation) in the G₀-S transition.

When serum-stimulated *tsJT16* cells were cultured at 40°C for various periods of time and the culture temperature was shifted down to 34°C, the entrance into the S phase was delayed for the same periods as for those cultured at 40°C (Fig. 3). This indicated that the cells remained at the execution point as long as they were maintained at 40°C. When the cells were cultured for

Fig. 2. Execution point of *tsJT16*. G₀-arrested *tsJT16* cells were stimulated with serum and cultured in the presence of [³H]thymidine at 34°C. At the indicated times, the culture temperature was increased to 40°C until the end of the experiments, 34 h after serum stimulation. The cultures were fixed and processed for autoradiography.

much longer periods at 40°C, they began to die.

When *tsJT16* cells grown at 34°C in a log phase were transferred to 40°C, they stopped growing and began to die (Fig. 4). To determine the cell phase where *tsJT16* was blocked at 40°C, the progression of cells in S, G₂, and M phases was analyzed. *tsJT16* cells were synchronized at the G₁-S boundary at 34°C by the successive block with hydroxyurea and excess thymidine. Upon release from the block, they passed through the S phase (Fig. 5a) and M phase (Fig. 5b) at both 34°C and 40°C, except that the second peak of the S phase (15 h after the removal of hydroxyurea) was abolished at 40°C. These results indicated that the progression of *tsJT16* cells in the cell cycle was not blocked at the S, G₂, and M phases but was probably blocked at the G₁ phase. The precise execution point in the G₁ phase of the growth cycle was not determined because of a viability loss at 40°C.

Induction of cell-cycle-dependent genes. Accumulation of cell-cycle-dependent mRNAs was analyzed by Northern blot of total cytoplasmic RNA from cells stimulated from G₀ with serum. *c-fos*, *c-myc*, and ODC were reported to be induced in serum-stimulated 3Y1 cells at both temperatures (8) and were selected as examples of immediate early-, early-, and late-inducible genes, respectively. In *tsJT16* cells, *c-fos* mRNA accu-

Fig. 3. Delay of cell entry into the S phase by culturing at 40°C. G₀-arrested *tsJT16* cells were stimulated with serum and cultured in the presence of [³H]thymidine. The cells were cultured at 40°C for 6 h (○) or for 12 h (△) after the stimulation and then maintained at 34°C, or cultured at 34°C throughout (□). After 36 h, they were fixed and processed for autoradiography.

mulated quickly at 34°C after serum stimulation with a peak at 30 min and then decreased (Fig. 6a). The accumulation of *c-myc* mRNA increased at 34°C with a broad peak at around 4 h (Fig. 6b). An accumulation of mRNA also occurred at 40°C with some differences in peak time. The accumulation of ODC mRNA began at 4 h and peaked at more than 8 h after serum stimulation at 34°C (Fig. 6c). It was unexpected that ODC mRNA also accumulated at 40°C with a peak later than 8 h (Fig. 6c), because the kinetic experiment showed that a progression of *tsJT16* cells in the G₀-S transition was blocked at 40°C at the execution point, 2–4 h after serum stimulation.

Induction of protein synthesis. Partial inhibition of protein synthesis was known to block progression of the G₁ phase in growing cells as well as the G₀-S transition in serum-stimulated G₀ cells (3, 4, 15, 17). To see whether the *ts* mutation of *tsJT16* results in an inhibition of protein synthesis, incorporation of [³H]leucine into acid-insoluble materials was measured after serum stimulation at both temperatures. No inhibition was observed at 40°C (Fig. 7).

We then analyzed cellular proteins synthesized 1–3 h after serum stimulation by 2-D PAGE. Although an increase or a decrease was observed after serum stimula-

tion in several [³⁵S]methionine-labeled protein spots, most of these changes were observed similarly in both samples from cells stimulated at 34°C and 40°C. Among them, one protein spot (p70) (pI 7.8 and molecular weight 70,000) was clearly observed in *tsJT16* cells stimulated at 34°C (Fig. 8a), but it was not detected or scarcely detected in samples from unstimulated cells (Fig. 8b) or those stimulated at 40°C (Fig. 8c). In 3Y1, a parental line of *tsJT16*, p70 increased following serum stimulation at both temperatures (Figs. 8d and e). An increase in p70 synthesis was likely to be transcriptionally controlled, because it was blocked when *tsJT16* cells were stimulated at 34°C in the presence of actinomycin D (Fig. 8f). These results indicated that an induction of some cell-cycle-dependent genes was intact but that of other genes such as p70 was blocked in *tsJT16* cells after the stimulation with serum at 40°C.

Fig. 4. Growth curve of *tsJT16*. *tsJT16* cells were plated and cultured at 34°C. On the day indicated by the arrow, the culture temperature was shifted to 40°C (●) or unchanged (○).

Fig. 5. The progression of the S and M phases at 34°C and 40°C. Exponentially growing *tsJT16* cells were treated at 34°C with 2.5 mM of thymidine for 24 h. After removal of thymidine, they were cultured for 10 h and treated with 1 mM of hydroxyurea. Hydroxyurea was removed at time 0 and the cells were cultured at 34°C (○) or 40°C (●). (a) Progression of the S phase. The cells were pulse-labeled with [³H]thymidine for 1 h at the indicated times and then measured for radioactivity. (b) Progression of the M phase. The frequency of mitotic cells was measured by counting about 300 cells under a microscope.

DISCUSSION

The results reported here are summarized as follows: 1) when G_0 -arrested *tsJT16* cells were stimulated for growth with serum, the execution point was determined to be within 4 h, indicating that the mutated function in *tsJT16* is required for the first 4 h or less for cells to transit from the G_0 to S phase; 2) progression of the G_1 phase in the growth cycle was temperature sensitive, while that of the S, G_2 , and M phases was not; 3) when G_0 -arrested *tsJT16* cells were stimulated for growth at 40°C, the accumulation of mRNAs from such cell-cycle-dependent genes as *c-fos*, *c-myc*, and ODC was almost identical with that observed at 34°C, while the synthesis of a protein, p70, was inhibited; and 4) induc-

tion of p70 was likely to be transcriptionally controlled. Our preliminary experiments suggested that p70 was a primarily induced protein following serum stimulation and that the failure of p70 synthesis at 40°C was due to the blockage of mRNA synthesis rather than the blockage of posttranscriptional processes (to be published elsewhere). These results suggest that 1) the mutated function of *tsJT16* operates in a signal transduction process from receptor binding of the growth factor at the cell membrane to the stimulation of the transcription of particular genes in nuclei; and 2) a stimulation of transcription occurs via at least two different signaling pathways: one induces *c-fos*, *c-myc*, and ODC (this process is intact in *tsJT16*), and the other p70 (this is mutated in *tsJT16*).

When the culture temperature of *tsJT16* cells growing in a log phase at 34°C was shifted up to 40°C, the cells could not continue to proliferate. The progression of the S, G_2 , and M phases was not temperature sensitive, and the cell phase where the mutated function is concerned is probably the G_1 phase in the growing cell cycle. The mutated function of *tsJT16* is likely to be concerned not only with signal transduction process soon after serum stimulation of G_0 -arrested cells, but also with the stage sensitive to the serum in the G_1 phase of the growing cell cycle. In 3T3 cells, the regulatory events for cell cycle progression are located at the restriction point (R-point) in the G_1 phase (12, 13). When

Fig. 6. Northern blot analysis of *c-fos*, *c-myc* and ornithine decarboxylase mRNAs. G_0 -arrested *tsJT16* cells were serum-stimulated and cultured for indicated periods at 34°C or 40°C. Cytoplasmic total RNA was extracted and analyzed as described in Methods (15 μ g/lane). (a) *c-fos*, (b) *c-myc* and (c) ornithine decarboxylase.

Fig. 7. Incorporation of [3 H]leucine into acid-precipitable materials. G_0 -arrested *tsJT16* cells were serum-stimulated (○, ●) or unstimulated (□, ■) and cultured for indicated periods at 34°C (○, □) or 40°C (●, ■). The cells were labeled with [3 H]leucine for 1 h. Each value indicates a mean of two determinations.

Fig. 8. Analysis of protein by two-dimensional gel electrophoresis. Cells were labeled with [35 S]methionine for the last 2 h, and proteins [5×10^5 cpm] were analyzed by two-dimensional polyacrylamide gel electrophoresis. Arrows indicate p70 protein. The enclosed area of the whole gel (upper photograph) is shown in the lower photographs. (a) *ts*JT16 serum-stimulated and cultured for 3 h at 34°C, (b) G_0 -arrested *ts*JT16, (c) *ts*JT16 serum-stimulated and cultured for 3 h at 40°C, (d) 3Y1 serum-stimulated and cultured for 3 h at 34°C, (e) 3Y1 serum-stimulated and cultured for 3 h at 40°C, and (f) *ts*JT16 serum-stimulated and cultured for 3 h at 34°C in the presence of 10 μ g/ml of actinomycin D.

growing 3T3 cells were transferred into serum-free medium before the R-point in the cell cycle, they did not pass through the R-point but entered the G_0 phase (5). When 3T3 cells were shifted into serum-free medium after the R-point, they were able to progress through the remaining G_1 , S, G_2 , and M phases and then arrested in the G_0 phase. Cell kinetic experiments using low concentrations of cycloheximide revealed the hypothesis that the commitment of cells to pass the R-point is dependent upon the synthesis and accumulation of a labile protein (4, 15). Croy and Pardee reported such a candidate protein with molecular weight 68,000 (p68) that was labile and accumulated in the G_1 phase

(6). The p70 that we showed here had a similar molecular weight and was also serum dependent and labile (unpublished data). It is of interest to see whether the failure of the synthesis of p70 at 40°C is a major cause of the blockage of the G_0 -S transition and of G_1 progression.

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(Received for publication, September 8, 1989
and in revised form, November 9, 1989)