A Nuclear Labile Protein, p70, is Expressed Exclusively during G₀-S Transition but not in G₁ Phase of a Cell Cycle *ts* Mutant, *tsJT16*

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ABSTRACT. *tsJT16* is a cell cycle temperature-sensitive (*ts*) mutant from a Fischer rat cell line. When it is growth-stimulated from G₀ phase it enters S phase at the permissive temperature (34°C) but not at the nonpermissive temperature (40°C). It induces a nuclear labile protein, p70, when it is stimulated from G₀ phase at 34°C, but not at 40°C. In growing cell cycle it progresses through the S, G₂ and M phases at both temperatures but fails to pass through G₁ phase at 40°C. Here we described that p70 was synthesized neither in the randomly growing cycle nor in the G₁ phase synchronously progressing from M phase. The cells synchronized at early G₁ phase by culturing in serum-free medium for 7.5 h from G₁/S boundary induced *c-fos* and *c-myc* following serum addition, but under the same condition p70 was not synthesized. These results indicate that the synthesis of p70 is not required for progression of the G₁ phase of the growing cycle and can be used as an exclusive marker of G₀-S transition.

In 3T3 cells, the regulatory events for cell cycle progression are located at the restriction point (R-point) in the late G₁ phase (9). When growing 3T3 cells were transferred into serum-free medium before the R-point in the cell cycle, they did not pass through R-point and entered G₀ phase (2, 14). When 3T3 cells were shifted into serum-free medium after R-point, they were able to progress through the remaining G₁, S, G₂ and M phases and were then arrested in the G₀ phase. Cell kinetic experiments using several cell lines treated with low concentrations of cycloheximide revealed the hypothesis that the commitment of cells to pass through the R-point regardless from M or G₀ phase was dependent on the synthesis and accumulation of labile proteins (1, 3, 6, 10, 11). Croy and Pardee reported that such a candidate protein with molecular weight 68,000 (p68) was synthesized and accumulated serum-dependently in G₁ phase (4). p68 was a labile protein with half life of about 2 h. Transformed 3T3 cells synthesized high levels of stable p68 and were free from R-point regulation.

tsJT16 is a G₀/G₁ *ts* mutant of cell cycle derived from Fischer rat fibroblasts and is characterized as follows (12, 13). 1) When it is stimulated from G₀ phase with serum, it enters S phase at the permissive temperature (34°C) but fails to do so at the nonpermissive temperature (40°C). 2) After serum stimulation from G₀

phase, the accumulation of mRNAs from such cell cycle-dependent genes as *c-fos*, *c-myc* and ornithine decarboxylase occurs almost in the same way at both temperatures. 3) The synthesis of a nuclear labile protein, p70, is induced at 34°C but not at 40°C following serum stimulation. 4) The synthesis of p70 is transcriptionally controlled. 5) The synthesis of p70 is maximum at around 2 h and continues for about 5 h after serum stimulation and diminishes before entering S phase, and 6) In addition to G₀-S transition, progression of G₁ phase in growing *tsJT16* is also temperature sensitive.

Both p68 of 3T3 and p70 of *tsJT16* are labile proteins with similar molecular weight, synthesized serum-dependently, accumulated in G₀-S transition phase, and are thought to be concerned with growth regulation. At 40°C, *tsJT16* cells failed to progress from M to S phase as well as from G₀ to S phase, and failed to synthesize p70 when stimulated from G₀ phase. p70 as well as p68 is a candidate protein that regulates G₀-S or G₁-S progression of the cell cycle. The purposes of the present paper are to determine 1) whether p70 is synthesized in G₁ phase of growing cell cycle, 2) whether the synthesis of p70 at G₁ phase, if it occurs, is temperature sensitive, and 3) whether p70 is synthesized at a high level in SV40-transformed *tsJT16* cells.

MATERIALS AND METHODS

Cells and cultures. A temperature-sensitive mutant of

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Fischer rat cell line, *tsJT16*, was cultured as described elsewhere (12). SV40-transformed clones were isolated as described previously (7). In this study they were cultured at 34°C (permissive temperature) unless otherwise indicated. CY1, a clone of adenovirus-transformed 3Y1, a gift from Dr. K. Shiroki, Institute of Medical Science, Tokyo University, was cultured at 37°C.

Analysis of cellular proteins and mRNAs. Labeling of cellular proteins with [³⁵S]methionine and analysis of p70 protein by 2-dimensional polyacrylamide gel electrophoresis were carried out as described elsewhere (12). Isolation and Northern blot analysis of cellular RNA were done as previously reported (12).

Synchronization of cells. *tsJT16* cells were growth-arrested at G₀ phase by culturing subconfluent cultures in serum-free medium for 2–3 days (12, 13).

For the mitotic synchronization, *tsJT16* was partially synchronized at G₁/S boundary by the treatment of randomly growing cells with 2.5 mM thymidine for 24 h. After wash out of thymidine, the cells were cultured for 8 h and then synchronized at M phase with 10 ng/ml of colcemid in calcium-free medium for 3 h. Mitotic cells were detached by pipetting and replated in fresh medium.

For the early G₁ synchronization, randomly growing *tsJT16*

cells were first synchronized partially at G₁/S boundary by culturing in the presence of 2.5 mM thymidine for 22 h. When they were released and cultured in serum-free medium for 7.5 h, they passed through S, G₂, and M phases and reached the early G₁ phase.

RESULTS

Growing *tsJT16* did not synthesize p70. Synthesis of p70 was hardly detected in G₀-arrested *tsJT16* cells (Fig. 1a). When growth of G₀-arrested cells was stimulated to grow with serum, synthesis of p70 was induced at 34°C (Fig. 1b). No growing *tsJT16* cells in log phase were found to synthesize p70 (Fig. 1c).

Transformed *tsJT16* did not synthesize p70. *tsJT16* cells were transfected with plasmid containing SV40 T antigen gene, and transformed foci were isolated. Growth of these transformed cells were temperature sensitive like their parental *tsJT16* (data not shown). No expression of p70 in growing transformants was detected at 34°C (Fig. 1d–f) or in CY1 cells, which are adenovirus-transformed 3Y1 (Fig. 2). These transformed cells were not growth-arrested even when they were cultured in serum-free medium, and we had no way to analyze

Fig. 1. Synthesis of p70 protein.

The cells were labeled with [³⁵S]methionine for 2 h and whole cell proteins were analyzed by two dimensional polyacrylamide gel electrophoresis. a) G₀-arrested *tsJT16*, b) *tsJT16* labeled from 1 through 3 h after serum stimulation from G₀ phase, c) randomly growing *tsJT16*, d) SV40-transformed *tsJT16* clone 2-2, e) SV40-transformed *tsJT16* clone 2-3, and f) SV40-transformed *tsJT16* clone 5-2. Arrow indicates p70.

Fig. 2. Synthesis of p70 in CY1 cells.

CY1 was labeled with [³⁵S]methionine for 2 h and whole cell proteins were analyzed by two dimensional polyacrylamide gel electrophoresis. a) CY1 cells cultured in serum-free medium for 2 days, b) CY1 cells serum-stimulated for 3 h from serum-deprived state and labeled from 1 through 3 h, and c) CY1 cells which were partially synchronized at G₁/S boundary by excess thymidine and then labeled from 1 through 3 h after the release.

p70 synthesis after the stimulation from G₀-phase.

tsJT16 did not synthesize p70 during G₁ phase from synchronized M phase. p70 was induced only transiently after serum stimulation of G₀-arrested *tsJT16* (13). If p70 was also synthesized transiently in the G₁ phase of the growing cycle, p70 synthesis might not be detected due to its dilution in randomly growing population. To see whether *tsJT16* cells synthesized p70 in G₁ phase, the cells were collected at mitosis and replated. They began to enter S phase after 6 h (Fig. 3). Synthesis of p70 during G₁ progression was monitored at 2 h-intervals by labeling cells for 2 h with [³⁵S]methionine. Again,

Fig. 3. Progression toward S phase of *tsJT16* from M phase. *tsJT16* was synchronized and collected at M phase. At various intervals after replating, they were labeled with [³H]thymidine for 1 h and processed for autoradiography.

synthesis of p70 was not undetected throughout the G₁ phase (Fig. 4).

Serum-stimulated tsJT16 at early G₁ phase did not synthesize p70. Along with p70 expression, *c-fos* and *c-myc* were also expressed transiently after serum stimulation of G₀-arrested *tsJT16* (12), but their expression in growing cells were at low or undetectable levels. When

Fig. 4. Synthesis of p70 in *tsJT16* synchronously progressing from M phase.

tsJT16 synchronously progressing from M phase was labeled with [³⁵S]methionine for 2 h a) from 1 to 3 h, b) from 3 to 5 h, c) from 5 to 7 h. d) G₀-arrested *tsJT16* stimulated with serum for 3 h and labeled for the last 2 h.

DISCUSSION

The findings in this paper may be summarized as follows: the synthesis of p70 was not detected even at 34°C in 1) randomly growing *tsJT16*; 2) randomly growing SV40-transformed *tsJT16*; 3) *tsJT16* in G₁ phase synchronously progressing from M phase; and 4) *tsJT16* in G₁ phase progressing from early G₁ phase. The present and the previous results (12, 13) indicate that p70 is synthesized exclusively in *tsJT16* cells growth-stimulated from G₀ phase at 34°C. These characteristics of p70 are quite different from those of p68 reported by Croy and Pardee (4) that is expressed in G₁ phase and is overexpressed in transformed cells. Although the induction of *c-fos* and *c-myc* is how used as a marker of growth induction, it was also observed in G₁ phase as shown in Fig. 5 and may be a marker of serum addition rather than that of growth induction from G₀ phase (8). By contrast, p70 could serve as a better marker of early G₀-S transition irrespective of its function. A G₀-specific *ts* mutant, *tsJT60*, which does not enter the S phase from G₀ phase at 40°C but runs the cell cycle normally at both temperatures (5), synthesized p70 after the stimulation with serum from G₀ phase at 40°C (data not shown).

tsJT16 cells synchronized at G₁/S boundary were cultured for 7.5 h in serum-free medium, they passed through the S, G₂ and M phases and reached the early G₁ phase. These cells in early G₁ phase induced *c-fos* and *c-myc* following serum-addition (Fig. 5), although the extent of induction was different from that in cells stimulated from G₀ phase. This finding is consistent with the previous results reported in 3Y1 cells (8). In contrast, p70 was not induced either *tsJT16* cells following serum-stimulation (Fig. 6), or in SV40-transformed *tsJT16* under the same condition (data not shown).

tsJT16 is a G₀/G₁ *ts* mutant that does not enter S phase at 40°C from either G₀ and M phases and is likely to be defective in one of the transduction processes of growth signal (12). When it is growth-stimulated from G₀ phase, p70 is induced transiently at 34°C but not at 40°C whereas other cell cycle dependent genes as *c-fos*, *c-myc* and ornithine decarboxylase are induced at both temperatures (12). Although the failure of the synthesis of p70 has been speculated to be a possible cause of blockage of the G₀-S transition, p70 is not synthesized in G₁ phase of growing cycle even at 34°C inspite of the

Fig. 6. Synthesis of p70 in *tsJT16* synchronized at early G₁ phase.

The cells were labeled with [³⁵S]methionine for the last 2 h and whole cell proteins were analyzed by two dimensional polyacrylamide gel electrophoresis. a) G₀-arrested *tsJT16*, b) *tsJT16* serum-stimulated for 3 h from G₀ phase, and c) *tsJT16* which has been synchronized at early G₁ phase in serum-free medium and stimulated with serum for 3 h.

temperature sensitivity of G₁ progression. By the analysis of silver-stained 2-dimensional gel pattern of 1%-Triton extracted nuclear proteins, p70 was present in *ts*JT16 stimulated with serum for 2 h from G₀ phase at 34°C but not in G₀-arrested and randomly growing *ts*JT16 cultures at 34°C, although quantitative data were not obtained because of its very low content (less than 0.01% of 1%-Triton extracted nuclear proteins by densitometric analysis of the stained gel). These results indicated that progression of G₁ phase is independent from p70 synthesis and that the absence of p70 expression in G₁ phase could not be a cause of G₁ block of *ts*JT16 cells at 40°C. At present, there are two possible explanations for that: 1) induction of p70 is essential for G₀-S transition but not for G₁ progression in growing cycle; and 2) it is not essential for either G₀-S transition and G₁ progression. A functional mutation in *ts*JT16 might also be related to the induction of genes other than p70 which are essential for the progression of G₁ periods and are induced temperature dependently. We are now investigating for these genes by differential hybridization of cDNA libraries prepared from mRNAs synthesized at 34°C and 40°C.

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