Journal of Cellular Biochemistry 55:503–512 (1994)

Induction of c-fos and c-jun mRNA at the M/G_1 Border Is Required for Cell Cycle Progression

Stephen C. Cosenza, Gladys Yumet, Dianne Robert Soprano, and Kenneth J. Soprano

Departments of Microbiology and Immunology (S.C.C., G.Y., K.J.S.), Biochemistry (D.R.S.), and the Fels Institute for Molecular Biology and Cancer (S.C.C., D.R.S., K.J.S.), Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Abstract The proto-oncogenes c-fos and c-jun have been shown in numerous model systems to be induced within minutes of growth factor stimulation, during the G_0/G_1 transition. In this report we use the mitotic shake-off procedure to generate a population of highly synchronized Swiss 3T3 cells. We show that both of these immediate-early, competence genes are also induced during the M/G_1 transition, immediately after completion of mitosis. While c-fos mRNA levels drop to undetectable levels within 2 hr after division, c-jun mRNA levels are maintained at a basal level which is ~30% maximum throughout the remainder of G_1. In order to access the functional significance of these patterns of c-fos and c-jun expression, antisense oligodeoxynucleotides specific to c-fos or c-jun were added to either actively growing Swiss 3T3 cells or mitotically synchronized cells, and their ability to inhibit DNA synthesis and cell division determined. Our results show that treatment of Swiss 3T3 cells with either c-fos or c-jun antisense oligodeoxynucleotides, while actively growing, during mitosis, or in early G_1, results in a reduction in ability to enter S and subsequently divide. This was also true if Swiss 3T3 cells were treated during mid-G_1 with c-jun antisense oligodeoxynucleotides. These results demonstrate that the regulation of G_1 progression following mitosis is dependent upon the expression and function of the immediate-early, competence proto-oncogenes c-fos and c-jun.

Key words: cell cycle, immediate-early competence genes, antisense, mitotic selection, Swiss 3T3 cells

The analysis of the temporal patterns of gene expression following the stimulation of growth in quiescent cells has long been accepted as a valuable tool for studying the genetic regulation of the cell cycle [for review see Denhardt et al., 1987]. Using a mechanical shake-off procedure to generate a highly synchronized population of mitotic cells, our laboratory identified the precise temporal patterns of expression of growth-associated genes such as JE, c-myc, ornithine decarboxylase, p53, vimentin, and calcyclin during the G_1 phase of active growth [Cosenza et al., 1991]. In the present work, we have extended these studies to include two important immediate-early competence genes, c-fos and c-jun. The proto-oncogenes c-fos and c-jun are members of a family of nuclear proteins which are growth factor responsive and function as components of the transcriptional transactivator complex AP-1 [Bochman et al., 1987; Chiu et al., 1988; Curran et al., 1985; Maki et al., 1987; Nishimura and Vogt, 1988; Rauscher et al., 1988a,b]. Both c-fos and c-jun mRNAs are rapidly and transiently induced early in G_1 when quiescent fibroblasts are stimulated to enter the cell cycle by treatment with a variety of agents including fetal bovine serum (FBS), purified growth factors, and the tumor promoter TPA [Bravo et al., 1986; Cochran et al., 1984; Greenberg and Ziff, 1984; Lamph et al., 1988; Ryseck et al., 1988; Ryder and Nathans, 1988]. The results presented in this paper show that both c-fos and c-jun mRNAs are also induced in actively proliferating Swiss 3T3 cells during early G_1, immediately after completion of mitosis. Moreover, while c-fos levels drop to undetectable amounts within 3 hr of mitosis, a basal level of c-jun mRNA was maintained throughout the remainder of G_1.

In order to access the functional importance of the patterns of c-fos and c-jun expression during active growth, antisense oligodeoxynucleotides specific to c-fos and c-jun were added to either actively proliferating or mitotically synchronized Swiss 3T3 cells, and their ability to

Received February 2, 1994; accepted March 7, 1994.

Address reprint requests to Dr. Kenneth J. Soprano, Department of Microbiology and Immunology, Temple University School of Medicine, 3400 North Broad Street, Philadelphia, PA 19140.

inhibit DNA synthesis and division determined. Our results show that if Swiss 3T3 cells are treated with antisense oligodeoxynucleotides specific for c-fos or c-jun while actively growing or during mitosis and early G_1, they exhibit a reduction in ability to enter S and subsequently divide. This was also true if Swiss 3T3 cells were treated with c-jun antisense oligodeoxynucleotides during mid-G_1. These results demonstrate that the regulation of G_1 progression following mitosis (i.e., in actively proliferating cells) is dependent at least in part upon the expression and function of these two immediate-early, competence genes.

METHODS

Cell Culture

Stock cultures of Swiss 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine *serum*, L-glutamine, and antibiotics at 37°C in a humidified atmosphere containing 10% CO_2.

Oligodeoxynucleotides

All antisense and sense oligodeoxynucleotides were synthesized by an ABI 380B synthesizer on a scale of 1.0 μmole per synthesis. The c-fos oligodeoxynucleotides were all 18mers corresponding to codons 3–8 of the mouse cDNA. The c-jun oligodeoxynucleotides were 18mers corresponding to codons 2–7 of the mouse cDNA. The sequences of all oligodeoxynucleotides were as follows: c-fos antisense ($5'$GGC GTT GAA ACC CGA GAA$3'$); c-fos sense ($5'$CCG CAA CTT TGG GCT CTT$3'$); c-fos mismatch ($5'$GGC *A*TT *T*AA *G*CC *A*GA$3'$); c-jun antisense ($5'$CGT TTC CAT CTT TGC AGT$3'$); c-jun sense ($5'$GCA AAG GTA GAA ACG TCA$3'$); c-jun mismatch ($5'$CGT *G*TC *A*AT *A*TT *G*GC AGT$3'$). The oligodeoxynucleotides were dried in a speed-vac, resuspended in water, ethanol precipitated, and resuspended in Tris-EDTA. The concentration was determined spectrophotometrically. Oligodeoxynucleotides were added directly to culture medium at a concentration of 80 μg/ml. This equals a final concentration of approximately 10 μM.

Synchrony

The large scale mitotic shake-off procedure of Borrelli et al. [1987] used to obtain highly synchronized Swiss 3T3 cells has been published in detail previously [Cosenza et al., 1991]. For the studies described in this report, mitotic Swiss 3T3 cells were plated onto laminin-treated coverslips contained in 60 mm tissue culture dishes. Extent of synchrony was routinely assayed for each experiment by determination of the mitotic index of cells at the time of harvest and at various times after plating.

Determination of Entry Into DNA Synthesis

Entry into S was determined in cells plated on glass coverslips by continuous labeling with 0.5 μCi/ml ^{3}H-thymidine or pulse labeling for 1 hr with 7.5 μCi/ml ^{3}H-thymidine. Following the labeling period, coverslips were harvested, fixed in cold absolute methanol, and prepared for autoradiography [Baserga and Malamud, 1969] as we have described previously [Cosenza et al., 1988, 1991; Carter et al., 1991; Owen et al., 1987]. The percent labeled nuclei was determined for each coverslip by analysis of at least 500 cells. All determinations were performed in triplicate.

Determination of Cell Growth

Cell growth and viability was determined by removal of cells from tissue culture plates by trypsinization, staining with trypan blue, and counting using a hemocytometer. All cell counts were done in triplicate.

RNA Isolation

Total cytoplasmic RNA was isolated by the method of Tushinski et al. [1977] as we have described previously [Cosenza et al., 1988, 1991; Carter et al., 1991; Owen et al., 1987]. RNA was quantitated by absorbance at 260 nm and purity was assessed by absorbance at 280 nm. Quantitation and purity of each sample was confirmed by analysis on agarose-formaldehyde gels stained with ethidium bromide.

Analysis of Gene Expression

The steady state levels of c-fos, c-jun, and β-2 microglobulin were determined by either RNAse protection assay or Northern blot hybridization using probes and methods that we have described in detail previously [Cosenza et al., 1988, 1991; Carter et al., 1991; Owen et al., 1987; Toscani et al., 1987]. Hybridization signals were quantitated by scanning appropriate exposures of autoradiographs with a Hoefer model 1650 scanning densitometer followed by integration of the resulting peaks with a Hoefer GS365

computer software system. Sample to sample variation in RNA concentration was normalized by determination of differences in the levels of β-2 microglobulin, a transcript known to remain constant throughout the cell cycle [Toscani et al., 1987; Owen et al., 1987; Cosenza et al., 1991].

RESULTS

Kinetics of Entry into S Phase After Replating Mitotically Synchronized Swiss 3T3 Cells

Swiss 3T3 cells were synchronized in mitosis by the large scale mechanical shake-off method which we have previously described [Cosenza et al., 1991]. We have previously shown that this method yields a virtually pure population (>90%) of mitotic cells, nearly all of which (>95%) complete mitosis within 30 min of plating into conditioned medium. These cells then progress through G_1 and begin to enter S at 5 hr after plating. The majority of cells undergo DNA synthesis by 8–9 hr. Figure 1 shows the kinetics of entry into DNA synthesis following plating of mitotic Swiss 3T3 cells into conditioned medium. These data confirm our previously reported kinetic results. It is clear that this method provides a highly synchronized population of cells which exhibit a G_1 phase of approximately 6–7 hr. This G_1 phase can be somewhat arbitrarily subdivided into early G_1 (hours 1–2), mid-G_1 (hours 3–5), and late G_1/S (hours 6–7).

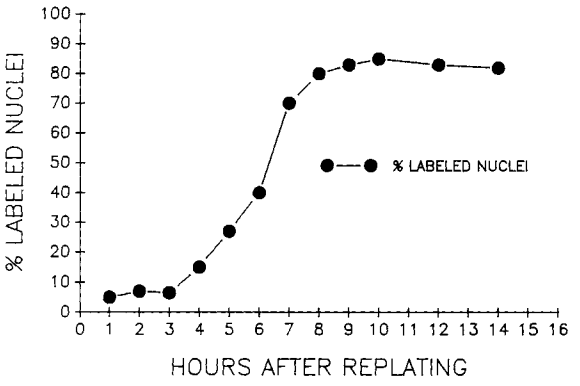

Fig. 1. Kinetics of entry into S phase after replating mitotically synchronized Swiss 3T3 cells. Swiss 3T3 cells synchronized by large scale mitotic shake-off were immediately replated onto glass coverslips in prewarmed conditioned medium containing 0.5 μCi/ml ^{3}H-thymidine. At the indicated times, coverslips were harvested, fixed, and prepared for autoradiography. The percentage of labeled nuclei was determined by analysis of at least 500 cells.

Expression of c-fos and c-jun During G_1 in Mitotically Synchronized Swiss 3T3 Cells

Expression of competence genes such as c-fos and c-jun were among the first growth-associated molecular events to be mapped in G_0/G_1 model systems [Bravo et al., 1986; Cochran et al., 1984; Greenberg and Ziff, 1984; Lamph et al., 1988; Ryseck et al., 1988; Ryder and Nathans, 1989]. One somewhat controversial issue has been whether competence genes play an important role in regulating G_1 progression during active growth. As a first step in addressing this question, we examined the expression of c-fos and c-jun during G_1 in our highly synchronized Swiss 3T3 cell model system. Total RNA was isolated at frequent time intervals following plating of mitotically selected cells. Steady state levels of c-fos and c-jun were determined by RNAse protection and Northern blotting, respectively. Expression of β-2 microglobulin was used as an internal, nongrowth-associated, normalizing control transcript. Figure 2A shows representative autoradiographs. Appropriate exposures of these autoradiographs were quantitated and the data plotted as percent maximum expression for each transcript in Figure 2B.

A close look at the patterns exhibited by each transcript shows slight differences in both the levels observed in mitotic cells and more striking differences in the pattern of expression during G_1, following maximum induction. Figure 2 shows that significant levels of both c-fos and c-jun mRNA (representing 25% and 50%, respectively, of maximum) were detected in mitotic cells. It is possible that a portion of these levels might reflect the presence of nonmitotic contaminating cells. However, this would not account for the entire c-fos and c-jun hybridization signal since the mitotic sample routinely contains no more than 5% nonmitotic cells. It can also be seen that for both c-fos and c-jun there is an immediate increase in steady state mRNA levels during early G_1, 1.0–1.5 hr after replating. Finally, it should be noted that although both genes were rapidly induced with similar kinetics following completion of mitosis and entry into G_1, the patterns of expression after the time of maximum induction differed. While the levels of c-fos were reduced to barely detectable amounts by 3 hr and remained low, the levels of c-jun dropped to a point about 25% of maximum and remained at that level through G_1. Levels of

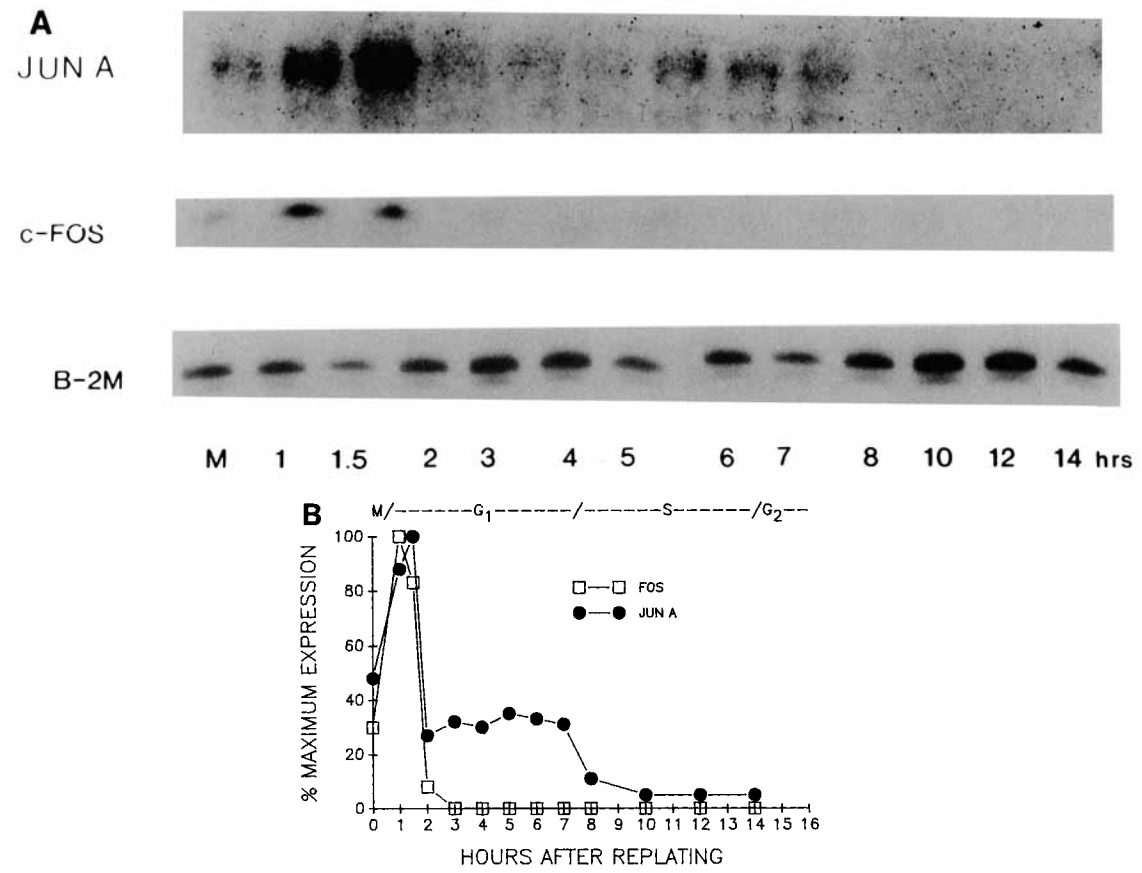

Fig. 2. Expression of c-fos and c-jun in mitotically synchronized Swiss 3T3 cells. Mitotically synchronized Swiss 3T3 cells were isolated by the large scale mitotic shake-off procedure and immediately replated into prewarmed conditioned medium. At the indicated time points, cells were harvested and total RNA isolated. **A:** Representative autoradiographs of (top) a Northern blot hybridized to a 650 bp oligolabeled probe of mouse c-jun; (middle) an RNAse protection assay using a ^{32}P-labeled cRNA probe of mouse c-fos; and (bottom) an RNAse protection assay using a ^{32}P-labeled cRNA probe of mouse β-2 microglobulin. **B:** Appropriate exposures of the autoradiographs shown in A were scanned densitometrically and quantitated using Hoeffer GS365 computer software. The relative integrated density of each band was determined for c-fos and c-jun and this value was divided by the relative integrated density value of the β-2 microglobulin band for the same sample. The resulting number was then expressed as the percentage of the highest value and plotted versus time.

c-jun were further reduced to barely detectable amounts as the cells entered S.

It could be argued that the process of attachment to the tissue culture plates induces an artifactual increase in c-fos and c-jun expression which is not related to the M/G_1 transition or G_1 progression. In order to directly address this possibility, we plated mitotic cells in serum-free medium. Under such conditions, mitotic cells attach to the culture dish but do not complete mitosis. After 2 hr, the cells were harvested, RNA isolated, and levels of c-fos and c-jun mRNA determined. No induction in c-fos or c-jun mRNA expression was observed in Swiss 3T3 cells that remained in M phase and failed to enter G_1 (data not shown).

These experiments show that there is, indeed, an immediate induction of competence genes such as c-fos and c-jun after mitosis.

c-fos and c-jun Antisense Oligodeoxynucleotide Treatment Inhibits the Growth of Swiss 3T3 Cells

Our initial experiments established that the competence genes c-fos and c-jun were maximally induced in actively proliferating cells, early in G_1, immediately after completion of mitosis. These results suggested that induction of these genes may in fact be important for the progression of cells through G_1 following mitosis as well as following growth arrest. In order to investigate this further, we next determined the effect of downregulating the expression of c-fos and

c-jun on cell division. We employed antisense oligodeoxynucleotides complementary to the sense strand of codons 2–7 for mouse c-jun and codons 3–8 for mouse c-fos. In addition, two types of control 18-mer oligodeoxynucleotides were used: (1) sense oligodeoxynucleotides complimentary to the antisense strand of c-fos and c-jun; and (2) c-fos and c-jun antisense oligodeoxynucleotides containing four mismatched bases. The sense controls were used to account for any non-specific detrimental effects on cell growth caused by addition of high concentrations of oligodeoxynucleotides to Swiss 3T3 cell cultures. The mismatch controls were used to confirm the specificity of the antisense treatment.

To determine the effect of these oligodeoxynucleotides on cell growth, 2.2×10^5 Swiss 3T3 cells were plated into 60 mm tissue culture dishes in fresh DMEM supplemented with 10% FBS. Twenty-four hours after plating, the cells were treated with oligodeoxynucleotides at a final concentration of 10 μM. A second treatment (10 μM) was added to any remaining plates at 48 hr after plating. At 24 hr intervals, two plates from each experimental group of cells were trypsinized and counted in a hemocytometer. Regardless of whether the treatment consisted of antisense, sense, or mismatch oligodeoxynucleotides, cell viability was always greater than 98% (data not shown).

As shown in Figure 3, the two treatments of 10 μM c-fos (solid circles) or c-jun (open triangles) antisense oligodeoxynucleotides had a significant effect on the proliferation of Swiss 3T3 cells. Within 24 hr after the second antisense treatment (3 d after plating), cell number was reduced compared to control treated cells (squares). Moreover, in contrast to the controls, the cell number did not continue to increase significantly during the next 2 d. Therefore, over the 5 d course of the experiment, there was a ~40% reduction in cell number when actively growing Swiss 3T3 cells were treated with antisense oligodeoxynucleotides specific for either c-jun or c-fos; however, when cells were treated with the same amount of sense or mismatch control oligodeoxynucleotides, no significant reduction in cell number was observed. In order to be able to compare inhibition of growth by antisense treatment to a more standard, well established method of growth inhibition, we also determined the effect of switching the untreated cells to medium containing 0.5% FBS. Figure 3

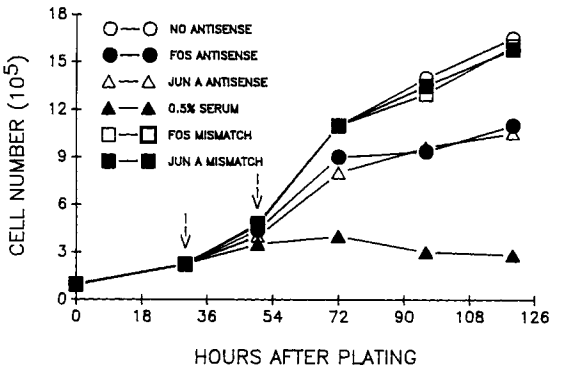

Fig. 3. Effect of c-fos and c-jun antisense oligodeoxynucleotide treatment on the growth of Swiss 3T3 cells. Actively proliferating Swiss 3T3 cells were plated at a concentration of 1.5×10^5 cells per 60 mm dish in fresh complete medium. At the times indicated by the arrows, the original plating medium was removed and replaced with fresh complete medium containing 10 μM of the appropriate oligodeoxynucleotide. In addition, one experimental group received fresh medium containing 0.5% FBS. After an additional 22 hr, the cells received a second 10 μM treatment of the appropriate oligodeoxynucleotide. Viable cell numbers were determined at each time point by counting in a hemocytometer. Data represent the mean of two experiments.

(solid triangles) shows that low serum treatment reduces growth more rapidly and, as a result, total cell number was reduced to a greater extent than by the antisense treatment (~80% vs. 40%).

c-fos and c-jun Antisense Oligodeoxynucleotide Treatment Inhibits Entry into DNA Synthesis by Actively Growing Swiss 3T3 Cells

We next determined the effect of antisense oligodeoxynucleotide treatment specific for c-fos and c-jun on the entrance of actively proliferating Swiss 3T3 cells into DNA synthesis. Actively growing Swiss 3T3 cells were plated onto coverslips in fresh DMEM containing 10% FBS. The cells were then treated with either 0.5% FBS or the appropriate oligodeoxynucleotide. At various times after treatment, coverslips were transferred to a separate culture dish and pulsed for 1 hr with 7.5 μCi ^{3}H-thymidine/ml and then harvested, fixed, and prepared for autoradiography. The results of this experiment are shown in Figure 4. In all three sets of controls, the percentage of labeled nuclei at any time during this experiment averaged ~53% (open circles). It can also be seen that treatment with either c-fos– or c-jun–specific antisense oligodeoxynucleotides (open triangles and solid circles) resulted in a steady decrease in the number of cells

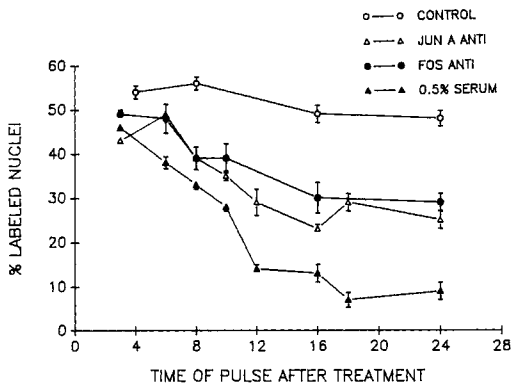

Fig. 4. Effect of c-fos and c-jun antisense oligodeoxynucleotide *treatment on entry* of Swiss 3T3 cells into DNA synthesis. Actively proliferating Swiss 3T3 cells were plated in fresh complete medium onto glass coverslips at a concentration of 5.0×10^5 cells per 100 mm dish. Twenty-four hours after plating, the original plating medium was removed, followed by pretreatment for 1 hr with prewarmed serum-free medium containing 10 μM of the appropriate oligodeoxynucleotide. After the 1 hr pretreatment, the medium was removed and replaced with fresh complete medium containing 10 μM of the appropriate oligodeoxynucleotide. At the indicated times, coverslips were removed and pulsed for 1 hr in complete medium containing 7.5 μCi/ml ^{3}H-thymidine, then harvested, fixed, and prepared for autoradiography. As controls, one experimental group of cells was incubated in only complete medium while a second group was incubated in medium supplemented with only 0.5% FBS. The data represent the mean percent labeled nuclei from three independently prepared coverslips ± SEM.

undergoing DNA synthesis. The extent of this reduction reached nearly 50% by 12–16 hr after treatment. Interestingly, both antisense oligodeoxynucleotides appeared to be equally successful at inhibiting entry into DNA synthesis. Once again, treatment with low serum (0.5%) was used as a positive control in this experiment (solid triangles). The rate of inhibition with low serum, although greater (~80% vs. ~50%), appeared to parallel that observed with antisense oligodeoxynucleotides, with maximum reduction occurring by 16 hr after treatment. Thus it would appear that the reduction in cell growth following treatment of actively proliferating Swiss 3T3 cells with either c-fos or c-jun antisense oligodeoxynucleotides occurs as a block in G_1 progression and entry into S. This is consistent with the possibility that c-fos and c-jun expression during G_1 is important for regulating cell cycle progression in actively growing cells.

c-fos and c-jun Antisense Oligodeoxynucleotide Treatment Alters the Entry of Mitotically Synchronized Swiss 3T3 Cells Into S

Since the DNA synthesis studies suggested G_1 progression and entry into S was altered in c-fos and c-jun antisense oligodeoxynucleotide-treated cells, and since maximum c-fos and c-jun expression was shown to occur during the M/G_1 transition, we next examined the effects on cell cycle progression of c-fos and c-jun antisense oligodeoxynucleotide treatment in mitotically synchronized Swiss 3T3 cells. We reasoned that this system would allow us to determine that the inhibition of entry into DNA synthesis was in fact a G_1-specific event. It would also permit a more precise mapping of the location during G_1 when treatment with c-fos and c-jun antisense oligodeoxynucleotides inhibits entry into S phase, and thus enable us to better correlate that time with the observed time of expression of both c-fos and c-jun.

For these studies, Swiss 3T3 cells were mitotically synchronized as we have previously described. It was suggested from both the expression data and the previous antisense studies that in order to be effective, the c-fos or c-jun antisense oligodeoxynucleotides would need to be present in the cell very early after division. Thus, it was necessary to modify the procedure used for plating the mitotic cells in order to ensure that the mitotic cells had sufficient time to take up the antisense oligodeoxynucleotides within 1 hr after division, the time of maximal expression of c-fos and c-jun. The modification consisted of plating the mitotic cells in prewarmed DMEM which contained the specific oligodeoxynucleotides but did not contain serum. Under these plating conditions, the mitotic cells attach but do not complete cytokinesis until serum is added. Thus, a 1 hr treatment with serum-free DMEM would result in an approximate 1 hr delay in the completion of cytokinesis and therefore allow sufficient time for the antisense oligodeoxynucleotides to enter the mitotic cells prior to induction of c-fos and c-jun mRNA during early G_1. It should be noted that we have found that the 1 hr treatment in serum-free DMEM results in only a comparable 1 hr delay in the entrance into DNA synthesis. It does not alter the rate of entry into S (compare Fig. 5 open circles to open squares).

Figure 5 shows the effects on entry into DNA synthesis of pretreating mitotic and early G_1 Swiss 3T3 cells with c-fos– and c-jun–specific antisense oligodeoxynucleotides. It can be seen that control cells preincubated for 1 hr in serum-free DMEM begin to enter S by 8 hr after plating. However, the cells treated during M and early G_1 (3 hr) with either c-fos or c-jun anti-

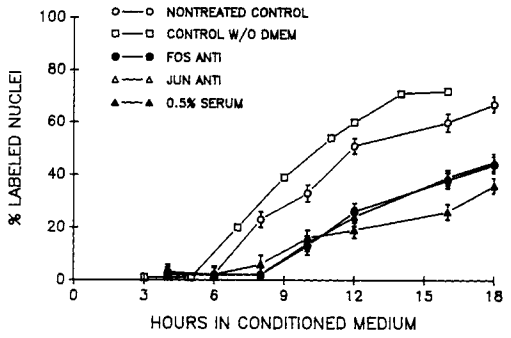

Fig. 5. Effect of c-fos and c-jun antisense oligodeoxynucleotide treatment on entry of mitotically synchronized Swiss 3T3 cells into DNA synthesis. Mitotically synchronized Swiss 3T3 cells were collected by large scale mitotic shake-off and plated onto laminin-treated coverslips in prewarmed serum-free medium containing 10 μM of the appropriate oligodeoxynucleotide. After 1 hr, the medium was removed and replaced with prewarmed conditioned medium containing 0.5 μCi/ml ^{3}H-thymidine. At the indicated times, coverslips were harvested, fixed, and prepared for autoradiography. In addition to the nontreated controls, two additional control groups were analyzed. One experimental group of cells was not pretreated with prewarmed serum-free medium but replated immediately into conditioned medium (control w/o DMEM). A second group of cells was treated with 0.5% FBS in place of oligodeoxynucleotides. The data represent the mean percent labeled nuclei from three independent experiments ± SEM.

sense oligodeoxynucleotides do not enter S until 10 hr. In addition, the antisense treatment resulted in a decrease in the total number of cells entering S phase. Both c-fos and c-jun antisense oligodeoxynucleotide treatment resulted in an approximately 60% reduction in the number of cells undergoing DNA synthesis, even by 18 hr after plating. Once again, treatment of mitotic cells with 0.5% serum was used as a positive control for this experiment. As can be seen in Figure 5 (solid triangles), incubation of mitotic and early G_1 cells (3 hr) in DMEM containing 0.5% serum resulted in a 2 hr lag in entry into DNA synthesis and an overall reduction in total number of cells eventually undergoing DNA synthesis, comparable to that observed following antisense treatment.

These data show that c-fos and c-jun expression and function during the first 2 hr following mitosis is required for cells to progress through G_1 and enter S.

c-jun but not c-fos Antisense Oligodeoxynucleotide Treatment of Mitotically Synchronized Swiss 3T3 Cells During Mid-G_1 Alters Entry Into S

Since both c-fos and c-jun mRNA were shown to accumulate to maximal levels very early in G_1 following mitosis, it was not surprising that c-fos– and c-jun–specific antisense oligodeoxynucleotide treatment of cells during the M/G_1 transition altered G_1 progression and entry into S. However, since a basal level of c-jun mRNA (~30% maximal) was maintained throughout the remainder of G_1, it was possible that the addition of antisense after the time of maximum expression could also result in a reduction in the number of cells entering S phase if continued expression of c-jun throughout G_1 is necessary for entrance into DNA synthesis. To test this possibility, we plated mitotically synchronized Swiss 3T3 cells into prewarmed conditioned medium. Within 30 min after plating, 95% of the cells had completed mitosis and presumably entered G_1. After 3 hr, during mid-G_1, antisense oligodeoxynucleotides specific for either c-fos or c-jun were added to the cultures along with ^{3}H-thymidine. The coverslips were harvested for autoradiography 9 hr later, at a time corresponding to the end of S phase. Figure 6 shows that the number of cells which entered DNA synthesis was modestly reduced (~30%) after the addition during mid-G_1 of 10 μM antisense oligodeoxynucleotides specific for c-jun but not c-fos. Since this experiment was repeated three times with comparable results, we feel confident in concluding that c-jun but not c-fos expression is important throughout G_1 for progression into S phase.

DISCUSSION

This is the first report to show that the immediate-early, G_0/G_1 competence genes c-fos and

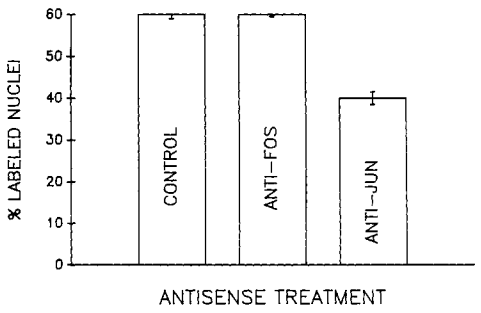

Fig. 6. Effect of c-fos and c-jun antisense oligodeoxynucleotide treatment during mid-G_1 on entry of mitotically synchronized Swiss 3T3 cells into DNA synthesis. Mitotically synchronized Swiss 3T3 cells were plated onto coverslips in prewarmed conditioned medium containing 0.5 μCi/ml ^{3}H-thymidine. After 3 hr, the plating medium was replaced with prewarmed conditioned medium containing 10 μM of the appropriate oligodeoxynucleotide. After an additional 9 hr, coverslips were harvested, fixed, and prepared for autoradiography. The data represent the mean percent labeled nuclei from three independent experiments ± SEM.

c-jun are also induced during the M/G_1 transition in actively proliferating Swiss 3T3 cells. However, the time a gene is expressed does not necessarily determine the time when that particular gene's function is needed. Thus, an important question raised by these results is whether this characteristic pattern of expression observed for c-fos and c-jun is important for progression of the cell out of mitosis, through G_1, into S. Follow-up experiments employing antisense technology provided the following additional new information concerning the role of immediate-early competence genes such as c-fos and c-jun in cell cycle progression: (1) Treatment of actively proliferating cells with antisense oligodeoxynucleotides specific for either c-fos or c-jun resulted in a reduced ability of Swiss 3T3 cells to enter S phase and subsequently divide. (2) In mitotically synchronized Swiss 3T3 cells, the presence of antisense oligodeoxynucleotides specific for either c-fos or c-jun during mitosis or up to 2 hr following division resulted in a decrease in the number of cells entering S phase. (3) Treatment of mitotically synchronized Swiss 3T3 cells with antisense oligodeoxynucleotides specific for c-jun in mid-G_1, 3 hr after division (1 hr after the time of maximum expression), also altered entry into S phase. These observations unequivocally establish a cell cycle regulatory role for induction of immediate-early competence genes such as c-fos and c-jun in nongrowth-arrested cells.

A number of other studies in the literature have suggested that c-fos and c-jun play a role in regulating growth. For example, Holt et al. [1986] showed that transfection of an expression plasmid producing high levels of c-fos antisense mRNA greatly decreased the growth rate of actively proliferating recipient cell lines. Likewise, Raibowol et al. [1988] showed that treatment with anti-c-fos antibody 9 hr after 3T3 cells were released from a double thymidine block resulted in a significant inhibition of cells entering the subsequent S phase. Finally, Kovary and Bravo [1991] showed that microinjection of antibodies specific for c-jun prior to stimulation of growth-arrested Swiss 3T3 cells prevented entry into DNA synthesis. Taken together, these studies suggested that c-fos and c-jun are required during G_1 in actively proliferating cells. Our present results confirm and extend this hypothesis to a more precise temporal location during the cell cycle when these genes function.

It is, of course, not surprising that inhibition of either c-fos or c-jun function during active proliferation would alter growth. Since it is known that both c-fos and c-jun are required to form a heterodimer in order to obtain maximum AP-1 transcriptional transactivating function [Chiu et al., 1988; Rauscher et al., 1988b], downregulating one of the proteins should produce phenotypes identical to what would be expected to be observed when the other or both are downregulated. Our data indicate that the time of expression and the function of these genes act synergistically with one another during the M/G_1 transition.

It should be noted that the similarities in the requirements for c-fos and c-jun during active proliferation diverge as the cells enter into mid-G_1. From the literature and from our own studies it is clear that c-fos is not needed during mid- to late G_1 [Holt et al., 1986; Nishikura and Murray, 1987; Riabowol et al., 1988], yet our results with c-jun–specific antisense oligodeoxynucleotide treatment during mid-G_1 resulted in a significant inhibition of cells entering DNA synthesis. This implies that c-jun is also needed during mid to late G_1. While the vast majority of the c-jun literature focuses on c-jun function during the G_0/G_1 transition, two studies suggest that c-jun functions later in G_1 as well. Carter et al. [1991] observed a second peak of c-jun mRNA induction just prior to when growth-arrested WI-38 cells and hamster ts13 cells enter S phase following serum stimulation. Likewise, Kovary and Bravo [1991] showed that microinjection of antibodies specific to c-jun during mid-G_1, as long as 7 hr after serum stimulation of Swiss 3T3 cells, led to inhibition of entry into S. Since c-fos mRNA and function are presumably not required during mid- and late G_1, it would appear that c-jun must complex with additional transcription cofactors during this time. Recent reports have shown that members of the ATF/CREB transcription factor family [Benbrook and Jones, 1990; Hai and Curran, 1991] and the Maf oncogene [Kataoka et al., 1994] can complex with c-jun and, as a result, permit binding and activation of modified AP-1 cis elements. It is tempting to speculate that by switching cofactors, it is possible to alter the transcriptional transactivation specificity of c-jun from early G_1 genes to late G_1 or S phase–specific genes. Interestingly, several S phase–specific genes contain modified AP-1 elements within their 5′ regulatory regions. These include hamster histone H3.2

[Sharma et al., 1989], topoisomerase I [Kunze et al., 1990], thymidylate synthase [Takeishi et al., 1989], thymidine kinase [Sauve et al., 1990], and PCNA [Travali et al., 1989]. Identification of the other cofactors as well as mid- and late G_1 target genes should provide information about why c-jun expression and function are required after the M/G_1 transition.

In all of our experiments, the extent of inhibition by antisense oligodeoxynucleotide treatment was less than that resulting from treatment with reduced serum. It is likely that this is due to a variety of factors including uptake of oligodeoxynucleotides, stability of the oligodeoxynucleotides once they have entered the cell, and the efficiency of the oligodeoxynucleotides in neutralizing the gene product [Marcus-Sekura, 1988]. Alternatively, this discrepancy may also reflect the existence of a certain amount of cellular growth-associated gene redundancy. The antisense treatment directly reduces only a single gene product. It is likely that more than one gene can carry out the function of c-fos or c-jun, albeit not necessarily as well. Hence, a reduced level of proliferation is observed. In contrast, low serum treatment results in the depletion of many gene products at once, including both c-fos and c-jun as well as other gene products which can substitute for the c-fos and c-jun. We would predict that simultaneous treatment with two or more antisense oligodeoxynucleotides to important growth-associated genes may inhibit growth to an extent more comparable to low serum. Obvious candidates for these other important genes would be the cyclins, c-myc, other members of the c-fos gene family (fra1, fra2, etc.), and other members of the jun gene family (jun B and jun D). Nevertheless, treatment with antisense oligodeoxynucleotides specific for a single immediate-early, competence gene consistently resulted in a significant reduction in cell number and entry into DNA synthesis.

The final question which remains to be answered is why competence genes are required for completion of the M/G_1 transition and progression through G_1. One possible source for an answer to this question can be obtained from the work of Zetterberg, Larsson, and colleagues [Zetterberg and Larsson, 1985; Larsson, et al., 1989]. These investigators showed that in both Swiss 3T3 cells and normal human fibroblasts there is a 3 hr period following mitosis (which they designate G_1pm) during which the cells require the competence growth factor PDGF. They hypothesized that it is during G_1pm that cells sense the growth conditions in the environment and decide whether to continue to proliferate or enter into a state of growth arrest. Since the time the cells require PDGF is also the time they must induce c-fos and c-jun, and since both c-fos and c-jun are known to be highly induced by PDGF treatment of quiescent cells, it is logical to hypothesize that similar growth factor–mediated signal transduction pathways are operating during G_1, regardless of whether the cell enters the phase of the cell cycle from mitosis or from G_0. Clearly, immediate-early, competence genes such as c-fos and c-jun are critical mediators of the molecular processes which must occur to initiate cell cycle traversal *in a nongrowing* cell and to maintain cell cycle traversal in an actively growing cell.

ACKNOWLEDGMENTS

This research was supported by grants to K.J.S. from the W. W. Smith Charitable Trust (C9104) and the National Institute of Health (NCI) (CA 56309). A preliminary report of a portion of this work was presented at the New York Academy of Sciences Meeting on Antisense Strategies.

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