Mitogenesis by v-Src: A Need for Active Oncoprotein Both in Leaving G₀ and in Completing G₁ Phases of the Cell Cycle¹

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Abstract
Activation of the tyrosine kinase of a temperature sensitive v-Src mutant of Rous sarcoma virus in quiescent Rat-1 cells leads to passage through the cell cycle. This is accompanied by a transient increase of the DNA binding activity of the transcription factor AP-1 which is not sufficient for the v-Src mediated cell cycle traverse. There is another need for v-Src later in the G1 phase of the cycle, and after completion of that event, cells are able to progress through DNA synthesis and division in the absence of either v-Src or other growth factors. When cells are exposed to v-Src activity for periods insufficient for it to behave as a complete mitogen, it can act as either a competence or progression factor in conjunction with appropriate purified growth factors.

Introduction
Although the pathways by which the v-src oncogene elicits its biological effects still await definition, the mitogenic response induced by pp60v-src has been clearly identified in both avian and rodent fibroblasts. Cells bearing temperature sensitive mutants of Rous sarcoma virus can be rendered quiescent by serum deprivation at the restrictive temperature and subsequent transit through the cell cycle induced simply by transfer to the permissive temperature (1–4). These studies led to the conclusion that v-Src can replace serum dependent processes occurring in the G₀–G₁ phases of the cell cycle that are necessary for DNA replication, and, since the effects of v-Src and serum were not synergistic, it was proposed that these two stimuli either acted at different positions in the cell cycle (2) or operated by convergent mechanisms (4).

There is evidence that v-Src stimulation of quiescent fibroblasts is accompanied by transient, increased transcription of a set of “immediate early genes” (5, 6). However, transcriptional activation of the classical immediate early genes, c-fos, c-jun, and c-myc, was not detectable on activation of pp60v-src in Rat-1 fibroblasts even though those genes could be induced by serum stimulation of the cells (4). Nonetheless, activation of v-Src did lead to an increase in the ability of nuclear extracts from those cells to bind to an oligonucleotide containing the AP-1 consensus sequence, implying a role for functional Fos and Jun family proteins in v-Src induction of cell cycling. There are many other data suggesting that the components of AP-1 are needed for quiescent cells to enter the cell cycle. Expression of antisense c-fos RNA prevented growth of 3T3 cells from reentering the cell cycle on mitogenic stimulation by PDGF (7), and microinjection of Fos antibodies up to 6–8 h after serum stimulation of quiescent rat fibroblasts (REF-52 cells) showed that Fos was required for at least 4 h to allow initiation of DNA synthesis (8). More recently, microinjection of specific antibodies to Fos and Jun family members demonstrated that both were required for entry into S phase (9), and the existence of different Fos/Jun complexes during G₀–G₁ transition has been described (10). In human fibroblasts, injection of oligonucleotides containing a consensus AP-1 binding site effectively prevented initiation of DNA synthesis after serum stimulation (11).

A previous report indicated that a short (2-h) “pulse” of v-Src activity was sufficient to induce DNA synthesis but not mitosis in NRK cells (3). This period of v-Src activity is approximately that needed to induce a marked increase in AP-1 DNA binding activity in Rat-1 cells (4), raising the question of whether this increased activity of AP-1 is sufficient for v-Src induced cell cycle progression. We now report tests of this postulate which show that the v-Src induced early increase in AP-1 activity is not sufficient to induce Rat-1 cell cycle progression. There is a further need for v-Src later in the G₁ phase of the cell cycle which then renders cells capable of progressing through DNA synthesis and division.

Results
Nature of the AP-1 Complex. We have previously shown that shifting Rat-1 cells bearing the temperature sensitive LA 29 v-src mutant of Rous sarcoma virus from a restrictive temperature (40°C) to the permissive temperature (35°C) activated the tyrosine kinase of pp60v-src within 10 min, and this was soon followed by a transient increase in protein binding to a consensus AP-1 oligonucleotide (4). Conversely, the kinase was inactivated equally rapidly on return to 40°C (data not shown). In Fig. 1A, it is shown that maximum AP-1 DNA binding activity was reached within 1 h after stimulation and had declined to that of the quiescent cells by 6 h. The basal level of AP-1 was higher in some experiments than in others but always increased on stimulation. This complex comigrated with the AP-1 DNA binding activity isolated from HeLa cells

¹ The abbreviations used are: PDGF, platelet derived growth factor; IGF-1, insulin-like growth factor 1; RB, retinoblastoma.

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pulses of 12 and 14 h, the cells initially entered S phase with the same kinetics as the control cells maintained at 35°C throughout the experiment (Fig. 3A). However, at approximately 18–20 h (about 6 h after the end of the pulse), thymidine incorporation in the pulsed cultures declined relative to that in controls, this being most marked in cultures receiving the shorter pulse.

Analysis by flow cytometry after v-Src pulses of 17, 19, and 21 h also showed that the proportion of cells in S plus G2-M phases started to decrease several hours after inactivating the kinase, whereas this proportion continued to increase in cells constantly exposed to v-Src (Fig. 3B). The mean percentages of cells in S plus G2-M phases in all pulsed populations relative to those maintained at 35°C throughout were plotted against the time interval after the shift to restrictive conditions (Fig. 3C). This showed that all pulsed populations deviated from controls between 6 and 7 h after the inactivation of the v-Src kinase. The data in Fig. 3, A–C, thus suggest that there is a point about 6–7 h prior to the onset of S phase at which cells become committed to DNA synthesis and no longer require the v-Src kinase. Cells that have not reached this point by the end of the v-Src pulse fail to reach S phase.

We sought to examine further the nature of the v-Src requirement by asking whether there was a need for v-Src tyrosine kinase throughout the period prior to commitment to S phase, or whether after an initial event (perhaps activation of AP-1 DNA binding), there were discrete later kinase dependent events. To do this, we performed a series of experiments in which v-Src kinase was activated for varying periods that were themselves insufficient to induce cell cycle progression, followed by a period of inactivation and finally reactivation at 16 h from the beginning of the experiment. The latter time was chosen since 16 h from the start is near the beginning of normal S phase in these cells (see Fig. 3A). Subsequent progression through the cycle was examined kinetically and compared with control cells exposed to v-Src throughout the experiment.

Three alternative results could be predicted: (a) the cells may pause at the stage of G1 reached by the end of the pulse, in which case further progression, compared to controls, would be delayed by 16 h minus the length of the pulse; (b) they may proceed or regress to a critical point in G1, in which case the delay compared to controls would be defined by this point, irrespective of the length of the pulse; or (c) they may return to G0, resulting in a 16-h delay compared to controls, again irrespective of the length of the pulse.

Several experiments using different pulse times (ranging from 2 to 8 h) were performed, and the cell cycle status was analyzed by flow cytometry. Statistical analysis (see "Materials and Methods") showed that the controls kept at 35°C throughout differed from the 2-, 4-, 5-, and 8-h pulsed data with a confidence of greater than 95%, whereas there were no significant differences between any pair of the v-Src pulsed data (all combinations were tried). Pulse times of 2, 4, 5, and 8 h all showed a similar delay in traverse through S plus G2-M phases relative to cells shifted to the permissive temperature at time zero and maintained there. Fig. 4 shows the results from a typical experiment in which both 4- and 8-h pulses of v-Src activity showed the same delay in reentering the cell cycle and subsequently followed the same kinetic be-

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Fig. 1. Gel retardation analyses of AP-1 DNA binding activity. A, time course of induction after temperature shift in the presence and absence of cold competitor oligonucleotide. B, effects of antisera: Lane 1, no antibody; Lane 2, nonimmune serum; Lane 3, anti-Jun serum; Lane 4, anti-Fos serum. Arrowheads, AP-1 specific bandshifts.

(data not shown) and was subject to competition by a 100-fold excess of cold oligonucleotide. Inclusion of a polyclonal Jun antiserum decreased the mobility of the complex, indicating the presence of Jun family members, whereas the anti-Fos serum used caused dissolution of the complex rather than supershift (Fig. 1B).

Is AP-1 Activity Sufficient for Cell Cycle Progression? Quiescent, morphologically normal Rat-1 LA29 cells were shifted from 40°C to 35°C for varying periods and then returned to 40°C for subsequent monitoring of cell cycle progression. Pulses of v-Src activity up to 7 h were not sufficient to initiate DNA synthesis (measured by uptake of [3H]thymidine) in these cells (see Fig. 2A). Measurement of the DNA content of pulsed versus control cells using flow cytometry also led to the conclusion that exposure of rat fibroblasts to the tyrosine kinase activity of pp60<sup>Src</sup>, for periods sufficient to induce increased AP-1 DNA binding activity, was not sufficient for cells to progress through the cycle. Fig. 2B illustrates the results of a typical experiment in which, after a 6-h v-Src pulse, 90% of the cells remained in G<sub>0</sub> with an identical profile to that of the quiescent cells (data not shown). For cells pulsed with v-Src for 17 h or for control cells maintained at 35°C, more than 50% were in either S or G2-M phases at a point 23 h after initiation of the stimulus (Fig. 2, C and D).

When Is v-Src Needed during the Cell Cycle? The above observations implied a need for v-Src kinase at some point between the 7-h pulse and the start of S phase. We therefore studied the effects of exposing quiescent cells to intermediate periods of v-Src tyrosine kinase activity and subsequently monitoring DNA synthesis by measurement of uptake of [3H]thymidine. After
Fig. 2. A, uptake of [3H]thymidine after temperature shift. ○, control cells shifted to 35°C at time zero; □, cells pulsed at 35°C for 0.5 h; ●, cells pulsed at 35°C for 1 h; ■, cells pulsed at 35°C for 7 h; X, control cells maintained at 40°C. Each point is the mean of 6 replicates. B, flow cytometric analysis after a 6-h pulse of v-Src. C, flow cytometric analysis after a 17-h pulse of v-Src. D, flow cytometric analysis of control cells shifted to and maintained at 35°C. Analyses were performed 23 h after shift.

behavior. The delay in recruitment into the S plus G2-M population was clearly less than 16 h, favoring the interpretation that the cells, although unable to progress through the cycle, paused at the same stage beyond quiescence and upon restimulation could proceed. It was not possible to make a precise temporal definition of this stage as the flow cytometric analysis measured cells in both S and G2-M phases. Furthermore, the pulsed cells seemed to show a slower rate of recruitment into S plus G2-M phases after reactivating v-Src, implying that they were less synchronous than the controls and might thus comprise discrete populations with different kinetics of reentry into the cycle.

To examine only the S phase population, we measured incorporation of thymidine after cells had been stimulated by temperature shift for 8 h, returned to the restrictive temperature for a further 8 h, and finally restimulated by a shift to 35°C at 16 h, when the control cultures were also shifted. Fig. 5 shows a typical result in which the "double-pulsed" cells gave a clearly biphasic response to initiation of DNA synthesis. Several such experiments were performed, all showing that the v-Src pulses generated two populations of cells. One group
had clearly returned to a point at or near Go, entering S phase in parallel with the controls shifted at 16 h from the start of the experiment. The second population reached a peak of DNA synthesis about 8 h earlier, suggesting that they had paused in a stable state midway through G1. We cannot determine whether only a subset of cells reached this point or whether the cells that had returned to Go represent a population in which this state proved to be unstable.

**Does Entry into S Phase Ensure Mitosis?** We next asked whether or not pulsed cells that entered S phase could complete their cycle and divide, by counting cell numbers over a period of several days after temperature shift. Mean values from six different experiments clearly demonstrated that either after a 17-h pulse of v-Src activity or on continuous exposure to the permissive temperature, many cells divided. As expected from the data shown in Fig. 3B, the 17-h pulsed cells increased in number by a mean of only 1.6-fold, whereas those left at 35°C continuously almost doubled by 65 h. At the end of the 17-h pulse, the cells had become refractile, but after return to the restrictive temperature, they reverted to, and divided as, morphologically normal fibro-
blasts. To confirm that mitosis was due entirely to v-Src and that there were no major effects from residual growth factors in the 0.2% serum containing medium, the experiment was modified so that, at the time of stimulation, the old medium containing 0.2% serum, in which the cells had been rendered quiescent, was removed, and the cells were washed and then shifted to 35°C in serum-free medium. Again, both pulsed and control cells were able to divide under serum-free conditions (data not shown).

**Cooperation between v-Src and Growth Factors.** Exit of cells from G0 and progression through the cycle have been classically resolved into two stages termed competence and progression (12), both stages being achieved as a result of the action of specific polypeptides. Since the experiments described above had also identified two periods in which v-Src kinase activity was needed prior to the start of DNA synthesis, we studied the role of this oncogenic protein both as a competence and as a progression factor by determining whether v-Src could synergize with growth factors whose roles in these phenomena have been defined. Doses of the classic competence factor PDGF and the classic progression factor IGF-1 which were mitogenic in Rat-1 LA29 cells at the restrictive temperature were 1 half-maximal unit/ml for PDGF and 10 ng/ml for IGF-1. Concentrations 10-fold lower than these were shown to be nonmitogenic.

Quiescent cells were exposed to the v-Src tyrosine kinase for periods of between 4 and 8 h, returned to the restrictive temperature, and treated with a nonmitogenic dose of IGF-1. S phase progression was monitored by thymidine incorporation over the following 24 h. Appropriate controls were provided by cells continuously exposed to v-Src and by cultures in which the IGF-1 was replaced by a nonmitogenic dose of PDGF. Typical results are shown in Fig. 6A. A 7-h pulse of v-Src was indeed able to make the cells competent for a nonmitogenic dose of IGF-1 to allow them to progress with kinetics identical to those of the positive controls, whereas the nonmitogenic PDGF had no progression effect.

Cells treated for 8 h with a mitogenic dose of PDGF, which was then washed out, were able to progress through the cycle on activation of v-Src kinase, but control cultures which received no further stimulus after removal of the PDGF were unable to progress to DNA synthesis (Fig. 6B). Cultures in which the v-Src was replaced with the nonmitogenic dose of IGF-1 also progressed with the same kinetics (data not shown). The 2-h delay in the peak of DNA synthesis for v-Src progression of PDGF competent cells, in the particular experiment illustrated, suggests that PDGF may not effect all of the changes wrought by v-Src in this early part of G1. However, as this delay was not always observed, it is difficult to comment on its significance. Nonetheless, it is clear that v-Src can act as a progression factor for competent Rat-1 cells in the absence of any other growth factors. In addition to our previous observation that the effects of v-Src and serum were not synergistic (4), we
before the onset of S phase is both necessary and sufficient for substantial DNA synthesis and mitosis.

There are similarities between this dissection of events and the steps defined biochemically (12–15) or kinetically (16, 17) in other cell systems using other growth stimuli. Studies with inhibitors have suggested that there are several protein kinase dependent restriction points in the G1 phase of normal but not transformed cells (13, 14), and it is postulated that these may differ from the previously defined growth factor dependent C, V, and W restriction points (12, 15). We cannot align these checkpoints with those that we have defined but they are likely to have common features, and their reversible perturbation by temperature sensitive v-Src will aid their analysis. However, the timing of the restrictions overcome by v-Src must be regarded as provisional. Cells maintained at 40°C show background thymidine incorporation, and the onset of S phase is relatively gradual (for example, Figs. 2A, 3A, and 5), as would be predicted by both transition probability (16) and continuum models (17) of cell cycle regulation. Thus, a few cells may continue cycling in “quiescent” cultures, and it is possible that some are blocked in mid-G1, a situation exaggerated by the effects of v-Src pulses, as shown in Fig. 5. If this is so, then the minimal length of complete G1 in cells stimulated from quiescence may be longer than our estimate of 16 h, and we cannot be precise about the positions of the pause and commitment points. Nonetheless, our observations already have the following implications.

1. A variety of data shows that AP-1 is essential for appropriately stimulated cells to reach S phase (7–11). This is consistent with our finding that a v-Src kinase, rendered incapable of transformation and mitogenesis by a mutation that prevents NH2-terminal myristylation, is also unable to increase AP-1 DNA binding (18). However, the high level of AP-1 DNA binding induced by a 2-h v-Src pulse (Fig. 1) is clearly insufficient for progress through cycle, although its effects are indistinguishable from those of an 8-h pulse which allows some cells to reach the pause point (Fig. 5), by which time AP-1 activity has declined. It has been shown in other cells that induction of c-fos expression does not ensure further progression through the cycle (19), and indeed, stimulation of the v-Src activity of quiescent Rat-1 LA29 cells is not accompanied by increased transcription of c-fos (4).

A recent report showed that comitogenic expression of c-fos and increased AP-1 activity did not lead to DNA synthesis in senescent human fibroblasts (20), and Roussel et al. (21) showed that a mutant colony stimulating factor-1 receptor, which cannot transmit a mitogenic signal in NIH 3T3 cells, can nonetheless induce expression of c-fos and junB but not c-myc. Enforced c-myc expression rescued receptor mediated proliferation in these cells, implicating c-myc in the mitogenic pathway (21), so it is significant that v-src mitogenesis in quiescent Rat-1 LA29 cells gives no detectable change in c-myc levels (4). Other events are needed during the first 4 h of v-Src activity, and these may comprise or include further AP-1 modifications. Jackson et al. (22) speculated that one of the competence inducing roles of PDGF is to stabilize the c-Fos component of AP-1, an effect only achieved when PDGF is present for 4 h. We are currently investigating the complex effects of v-Src on AP-1 (18).

Discussion

The single integrated provirus in the Rat-1 LA29 cell line specifies sufficient v-Src tyrosine kinase to impel these cells from quiescence and through the cycle (4). This oncoprotein thus substitutes for mitogenic growth factors, presumably by usurping or obviating the pathways through which these mitogens function. The rapidly reversible v-Src temperature sensitivity makes it a potent tool to determine when its tyrosine kinase is needed during cell cycling and to correlate these needs with changes in cellular biochemistry and the action of other mitogens.

Pulses of v-Src activity reveal two periods during which the v-Src kinase is required for quiescent cells to reach S phase. The beginning and end of each period define four restrictions which v-Src must overcome for cells to progress through the cycle (shown diagrammatically in Fig. 7): (1) v-Src activity is essential for serum deprived cells to leave the quiescent state. (2) Pulses of 2, 4, 5, or 8 h, however, have indistinguishable consequences, promoting passage through the cycle to a relatively stable “pause” point about 8 h before the onset of DNA synthesis (Figs. 4 and 5). (3) This point is only passed if there is further v-Src activity, but once cells are within about 6 h of starting S phase, the v-Src function is no longer needed. At this point (4), the cells are committed to DNA synthesis (Fig. 3) and, apparently, subsequent mitosis. The requirements for v-Src at points 3 and 4 are distinct in their consequences for further passage through cycle, but it is possible that points 1 and 2 are close and may define an identical need for v-Src activity. Our conclusions differ from those of Durkin and Whitfield (3), who found that short pulses of v-Src could lead to DNA synthesis, but longer pulses were needed for mitosis. However, the G1 phase in their NRK cells was only about 10 h, possibly compressing and overlapping the series of events that we describe. Indeed, their data are consistent with our conclusion that a pulse of v-Src up to about 6 h
2. The nature and temporal position of the pause point 3 in Fig. 7 recalls the V-point defined in BALB/c 3T3 cells (12, 15). However, unlike the V-point, the pause point may be unstable, since some cells may revert to a state temporally indistinguishable from quiescence (Fig. 5). This behavior is reminiscent of the restriction point induced in BALB/c 3T3 cells by the inhibitor methylglyoxal bis guanylylhydrazone (23) and of the indeterminate A state that the transition probability model of Brooks et al. (16) postulates shortly before the start of S phase. On the other hand, the indistinguishable behavior of cells pulsed with v-Src for between 2 and 8 h (Fig. 4) and the subsequent generation of a biphasic pattern of DNA synthesis (Fig. 5) is hard for us to reconcile with the continuum model of cell cycle progression (17). Nonetheless, although v-Src may only lead to temporary progression through restriction points 1-3 in Fig. 7, it can induce a state of competence for a time, as demonstrated by the ability of a nonmitogenic dose of IGF-1 to progress v-Src pulsed cells into S phase (Fig. 6). This capacity apparently does not depend on v-Src acting as a complete mitogen, for Han et al. (24) have evidence for v-Src acting as a competence factor for epidermal growth factor in BALB/c 3T3 cells, a cell line in which v-Src appears to be a poor mitogen.

It is also interesting to note that although the mitogenic activity of v-Src is not synergistic with serum growth factors in rat fibroblasts (4), in quail neuroretinal cells, both v-Src kinase activity and serum growth factors are needed to induce proliferation, suggesting the existence of two complementary mitogenic pathways in the neural cells (25). Moreover, at least some of the effects of v-Src in these cells seem to operate in mid- to late G1 phase of the cell cycle.

The need for v-Src activity in mid-G1 is interesting but not unexpected. Passage from G1 into S phase is a checkpoint influenced by a number of regulatory proteins, such as p53 (26). In the case of the retinoblastoma gene product, p110, transitions in phosphorylation status have been shown to modulate regulation by the protein (27). These transitions are probably controlled by cyclin dependent kinases and protein phosphatases (28). There is evidence that cells are sensitive to the inhibitory effect of unphosphorylated RB protein at a point in G1 about 6–10 h before S phase (29), and it is at about this period that the G1 specific type D cyclin, PRAD 1, is induced by serum in human fibroblasts (30) and by PDGF in BALB/c 3T3 mouse cells (31). The activity of v-Src at the same stage of G1 might effect these alterations in the absence of the usual growth stimulatory signals.

It is more surprising that, once restriction point 4 is passed (Fig. 7), cells can divide without further stimulus. The ability of cells to pass the G2-S and G2-M checkpoints is controlled by some common components, but each has its specific regulatory features to ensure that downstream events do not occur before the completion of appropriate previous requirements (reviewed in Refs. 32 and 33). The ability of v-Src expression, which is limited to G1, to override G2-M regulation suggests that the latter checkpoint is not of major significance in these cells, bearing out the tendency of serum deprived cells to arrest in G0 after mitosis.

In summary, our study has shown that the v-Src oncoprotein acts at more than one point in the G1-G2 phases of the cell cycle. It is likely that multiple and probably phase specific signaling pathways are involved, and their further elucidation should help to understand both normal cell cycle regulation and its perturbation in oncogenesis.

Materials and Methods

Materials. [methyl-3H]Thymidine and human recombinant IGF-1 were purchased from Amersham International, and 50% pure PDGF was obtained from Collaborative Research.

Cell Culture. Rat-1 LA29 cells (34) were grown in Dulbecco’s modified Eagle’s medium, supplemented with 5% newborn calf serum, at either 35°C (permissive) or 40°C (restrictive) temperatures. For quiescence, 1 × 10⁴ cells were plated in 96-well microtiter plates and grown at 40°C overnight. Medium was replaced with Dulbecco’s modified Eagle’s medium containing 0.2% newborn calf serum, and the cultures were maintained at 40°C for a further 48 h. Stimulation was achieved by shifting to 35°C or by addition of growth factors at either temperature, as described in individual experiments.

Flow Cytometric Determination of Cell Cycle Status. Analysis of the cell cycle status of Rat-1 LA29 cell cultures was performed by the detergent-trypsin method of Vindelov et al. (35). Cells (10⁴) were plated in 60-mm dishes, and quiescence was induced as described above. At the end of the experiment, cells were harvested by trypsinization, washed in phosphate buffered saline, and resuspended in 100 μl of citrate buffer (250 mM sucrose-5% (v/v) dimethyl sulfoxide-40 mM trisodium citrate, pH 7.6) and added to 900 μl of a solution of 30 μg/ml trypsin in stock buffer [0.1% (w/v) Nonidet P-40-1.5% (w/v) spermine tetrahydrochloride-3.4 mM trisodium citrate-0.5 mM Tris-HCl, pH 7.6]. After a 10-min incubation at room temperature, 750 μl of a solution of 500 μg/ml soybean trypsin inhibitor-100 μg/ml ribonuclease in stock buffer were added, and the mixture was incubated for a further 10 min at room temperature. Finally, 750 μl of an ice-cold solution of 416 μg/ml propidium iodide-1.16 mg/ml spermine tetrachloride, also in stock buffer, were added to the extracts, and the samples were incubated on ice for a further 15 min. Samples were analyzed on a Becton-Dickinson FACScan flow cytometer. Data, based on 10,000 events collected in linear mode, were acquired using the Consort 30 program and analyzed using either polynomial or sum of broadened rectangles curve fitting programs. Statistical comparisons between data sets from different experiments were made by Dr. W. Millar, using either the Friedman or Wilcoxon signed pair tests (36).

Measurement of DNA Synthesis: Uptake of [3H]Thymidine. Cells were pulse labeled for 1 h by addition of [methyl-3H]thymidine to a final concentration of 10 μCi/ml. Medium was removed by aspiration, and after trypsinization, the cells were collected on glass fiber filter mats using an LKB automatic cell harvester prior to liquid scintillation counting in a Pharmacia betaplate counter. In each experiment, six replicate wells were labeled for each point, and mean values were plotted. All experiments were repeated several times.

Nuclear Extracts and Gel Retardation Analyses. Nuclei were prepared, and gel retardation analyses were performed exactly as described previously (4).

Antisera. Antisera used were rabbit polyclonal sera. Antiserum 948 was raised against the carboxy-terminal
region of c-Jun (37), and antiserum 348 was raised against a ttp-Fos fusion protein (38). Both were the kind gifts of D. A. F. Gillespie.

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