MOLECULAR AND CELLULAR BIOLOGY, Nov. 1992, p. 5015–5023
0270-7306/92/115015-09$02.00/0

Vol. 12, No. 11

Existence of Different Fos/Jun Complexes during the G_0-to-G_1 Transition and during Exponential Growth in Mouse Fibroblasts: Differential Role of Fos Proteins

KARLA KOVARY AND RODRIGO BRAVO*

Department of Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, P.O. Box 4000, Princeton, New Jersey 08543-4000

Received 19 May 1992/Returned for modification 2 July 1992/Accepted 16 August 1992

We have determined the different Fos/Jun complexes present in Swiss 3T3 cells either following serum stimulation of quiescent cells or during exponential growth by immunoprecipitation analyses. We have shown that while c-Fos is the major Fos protein associated with the Jun proteins (c-Jun, JunB, and JunD) soon after serum stimulation, at later times Fra-1 and Fra-2 are the predominant Fos proteins associated with the different Jun proteins. During exponential growth, the synthesis of Fra-1 and Fra-2 is maintained at a significant level, in contrast to c-Fos and FosB, which are expressed at very low or undetectable levels. Consequently, Fra-1 and Fra-2 are the main Fos proteins complexed with the Jun proteins in asynchronously growing cells. To determine whether the Fos proteins are differentially required during the G_0-to-G_1 transition and exponential growth for the entrance into S phase, we microinjected affinity-purified antibodies directed against c-Fos, FosB, Fra-1, and Fra-2. We have found that while the activities of c-Fos and FosB are required mostly during the G_0-to-G_1 transition, Fra-1 and Fra-2 are involved both in the G_0-to-G_1 transition and in asynchronous growth.

A number of studies have demonstrated that the transcription factor AP-1, originally discovered in human cells as an activity that selectively binds to enhancer elements in the promoter region of simian virus 40 and of many other vertebrate genes (1, 19), consists of several distinct proteins, including those encoded by the proto-oncogenes c-*jun* and c-*fos* and other members of the *jun* and *fos* gene families (for reviews, see references 2 and 40). The three Jun proteins, c-Jun, JunB, and JunD, can form homodimers and bind to an AP-1 site, but they differ in their binding affinities (33), and it has been demonstrated, at least for c-Jun and JunB, that they have different transcriptional and biological activities (4, 36, 37). The Fos proteins, c-Fos, FosB, Fra-1, and Fra-2, differ from the Jun proteins in that they do not form homodimers and have no intrinsic specific DNA binding activity (6, 9, 24, 39, 42). However, the binding affinity and transcriptional activation of the Jun proteins are dramatically increased upon dimerization with Fos proteins (6, 9, 23, 28, 29, 33, 39, 42). The complexity arising from the possible associations between different Jun and Fos proteins has been further increased by the findings that Fos proteins differentially affect the activity of the Jun proteins (7, 33, 39). Interestingly, the members of the *jun* (16, 30–32, 34) and *fos* (5, 8, 15, 20, 22, 26, 42) families belong to the set of genes that are rapidly induced by growth factors in quiescent fibroblasts (3, 10, 17), indicating that the Jun and Fos proteins may play an important role in the regulation of cellular proliferation. The notion that AP-1 activity is required in cell growth is supported by the observation that inhibition of Fos and Jun activities either by the expression of antisense RNA (11, 25, 38) or by microinjection of antibodies (13, 29) inhibits induction of cell proliferation and cell cycle progression. Further evidence that Fos and Jun proteins are involved in the control of cell growth is the observation that deregulated expression of c-Fos, FosB, Fra-2, and c-Jun can induce malignant cell transformation (14, 18, 21, 26, 35, 36, 41).

Former studies have demonstrated that following serum stimulation, the Fos proteins have distinct patterns of expression, with c-Fos and FosB being expressed transiently, whereas Fra-1 and Fra-2 are synthesized later and for more prolonged times (5, 12, 22, 26). These data suggest that the different Fos proteins could have differential roles in cellular proliferation. Thanks to the generation of specific antibodies against the different Fos proteins, we have done comparative studies among the Fos proteins to gain an insight into the possible differential regulatory activities exerted by them that are implicated in normal cell growth control. We have previously demonstrated that following serum stimulation of quiescent Swiss 3T3 cells, different Jun (c-Jun, JunB, and JunD) and Fos (c-Fos and FosB) proteins coexist for several hours, generating different Jun/Fos complexes in the cell (12). The relative proportion of the different heterodimers formed at any time is governed by the amount of each Jun and Fos protein present at that moment. In this report, we have extended these studies to Fra-1 and Fra-2. We have investigated the changes in the expression of Fra-1 and Fra-2 in Swiss 3T3 cells under two different growth conditions, the transition from G_0 to G_1 and exponential growth, and compared them with those of c-Fos and FosB. The complexes formed between Fos and Jun proteins during both cell growth conditions have also been determined. These studies have been complemented by microinjection of Swiss 3T3 cells with affinity-purified polyclonal antibodies that are specific for each Fos protein or that recognize all Fos proteins to determine their requirement for cell cycle progression by monitoring DNA synthesis. From the results obtained, we conclude that there is a differential requirement of the Fos proteins during the G_0-to-G_1 transition and during exponential growth.

* Corresponding author.

MATERIALS AND METHODS

Cell culture. Swiss 3T3 cells were routinely grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics (100 U of penicillin per ml and 50 μg of streptomycin per ml). Confluent cells were made quiescent by incubating them for 48 h in DMEM containing 2.5% FCS. For stimulation, quiescent cells were incubated in DMEM containing 20% FCS for the indicated periods of time.

Cell labeling and immunoprecipitation. (i) Cell labeling. For pulse-labeling experiments with [^{35}S]methionine, Swiss 3T3 cells grown in 24-well plates were rinsed twice with DMEM lacking methionine and then labeled for different times (15 to 60 min) with 800 μCi of [^{35}S]methionine per ml in methionine-free medium at the indicated times. For continuous labeling, quiescent Swiss 3T3 cells were rinsed twice with DMEM minus methionine and then labeled for different times with 800 μCi of [^{35}S]methionine per ml in DMEM without methionine supplemented with 20% dialyzed FCS. In all experiments, cells from 2 wells (24-well plates) were labeled per point.

(ii) Immunoprecipitation. For the preparation of native cellular lysates (nondenaturing conditions), the labeling medium was removed, and the cells were washed briefly with cold phosphate-buffered saline (PBS) and lysed on ice by the addition of RIPA buffer (10 mM Tris-HCl [pH 7.5], 1% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.25 mM phenylmethylsulfonyl fluoride). For denatured cell extracts, cells were lysed in denaturing buffer (50 mM Tris-HCl [pH 7.5], 0.5% SDS, 70 mM β-mercaptoethanol), boiled for 10 min, and then diluted by adding 4 volumes of RIPA buffer without SDS. The lysates (final volume, 1 ml) were incubated with antiserum (3 μl) for 1 h on ice and then incubated with 15 μl of protein A-Sepharose CL-4B (Pharmacia) for 3 h on a roller system at 4°C. The immunocomplexes with the protein A-Sepharose beads were washed twice with buffer A (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40), once with buffer B (10 mM Tris-HCl [pH 7.5], 500 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40), and once with buffer C (10 mM Tris-HCl [pH 7.5]). The samples were boiled in 2× Laemmli sample buffer and then run overnight on a 12.5% acrylamide-bisacrylamide gel (200:1) at 12 mA per gel. Fixed gels were incubated twice for 1.5 h in dimethyl sulfoxide, then incubated for 3 h in 20% 2,5-diphenyloxazole (PPO) in dimethyl sulfoxide, and finally washed for 30 min in water. Gels were dried and exposed to Kodak X-Omat AR film at −70°C for different times.

All lysates were initially preabsorbed with 3 μl of rabbit preimmune serum and incubated at 4°C for 1 h in the presence of 15 μl of protein A-Sepharose beads. The supernatants of these incubations were then used for immunoprecipitation with the different antibodies. The same cellular lysate was used for sequential immunoprecipitation of the different Fos proteins.

Antibodies. (i) Generation. The polymerase chain reaction product of the mouse *fra*-2 cDNA (coding for the full-length protein, amino acids 1 to 326) was cloned into the expression vector pEx1 (β-galactosidase), and the bacterially expressed fusion protein obtained was purified and injected into rabbits. The specificity and cross-reactivity of the generated Fra-2 antibodies were tested by immunoprecipitation of the different Fos proteins in vitro translated as described previously (12, 13). Generation and specificities of antibodies against c-Fos, FosB, Fra-1, c-Jun, JunB, and JunD have been described elsewhere (12, 13).

(ii) Immunoaffinity purification. Specific antibodies against the different Fos and Jun proteins were immunopurified by using columns of Affi-Gel 15 beads (Bio-Rad Laboratories) covalently bound with bacterially expressed fusion proteins of either Fos or Jun as described elsewhere (13). Briefly, the PBS-dialyzed immunoglobulin fraction obtained by ammonium sulfate precipitation of each antiserum was repeatedly passed through different columns. The columns containing the different antigens were used in the following order: first, the bacterial protein portion of the corresponding fusion protein (β-galactosidase or MS2 polymerase); second, the fusion proteins whose mammalian protein portion belongs to the same protein family (either Fos or Jun) in order to eliminate cross-reactivities; and last, the specific Fos or Jun antigen. Antibodies were eluted with 0.05 M glycine buffer (pH 2.3, containing 0.15 M NaCl) directly into 0.5 M sodium phosphate buffer (pH 7.7) and concentrated by centrifugation in Centricon 30 microconcentrators (Amicon Corp.) at 4°C.

Microinjection and immunofluorescence. Swiss 3T3 cells grown on coverslips (1 by 1 cm) were microinjected with antibodies into the cytoplasm, using the AIS automated microinjection system (Zeiss) as described previously (27). Single antibodies were injected at a concentration of 5 mg/ml. Combinations of two different anti-Fos antibodies were injected at a final concentration of 3 mg/ml each. For each assay, 100 to 200 cells were microinjected in duplicate. To detect DNA synthesis, 5-bromodeoxyuridine (Sigma) at a final concentration of 100 μM was added to the medium 6 to 14 h before cells were fixed. For indirect immunofluorescence, cells were fixed with cold methanol (4°C) for 10 min, rehydrated in PBS, and incubated for 30 min in 1.5 M HCl to denature the DNA. After the coverslips were washed several times with PBS, the cells were first incubated with a mouse antibromodeoxyuridine monoclonal antibody (1:50; Becton Dickinson) for 30 min at room temperature, washed several times with PBS, and then incubated for another 30 min (room temperature) with a mixture of a goat polyclonal anti-rabbit immunoglobulin antibody conjugated with fluorescein isothiocyanate (1:100; DAKO) to visualize the injected cells and a donkey polyclonal antibody anti-mouse immunoglobulin conjugated with Texas red to detect DNA synthesis (1:50; Amersham). After several washes in PBS, the coverslips were mounted on slides with Fluoromount G (Southern Biotechnic). DNA synthesis inhibition was calculated by determining the percentage of injected cells that were not in DNA synthesis after microinjection.

RESULTS

Fra-1 and Fra-2 synthesis during the G_0-to-G_1 transition. We have previously demonstrated that in Swiss 3T3 cells during the first hours of transition from G_0 to G_1, there is a rapid but transient increase in the rate of synthesis of c-Fos and FosB, reaching a peak between 30 to 60 min and decreasing to undetectable levels after 2 h (12). In contrast, it has been shown in other fibroblast cell lineages that although the rates of synthesis of the other two members of the Fos family, Fra-1 and Fra-2, are also rapidly induced following serum stimulation, their synthesis remain at a significant level for several hours (5, 26). We have extended these observations to Swiss 3T3 cells (Fig. 1). Following stimulation with 20% FCS and at the indicated times after 30 min of labeling with [^{35}S]methionine, denatured cellular

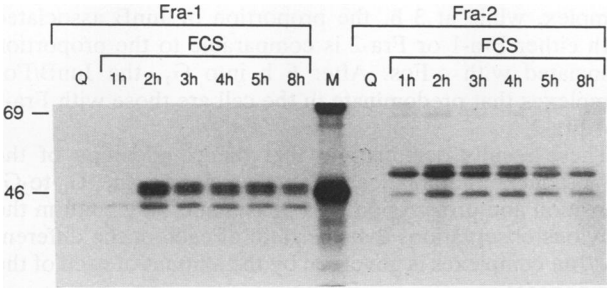

FIG. 1. Time course of induction of Fra-1 and Fra-2 during the G_0-to-G_1 transition. Quiescent Swiss 3T3 fibroblasts stimulated with 20% FCS were labeled with [^{35}S]methionine in the presence of serum for 30 min before the indicated times and lysed in denaturing conditions, and Fra-1 and Fra-2 were detected by sequential immunoprecipitations of the same cellular lysate, using polyclonal antisera as described in Materials and Methods. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Lane Q, immunoprecipitates of quiescent cells labeled with [^{35}S]methionine for 30 min in serum-free medium; lane M, molecular weight markers. Sizes are indicated in kilodaltons on the left.

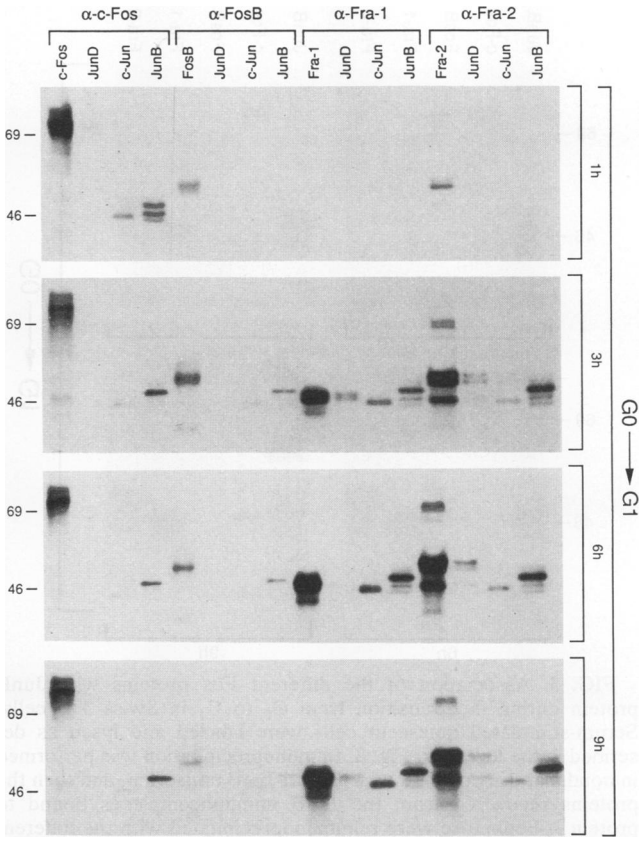

FIG. 2. In vivo association of Fos proteins with different Jun proteins during the transition from G_0 to G_1 in Swiss 3T3 cells. Quiescent cells stimulated with 20% dialyzed FCS in the presence of [^{35}S]methionine were continuously labeled for the indicated periods of time and then lysed under nondenaturing conditions (RIPA buffer). The Fos proteins were immunoprecipitated sequentially from the same cellular lysates by using first anti-c-Fos antibody and then anti-FosB, anti-Fra-1, and finally anti-Fra-2 antisera. The Fos immunocomplexes recovered by incubation with protein A-Sepharose were washed, dissociated by boiling in denaturing conditions, diluted five times with RIPA buffer without SDS, and then reimmunoprecipitated with anti-JunD, anti-c-Jun, and anti-JunB antisera, in sequential order. The immunocomplexes were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Sizes are indicated in kilodaltons on the left.

lysates were prepared and immunoprecipitated sequentially with anti-Fra-1 and anti-Fra-2 antibodies. A significant increase in Fra-1 and Fra-2 synthesis can be observed during the first hour of the transition, with Fra-2 presenting the most rapid increase in synthesis. The level of expression of Fra-1 and Fra-2 after serum stimulation is 10- to 20-fold higher than that in quiescent cells and remains at this level for at least 8 h. These results demonstrate that in Swiss 3T3 cells, as in other fibroblast cell lines, Fra-1 and Fra-2 are synthesized at a time in the transition from G_0 to G_1 when neither c-Fos nor FosB synthesis is detectable any longer.

Fos/Jun complexes during the transition from G_0 to G_1. Since the different Fos family members are expressed in a diversified manner throughout the initial stages of the G_0-to-G_1 transition, it is expected that the composition of the AP-1 complex during this period is also continuously changing. This conclusion is in part supported by our previous results showing that after serum stimulation, the newly synthesized c-Fos, FosB, and Jun proteins rapidly form Fos/Jun heterodimers and that the proportion of the different complexes formed is dependent on the relative amount of each Fos and Jun protein. This finding, together with the significant differences in expression of c-Fos and FosB versus Fra-1 and Fra-2, prompted us to determine which Fos/Jun complexes exist and prevail during the different stages of the transition from G_0 to G_1. Quiescent Swiss 3T3 cells stimulated with 20% FCS and continuously labeled for 1, 3, 6, and 9 h were lysed under native conditions, and the different Fos/Jun complexes were sequentially immunoprecipitated with specific anti-Fos antibodies from the same cell lysate. These immunocomplexes were dissociated, and the Jun proteins present in the complexes were identified by immunoprecipitation with specific Jun antibodies (Fig. 2). As we have previously shown (12), the dominant form of Fos protein at very early times of the transition from G_0 to G_1 (1 h) is c-Fos. Of the Jun proteins, JunB is the major form associated with c-Fos, since it is the predominant Jun protein synthesized by Swiss 3T3 cells (12). After 1 h into G_1, newly synthesized FosB and, to a greater extent, Fra-1 and Fra-2 molecules start to accumulate in the cell and to compete for the association with Jun proteins. At 3 h, the predominant Fos proteins are Fra-1 and Fra-2. Consequently, the proportion of Jun proteins complexed with these two proteins is higher than that observed for c-Fos and FosB. The dominance of the Fra-1/Jun and Fra-2/Jun complexes with respect to the other Fos/Jun complexes becomes more dramatic at later times following serum stimulation.

Interestingly, although no synthesis of c-Fos and FosB can be detected after 3 h of serum stimulation, it is still possible to find a significant amount of c-Fos and, to a lesser extent, FosB associated with Jun proteins at 9 h. The lasting presence of c-Fos agrees with our previous observations that c-Fos exhibits a biphasic curve of decay, being more stable at a time in the transition when the majority of the c-Fos molecules are associated with Jun proteins (12).

We have consistently observed differences in the relative amounts of c-Jun and JunB associated with Fra-1 and Fra-2 (Fig. 2). At different times in the transition from G_0 to G_1, c-Jun is preferentially associated with Fra-1, while JunB is associated with Fra-2.

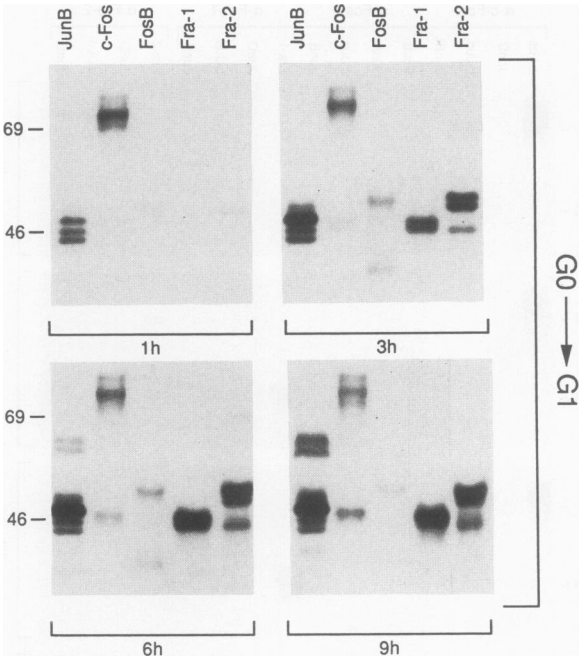

FIG. 3. Association of the different Fos proteins with JunB protein during the transition from G_0 to G_1 in Swiss 3T3 cells. Serum-stimulated quiescent cells were labeled and lysed as described in the legend to Fig. 2. Immunoprecipitation was performed in nondenaturing conditions with anti-JunB antiserum, and then the proteins recovered from the JunB immunocomplexes bound to protein A-Sepharose were reimmunoprecipitated with the different anti-Fos antibodies in sequential order. Sizes are shown in kilodaltons on the left.

To further confirm that the Fos/Jun complexes continuously change following serum stimulation, JunB was immunoprecipitated under native conditions to identify which Fos proteins are associated in vivo with this Jun molecule following induction of cell proliferation. JunB was chosen from the Jun family because it is the most abundant Jun protein present in Swiss 3T3 cells and because the anti-JunB antibody does not disrupt the Jun/Fos complex (12). The results shown in Fig. 3 are similar to those obtained for the immunoprecipitation of the Fos proteins in nondenaturing conditions (Fig. 2). During the first hour following serum stimulation, JunB associated with c-Fos is the dominant complex, while at 3 h, the proportion of JunB associated with either Fra-1 or Fra-2 is comparable to the proportion associated with c-Fos. After 6 h into G_1, the JunB/Fos complexes that predominate in the cell are those with Fra-1 and Fra-2.

These results demonstrate that the composition of the AP-1 complex dramatically changes during the G_0-to-G_1 transition and progression to S phase, and they confirm the previous observations that the ratio of each of the different Fos/Jun complexes is governed by the amount of each of the components.

Fos expression and complex formation with Jun proteins during asynchronous growth. The observation that Fra-1 and Fra-2 are synthesized for several hours following serum stimulation prompted us to investigate whether their expression is maintained during exponential growth and, if so, whether they are complexed with the Jun proteins. For this purpose, cells plated for 48 h (refed after 24 h of plating) were labeled for 3 h with [^{35}S]methionine and lysed under native conditions, and the different Fos-Jun complexes were then sequentially immunoprecipitated with specific anti-Fos antibodies as described above. The results shown in Fig. 4A demonstrate that the synthesis of both Fra-1 and Fra-2 is high in asynchronously growing cells. Several forms of Fra-1 and Fra-2 can be observed. Immunoprecipitations performed from exponentially growing cells labeled with [^{35}S]methionine or $^{32}P_i$ and then subjected to alkaline phosphatase treatment demonstrate that the various forms of Fra-1 and Fra-2 are due to different degrees of phosphorylation (not shown). Of note, FosB is undetectable and c-Fos is barely detectable in asynchronously growing cells. The weak expression of c-Fos observed is triggered by the experimental conditions (see below).

Fra-1 and Fra-2 are both complexed with the Jun proteins in exponentially growing cells (Fig. 4A), and the level of c-Jun and JunB associated with these Fos proteins is comparable to that observed at later times following serum stimulation of quiescent cells (Fig. 2). Interestingly, it can also be observed in exponentially growing cells that JunB binds preferentially to Fra-2 and that c-Jun binds preferentially to Fra-1. Results of similar experiments using anti-JunB antibodies to immunoprecipitate the JunB/Fos complexes demonstrate that a significant amount of Fra-1 and Fra-2 is complexed with JunB in exponentially growing cells (Fig. 4B). Comparison of the total amounts of c-Jun, JunB, Fra-1, and Fra-2 indicates that a high percentage of these proteins is associated as Jun/Fos complexes during asyn-

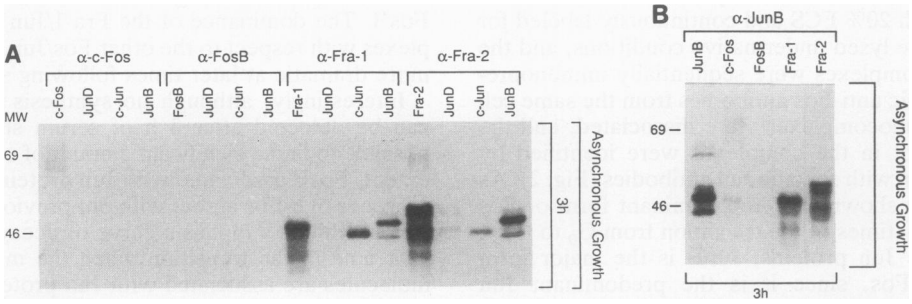

FIG. 4. In vivo associations of Fos proteins with different Jun proteins (A) and of JunB with different Fos proteins (B) during asynchronous growth of Swiss 3T3 cells. Exponentially growing cells in 10% FCS–DMEM were labeled with [^{35}S]methionine for 3 h in the presence of 10% dialyzed FCS and lysed under nondenaturing conditions (RIPA buffer). Then immunoprecipitations were done in sequential order; immunoprecipitation with either the different anti-Fos (A) or JunB (B) antibodies was followed by immunoprecipitation of the recovered Fos or JunB-associated proteins with anti-Jun or anti-Fos antibodies, respectively, as described in the legend to Fig. 2.

chronous growth (not shown). Moreover, the same post-translation modified forms of Fra-1 and Fra-2 observed in Fig. 4A can be detected in the heterodimers with JunB. This observation indicates that there is no particular preference of JunB for any of the modified forms of Fra-1 and Fra-2 in complex formation.

Steady-state levels of Fos proteins during different growth conditions. The results shown in Fig. 2 to 4 represent de novo protein synthesis and therefore provide no information on the amount of each protein already present in the cells before labeling. To verify how much of each Fos protein is present in the cells under different growth conditions, indirect immunofluorescent staining of the Fos proteins was performed with immunopurified antibodies. Figure 5A shows the immunofluorescence pattern of quiescent Swiss 3T3 cells serum stimulated for different periods of time. In quiescent cells (Fig. 5A, column Q), c-Fos and FosB are undetectable, while a very weak immunofluorescent signal can be seen for Fra-1 and Fra-2. Following stimulation with 20% FCS, a strong immunofluorescent signal is detected for c-Fos at 1 h that decays thereafter but still remains detectable in the cells after 6 h. In contrast, FosB is detected after 1 h and only for a short period of time. Fra-1 and Fra-2 immunofluorescence, on the other hand, becomes significant at 1 h following serum stimulation, increasing thereafter at least up to 9 h. The immunofluorescent signal shown by Fra-1 and Fra-2 after 9 h of serum stimulation is severalfold higher than that observed for c-Fos, demonstrating that indeed Fra-1 and Fra-2 are the predominant Fos molecules at later times of the G_0-to-G_1 transition. These results show that the amount of each Fos protein that can be detected by indirect immunofluorescence in the cells at different stages of the transition from G_0 to G_1 is comparable to the amount of each Fos protein detected by continuous cell labeling with [^{35}S]methionine as shown in Fig. 2.

Immunofluorescence of the Fos proteins in asynchronously growing cells carried out 24 h after the last medium change (Fig. 5B, column t_0) shows that Fra-1 and Fra-2 are the only Fos proteins present at significant levels in growing cells. However, as shown in Fig. 4A, c-Fos is weakly detected in immunoprecipitates of asynchronously growing cells labeled for 3 h with [^{35}S]methionine. To determine whether this expression of c-Fos is due to the change of medium required for the labeling, growing cultures were refed with fresh medium and immunofluorescence of Fos proteins was carried out at the indicated times. As shown in Fig. 5B, there is indeed a strong but very short induction of c-Fos expression that lasts for less than 2 h, explaining the presence of c-Fos in the immunoprecipitates of labeled cells. Conversely, FosB is not detected after the change of medium, while the expression of Fra-1 and Fra-2 is slightly increased.

The results obtained by indirect immunofluorescent staining of Fos proteins during different growth conditions support the notion that c-Fos and FosB are expressed mainly during the early stages of the transition from quiescence to G_1, while Fra-1 and Fra-2 are expressed in both, i.e., during the transition from G_0 to G_1 and during asynchronous growth.

Fos protein requirements for the entrance into S phase under different growth conditions. The expression of Fos proteins during the G_0-to-G_1 transition and asynchronous growth indicates that in Swiss 3T3 cells, these proteins could have differential roles in cell proliferation. Therefore, it was of interest to investigate whether the entry into S phase of serum-stimulated and exponentially growing cells depends on the activity of the different Fos proteins. For this purpose, cytoplasmic microinjections of immunopurified antibodies against each individual Fos protein either alone or in combinations were performed. Swiss 3T3 cells were grown in both growth conditions, and the effects of the antibodies on DNA synthesis were determined. We have previously demonstrated that individual neutralization of c-Fos, FosB, and Fra-1 during the transition from G_0 to G_1 blocks only 30 to 50% of the injected cells from entering the S phase (13). In these studies, we have extended these observations by including the microinjection of anti-Fra-2 antibodies (Table 1). The results show that serum-stimulated cells do not depend exclusively on the activity of any single Fos protein in order to progress through G_1 and start DNA synthesis. The highest inhibition was obtained with anti-c-Fos antibody (46%), and the lowest was obtained with anti-Fra-1 antibody (30%). Both anti-FosB and anti-Fra-2 antibodies showed an intermediate effect (36%). On the other hand, if all Fos proteins were neutralized at once (anti-Fos family antibodies), more than 85% of the injected cells became blocked in the G_0-to-G_1 transition and did not enter the S phase. In contrast to what is observed in serum-stimulated cells, microinjection of the different anti-Fos antibodies into exponentially growing cells shows that anti-Fra-1 is the most effective in preventing DNA synthesis (32%), and anti-Fra-2 antibodies are effective to a lesser extent (26%), compared with anti-c-Fos and anti-FosB antibodies, which inhibit only 12 and 10%, respectively. When asynchronously growing cells are microinjected with the anti-Fos family antibodies, nearly 45% of the injected cells are inhibited from entering S phase, which is significantly higher than the DNA synthesis inhibition observed by neutralizing either Fra-1 or Fra-2. These results show that either during the transition from G_0 to G_1 or during asynchronous growth, the functional elimination of a single member of the Fos family is less effective in preventing DNA synthesis than is the simultaneous inhibition of two or more different Fos molecules, and they suggest that the lack of activity of any given member of the Fos family is in part compensated for by the remaining Fos proteins.

Since Fra-1 and Fra-2 are present in both serum-stimulated and exponentially growing cells, while c-Fos and FosB are restricted mainly to the transition from G_0 to G_1, we decided to investigate whether these Fos combinations differ in temporal importance for the entrance into S phase. For this purpose, combinations of anti-c-Fos and anti-FosB antibodies or anti-Fra-1 and anti-Fra-2 antibodies were microinjected into G_0/G_1 and exponentially growing cells. The percentage of DNA synthesis inhibition obtained was compared with the percentage of inhibition that resulted from the injection of anti-Fos family antibodies (maximum DNA synthesis inhibition due to anti-Fos antibodies, which was normalized to 100% in order to facilitate the comparisons [Table 1]). As expected from the protein data, the simultaneous neutralization of c-Fos and FosB had a stronger effect on DNA synthesis in serum-stimulated quiescent cells (82% of inhibition) than in growing cells, in which the level of inhibition was approximately 30%. Conversely, the simultaneous inactivation of Fra-1 and Fra-2 was more effective in preventing DNA synthesis in growing cells (72% of inhibition), while in G_0/G_1 cells, less than 45% of the injected cells were affected. These results indicate that there is indeed a differential requirement of Fos proteins for cell cycle progression and for entering S phase during the induction of cell proliferation and exponential growth.

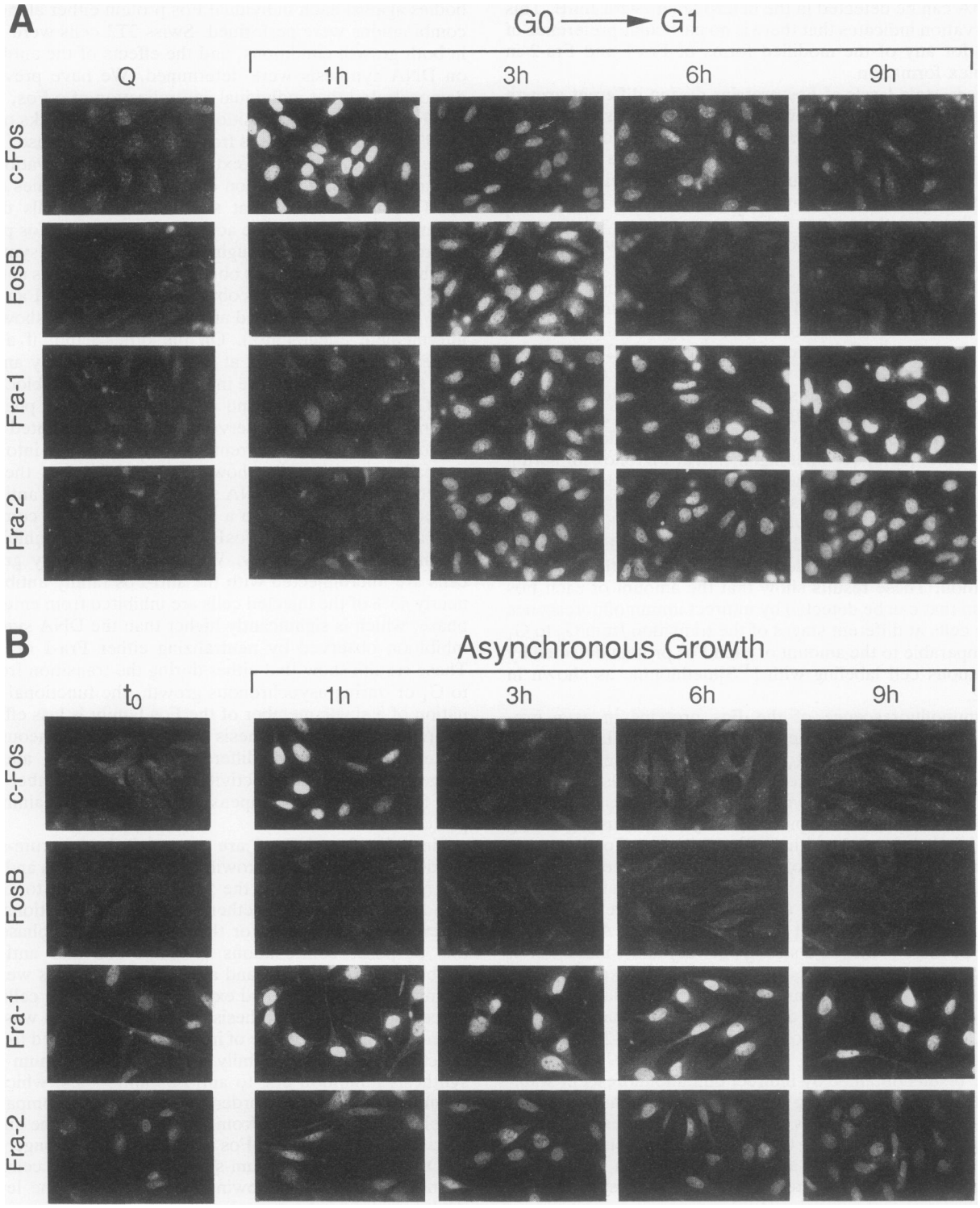

FIG. 5. Expression of the Fos proteins during the transition from G_0 to G_1 (A) and during asynchronous growth (B) of Swiss 3T3 cells visualized by indirect immunofluorescent staining. (A) Immunofluorescence of quiescent cells (Q) stimulated with 20% FCS for the indicated periods of time; (B) immunofluorescence of exponentially growing cells before (t_0) and after being refed with 10% FCS for different periods of time. Cells were fixed with cold methanol (4°C) and washed with PBS, and immunofluorescence staining was performed with anti-Fos antibodies as described in Materials and Methods.

TABLE 1. Microinjection of anti-Fos antibodies into Swiss 3T3 cells in different growth conditions[a]

Immunoglobulin injected	% inhibition of DNA synthesis (avg ± SD)[b]	
	G_0-to-G_1 transition	Asynchronous growth
Control	13.6 ± 1.2 (15.5)	4.0 ± 2.1 (9.0)
Anti-c-Fos	47.0 ± 12.1 (53.7)	12.4 ± 4.8 (27.9)
Anti-FosB	36.6 ± 10.2 (41.8)	9.9 ± 4.3 (22.2)
Anti-Fra-1	30.5 ± 9.5 (34.9)	32.1 ± 6.2 (71.9)
Anti-Fra-2	36.1 ± 9.9 (41.3)	26.1 ± 7.3 (58.7)
Anti-Fra-1 + anti-Fra-2	37.5 ± 6.7 (42.9)	32.1 ± 1.7 (72.1)
Anti-c-Fos + anti-FosB	71.5 ± 13.1 (81.7)	13.6 ± 4.7 (30.6)
Anti-Fos Family	87.5 ± 9.5 (100)	44.5 ± 7.8 (100)

[a] Antibodies were microinjected into Swiss 3T3 cells in two growth conditions: quiescence and exponential growth. To determine the effects of the antibodies during the transition from G_0 to G_1, quiescent cells (48 h in 2.5% FCS) were microinjected into the cytoplasm with the antibodies and then immediately stimulated with 20% FCS. Cells were fixed 24 h later with cold methanol. For exponential growth, cells were plated at a density of 2×10^3 to 3×10^3 cells per cm^2 in DMEM containing 10% FCS. Cells were microinjected into the cytoplasm with the different antibodies 48 h after plating and fixed 30 h later with cold methanol. DNA synthesis was checked by bromodeoxyuridine incorporation. Immunofluorescent staining of the positive cells was done as described in Materials and Methods.

[b] Numbers in parentheses indicate the percentage of inhibition with respect to the value for the anti-Fos family antibody (normalized to 100%) in order to facilitate comparison.

DISCUSSION

The *fos* family genes are transcribed differentially during the transition of fibroblasts from G_0 to G_1, with *fra*-1 and *fra*-2 being expressed later and showing a more prolonged kinetics of transcription than c-*fos* and *fos*B (5, 8, 15, 16, 22, 26, 42). Therefore, concomitant changes in the composition of the AP-1 complexes formed during the different stages of the transition from G_0 to G_1 are expected. However, qualitative changes in the composition of the Fos proteins present in AP-1 complexes have never been thoroughly investigated, possibly because of the lack of specific antibodies against the different Fos proteins. The generation of specific antibodies against c-Fos, FosB, Fra-1, and Fra-2 enabled us to investigate their levels of expression and complex formation with Jun proteins as well as their requirements for cell cycle progression in Swiss 3T3 cells under different growth conditions.

The results of the immunoprecipitation experiments of the complexes formed between Fos and Jun proteins during the transition from G_0 to G_1 show that all possible Fos/Jun combinations are present nearly throughout the transition but in different proportions (Fig. 2). For example, in the first hour of the G_0-to-G_1 transition, the most abundant complexes are those between c-Fos and Jun proteins, while in late G_1 (9 h), Fra-1 and Fra-2 are the major Fos proteins found associated with the Jun proteins. These results are in agreement with the observation that the amount of a specific heterodimer formed at any given period of time between distinct Fos and Jun proteins depends on the relative amount of each protein present at that moment (12). However, our studies have shown for the first time that heterodimer formation could have certain specificity such as that observed for the association of c-Jun with Fra-1 and JunB with Fra-2 (Fig. 2). The continuous qualitative and quantitative changes of the different Jun homodimers and Fos/Jun heterodimers during the G_0/G_1 transition may provide the cell with a very fine tuning mechanism for controlling gene expression. This notion is supported by the observations that the Jun proteins differ in their binding and transcriptional activities (4, 33, 37), which are differentially affected by Fos proteins (33, 39). Taken together, these data strongly suggest that in fibroblasts following serum stimulation, not only the total AP-1 composition but also its functional properties are rapidly changing as a result of the variation in its components.

Fos protein expression is not restricted only to the transition from G_0 to G_1. We have demonstrated that during exponential growth, two of the Fos proteins, Fra-1 and Fra-2, are continuously synthesized (Fig. 4), indicating that in growing cells there is a nearly constant amount of these proteins (Fig. 5B). It is important to mention that although c-Fos is not detectable in asychronously growing cells, it is transiently induced after the medium is changed (Fig. 5B). The c-Fos protein synthesized by growing cells remains detectable for less than 2 h, in contrast to the lasting presence of c-Fos in serum-stimulated quiescent cells. Since in growing cells the majority of the Jun molecules are associated with Fra-1 and Fra-2 proteins (Fig. 4), it is possible that most of the c-Fos molecules transiently synthesized during exponential growth are not able to associate with Jun proteins, and therefore the c-Fos protein could be less stable under these conditions, similar to the situation of c-Fos during the first hour of the transition G_0 to G_1, when a small number of Jun molecules are present (12).

Our results demonstrate that Fos proteins are differentially distributed in a quantitative and qualitative manner during the different stages of the transition from G_0 to G_1 and during asynchronous growth. c-Fos and FosB expression is restricted mainly to the onset of cell proliferation, while Fra-1 and Fra-2, apart from being expressed during the induction of cell proliferation, are the major Fos proteins present in growing cells. Recently, it has been shown that both Fra-1 and Fra-2 can have a stimulatory or inhibitory effect on Jun activity, depending on the partner in the heterodimer. c-Jun activity is inhibited by Fra-1 and Fra-2, while JunD activity is stimulated (39). Moreover, Fra-1 and Fra-2 have an inhibitory effect on the transcriptional stimulation of c-Fos/c-Jun heterodimers. These results, together with the finding that Fos proteins are differentially expressed during the induction of cell proliferation and exponential growth, strongly indicate that the regulatory properties of AP-1 differ under these growth conditions and suggest that the repertoires of genes controlled by AP-1 in the two cases should, in part, be different.

The results of the microinjection of anti-Fos antibodies (Table 1) into Swiss 3T3 cells show that Fos proteins are required both during the transition from G_0 to G_1 and during exponential growth for the entrance into S phase. Nevertheless, there are temporal differences in the kind of Fos protein required for cellular proliferation in either growth condition. Microinjection of anti-c-Fos and anti-FosB antibodies demonstrated that c-Fos and FosB, although they are the most important Fos proteins required by serum-stimulated quiescent cells in order to initiate events leading to DNA synthesis, play a minor role in exponentially growing cells. On the other hand, microinjection of anti-Fra-1 and anti-Fra-2 antibodies showed that the activities of Fra-1 and Fra-2, while in part required for cell cycle progression of serum-stimulated cells, are most important for cell division during exponential growth.

Proliferating cells normally have a shorter G_1 period than do cells that are exiting quiescence, and it is assumed that this is because proliferating cells do not require the early G_1 events that are necessary for the transition from G_0 to G_1. It

has been suggested that those G_1 events, absent in growing cells, would correspond to the first hours of the transition from G_0 to G_1. Since the synthesis of c-Fos and FosB occurs in this period of the transition, it is possible to infer that both Fos proteins play an important role in the control of the initial G_1 events that would be unique to the transition from G_0 to G_1. We have consistently observed that a small but significant fraction of the cells from an asynchronous population microinjected with either anti-c-Fos or anti-FosB antibodies are inhibited to enter S phase. Most probably this corresponds to the fraction of cells in an asynchronous culture that normally leave the cell cycle and enter G_0. It is important to mention that the medium is changed after microinjection, which stimulates a small fraction of cells in G_0 to reenter the cell cycle. If the activities of either c-Fos or FosB have been neutralized by the microinjection of antibodies, then the cells will not be able to reach S phase, explaining the inhibition observed with these antibodies in asynchronous cultures. On the other hand, considering the patterns of expression of Fra-1 and Fra-2, their activities are most likely implicated with cell cycle progression, and therefore these proteins will be required both during the G_0-to-G_1 transition and during exponential growth. Although Fos proteins could have different specific functions, our microinjection experiments indicate that the missing activity of any given Fos protein can be partially compensated for by the function of the other Fos family proteins, suggesting that they share some common activities (Table 1) (13). These results could explain, in part, why the constitutive overexpression of any of the Fos proteins in fibroblast cells efficiently induces malignant transformation (13a, 14, 18, 21, 26, 35, 41). It will be of interest to determine whether either c-Fos or FosB can compensate for the inhibition of Fra-1 and Fra-2 activities in exponentially growing cells.

Since the activity of Fra-1 and Fra-2 is required for the continuous proliferation of Swiss 3T3 cells, and considering that the overexpression of either of these proteins induces malignant transformation in fibroblasts, a tight control of the level and activities of Fra-1 and Fra-2, in order to keep cells under normal growth, is expected. This strict control is supported by the fact that the synthesis, posttranslational modifications, and stability of both proteins rapidly decrease in exponentially growing cultures following serum deprivation (not shown). Considering that an asynchronously growing culture is a heterogeneous cellular population composed of cells distributed along the different phases of the cell cycle, it is possible that some of the posttranslational modifications of Fra-1 and Fra-2 observed in exponentially growing cells are cell cycle regulated. This hypothesis is currently being investigated.

ACKNOWLEDGMENTS

We thank Richard Metz and Heather Macdonald-Bravo for critical review of the manuscript and Josephine Vittor for its preparation.

REFERENCES

1. **Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin.** 1987. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell **49**:729–739.
2. **Angel, P., and M. Karin.** 1991. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim. Biophys. Acta **1072**:129–157.
3. **Bravo, R.** 1990. Growth factor inducible genes in fibroblasts, p. 324–343. *In* A. Habenicht (ed.), Growth factors, differentiation factors and cytokines. Springer-Verlag, Berlin.
4. **Chiu, R., P. Angel, and M. Karin.** 1989. Jun-B differs in its biological properties from, and is a negative regulator of, c-Jun. Cell **59**:979–986.
5. **Cohen, D. R., and T. Curran.** 1988. *fra*-1: a serum-inducible, cellular immediate early gene that encodes for a *fos*-related antigen. Mol. Cell. Biol. **8**:2063–2069.
6. **Cohen, D. R., P. C. P. Ferreira, R. Gentz, B. R. Franza, Jr., and T. Curran.** 1989. The product of a *fos*-related gene, *fra*-1, binds cooperatively to the AP-1 site with Jun: transcription factor AP-1 is comprised of multiple protein complexes. Genes Dev. **3**:173–184.
7. **Dobrzanski, P., T. Noguchi, K. Kovary, C. A. Rizzo, P. S. Lazo, and R. Bravo.** 1991. Both products of the *fos*B gene, FosB and its short form, FosB/SF, are transcriptional activators in fibroblasts. Mol. Cell. Biol. **11**:5470–5478.
8. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-*fos* proto-oncogene. Nature (London) **311**:433–438.
9. **Halazonetis, T. D., K. Georgopoulos, M. E. Greenberg, and P. Leder.** 1988. c-*jun* dimerizes with itself and with c-*fos*, forming complexes of different DNA binding affinities. Cell **55**:917–924.
10. **Herschman, H. R.** 1991. Primary response genes induced by growth factors and tumor promoters. Annu. Rev. Biochem. **60**:281–319.
11. **Holt, J. T., T. V. Gopal, A. D. Moulton, and A. W. Nienhuis.** 1986. Inducible production of c-*fos* antisense RNA inhibits 3T3 cell proliferation. Proc. Natl. Acad. Sci. USA **83**:4794–4798.
12. **Kovary, K., and R. Bravo.** 1991. Expression of different Jun and Fos proteins during the G_0-to-G_1 transition in mouse fibroblasts: in vitro and in vivo associations. Mol. Cell. Biol. **11**:2451–2459.
13. **Kovary, K., and R. Bravo.** 1991. The Jun and Fos protein families are both required for cell cycle progression in fibroblasts. Mol. Cell. Biol. **9**:4466–4472.

13a. **Kovary, K., C. A. Rizzo, and R. Bravo.** Unpublished data.

14. **Kovary, K., C. A. Rizzo, R.-P. Ryseck, T. Noguchi, C. Raynoschek, J.-M. Pelosin, and R. Bravo.** 1991. Constitutive expression of FosB and its short form, FosB/SF, induces malignant cell transformation in Rat-1A cells. New Biol. **3**:870–879.
15. **Kruijer, W., J. A. Cooper, T. Hunter, and I. M. Verma.** 1984. Platelet-derived growth factor induces rapid but transient expression of the c-*fos* gene and protein. Nature (London) **312**:711–716.
16. **Lamph, W. W., P. Wamsley, P. Sassone-Corsi, and I. M. Verma.** 1988. Induction of proto-oncogene JUN/AP-1 by serum and TPA. Nature (London) **334**:629–631.
17. **Lau, L. F., and D. Nathans.** 1991. Genes induced by serum growth factors, p. 165–201. *In* P. Cohen and J. G. Foulkes (ed.), The hormonal control regulation of gene transcription. Elsevier, New York.
18. **Lee, W., C. Lin, and T. Curran.** 1988. Activation of the transforming potential of the human *fos* proto-oncogene requires message stabilization and results in increased amounts of partially modified *fos* protein. Mol. Cell. Biol. **8**:5521–5527.
19. **Lee, W., P. Mitchell, and R. Tjian.** 1987. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell **49**:741–752.
20. **Matsui, M., M. Tokuhara, Y. Konuma, N. Nomura, and R. Ishizaki.** 1990. Isolation of human *fos*-related genes and their expression during monocyte macrophage differentiation. Oncogene **5**:249–255.
21. **Miller, A. D., T. Curran, and I. M. Verma.** 1984. c-fos protein can induce cellular transformation: a novel mechanism of activation of a cellular oncogene. Cell **36**:51–60.
22. **Müller, R., R. Bravo, J. Burckhardt, and T. Curran.** 1984. Induction of c-*fos* gene and protein by growth factors precedes activation of c-*myc*. Nature (London) **312**:716–720.
23. **Nakabeppu, Y., and D. Nathans.** 1991. A naturally occurring truncated form of FosB that inhibits Fos/Jun transcriptional activity. Cell **64**:751–759.
24. **Nakabeppu, Y., K. Ryder, and D. Nathans.** 1988. DNA binding activities of three murine *jun* proteins: stimulation by fos. Cell **55**:907–915.
25. **Nishikura, K., and J. M. Murray.** 1987. Antisense RNA of

proto-oncogene c-*fos* blocks renewed growth of quiescent 3T3 cells. Mol. Cell. Biol. 7:639–649.
26. **Nishina, H., H. Sato, T. Suzuke, M. Sato, and H. Iba.** 1990. Isolation and characterization of *fra*-2, an additional member of the *fos* gene family. Proc. Natl. Acad. Sci. USA 87:3619–3623.
27. **Pepperkok, R., C. Schneider, L. Philipson, and W. Ansorge.** 1988. Single cell assay with automated capillary microinjection system. Exp. Cell Res. 178:369–376.
28. **Rauscher, F. J., P. J. Voulalas, B. R. Franza, and T. Curran.** 1988. Fos and Jun bind cooperatively to the AP-1 site: reconstitution *in vitro*. Genes Dev. 2:1687–1699.
29. **Riabowol, K. T., R. J. Vosatka, E. B. Ziff, N. J. Lamb, and J. R. Feramisco.** 1988. Microinjection of *fos*-specific antibodies blocks DNA synthesis in fibroblast cells. Mol. Cell. Biol. 8:1670–1676.
30. **Ryder, K., A. Lanahan, E. Perez-Albuerne, and D. Nathans.** 1989. Jun-D: a third member of the Jun gene family. Proc. Natl. Acad. Sci. USA 86:1500–1503.
31. **Ryder, K., L. F. Lau, and D. Nathans.** 1988. A gene activated by growth factors is related to the oncogene v-*jun*. Proc. Natl. Acad. Sci. USA 85:1487–1491.
32. **Ryder, K., and D. Nathans.** 1988. Induction of proto-oncogene c-*jun* by serum growth factors. Proc. Natl. Acad. Sci. USA 85:8464–8467.
33. **Ryseck, R.-P., and R. Bravo.** 1991. c-JUN, JUNB and JUND differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins. Oncogene 6:533–542.
34. **Ryseck, R.-P., S. I. Hirai, M. Yaniv, and R. Bravo.** 1988. Transcriptional activation of c-*jun* during the G0/G1 transition in mouse fibroblasts. Nature (London) 334:535–537.
35. **Schuermann, M., K. Jooss, and R. Muller.** 1991. *fos*B is a transforming gene encoding a transcriptional activator. Oncogene 6:567–576.
36. **Schutte, J., J. D. Minna, and M. J. Birrer.** 1989. Deregulated expression of human c-*jun* transforms primary rat embryo cells in cooperation with an activated c-Ha-ras gene and transforms Rat-1a cells as a single gene. Proc. Natl. Acad. Sci. USA 86:2257–2261.
37. **Schutte, J., J. Viallet, M. Nau, S. Segal, J. Fedorko, and J. Minna.** 1989. *jun*-B inhibits, and c-*fos* stimulates the transforming and trans-activating activities of c-*jun*. Cell 59:987–997.
38. **Smith, M. J., and E. V. Prochownik.** 1992. Inhibition of c-*jun* causes reversible proliferative arrest and withdrawal from the cell cycle. Blood 79:2107–2115.
39. **Suzuki, T., H. Okuno, T. Yoshida, T. Endo, H. Nishina, and H. Iba.** 1992. Difference in transcriptional regulatory function between c-Fos and Fra-2. Nucleic Acids Res. 19:5537–5542.
40. **Vogt, P. K., and T. J. Bos.** 1990. Jun: oncogene and transcription factor. Adv. Cancer Res. 55:2–36.
41. **Yen, J., R. M. Wisdom, I. Tratner, and I. M. Verma.** 1991. An alternative spliced form of FosB is a negative regulator of transcriptional activation and transformation by Fos proteins. Proc. Natl. Acad. Sci. USA 88:5077–5081.
42. **Zerial, M., L. Toschi, R.-P. Ryseck, M. Schuermann, R. Müller, and R. Bravo.** 1989. The product of a novel growth factor activated gene, *fos* B, interacts with JUN proteins enhancing their DNA binding activity. EMBO J. 8:805–813.