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E1A + cHa-*ras* Transformed Rat Embryo Fibroblast Cells Are Characterized by High and Constitutive DNA Binding Activities of AP-1 Dimers With Significantly Altered Composition

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Transcription factors of the AP-1/ATF family, including c-Fos, c-Jun, and ATF-2, play an important role in the regulation of cell proliferation and differentiation, and changes in their levels and/or activities may contribute to oncogenesis. We analyzed the alterations of AP-1/ATF transcription factors upon immortalization and transformation in a panel of cell lines derived from rat embryo fibroblast (REF) cells. The tumorigenic E1A + cHa-*ras* cells are characterized by high and constitutive DNA binding activities of AP-1, in contrast to nontransformed cells and the E1A cells. The expression of c-*fos* and c-*jun* genes was affected differently by the oncogenic transformation. By using antibodies to c-Jun and c-Fos proteins in electrophoretic mobility shift assays (EMSA), we showed that E1A + cHa-*ras* transformants did not contain c-Fos under any condition of cell cultivation and growth factor stimulation, whereas c-Jun was constitutively upregulated. In the absence of c-*fos* gene expression, c-Fos protein appears to be replaced by proteins of Fos family (Fra-1) and ATF family (ATF-2 and ATFa). To determine the possible mechanisms of c-*fos* downregulation in E1A + cHa-*ras* transformants we have obtained populations of geneticin-resistant clones containing integrated reporter construct −711*fos*-CAT and its mutants in serum-responsive element (SRE) and cAMP-responsive element (CRE). Data obtained show that the mutations within the SRE lead to a manifold activation of *fos*-CAT expression. This allows to suggest that c-*fos* downregulation in E1A + cHa-*ras* transformants is provided by a negative control mediated through the SRE regulatory region. The profound differences in regulation and composition of transcription factors of the AP-1 family probably play a pivotal role in the transformation of REF cells by E1A and cHa-*ras* oncogenes.

E1A and cHa-*ras* oncogenes *fos* and *jun* expression AP-1 transcription factors

STIMULATION of quiescent normal cells to proliferation by growth factors initiates their transition from phase G_0 to G_1 of the cell cycle and induces the transcription of a large number of so-called immediate-early genes and genes involved in signal transduction (13,24). The first group includes the proto-oncogenes c-*jun* and c-*fos*, the products of which are members of the AP-1 transcription factors family. The AP-1 factors play an important role in the regulation of cell proliferation and differentiation in re-

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sponse to various stimuli. Transcription factors of this family constitute Fos/Jun heterodimers, Jun/Jun homodimers, or Jun heterodimers with members of the ATF/CREB family (i.e., Jun/ATF-2, Jun/ATF-3, and Jun/ATFa) (1,6,7,19,21,26). Depending on the composition of the dimers, these proteins are capable of binding to specific subsets of AP-1-regulatory elements and respond to different stimuli (21,26,40). The 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-responsive element (TRE) of the collagenase gene promoter (coll-TRE) is bound preferably by Jun/Fos and Jun/Jun dimers, and is activated mainly by mitogenic stimuli. Similar AP-1-responsive elements have been identified in the promoters of many genes activated upon the stimulation of cells by growth factors or TPA (3,30). Therefore, the Fos and Jun proteins possibly play an important role in the control of cell proliferation and transformation (9,27,48,49). The TREs of the c-*jun* gene itself (*jun1*-TRE and *jun2*-TRE) are preferentially targeted by Jun/ATF-2 dimers, and can be activated by UV light and DNA-damaging agents, such as MMS (19,46,47). The ATF-containing complexes may thus be involved primarily in various stress responses. It is possible that perturbations in the levels of the different protein complexes and/or in the regulation of their activities by genetic mutation or by viruses may have profound consequences for the cell proliferation and differentiation control and thereby contribute to malignant cell transformation. This suggestion is supported by the finding that various viral and cellular nuclear oncoproteins, such as human adenoviruses E1A proteins, SV40 Large T, c-*myc*, and several cytoplasmic oncoproteins (cHa-*ras*, c-Raf, or polyoma middle T), indeed influence transcription of a number of cellular genes via AP-1-responsive DNA elements by modulating the composition and/or activities of AP-1 complexes (3,9,16,17,49). These oncoproteins belong to distinct subgroups: the nuclear oncoprotein (e.g., E1A), which can immortalize primary cells (5), and oncoproteins such as cHa-Ras, which can cooperate with the immortalizing proteins for full oncogenic transformation (35,39,48,50). Accordingly, distinct changes in the composition of AP-1 protein complexes or in the activities of their constituents may contribute to the immortalization process, and additional changes may be necessary for full transformation. In this study, we show that E1A + cHa-*ras* transformed cells are characterized by high and constitutive DNA binding activity of the AP-1 complex. In these cells, c-*fos* gene is downregulated and c-*jun* gene is upregulated. Moreover, significant changes of the AP-1 complex composition have been detected: c-Fos appears to be replaced by Fra-1 protein and factors of the ATF family (ATF-2, ATFa). The expression of *fos*-CAT mutants integrated in E1A + cHa-*ras* transformants allows to suggest that downregulation of c-*fos* gene expression is likely to be mediated through the SRE regulatory region of c-*fos* gene promoter.

MATERIALS AND METHODS

Cell Lines

Rat embryo fibroblasts (REF) immortalized by stable transfection of the Ad5 E1A oncogene or transformed by a combination of E1A + cHa-*ras* oncogenes have been described earlier (36). In contrast to the E1A-immortalized cells, E1A + cHa-*ras* cells display an increased saturation density and form colonies in soft agar. When injected into nude mice, E1A + cHa-*ras* cells give rise to tumors within a few weeks. E1A + E1B19kD cell lines have been established by cotransfection of primary REF cells with expression vectors encoding for Ad5 E1A and Ad5 E1B19kD (43). The REF cells (second passage) and the cell lines were grown in DMEM supplemented with 10% fetal calf serum (FCS; Gibco or Biolot). Cells were serum starved for 48 h in the presence of 0.5% FCS and stimulated by addition of 10% FCS, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA, 50 ng/ml, Sigma), epidermal growth factor (EGF, 100 ng/ml, Serva), dibutyryl cAMP (dbcAMP, 0.001 M, Sigma) for 1 h.

Nuclear Extracts

Nuclear extracts were prepared by using a protocol that has already been described (37). Briefly, 5×10^6 cells were resuspended in 1.5 ml of PBS solution and centrifuged, after which the pellet was resuspended in 800 µl of cold hypotonic solution (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) for 15 min. Subsequently, 50 µl of 10% NP-40 was added and the mixture was vigorously shaken. Sedimented nuclei were gently shaken in a solution consisting of 20 mM HEPES, pH 7.9, 0.42 M NaCl, 1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, and other protease inhibitors for 15 min at 0°C. Subsequently, the extract was cleared by centrifugation. The nuclear extracts were stored at 70°C in 10-µl aliquots. Protein concentration was determined according to Bradford's method (12).

Oligonucleotides

The oligonucleotides used in this work are as follows. The TRE from the promoter of the human collagenase I gene, 5′-AGCATGAGTCAGCC-3′ (coll-

TRE); a mutated TRE derived from the same promoter, 5′-AGCTGGAGTCAGCC-3′; one of the TREs of the c-*jun* gene, 5′-AGCTAGCATTACCT CATCCC-3′ (*jun2*-TRE). The oligonucleotides were labeled with [^{32}P]dNTPs with the Klenow fragment of *E. coli* DNA-polymerase I or phosphorylated by polynucleotide kinase with [γ-^{32}P]ATP.

Electrophoretic Mobility Band Shift Assay (EMSA)

The incubation reaction mixture (10 µl) consisted of 10 mM HEPES, pH 7.9, 1 mM DTT, 1 mM EDTA, 8 mM $MgCl_2$, 10% glycerol, 2 µg of nuclear extracts, 1 µg poly(dI-dC). The mixture was incubated for 20 min at 4°C followed by addition of labeled oligonucleotides (30,000 cpm/ng) for a 20-min period. Specific and nonspecific oligonucleotides were used in competition experiments at a 100-fold molar excess. DNA–protein complexes were separated by electrophoresis in a 5% polyacrylamide gel (30:1) in 1× TBE buffer, pH 8.3. Gels were transferred to filter paper, dried, and exposed to X-ray film. EMSA experiments with specific antibodies (a supershift analysis) were carried out as follows: nuclear extracts were incubated in the presence of 2 µl PBS, 2 µl nonimmune serum (NIS), or specific antibodies for 2 h on ice, before addition of the labeled oligonucleotides. Antibodies used in these supershift experiments were purchased from Santa Cruz Biotechnologies: c-Fos (#sc-52x, #sc-413x), c-Jun (#sc-45x), ATF-2 (#sc-187x), JunD (#sc-74x), ATF-3 (#sc-188), Fra-1 (#sc-183x). Mouse monoclonal 3C12 antibody against full-length ATFa3 was a kind gift of B. Chatton (14). Rabbit polyclonal antibodies to FosB, (83–138 aa), Fra-1 (1–82 aa), and Fra-2 (200–252 aa) were kindly supplied by M. Yaniv (29).

RNA Analysis

Total cellular RNA was prepared as described (10, 36a). For Northern blots, total RNA (20–30 µg/lane) was denatured and electrophoresed on 1.5% agarose gels containing 2.2 M formaldehyde. The amount and quality of the RNA samples were checked by staining the gels with ethidium bromide (data not shown). Gels were capillary blotted onto nylon membranes (Gene Screen Plus, NEN) and baked for 2 h at 80°C. The DNA fragments used as probes are a 1.1 kb *Pst*I fragment of the v-*fos* gene and a 1.0 kb *Pst*I fragment of the c-*jun* gene. The DNA fragments were nick-translated with [^{32}P]dNTPs. Hybridization was performed in rotating cylinders for 36 h at 68°C in a solution consisting of 3× SSC, 1× Denhardt, 0.5% SDS, and denatured *E. coli* DNA (50 µg/ml). Membranes were washed, dried, and autoradiographed at −70°C in the presence of an intensifying screen (Curix MR 600).

Populations of Clones With Integrated Plasmid Constructs

E1A + cHa-*ras* cells were cotransfected with a selectable vector pSVneo conferring geneticin resistance and the following plasmids: *fos*-CAT (−711*fos*-CAT) or point mutants in SRE site [substitution G > A in positions of −319 and −304 nucleotides (11)]; a deletion mutant of CRE site (−711*fos*Δ-65/−52CAT) (22); *jun*-CAT (−1600/+170m*jun*-CAT) and its mutants in *jun1*-TRE (−1600/+170Δ1) or *jun2*-TRE (−1600/+170Δ2) (44). The ratio of selectable/reporter plasmids was 1:5. Following the transfection, cells were grown for 2 days without antibiotic, then were plated on a selective medium containing 400 µg/ml of geneticin (G-418, Gibco). Selection was continued until the visible clones could be seen. All clones were pooled in a population containing the corresponding reporter construct. The populations of clones were used for obtaining cell extracts to monitor for the CAT activity. Cell extracts were normalized with respect to total protein measured by Bradford's method (12).

Determination of CAT Activity

CAT activity was determined according to a method previously described (18) by using [^{14}C]-chloramphenicol (Amersham) as an acceptor of acetyl groups. Acetylated products were separated by thin-layer chromatography on silicagel-covered plates (Merck) in a mixture of chloroform/methanol (95:5). Dried plates were exposed to Kodak X-ray films. All experiments were repeated not less than three times. Fold induction was calculated as the increase in CAT activity relative to that obtained with the control (wild-type) reporter plasmid. Values are given as the averages of the three independent experiments, and errors show standard deviations.

Western Blot Analysis

After removing medium, cells were rinsed with cold PBS. RIPA buffer (0.6 ml) was added to a 100-mm petri dish (RIPA buffer composition: PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors including 1 mM PMSF, pepstatin A, leupeptin, aprotinin, trypsin inhibitor, 100 nM sodium orthovanadate). Cells were scrapped with a rubber scraper, transferred to a microfuge tube, vigorously vortexed, and centrifuged for 15 min at 12,000 rpm. Protein concentration in supernatant was measured by Bradford's method (12). Proteins were sepa-

rated by electrophoresis in 7.5–10% polyacrylamide gel and then transferred from the gel to Immobilon P membrane (Millipore). The membranes were incubated with primary antibodies: to c-Fos (sc-52), to c-Jun (sc-45), to JunD (sc-74) (Santa Cruz Biotech.). After washing, binding primary antibodies were detected with horseradish-conjugated secondary antibodies and revealed using the enhanced chemiluminescence method (ECL) following the manufacturer's instructions (Amersham).

RESULTS

*High and Constitutive DNA Binding Activity of AP-1 Complexes in E1A + cHa-*ras *Transformed Cells*

We analyzed DNA binding activities of the AP-1 complexes, their composition and regulation by growth factors in REF cells immortalized by E1A, or transformed by a combination of E1A and cHa-*ras* oncogenes. An electrophoretic mobility-shift analysis (EMSA) of AP-1 transcription factors in nuclear extracts of cells grown in the presence of 10% FCS was performed with the TRE of the human collagenase I gene promoter (coll-TRE) or one of the TREs of the c-*jun* gene (*jun2*-TRE) as probes. These oligonucleotides were chosen because they differ in their affinities for various AP-1 dimers (47). The *jun2*-TRE binds mainly Jun/ATF dimers, whereas the coll-TRE binds preferably Fos/Jun complexes (40,47). Slower and faster migrating I and II complexes can be resolved with the used oligonucleotide probes (20,46,47) (Fig. 1A). Competition experiments with cold nonmutated and a mutated oligonucleotide confirmed the specificities of the observed binding (results not shown). Two important differences between E1A + cHa-*ras* cells and REF and E1A cells could be observed. First, the slower migrating complex formed on the *jun2*-TRE (i.e., complex I) was found to be low in untransformed REF and E1A-immortalized cells, but was drastically increased in E1A + cHa-*ras* transformed cells. The amount of the faster migrating complex II bound to the *jun2*-TRE was also increased in the E1A + cHa-*ras* transformed cells, albeit to a lesser extent. These results suggest that E1A + cHa-*ras* cells contain significantly higher levels of ATF-containing dimers (complex I). As for the coll-TRE bound complexes, the amount of complex II was clearly increased upon transformation. Taken together, these results suggest that E1A+ cHa-*ras* cells contain significantly higher levels of both dimer types (see also below).

The AP-1 binding activity has also been analyzed with the coll-TRE as a probe in nuclear extracts of REF cells and E1A + cHa-*ras* transformants grown under various conditions (Fig. 1B, C). Serum-starved cells (0.5% FCS for 48 h) were stimulated for 1 h with 10% FCS, TPA, EGF, or dbcAMP. As shown in Fig. 1B, in serum-starved REF cells (lane 2) the AP-1 DNA binding activity was very low, but could be stimulated by addition of 10% FCS, EGF, or TPA. The inducibility of AP-1 DNA binding activity was also observed in E1A-immortalized cells (results not shown). In contrast to the REF and E1A cells, E1A + cHa-*ras* transformants reveal high levels of AP-1 complexes, which cannot be up- or downregulated by addition or removal of serum or by treatment with TPA, EGF, or dbcAMP (Fig. 1C).

The c-jun *Gene Is Constitutively Expressed at High Levels, Whereas* c-fos *Is Downregulated and Cannot Be Induced in E1A + cHa-*ras *Cells*

We have earlier analyzed by Northern blot hybridization the expression of c-*fos* and c-*jun* genes, whose products are the constituents of the AP-1 complexes (36a). Total RNA was prepared from REF cells, E1A-immortalized, and E1A + cHa-*ras* transformed cells grown in the presence of 10% FCS or 0.5% FCS (serum starvation) and after stimulation of serum-starved cells with 10% FCS and TPA (Fig. 2). The results showed that the expression of c-*fos* and c-*jun* genes was found to be under stringent control in normal REF cells and that these genes could be activated by growth factors as expected from data in Fig. 1B. c-*fos* and c-*jun* genes were also found to be activated by serum in E1A-immortalized cells, although the noninduced levels of c-*jun* gene expression were somewhat higher than in REF cells (Fig. 2, middle). In E1A + cHa-*ras* transformed cells, which have very high level of AP-1 DNA binding activity (Fig. 1), the c-*fos* gene expression could not be detected under any conditions of cell cultivation, whereas c-*jun* was expressed at a high and constitutive level (Fig. 2, right). Thus, the expression of c-*fos* and c-*jun* genes was found to be affected to a different extent in E1A + cHa-*ras* transformed cells: c-*fos* was downregulated and could not be stimulated by any growth factor, whereas c-*jun* was constitutively upregulated.

Because the most significant increases in expression of immediately early genes, such as c-*fos*, can be detected upon serum stimulation of quiescent cells, we performed the EMSA after incubation of nuclear extracts prepared from serum-stimulated cells with c-Fos-specific antibodies. We used the concentrated antibodies (see Materials and Methods), which are able to form high molecular complexes with the transcription factors, giving rise to the supershifted

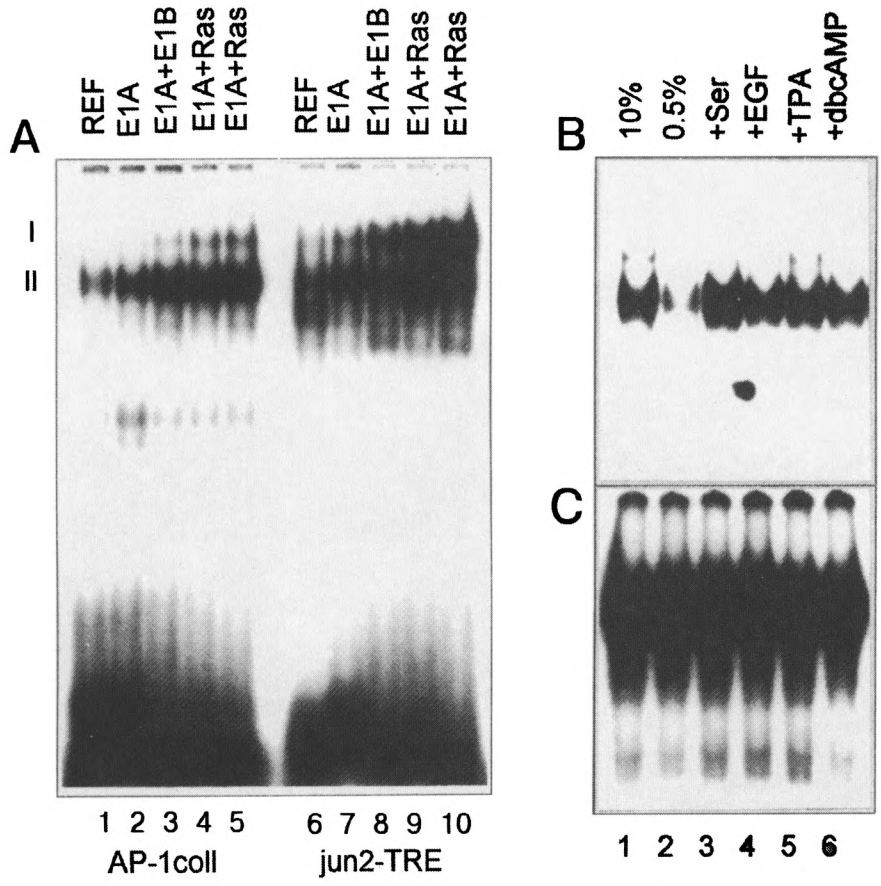

FIG. 1. (A) Changes of AP-1 complexes I and II detected in nuclear extracts of REF, E1A-immortalized, E1A + E1B19kD, and E1A + cHa-*ras* cells with the coll-TRE or *jun2*-TRE probes in electrophoretic mobility shift assays (EMSA). Nuclear extracts were prepared from cells grown in medium supplemented with 10% FCS. The positions of the slower migrating complex (I) and the faster complex (II) have been indicated. (B, C) Regulation of AP-1 DNA binding activity in REF cells (B) and E1A + cHa-*ras* transformants (C). Cells were serum starved in the presence of 0.5% FCS for 48 h and stimulated by addition of 10% FCS, EGF, TPA, or dbcAMP for 1 h (see Materials and Methods). Nuclear extracts were isolated and used in EMSA. The labeled coll-TRE oligonucleotide was used as a probe.

bands upon electrophoresis (Fig. 3). It was found that c-Fos protein can indeed be detected in the serum-stimulated REF and E1A-immortalized cells (supershifted complexes marked by SC). Under the same conditions, in nuclear extracts of E1A + cHa-*ras* or E1A + E1B19kD transformed cells the supershifted complexes were not detected (Fig. 3). A band marked by a star does represent the complex I described in Fig. 1A, and it migrates actually faster than the supershifted Fos-specific complex detected in REF and E1A cells. In agreement with these data, c-Fos proteins were also not detected by Western blot both in nonstimulated and stimulated E1A + cHa-*ras* cells (Fig. 4B, lanes 3 and 4), whereas in REF cells c-Fos proteins were readily seen after serum stimulation (Fig. 4B, lanes 1 and 2). In accordance with our Northern blot hybridization data, the amount of c-Jun protein was found to be increased in exponentially growing E1A + cHa-*ras* transformants (Fig. 4A, lane 4). Correspondingly, antibodies raised against c-Jun protein caused the formation of a supershifted band when incubated with nuclear extracts of not only serum-stimulated REF cells but also E1A + cHa-*ras* transformants growing in the presence of 10% FCS (Fig. 5A, lanes 3 and 7). Interestingly, the amount of JunD proteins measured by the immunoblot procedure was very similar in all investigated cell lines (Fig. 4A, top panel). But JunD-supershifting antibodies revealed detectable amount of JunD-containing AP-1 complexes both in nonstimulated and serum-stimulated REF and E1A cells (Fig. 5B), but barely in E1A + cHa-*ras* transformants (Fig. 5C, lanes 3 and 4 the supershifted complexes). Thus, though JunD protein is present in comparable amounts in REF and E1A + cHa-*ras* cells (Fig. 4A), the content of JunD-containing AP-1 complexes appears to be low in E1A + cHa-*ras* transformants compared with normal REF cells.

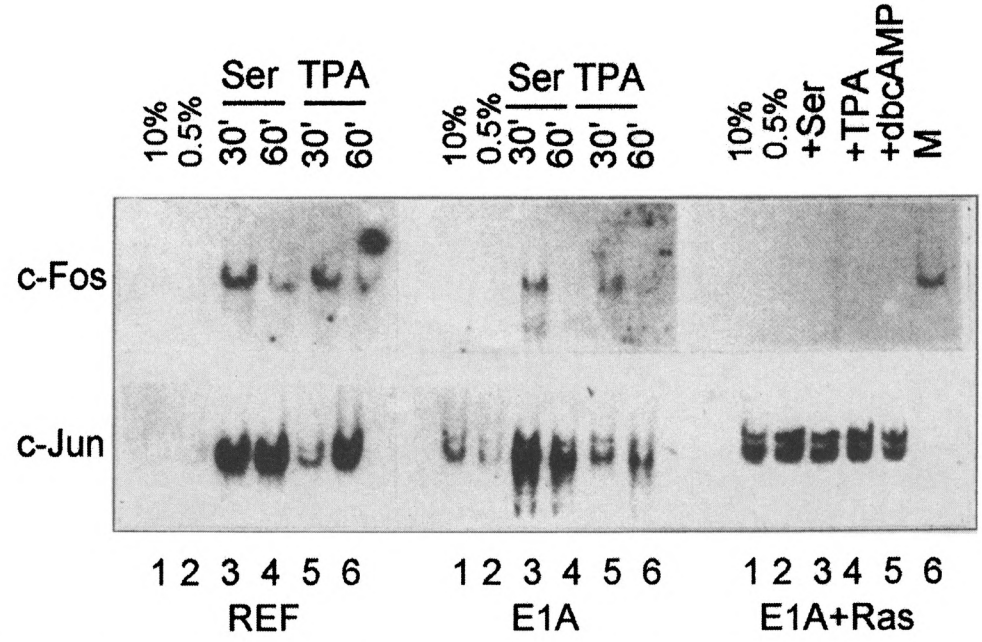

FIG. 2. Northern blot hybridization of RNA isolated from REF cells, E1A-immortalized cells, and E1A + cHa-*ras* transformed cells with DNA probes specific for the c-*fos* and the c-*jun* genes (36a). REF and E1A cells were grown in DMEM supplemented with 0.5% FCS for 48 h (serum starvation) and subsequently were stimulated by addition of 10% FCS and TPA during 30 and 60 min. E1A + cHa-*ras* cells were stimulated with 10% FCS, TPA, and dbcAMP during 30 min. In the lane indicated with "marker," a control RNA isolated from serum-stimulated E1A cells was hybridized with the c-*fos* probe.

*c-Fos Appears to be Replaced by Fra-1 Protein in E1A + cHa-*ras *Transformants*

Because c-Fos protein was found to be absent in E1A + cHa-*ras* transformants, we also checked other proteins of the Fos family by incubating the nuclear extracts with antibodies to FosB, Fra-1, and Fra-2 transcription factors. Data presented in Fig. 6A show that the FosB protein expression appears to follow by the same mode as for c-Fos: exponentially growing REF cells (10% FCS, Fig. 6, lane 2)) do not practically reveal FosB in AP-1 complex, whereas serum stimulation leads to FosB accumulation (Fig. 6A, lane 5). However, AP-1 complexes from serum-stimulated E1A + cHa-*ras* transformants did not contain FosB protein (Fig. 6, lane 11). The Fra-2 protein can be detected in unstimulated REF cells and in less amount in stimulated REF and E1A cells (Fig. 6, lanes 3, 6, and 9), but not in AP-1 complex of E1A + cHa-*ras* transformants (no supershifted complexes, no reduction of the AP-1 complex intensity, Fig. 6, lane 12). Quite different data were obtained with antibodies specific to Fra-1 protein (Fig. 6B, C). If unstimulated (Fig. 6B) and stimulated (Fig. 6C) REF and E1A cells contain little but detectable quantity of the factor, the amount of Fra-1 in E1A + cHa-*ras* transformants was found to be significantly increased (Fig. 6B, C, supershifted complexes). Thus, in the absence of c-*fos* gene expression, c-Fos protein appears to be replaced by Fra-1, and Fra-1 becomes a predominant component of the Fos family presented in the AP-1 complex of E1A + cHa-*ras* cells.

*E1A + cHa-*ras *Transformants Contain High Levels of ATF-Containing Dimers*

To study whether AP-1 complex of E1A + cHa-*ras* transformants does contain, except of Fra-1 and Jun, the proteins of the ATF family, we used antibodies specific for ATF-2, ATF-3, and ATFa factors and the labeled *jun2*-TRE as a probe in supershift EMSA experiments. Data presented in Fig. 7 show that E1A + cHa-*ras* cells do contain significant amount of ATF-2 and ATFa factors, which are the components of complex I. One can see the supershifted complexes concomitant with the reduction of the complex I intensity (Fig. 7A, B). No supershifted complexes could be detected with ATF-3-specific antibodies, although ATF-3 factor could be readily detected with the same antibodies in control extracts (HER cells transformed by E1 region of Ad5) (19) (results not shown). Thus, the composition of AP-1 complexes of E1A + cHa-*ras* cells is changed from c-Fos/c-Jun and Jun/Jun dimers to those composed of Fra-1/ATF/Jun.

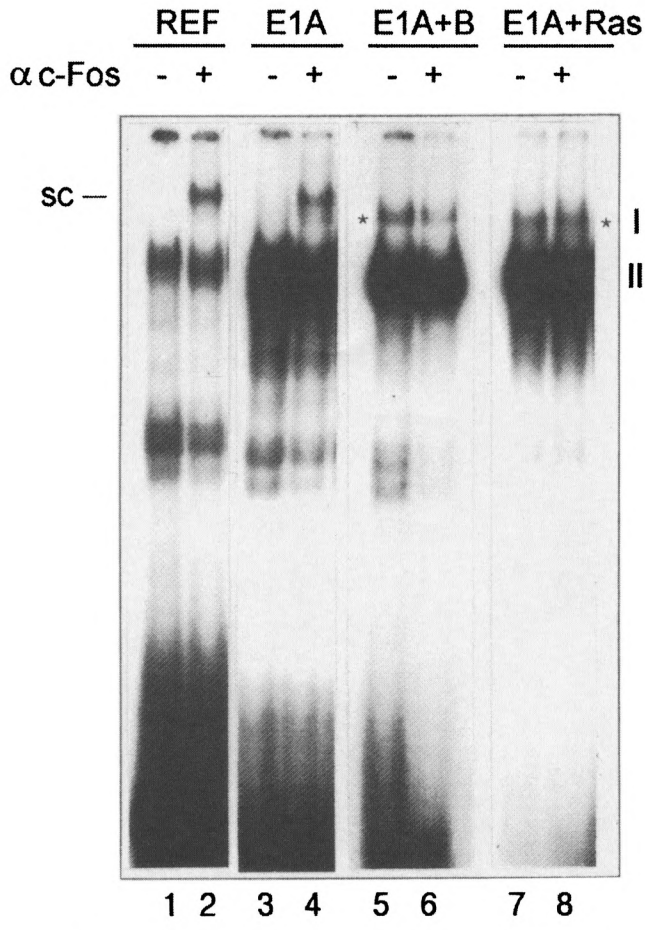

FIG. 3. c-Fos protein is a component of AP-1 complex in nuclear extracts of serum-stimulated REF (lanes 1 and 2) and E1A-immortalized cells (lanes 3 and 4) but not in serum-stimulated E1A + E1B19kD (lanes 5 and 6) and E1A + cHa-*ras* transformants (lanes 7 and 8). Cells were serum starved for 48 h (0.5% FCS), then subsequently stimulated by addition of 10% FCS for 1 h. Nuclear extracts (2 μg) were incubated with 2 μl of PBS or with c-Fos-specific antibodies (Santa Cruz sc-52x). Subsequently, labeled coll-TRE was added followed by incubation for 20 min. SC indicates supershifted AP-1 complexes containing the c-Fos; asterisks indicate the position of complex I in relation to supershifted complex (SC).

Negative Regulation of c-fos *Gene Promoter in E1A + cHa-*ras *Cells Is Likely to Be Mediated Through the SRE Site*

To determine the possible mechanisms of c-*fos* downregulation we stably integrated a plasmid construct containing a fragment of c-*fos* gene promoter (–711/+45) linked to a reporter bacterial CAT gene (–711*fos*-CAT). To study the effects produced by mutations in the c-*fos* promoter, we used several mutant constructs: point mutants in serum response element (SRE) (G > A transitions at positions –319 at the TCF binding site and –304 nt at the SRF binding site), a deletion mutant of cAMP-responsive element (CRE) (–711*fos*Δ-65/–52CAT). The used mutations inhibited the serum-stimulated *fos*-CAT activity differently, in particular, in the TCF binding site to 80–90%, in the SRF binding site to 60% (11), and in the CRE site up to 70–80% (22). All plasmids were introduced into E1A + cHa-*ras* cells by cotransfecting with a selectable vector pSVneo conferring geneticin resistance to antibiotic G-418. The formed geneticin-resistant clones were pooled and used to monitor the levels of CAT activity. The CAT assay given in Fig. 8 represents one of three independent experiments with each mutant construct. The averages and standard deviations are given below in the text. As expected, the activity of wild-type –711*fos*-CAT construct was very low in E1A + cHa-*ras* cells that was in clear agreement with the lack of c-*fos* gene expression in these cells (see Fig. 2). A deletion of the CRE site (ΔCRE) only slightly affected the activity of –711*fos*-CAT (1.55 ± 0.4-fold activation). The fact that the CRE mutation did not lead to any significant

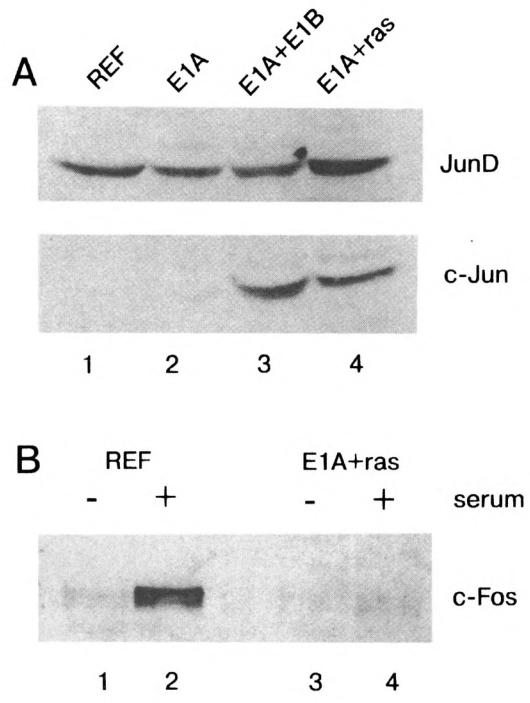

FIG. 4. (A) Western blot analysis of total proteins from REF, E1A, E1A + E1B19kD, and E1A + cHa-*ras* cells probed with antibodies to JunD and c-Jun proteins. Cells were grown in the presence of 10% FCS and lysed with a full RIPA buffer (see Materials and Methods). Proteins were separated by electrophoresis in 10% polyacrylamide gel and stained by antibodies specific to JunD and c-Jun proteins (Santa Cruz sc-74 and sc-45). (B) c-Fos protein is not serum stimulated in E1A + cHa-*ras* transformants. REF (lanes 1, 2) and E1A + cHa-*ras* (lanes 3, 4) cells were serum starved (0.5% FCS for 48 h, lanes 1 and 3), then serum stimulated with 10% FCS for 1 h (lanes 2 and 4). Proteins were separated by electrophoresis and visualized with antibodies specific to c-Fos protein (Santa Cruz sc-52).

increase in expression suggests that the c-*fos* promoter repression was not based on this element in E1A + cHa-*ras* transformants. On the other hand, both mutations within the SRE site (at −319 and −304 nt positions) caused a several fold increase of *fos*-CAT activity (mTCF: 13.9 ± 0.8- and mSRF: 33.9 ± 0.1-fold activation, respectively) (Fig. 8A). Because these mutations affect the TCF and SRF binding to the SRE (45,52), this allows to suggest that negative regulation of c-*fos* promoter in E1A + cHa-*ras* cells is mediated through the SRE-SRF/TCF ternary complex. To prove that c-*fos* promoter mutants behave properly in other cell lines, we transiently transfected the plasmids into REF and NIH 3T3 cells. The cells were grown in 10% FCS (c-*fos* is repressed) or serum starved in 0.5% FCS and then stimulated with 10% FCS for 1–3 h (c-*fos* is induced). Data presented in Fig. 8B show that under conditions of c-*fos* downregulation the mutations affecting TCF and SRF binding caused elevated *fos*-CAT expression (Fig. 8B, lanes 2 and 3). In contrast, the mutations inhibited serum-stimulated c-*fos* promoter expression (Fig. 8B, lanes 5 and 6).

To confirm a functional significance of Jun/ATF heterodimers that are in abundance in nuclear extracts of E1A + cHa-*ras* cells, we stably integrated by similar method the the CAT constructs driven by c-*jun* gene promoter: wild-type −1600/+740*jun*-CAT, mutants in *jun1*-TRE (−1600/+740Δ1) and in *jun2*-TRE (−1600/+740Δ2) (44). As expected, activity of wild-type *jun*-CAT was very high in E1A + cHa-*ras* cells; the results obtained for *jun1*-TRE mutation (Δ1) showed that this AP-1 site contributed little to c-*jun* expression because there can be even seen slight activation (1.14 ± 0.02). On the other hand, *jun2*-TRE mutation (Δ2) practically turned the c-*jun* promoter off (Fig. 8A). Thus, the *jun2*-TRE element is functionally significant for the expression of c-*jun* promoter in E1A + cHa-*ras* cells, and Jun/ATF heterodimers that fail to bind to the mutant *jun2*-TRE site are not capable of effectively using another AP-1 element, *jun1*-TRE, to facilitate the transcription of c-*jun* promoter in these cells.

DISCUSSION

Changes in the levels and/or activities of AP-1/ATF factors by genetic mutation or by the action of viral proteins may contribute to oncogenesis. In this article, we show that REF cells transformed by Ad5 E1A and cHa-*ras* oncogenes are characterized by high and constitutive DNA binding activity of AP-1 complexes when various AP-1 elements have been used as probes in EMSA: coll-TRE and *jun2*-TRE. Also, we observed significant changes in the composition of AP-1 complexes: c-*fos* was no longer expressed or stimulated by serum in E1A + cHa-*ras* transformants, whereas some other members of Fos family (FosB, Fra-2) contributed very little, if at all, to the AP-1 complex in these cells. In contrast, Fra-1 factor, another member of the Fos family, was found to be accumulated in the AP-1 complex of E1A + cHa-*ras* transformants. It appears that c-Fos/c-Jun heterodimers, which are specific for the $G_0 > G_1$ transition of the cell cycle, are replaced by the complexes containing Fra-1 factors. Because Fra-1 protein was found to be accumulated in NIH 3T3 cells transformed by a single cHa-*ras* oncogene (33), one may suggest that the E1A oncogene does contribute little to elevation of the Fra-1 content of E1A + cHa-*ras* cells (see also Fig. 6B, C).

The above-mentioned shift in the AP-1 composi-

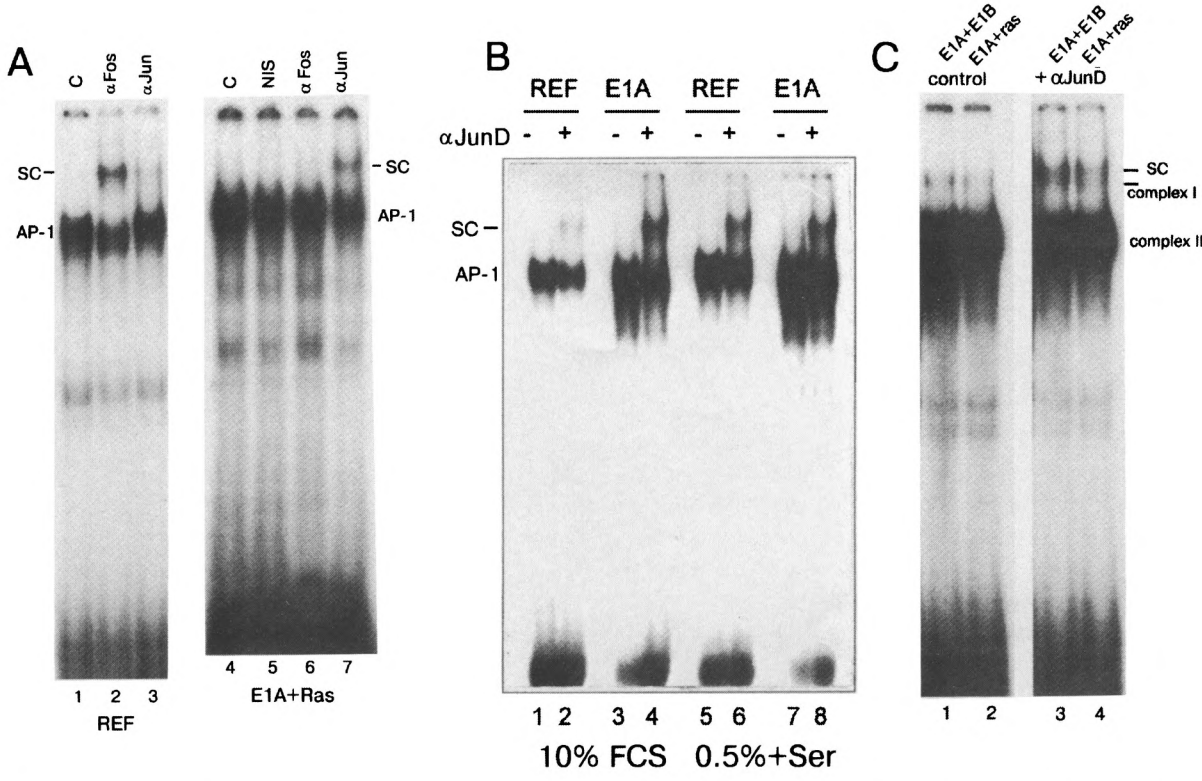

FIG. 5. (A) c-Jun proteins are present in AP-1 complex of exponentially growing E1A + cHa-*ras* cells. Nuclear extracts of serum-stimulated REF cells (lanes 1–3) and exponentially growing E1A + cHa-*ras* cells (lanes 4–7) were incubated with antibodies to c-Jun protein (Santa Cruz #sc-45x, lanes 3 and 7) and c-Fos protein (Santa Cruz sc-52x, lanes 2 and 6). NIS: nonimmune serum; SC: supershifted AP-1 complexes formed with the specific antibodies. (B, C) JunD proteins are detected in AP-1 complex of nuclear extracts from normal and transformed cells by the EMSA supershift analysis. (B) REF and E1A cells were grown in the presence of 10% FCS (lanes 1–4) or serum starved (0.5% FCS for 24 h) and then serum stimulated with 10% FCS for 1 h (lanes 5–7). Nuclear extracts were incubated with PBS (lanes 1, 3, 5, 7) or with JunD-specific antibodies (lanes 2, 4, 6, 8, Santa Cruz sc-74x), and complexes were separated in 5% gel. SC: AP-1 complexes supershifted by JunD-specific antibody. (C) Nuclear extracts from E1A + E1B19kD cells (lanes 1 and 3) and E1A + cHa-*ras* cells (lanes 2 and 4) were incubated with PBS (control, lanes 1 and 2) or with JunD-specific antibodies (lanes 3 and 4). Two horizontal lines are given to discriminate mobilities of supershifted AP-1 complexes (SC) and the complex I that migrates faster. The labeled coll-TRE oligonucleotide was used as a probe.

tion may have profound effects on the regulation of gene expression. The c-Jun/c-Fos and c-Jun/ATF complexes have distinct DNA binding specifities and influence on different sets of target genes. In addition, the c-Jun/c-Fos and the c-Jun/ATF complexes are differentially affected by various stimuli. The first mentioned complexes are stimulated mainly by mitogenic agents, whereas the latter are predominantly activated by stress- and DNA-damaging agents. Moreover, these complexes are affected differently by E1A. Whereas E1A oncogene represses transcription mediated by c-Jun/c-Fos complexes, possibly by interference with their DNA binding activities, and by squelching the transcriptional coactivator p300, E1A stimulates transcription via c-Jun/ATF heterodimers (2,4,20). Taking into account that the E1A + cHa-*ras* cells contain high and constitutive levels of Jun/ATF complexes and no c-Fos-containing complexes under any of the tested growth conditions, one can expect that especially genes containing regulatory elements resembling the *jun1*-TRE and the *jun2*-TRE will be activated. Which target genes are important for transformation by E1A + cHa-*ras* largely remain to be determined; however, it seems highly probable that high and constitutive expression of the c-*jun* gene (and probably *fra*-1) may contribute to transformation by E1A + cHa-*ras* oncogenes. Consistently, fibroblasts from c-*jun* gene knock-out mice cannot be transformed by the cHa-*ras* oncogene, whereas ectopic expression of c-*jun* leads to partial transformation of NIH 3T3 cells (27).

Concerning the role of another immediate-early gene, c-*fos*, in oncogenic transformation, the situation is more complex. In fibroblast cells the formation of transformed foci by various oncogenes was found to be independent of c-*fos* gene expression (c-*fos* +/+

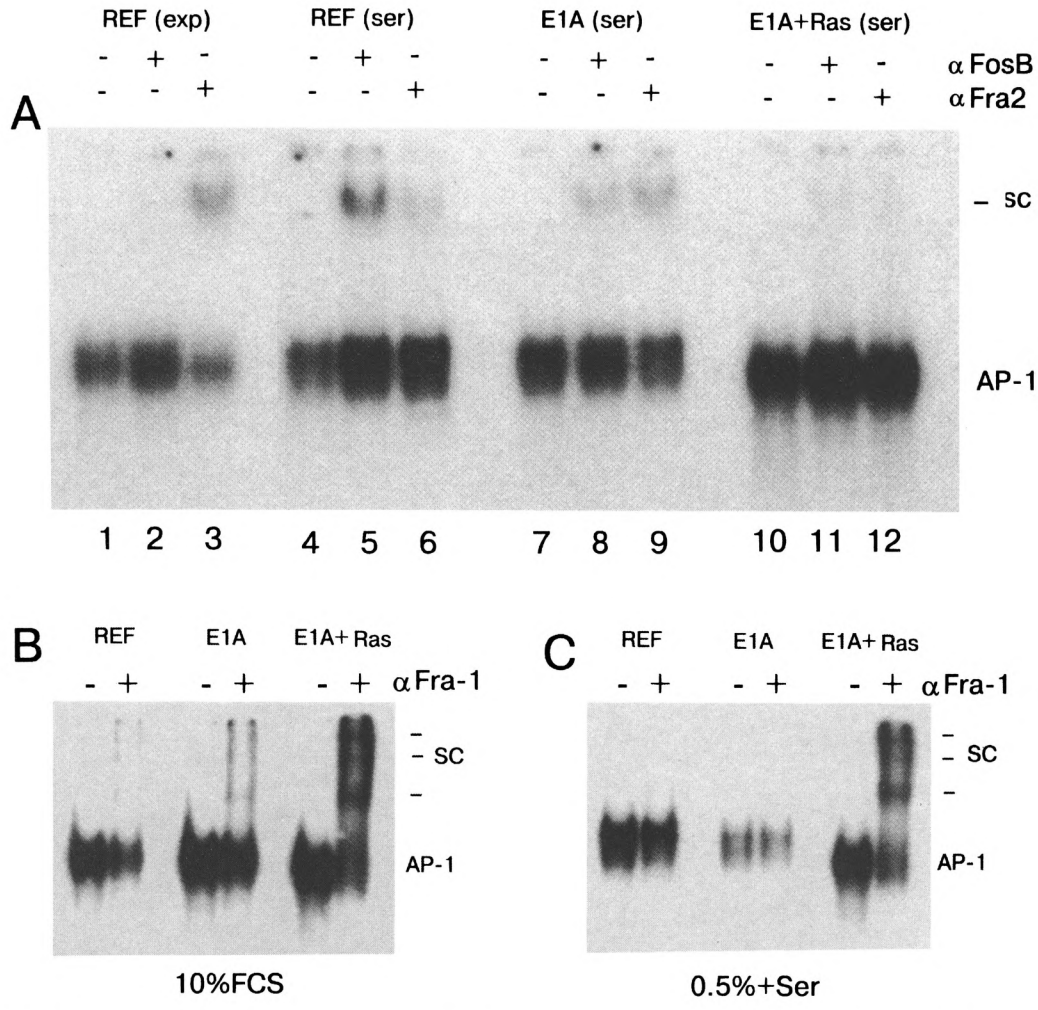

FIG. 6. (A) FosB and Fra-2 proteins in AP-1 complexes of normal and transformed cells. Nuclear extracts have been isolated from exponentially growing REF cells (10% FCS, lanes 1–3) or REF cells stimulated by 10% FCS for 1 h (lanes 4–6), from serum-stimulated E1A cells (lanes 7–9), and serum-stimulated E1A + cHa-*ras* cells (lanes 10 and 12). Nuclear extracts were incubated with antibodies to FosB and Fra-2 proteins (29). The labeled coll-TRE oligonucleotide was used as a probe. SC: high molecular complexes (supershifts) formed in the presence of the specific antibodies. (B, C) Fra-1 protein is accumulated in nuclear extracts of E1A + cHa-*ras* transformed cells. Nuclear extracts have been obtained from exponentially growing (B) and serum-stimulated (C) REF, E1A, and E1A + cHa-*ras* cells. In both panels, lanes (–) without addition of Fra-1-specific antibodies, lanes (+) added Fra-1 specific antibodies (Santa Cruz sc-183x). The labeled coll-TRE oligonucleotide was used as a probe. Horizontal lines indicate the positions of supershifted complexes (SC).

vs. c-*fos* –/– cells), suggesting that active c-*fos* is not obligatory, at least for initial steps of transformation (25), but c-*fos* is required for malignant progression of keratinocytes (41). On the other hand, rat fibroblast cell line 208F can be morphologically transformed by inducible continuous expression of a single c-*fos* gene (34). These authors showed that the c-*fos* expression was not sufficient, however, to stimulate cell cycle progression, implying that other genes should be switched to start cell proliferation. Other experiments suggest that the noninducibility or attenuation of c-*fos* expression upon transformation by Ha-Ras may be a more general phenomenon (32,51). For example, in rat 3Y1 cells transformed by cytoplasmic oncogenes (v-*src*, v-*sis*, and v-*raf*) serum inducibility of several immediate early genes, including c-*fos*, was shown to be attenuated (51). In mouse NIH 3T3 cells, introduction of cHa-*ras* also results in loss of the c-*fos* inducibility (37). Apparently, downregulation and noninducibility of c-*fos* upon oncogenic transformation is not limited to the REF cell system and may contribute to a shift in balance of transcription factors and thereby may contribute to oncogenic transformation. It has been suggested that ATFa and c-Fos might have functional antagonistic property (14). The c-Jun heterodimers with ATF-2 and c-Fos

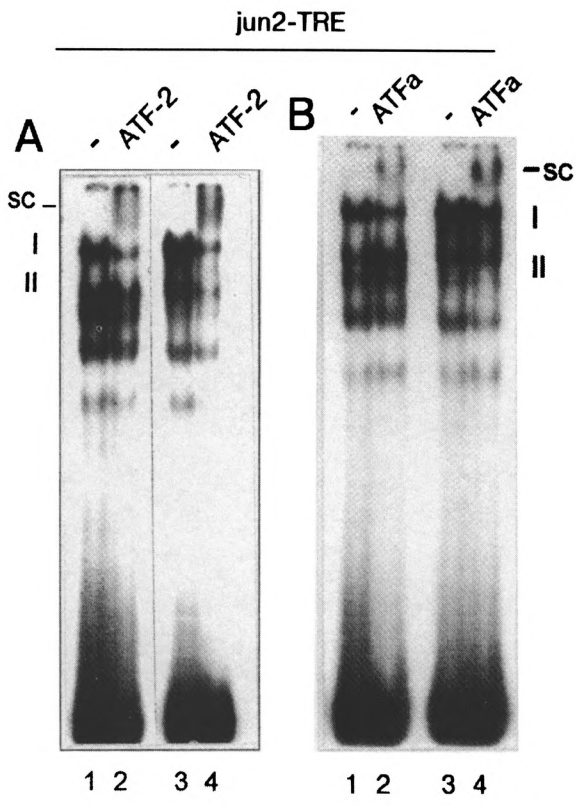

FIG. 7. Detection of ATF-containing complexes with the *jun2*-TRE probe in supershift EMSA experiment. (A) ATF-2-containing AP-1 complexes in nuclear extracts of E1A + E1B19kD (lanes 1 and 2) and E1A + cHa-*ras* (lanes 3 and 4) cells were detected with the *jun2*-TRE-labeled probe and ATF-2-specific antibodies (lanes 2 and 4). (B) ATFa-containing AP-1 complexes were detected in nuclear extracts of E1A + E1B19kD cells (lanes 1 and 2), and E1A + cHa-*ras* cells (lanes 3 and 4) with ATFa-specific antibodies (14) (lanes 2 and 4) and *jun2*-TRE-labeled probe. I and II indicate the AP-1 complexes, and SC indicates the supershifted complexes.

can regulate positively and negatively for the urokinase gene enhancer, respectively (15). In the E1A + cHa-*ras* cells, the absence of c-Jun/c-Fos complexes and constitutively high levels of Jun/ATF may thus even further increase activation of certain genes via ATF-responsive elements.

As for Fra-1 expression in E1A + cHa-*ras* transformants, its expression can be mediated through an AP-1 site located in the intron I of the gene (8) due to high levels of Jun proteins in the transformants. Fra-1/Jun complexes, in turn, can modulate the c-*fos* transcription via the AP-1 site located downstream of the SRE in c-*fos* promoter. This suggestion is supported by the results evidencing that ectopic expression of *fra*-1 and c-*jun* genes is able to suppress c-*fos* response on serum stimulation (33); moreover, NIH 3T3 clones overexpressing both c-Jun and Fra-1 proteins display enhanced transformed properties (33). The experiments with c-*fos* promoter mutants presented in this study showed that negative regulation of c-*fos* promoter in E1A + cHa-*ras* cells was mediated through the SRE site. The SRE element of c-*fos* promoter is a target for negative factors in exponentially growing normal and minimally transformed cells, and in vivo titration by exogenous SRE can relieve the c-*fos* repression (28,38). It remains to determine whether the c-*fos* repression in E1A + cHa-*ras* transformants is mediated by a yet unidentified factor or the repression is provided by a modification of preexisting factors on a level of phosphorylation/dephosphorylation of ternary complex SRE/SRF/TCF (23,52). Indeed, the SRE mutations used in the present work are located in those parts of the SRE, which are responsible for interaction with TCF and SRF factors. Therefore, one may suggest that in E1A + cHa-*ras* transformants the complex SRF-TCF permanently bound to the SRE (23) functions as a repressor. The repression can be provided, at least partly, by dephosphorylation of the TCF factor. Indeed, treatment of E1A + cHa-*ras* population bearing integrated wild-type *fos*-CAT construct with okadaic acid, an inhibitor of protein phosphatases, particularly PP2A (42), led to twofold activation of the *fos*-CAT expression. This might imply that *fos* promoter repression was reversible and was dependent on the phosphorylation/dephosphorylation equilibrium. We have noticed also that irradiation of wild-type *fos*-CAT-bearing E1A + cHa-*ras* population by UV light, which is known to activate the c-*fos* transcription due to phosphorylation of TCF factor (3), stimulated of *fos*-CAT expression (unpublished).

Antibodies to c-Fos, FosB, and Fra-2 proteins did not reveal these factors in the AP-1 complex of E1A + cHa-*ras* transformants, in contrast to Fra-1 factor, which was found to be significantly accumulated. A sequence analysis of promoters of c-*fos*, *fosB*, and *fra*-1 genes shows that only *fra*-1 gene promoter seems not to have the SRE (31). If c-*fos* (and *fosB*) repression is indeed mediated through the SRE regions of these genes, the absence of the SRE in promoter of *fra*-1 gene can give an explanation of why *fra*-1 gene is not downregulated in E1A + cHa-*ras* transformants.

In summary, it appears likely that E1A proteins alter the c-*jun* gene expression and the *trans*-activating capacities of ATF-containing factors, whereas E1A + cHa-*ras* promotes the maintenance of high levels of factors of the AP-1 family after transformation, which cannot be regulated by mitogenic stimuli. In addition, the shift from c-Jun/c-Fos towards constitutive levels of Fra-1/Jun/ATF-containing complexes may have profound consequences for the transcriptional regulation of relevant target genes.

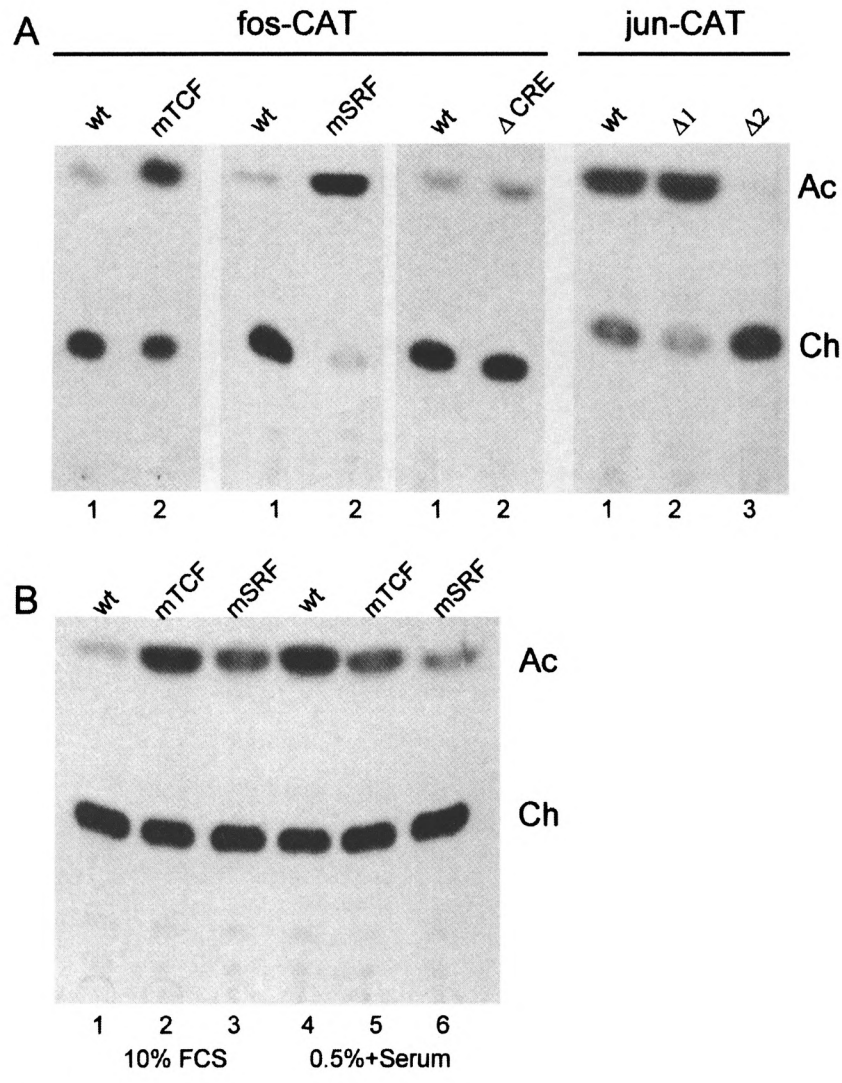

FIG. 8. (A) Chloramphenicol acetyltransferase activity of wild-type *fos*-CAT and *jun*-CAT constructs and their mutants stably integrated into E1A + cHa-*ras* cells. Cell lysates were prepared from populations of clones containing integrated wild-type constructs or mutant derivatives. The levels of CAT activity were measured for wild-type *fos*-CAT (wt), a mutant in the TCF binding site (mTCF), a mutant in the SRF binding site (mSRF), a mutant in the CREB binding site (ΔCRE). The right panel of CAT assays is for wild-type *jun*-CAT construct (wt) and two mutants: Δ1 and Δ2 lacking the functional *jun1*-TRE and *jun2*-TRE, respectively (45). Ch and Ac: nonacetylated and acetylated forms of [^{14}C]chloramphenicol. (B) Mutants affecting TCF and SRF binding cause overexpression of *fos*-CAT construct in REF cells grown in 10% FCS (c-*fos* is repressed). Cells were transiently transfected with wild-type *fos*-CAT (wt) or its mutants at the TCF (mTCF) and SRF (mSRF) binding. At 16 h after transfection, cells were either grown in 10% FCS (lanes 1–3) or serum starved in 0.5% FCS and then stimulated with 10% FCS for 1–3 h (lanes 4–6).

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