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Analysis of AP-1 Function in Cellular Transformation Pathways

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To understand the role of endogenous AP-1 activity in cellular transformation induced by oncogenes, we have made use of a *fos* mutant (sup*fos-1*) and a *jun* mutant (sup*jun-1*), either of which can function as a transdominant inhibitor of AP-1-mediated transcriptional regulation. Chicken embryo fibroblasts (CEF) infected with a series of transforming retroviruses were doubly infected with retrovirus carrying sup*fos-1* or sup*jun-1*, and suppression of cellular transformation was monitored in terms of reversion to normal cellular morphology or acquisition of anchorage-dependent growth. Cellular transformation induced by several exogenously expressed transforming genes of the *fos* or *jun* family was efficiently suppressed, as expected. CEF transformed by v-*src*, v-*yes*, v-*fps*, c-Ha-*ras*, and N-terminally truncated c-*raf* were also induced to revert to the normal phenotype by these transdominant mutants, suggesting that functional transcription factor AP-1 activity is essential for the cellular transformation induced by these oncogenes. The suppression is not attributable to nonspecific inhibition of cellular proliferation, because CEF transformed by v-*ros* or v-*myc* were not induced to revert to the normal phenotype. We next analyzed changes in all known components of chicken AP-1 induced by v-*src*, c-Ha-*ras*, or activated c-*raf* transformation. The levels of both Fra-2 and c-Jun expression were elevated two- to fourfold, and hyperphosphorylation of Fra-2 was also observed. We further showed that Fra-2–c-Jun heterodimer is mainly responsible for the elevated AP-1 DNA-binding activity in these transformed cells, and we propose that this heterodimer play a crucial role in the transformation induced by these oncogenes.

The c-*fos* and c-*jun* proto-oncogenes were originally identified as the cellular counterparts of the viral oncogenes carried by Finkel-Biskis-Jinkins (FBJ) murine sarcoma virus (11) and avian sarcoma virus 17 (21), respectively. c-*fos* belongs to a multigene family that includes *fra-1* (9), *fra-2* (23, 27), and *fosB* (42), and the *fos* gene family codes for nuclear proteins that dimerize with the Jun family proteins, such as c-Jun (26), JunB (33), and JunD (16, 32), to form the transcription factor complex AP-1. Dimerization occurs specifically through a leucine zipper structure: Jun family members can form low-affinity homodimers and high-affinity heterodimers with the Fos family, whereas Fos-related proteins do not form stable homodimers (10, 25). Although these hetero- and homodimers bind to similar DNA-binding sites (TGACTCA, AP-1-binding sites) through the basic domains of both proteins, which are juxtaposed by the dimerization, each dimer was shown to have a distinct transcriptional regulatory function, so that transcription can be positively and negatively modulated (37).

High-level expression of most members of the *fos* or *jun* gene family has been reported to cause cellular transformation of chicken embryo fibroblasts (CEF) (17, 27, 36). JunD has no transforming activity, but it can acquire transforming potential by spontaneous mutation (15, 18). These results indicate that uncontrolled expression and qualitative change of any component of AP-1 can induce cellular transformation.

Logarithmically growing CEF express c-Jun and Fra-2 at relatively high levels (36, 41), and basal-level expression of JunD was also reported in these cells (14). In c-*fos*-overexpressing cells, cellular transformation seems to be mediated by the heterodimeric complex of exogenous c-Fos and endogenous c-Jun (38). In the case of transforming *junD* mutants, it was suggested that the heterodimeric complexes of endogenous Fra-2 and JunD mutants play a crucial role in cellular transformation (18).

In our previous attempt to suppress specifically the transcriptional function of c-*jun*, we constructed a transdominant mutant designated sup*fos-1* (28). sup*fos-1* was originally described as a nontransforming v-*fos* mutant (40) which has an insertion of four amino acids between the basic domain and the leucine zipper structure (Fig. 1), and its gene product was shown, both in vitro and in vivo, to form a heterodimer with c-Jun that lacks the specific AP-1 DNA binding (28). This mutant, when introduced by retrovirus vectors, efficiently suppresses c-*jun* transformation by sequestering the unstable c-Jun homodimer into the stable and nonfunctional supFos1–Jun heterodimer in the cells. Interestingly, nuclear extracts from the sup*fos-1*-expressing CEF reduced endogenous AP-1 DNA-binding activity (mainly contributed by Fra-2–Jun) to an almost nondetectable level by competing out endogenous Fra-2 (27), suggesting that this mutant can also function as an inhibitor of Fos family proteins. Several *fos* or *jun* mutants have also been reported by other groups to function as transdominant suppressors by a mechanism similar to that used by sup*fos-1* (38). Some of the transdominant mutants, however, function without the leucine zipper structure, possibly by a squelching mechanism (38), while others retain both dimer-forming and DNA-binding activities but lack the transactivation domain (6, 13, 20).

Most of the *fos* and *jun* family members belong to the

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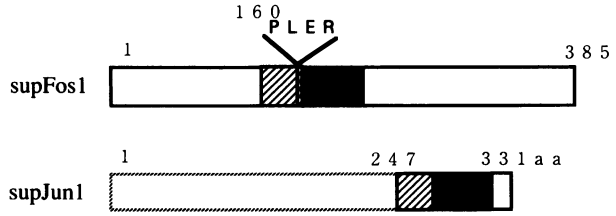

FIG. 1. Protein structures of supFos-1 and supJun-1. supFos-1 is a derivative of v-Fos (FBJ), which has an insertion composed of four amino acids (aa) between the basic domain (striped box) and the leucine zipper motif (black box). supJun-1 is an N-terminally truncated mutant of c-Jun (human) and starts from an internal methionine 247.

category of immediate-early genes and are promptly induced by several external stimuli as well as transient expression of such oncogenes as *src*, *ras*, and *raf*, which are believed to be located upstream of *fos* and *jun* family members in the signal transduction pathway (2). It is noteworthy that cellular transformation induced by constitutive expression of these oncogenes seems to require functional endogenous c-Fos or c-Jun. This was suggested by the reversion of transformed cells to the normal phenotype following introduction of antisense RNA (19) for the *fos* gene, anti-Fos antibody (31), or a transdominant mutant of *fos* or *jun*. Because AP-1 activation by several oncogenes has been studied in a number of different cell lines by using either stable or transient transfections, it is not easy to develop a general model from these observations. It seems necessary to perform extensive screening of oncogenes which require endogenous AP-1 for their transforming activity and detailed analysis of the induced changes in all of the AP-1 components, using the same cell system.

In the first part of this report, we introduce a new transdominant suppressor, sup*jun-1*, and show that both this mutant

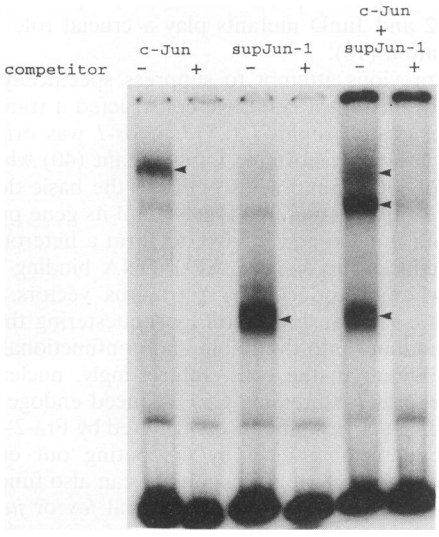

FIG. 2. supJun-1 binds to AP-1-binding sites as a homodimer or heterodimer with c-Jun. Each protein was synthesized separately, and mixtures were incubated for 30 min at 37°C. DNA-binding activity was determined by gel shift analysis using 0.2 ng of a ^{32}P-labeled oligonucleotide containing the AP-1-binding site (FSE2) and 4 μg of reticulocyte lysate mixture in the presence (+) or absence (−) of 20 ng of unlabeled oligonucleotide as a specific competitor. Arrowheads indicate positions of specific AP-1 DNA-binding activity.

and sup*fos-1* can function as general inhibitors of AP-1. By introduction of the dominant negative mutants into CEF transformed by a series of oncogenes that do not belong to the *fos* or *jun* family, we were able to categorize the oncogenes into two groups; one group requires functional AP-1 activity for transforming activity, while the other does not. We have further analyzed all known components of the AP-1 complex in CEF transformed by oncogenes that belong to the former group in an attempt to understand the molecular mechanisms by which these oncogenes affect endogenous AP-1 activity.

MATERIALS AND METHODS

Plasmid construction. The 0.8-kb *Sma*I fragment containing the entire cDNA of human c-H-*ras* was isolated from pSPT-Hras (from the Japanese Cancer Research Resources Board) (30), ligated to *Bam*HI linkers, and inserted into the unique *Bgl*II site of pDS3 (17) to generate pHras. pND-raf was generated from the human c-*raf-1* cDNA, p627 (from JCRB) (5), by PCR using a 5′ primer that covers the artificial methionine codon preceded by an artificial Kozak consensus sequence and a *Bam*HI site (5′-AGGGATCCACCATGGAA AGAGAGCGGCACCAGT-3′) and a 3′ primer that contains the stop codon (5′-CCGGATCCCTACTAGAAGACAGGCA GCCTCGG-3′). The PCR product was digested with *Bam*HI and inserted into the unique *Bgl*II site of pDS3. pND-raf encodes the same amino acid sequence as the 20A clone constructed by Stanton et al. (35), which lacks the N-terminal 313 amino acids of human c-*raf*. The 0.5-kb *Nae*I-*Eco*T4I fragment of pHJ (gift from R. Tjian) (4) was filled in, ligated to *Bgl*II linkers, and inserted into the *Bgl*II site of pDS3 to generate psupjun-1, which produces an N-terminally truncated Jun from the internal methionine 247. The truncated Jun retains the entire basic domain and the intact leucine zipper structure, as shown in Fig. 1. The 0.83-kb *Eco*RI-*Bsp*MI fragment of pSPfra-1 (rat) (9) was filled in and ligated with blunt-ended *Bgl*II-cut pDS3 to generate pF1R1, which carries the entire rat *fra-1* gene.

For RNA probe synthesis, four DNA templates were constructed. The 450-bp *Bst*X1-*Eco*RI fragment of pJun (gift from P. Vogt) (21) encoding the C-terminal half of the v-*jun* gene was cloned into pBlueskm to generate pjunRPA, which was linearized at the *Pvu*II site and used as a template. The 260-bp *Pst*I-*Sac*I fragment of chicken v-*fos* (12) was inserted into pSPT19 to generate pfosRPA, which was linearized at the *Nde*I site and used as a template. The 250-bp *Bst*EII-*Nco*I fragment of chicken *fra-2* (26) was filled in and ligated to pSP64, which was doubly digested with *Eco*RI and *Bam*HI and blunt ended, to generate pfra2RPA. The pfra2RPA was linearized at the *Pvu*II site and used as a template. The chicken *junD* construct was generated by PCR, using the published sequence (14). The 245-bp *Sac*II-*Taq*I fragment of the PCR product was inserted between the *Acc*I and *Sac*II sites of pBlueskm to generate the pjunDRPA. pjunDRPA was linearized at the internal *Dde*I site and used as a template. For Northern (RNA) blotting of the chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, the c-DNA that encodes the entire GAPDH was generated by the reverse transcription RT-PCR technique, using the published sequence (29).

Cells and viruses. CEF were prepared, grown, and infected with viruses as reported previously (28). For metabolic labeling, CEF were grown in 60-min-diameter dishes and labeled with 500 μCi of [^{35}S]methionine for 60 min. For the production of recombinant viruses such as sup*jun-1* (carrying N-terminally truncated *jun*), ND-*raf* (carrying N-terminally deleted *raf*), and H-*ras* (carrying normal c-Ha-*ras*) viruses, psupjun-1 and pHras were completely digested with *Sal*I and ligated to the *Sal*I

digest of pREP to form the structure of the replication-competent provirus (subgroup A); pND-raf was partially digested with *Sal*I, and a 1.3-kb fragment that covers the *raf* sequence was isolated and used for ligation with the *Sal*I digest of pREP. Ligated DNAs (2 μg) were transfected into CEF as described previously (17), and replication-competent virus stocks were collected from the culture 5 or 6 days after transfection. Viruses were never propagated before use to minimize genetic changes of the virus genome. For production of the subgroup B virus, pREP was substituted with pREP-B. Similar viruses containing the human c-*jun* gene (JH-1 [36]), the mouse c-*fos* gene (FM4 [17]), the chicken *fra-2* gene (F2C1 [27]), the v-*src* gene of Rous sarcoma virus (N4 [17]), a transdominant negative mutant of v-*fos* (sup*fos-1* [28]), and no oncogene (DS3 [17]) have been described previously. The natural avian retroviruses Rous sarcoma virus Prague C, Fujinami sarcoma virus (Rous-associated virus-1 [RAV-1]), Y73-associated virus (YAV), UR2 (RAV-1), and MC29 (RAV-1 or RAV-2) were also used to introduce the v-*src*, v-*fps*, v-*yes*, v-*ros*, and v-*myc* genes, respectively.

Colony formation. CEF that were sequentially infected with two species of viruses were trypsinized 4 days after the second infection. Approximately 3,000 doubly infected CEF were mixed with 3×10^5 freshly prepared CEF feeder cells and seeded in suspension in soft agar (0.4%) on top of a bed of hard agar (0.8%) as discussed previously (28). Colonies, which were formed after 2 weeks of incubation at 38.5°C, were counted (average of three plates) at 3 weeks after seeding.

Gel shift analysis. Gel shift analysis using proteins synthesized in rabbit reticulocyte and using a ^{32}P-labeled 62-bp double-stranded DNA probe containing the FSE2 AP-1 DNA-binding site was described previously (37).

The nuclear extracts were prepared from infected CEF as described previously (28). Gel shift analysis of the nuclear extract was done as described previously (28, 41), using a ^{32}P-labeled 65-bp *Hin*dIII-*Ava*I fragment of pco1I, which has an insertion of an oligonucleotide containing the AP-1 DNA-binding site of the human collagenase gene at the *Bam*HI site of pUC119. In some experiments, the antiserum was added to the mixture 15 min before addition of the labeled probe. The samples were analyzed by electrophoresis on a nondenaturing 5% polyacrylamide gel at 4°C, and shifted bands were detected by autoradiography.

Immunoprecipitation and Western blot (immunoblot) analysis. ^{35}S-labeled cell lysates were prepared under denaturing conditions (boiling in 2.0% sodium dodecyl sulfate [SDS]) and immunoprecipitated as described previously (17, 40). Polyclonal antisera raised against Fos peptide 1 (40) and Fra-2 peptide 2 (27) have been described previously.

The total cell extracts prepared under denaturing conditions (80 μg of each) were resolved by electrophoresis on an SDS–10% polyacrylamide gel, and the proteins were transferred to a polyvinylidene difluoride membrane (Millipore Immobilon P). Immunoblots were treated with 5% dried milk and then incubated with affinity-purified anti-Jun antiserum. The filter was incubated with anti-rabbit immunoglobulin G conjugated to horseradish peroxidase, and the protein bands were visualized by the ECL Western Blotting Detection System (Amersham).

RNA preparation and RNase protection analysis. Total RNA was extracted and purified from infected CEF by the acid guanidium-thiocyanate-phenol chloroform method (22). RNA probes for RNase protection analysis and RNA molecular weight markers were synthesized from the linearized template DNA (0.5 μg) by T7 or SP6 RNA polymerase (30 U) in 40 mM Tris Cl (pH 8.0)–8 mM $MgCl_2$–2 mM spermidine–50 mM NaCl–10 mM dithiothreitol–1 mM ATP, GTP, and UTP–10 μM CTP–50 μCi of [γ-^{32}P]CTP (3,000 Ci/mmol)–20 U of RNase inhibitor at 37°C for 30 min. RNA probes were purified by polyacrylamide gel electrophoresis and hybridized to 10 μg of total cellular RNA in 80% formamide–40 mM piperazine-*N*,*N*-bis(2-ethanesulfonic acid) (PIPES)–400 mM NaCl at 45°C for 12 h. After digestion with 40 μg of RNase A per ml and 2 μg of RNase T_1 per ml at 42°C, samples were analyzed in 6 M urea–polyacrylamide gels and detected by autoradiography.

RESULTS

Another highly effective transdominant mutant, supJun-1. To obtain effective transdominant mutants other than sup*fos-1* (Fig. 1), we tested some nontransforming mutants of *fos* or *jun* described here or previously (40) for the ability to suppress c-*jun* transformation, using the method previously described for sup*fos-1* (28). To introduce c-*jun* and the candidate mutant into a single cell, we used replication-competent retrovirus vectors as described previously (28). Two sets of vectors that differed in subgroup specificity (A or B) were created for each gene. CEF are resistant to infection by retroviruses of the same subgroup, but they can be superinfected by retroviruses of a different subgroup. Thus, it was possible to obtain large populations of doubly infected CEF expressing both c-Jun and the candidate mutant at similar levels without any selection procedure.

One mutant, designated sup*jun-1*, which lacks the entire transactivation domain (Fig. 1) was shown to have an equivalent or slightly enhanced suppressing activity compared with sup*fos-1*; CEF infected with sup*jun-1* (A) (subgroup A) is resistant to morphological transformation after superinfection with JH-1 (B) (carrying human c-Jun). This suppression was never observed when sup*jun-1* (A) was substituted with DS3(A) (control vector). At the same time, JH-1(A)-transformed CEF reverted to the normal phenotype upon superinfection with supJun-1 (B). The suppression was clearly quantitated by analyzing colony-forming activity in soft agar (Table 1). Similar results were obtained when JH-1 was substituted for T2 and T3 viruses (Table 1), which encode transforming *junD* mutants.

Although supJun-1 protein lacks all regions that have been reported to function as transactivation domains (3), it should have specific DNA-binding activity, because it retains the entire basic region and the intact leucine zipper structure. This was confirmed by gel shift analysis (Fig. 2) of the mixture of supJun-1 and wild-type Jun which were produced in vitro; three specific band shifts were generated by the supJun-1 homodimer, the supJun1–c-Jun heterodimer, and the c-Jun homodimer. This result is in a clear contrast to supFos-1, which was shown to have no DNA-binding activity (28), and we decided to compare the two mutants in a further analysis.

Either sup*fos-1* or sup*jun-1* can function as a general inhibitor of AP-1 activity. We next tested whether sup*fos-1* or sup*jun-1* can suppress not only c-*jun* transformation but also transformation induced by oncogenes that belong to the *fos* family. We previously reported the full transforming activity of FM4 and F2C1 viruses, which carry c-*fos* (mouse) (17) and *fra-2* (chicken) (27), respectively. The *fra-1*-carrying virus constructed here (F1R1) exhibited similar transforming activity, as judged from the titer of transforming virus in the virus stock or its efficiency of colony formation in soft agar (data not shown). As is the case with c-*jun*, the transformation induced by c-*fos*, *fra-1*, and *fra-2* was efficiently suppressed by subsequent infection with sup*fos-1* or sup*jun-1* when judged by colony-forming activity (Table 1) and cellular morphology

TABLE 1. Suppression of colony formation by sup*fos-1* and sup*jun-1* viruses

Virus[a]	Oncogene	Ratio[b]			
		sup*fos-1*		sup*jun-1*	
		Oncogene→sup*fos-1*[c]	sup*fos-1*→oncogene[d]	Oncogene→sup*jun-1*	sup*jun-1*→oncogene
FM4	c-*fos*	ND	0.28	0.25	0.18
F1R1	*fra-1*	ND	0.35	ND	ND
F2C1	*fra-2*	ND	0.33	ND	0.13
JH-1	c-*jun*	ND	0.25	0.10	0.05
T2	*junD* mutant	0.65	0.56	ND	0.25
T3	*junD* mutant	ND	0.31	ND	0.30
N4	v-*src*	ND	0.25	0.32	0.17
PrC	v-*src*	ND	0.27	0.32	0.20
Y73	v-*yes*	0.36	ND	0.23	ND
FSV	v-*fps*	0.13	ND	0.18	ND
H-*ras*	c-H-*ras*	0.40	0.28	0.18	0.09
ND-*raf*	N-terminally deleted *raf*	0.31	0.24	0.14	0.06
MC29	v-*myc*	0.90	1.1	0.98	0.96
UR2	v-*ros*	1.07	ND	0.86	ND

[a] PrC, Rous sarcoma virus Prague C; FSV, Fujinami sarcoma virus.
[b] The number of colonies formed by sup*jun-1*(A)/FM4(B)-infected CEF as shown in Fig. 4D was divided by that formed by DS3(A)/FM4(B)-infected CEF (Fig. 4A). The ratio shown is the average of three independent experiments. ND, not determined.
[c] The experimental protocol in which transforming virus was infected and sup*fos*1 was infected subsequently.
[d] The experimental protocol in which sup*fos*1 was infected first and transforming virus was infected subsequently.

(data not shown). The overall results were reproducible even when the order of introduction of the transdominant mutant and *fos* family oncogene was changed (Table 1). For example, when DS3(A)-infected CEF were subsequently infected with a c-*fos* virus [FM4(B)], cells were clearly transformed, assuming a polygonal cellular morphology with a high saturation density (Fig. 3B). When CEF were initially infected with sup*jun-1*, however, subsequent infection with c-*fos* virus did not induce cellular transformation (Fig. 3G), and the cells exhibited a morphology similar to that of CEF infected with DS3(A) alone. These results further suggest that either of these transdominant mutants can function as a general inhibitor of both the Fos and Jun family proteins (AP-1).

Screening of other oncogenes whose transforming activity

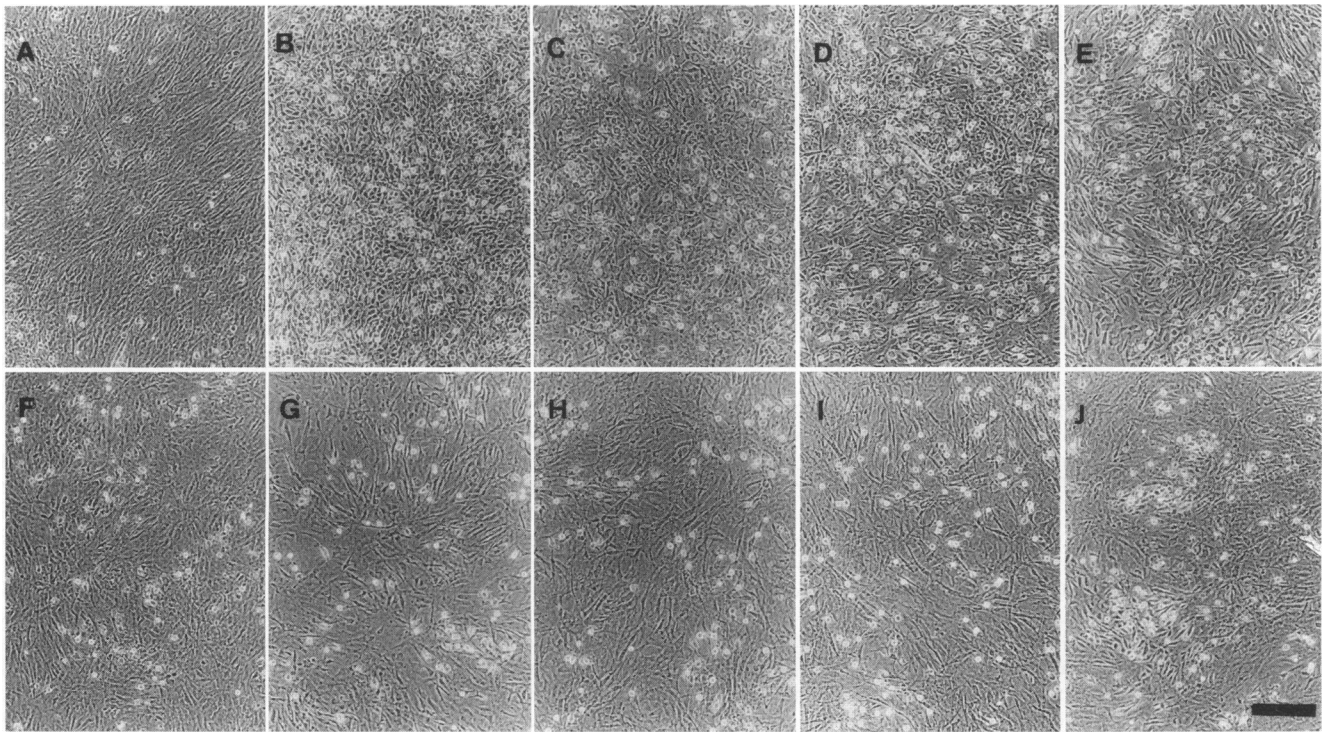

FIG. 3. Cellular morphology of doubly infected CEF. CEF were first infected with DS3(A) (A to E) or sup*jun-1*(A) (F to J) 4 days later superinfected with FM4(B) (B and G), H-*ras*(B) (C and H), ND-*raf*(B) (D and I), or N4(B) (encoding v-*src*) (E and J) and kept under minimal essential medium containing soft agar for 5 days before being photographed. The bar corresponds to 100 μm.

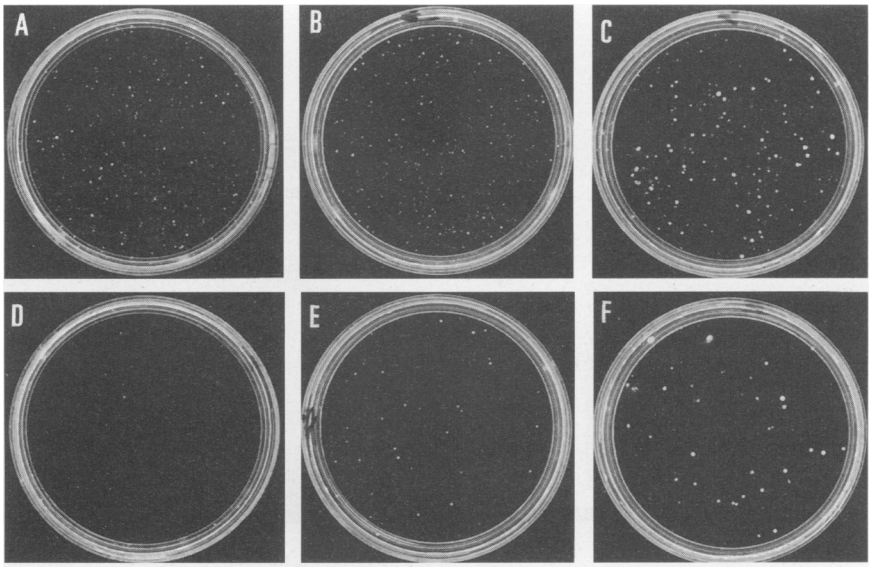

FIG. 4. sup*jun-1*-infected CEF are resistant to transformation by several oncogenes. CEF were first infected with DS3(A) (A to C) or sup*jun-1*(A) (D to F) and 4 days later superinfected with FM4(B) (A and D), H-*ras*(B) (B and E), or N4(B) (C and F). At 4 days after superinfection, they were trypsinized, seeded into soft agar (60-mm-diameter plates), and propagated for 3 weeks at 38.5°C before being photographed.

was suppressed by the transdominant mutants. Using these inhibitors of AP-1, we next screened CEF transformed by a series of naturally isolated or recombinant transforming retroviruses to test whether endogenous AP-1 function is essential for their transformation. Among the recombinant viruses, H-*ras* and ND-*raf* were constructed in this study and encode the normal human Harvey c-*ras* gene or N-terminal truncated human c-*raf*, respectively. Both viruses transformed CEF moderately, as judged from the morphological changes (Fig. 3C and D) and titer of focus formation [around 10^5 focus-forming units/ml for H-*ras*(A) or ND-*raf*(A)]. These results are in good agreement with those observed in murine cell lines (8, 35).

As shown in Table 1 and Fig. 4, sup*fos-1* and sup*jun-1* can efficiently suppress colony formation in soft agar caused by v-*src*, v-*yes*, v-*fps*, c-Ha-*ras*, and ND-*raf*, though in all of these cases, suppression by sup*jun-1* was stronger than suppression by sup*fos-1*. On the other hand, colonies formed by v-*myc* or v-*ros* were never suppressed by superinfection with either of these transdominant mutants. The reduction of anchorage-independent growth of the doubly infected CEF is in good agreement with the reversion to the normal cellular morphology in monolayer cultures, some examples of which are shown in Fig. 3 (compare Fig. 3C to E with Fig. 3H to J, respectively). In the v-*src*-introduced culture, however, partially refractile cells were still detectable (Fig. 3J), and their population increased gradually when the cultures were maintained for longer periods of time. It is also noteworthy that CEF transformed by v-*myc* or v-*ros* did not revert to the normal morphology upon superinfection with these two transdominant mutants (data not shown).

Analysis of the AP-1 components in CEF transformed by several oncogenes. As discussed above, we categorized seven oncogenes into two groups by using transdominant mutants; five (v-*src*, v-*yes*, v-*fps*, ND-*raf*, and c-H-*ras*) require endogenous AP-1 activity for their transforming activity, while two (v-*ros* and v-*myc*) do not. Since either of the two transdominant mutants used in this study can function as a general inhibitor of AP-1, we next analyzed all known chicken AP-1 components (c-*fos*, *fra-2*, c-*jun*, and *junD*) in cells transformed by several oncogenes to examine the molecular mechanisms by which AP-1 is involved in cellular transformation.

From logarithmically growing CEF, we prepared total cell extracts and analyzed the absolute amounts of endogenous Jun and JunD by immunoblotting with an anti-Jun antiserum that is cross-reactive to JunD (Fig. 5A). Compared with DS3-infected or uninfected CEF, CEF transformed by v-*src*, v-*fps*, and v-*ros* expressed three- to fourfold more c-Jun, while about twofold-greater expression was observed in v-*yes*-, c-Ha-*ras*-, and ND-*raf*-transformed CEF. It should be pointed out that the c-*jun*-transformed CEF expressed much higher levels of exogenous human c-Jun (about five times the endogenous c-Jun level). On the other hand, v-*myc*-transformed CEF expressed a similar level of c-Jun, comparable to DS3-infected or uninfected CEF. The JunD bands were too weak compared with c-Jun bands to allow comparison of the expression levels among these cells quantitatively.

For analysis of endogenous c-Fos and Fra-2, logarithmically growing CEF were metabolically labeled with [^{35}S]methionine. From cellular lysates prepared under denaturing conditions, proteins were immunoprecipitated with the anti-Fra-2 antiserum, which specifically precipitates Fra-2 (Fig. 5B), and the anti-Fos antiserum, which precipitates all Fos family proteins. None of the logarithmically growing CEF showed any detectable c-Fos expression (data not shown), but basal expression of Fra-2 was detectable in all cells. In CEF transformed by v-*src*, ND-*raf*, and c-H-*ras*, Fra-2 formed a broad band of 41 to 46 kDa, compared with a sharper, thin band of 41 to 42 kDa detected in DS3-infected CEF. After bacterial alkaline phosphatase treatment of the immunoprecipitates, the protein bands of all cells migrated as sharp 41-kDa bands, indicating that the slowly migrating population of the protein represents hyperphosphorylated forms (Fig. 5B) as we have previously observed in Fra-2 immediately after growth stimulation (39). Phosphorylation of Fra-2 was most intensive in v-*src*-transformed cell and was also clear in ND-*raf*- or c-Ha-*ras*-transformed CEF. By comparing the dephosphorylated form of

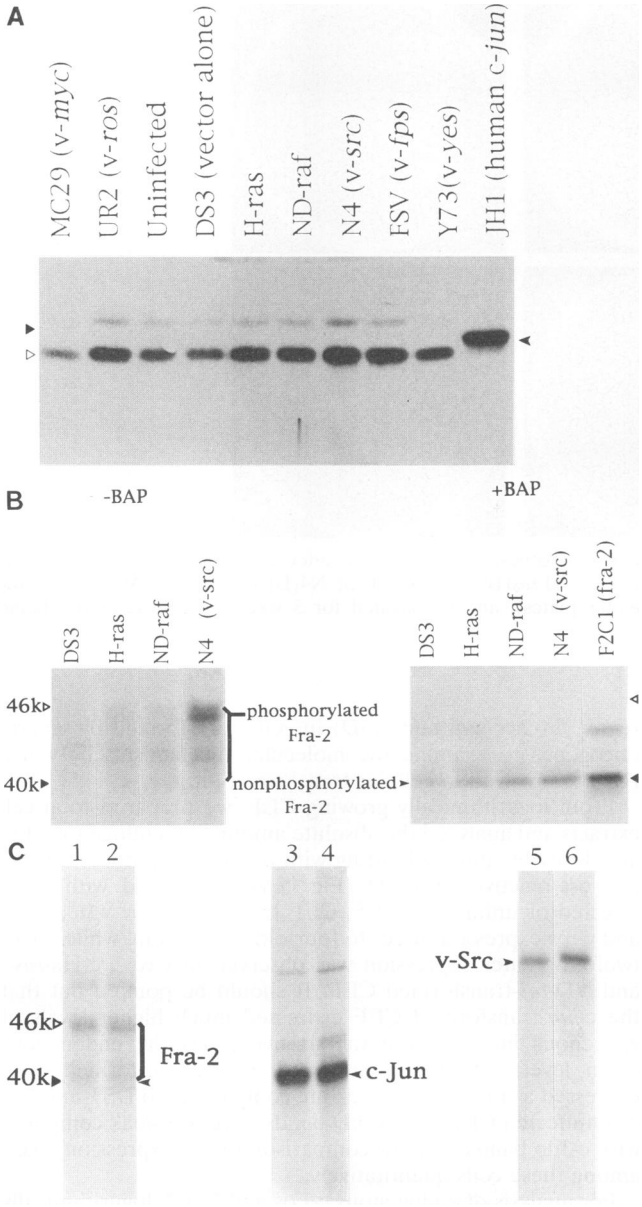

FIG. 5. Expression of Jun (A and C) and Fra-2 (B and C) in infected CEF. (A) Total cell lysate (80 μg of protein each) was resolved on an SDS–10% polyacrylamide gel, and proteins were detected with anti-Jun antiserum, using the ECL Western Blotting Detection System. FSV, Fujinami sarcoma virus. The open and closed triangles indicate the positions of endogenous chicken c-Jun and JunD, respectively. The arrowhead indicates the position of human c-Jun. (B) CEF were labeled with [^{35}S]methionine for 60 min. Cell lysates were prepared under denaturing conditions and treated with anti-Fra-2 peptide 2. Immunoprecipitates containing the same amount of radioactivity were incubated without or with bacterial alkaline phosphatase (BAP), resolved on an SDS–10% SDS polyacrylamide gel, and visualized by fluorography after exposure of X-ray films for 32 days (−BAP) or 12 days (+BAP). (C) sup*jun-1*(A)-infected (lanes 2, 4, and 6) or DS3(A)-infected (lanes 1, 3, and 5) CEF were superinfected with N4(B) as shown in Fig. 3J or E. After labeling with [^{35}S]methionine for 1 h, Fra-2, c-Jun, and v-Src were immunoprecipitated with anti-Fra-2 peptide 2 (lanes 1 and 2), anti-Jun (lanes 3 and 4), and anti-v-Src (lanes 5 and 6) antisera, respectively, resolved on an SDS–10% polyacrylamide gel, and visualized by fluorography.

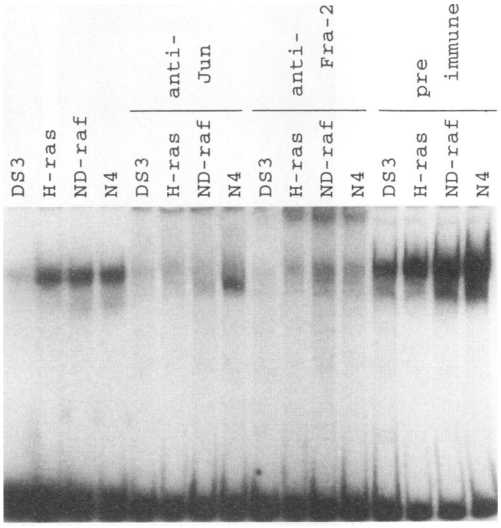

FIG. 6. Endogenous AP-1 DNA-binding activity in nuclear extracts from infected CEF. The AP-1-binding activity present in extracts prepared from CEF infected with DS3, H-*ras*, ND-*raf*, or N4 (carrying v-*src*) was determined by gel shift assays using a ^{32}P-labeled DNA probe containing the AP-1-binding site of the collagenase gene. Some of the nuclear extracts were treated with anti-Jun or anti-Fra-2 peptide 1 antiserum as well as preimmune serum.

Fra-2 protein, we can roughly estimate that the expression levels in CEF transformed by v-*src*, H-*ras*, and ND-*raf* are about three-, two-, and twofold greater than those in DS3-infected CEF, respectively. Although v-*myc*-transformed cells expressed low levels of nonphosphorylated or hypophosphorylated forms of Fra-2 as in DS3-infected CEF, v-*ros*-transformed CEF produced elevated levels of highly phosphorylated Fra-2 as in v-*src*-transformed CEF (data not shown). In the CEF transformed by exogenously expressed *fra-2*, the Fra-2 expression level was increased about sevenfold (Fig. 5B).

When sup*jun-1*-infected CEF were superinfected with N4 (Fig. 3J), the cells were metabolically labeled with [^{35}S]methionine and Fra-2 and Jun expression was analyzed by immunoprecipitation. The results indicated that expression levels of Fra-2 and Jun as well as the extent of Fra-2 hyperphosphorylation were not affected by the expression of supJun-1 (Fig. 5C; compare lane 1 and 2 or lanes 3 and 4), indicating that supJun-1 suppresses the transformation not by affecting the components of endogenous AP-1. We have also observed that sup*jun-1* does not affect expression of the exogenous v-*src* gene (Fig. 5C; compare lane 5 and 6).

We next determined the AP-1 DNA-binding activity in v-*src*-, ND-*raf*-, and c-Ha-*ras*-transformed CEF, using gel shift analysis of nuclear extracts (Fig. 6). When a DNA probe containing the AP-1 site from the human collagenase gene was used, DS3-infected CEF generated a thin band of gel shift complex, while greatly enhanced bands with the same mobility were observed when CEF expressing v-*src*, ND-*raf*, or c-Ha-*ras* were used. All of the shifted bands disappeared upon addition of either the anti-Jun or the anti-Fra-2 antiserum but were insensitive to a preimmune serum. The apparently increased AP-1-binding activity in the presence of preimmune serum is possibly attributable to a carrier effect of increased protein concentration. This result indicates that the Fra-2–c-Jun complex is mainly responsible for the band shifts and also for the enhancement of DNA-binding activity in these transformed cells.

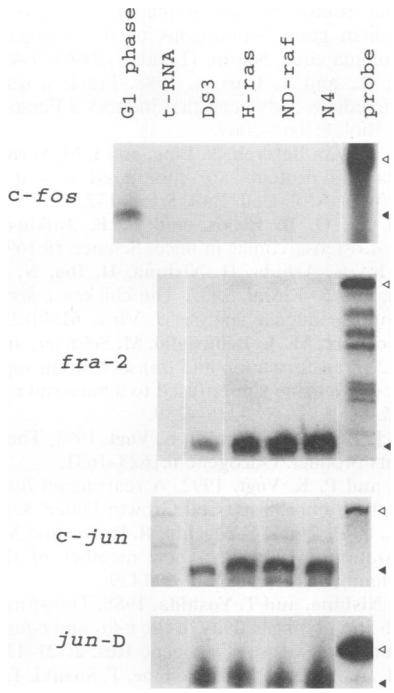

FIG. 7. Expression levels of mRNAs that encode AP-1 components in infected CEF. Levels of *fos*, *fra-2*, c-*jun*, and *junD* transcripts in CEF infected with DS3, H-*ras*, ND-*raf*, and N4 (carrying v-*src*) viruses were analyzed by RNase protection assay. Open triangles indicate positions of untreated probes; closed triangles correspond to positions of probes protected by the transcripts. Samples were analyzed in a 3.5% (*fos*, *jun*, and *fra-2* transcripts) or 5% (for *jun*D) polyacrylamide gel.

Elevated levels of Fra-2 and Jun proteins result from regulation at the transcriptional level. CEF transformed by v-*src*, ND-*raf*, and c-Ha-*ras* expressed elevated levels of Fra-2 and c-Jun proteins. We next tested the expression levels of c-*fos*, *fra-2*, c-*jun*, and *junD* mRNAs by RNase protection assay. As shown in Fig. 7, c-*fos* mRNA was undetectable in all of the logarithmically growing cells, while growth-stimulated CEF expressed it clearly. The *fra-2* and c-*jun* mRNA levels were elevated two- to fourfold in v-*src*, ND-*raf*-, or c-Ha-*ras*-transformed cells. In RNase protection assays, intactness and quantity of the RNA samples were tested by ethidium bromide staining of the gel or by Northern blot analysis using a probe for a housekeeping gene encoding GAPDH. This result suggest that the enhancement of Fra-2 and c-Jun expression in these cells is mainly regulated at the transcriptional level. Although *junD* mRNA expression in v-*src*-transformed cells was slightly higher, the *junD* mRNA levels were similar among other cells.

DISCUSSION

Although supFos-1 was initially designed as a specific suppressor of Jun family proteins by forming a nonfunctional and stable complex of supFos-1 and Jun (28), we have shown here that it can also efficiently suppress the transformation induced by *fos* family proteins. Since heterodimer formation with endogenous c-Jun is thought to be essential for the transformation by exogenously expressed Fos family proteins (38), sup*fos-1* would function as a competitive inhibitor of exogenous Fos in this case. Like sup*fos-1*, sup*jun-1* suppressed either *fos* or *jun* transformation, and it is noteworthy that sup*jun-1* was a more potent suppressor of either *fos* or *jun* transformation (Table 1). This stronger suppression might be attributable to the direct blocking of transcription by the AP-1 DNA-binding activity of supJun-1. On the other hand, non-DNA-binding heterodimers formed by supFos-1 are passively removed from the transcriptional system. supJun-1 forms homodimers and heterodimers with c-Jun, as shown in vitro (Fig. 1), and stable heterodimeric complexes with Fra-2 were detected in CEF (our unpublished result). We would expect only a very limited transactivation activity in all of these homo- or heterodimers, because the in vitro transcriptional analysis indicated that a similar truncated Jun showed only marginal transcriptional activity even in combination with full-length Fos (1). supJun-1 shares some properties with Δ9 (20) and Tam-67 (6), which were previously reported as transdominant mutants of Jun that lack transactivation domains. Both of the mutants were shown to have inhibitory effects on AP-1-mediated transcription in transient expression systems. Our model of the inhibition mechanism favors the quenching mechanism proposed by Brown et al. (6).

We found that cellular transformation induced by such oncogenes as *src*, *fps*, *yes*, *ras*, and *raf* was suppressed by both of the transdominant mutants. Therefore, we decided to analyze the amounts of all known chicken AP-1 components (c-Fos, Fra-2, c-Jun, and JunD) in transformed cells induced by each of 13 oncogenes. The expression level of either Fra-2 or c-Jun in these transformed CEF was about two to four times that of the untransformed CEF, but we think that the elevation of either protein alone is not sufficient to cause the cellular transformation; the amounts of exogenously expressed Fra-2 and c-Jun that induce cellular transformation are about seven and five times, respectively, that of the endogenous protein in infected CEF. Elevation of both proteins, however, would contribute synergistically to the enhancement of AP-1 DNA-binding activity (Fig. 6) and possibly also to the induction of cellular transformation. Although we did not detect elevated expression from a reporter plasmid containing a single AP-1-binding site in these transformed CEF, three- to fivefold elevation was detected when a reporter with three tandem repeats of an AP-1 site was used instead (our unpublished results).

Cellular transformation induced by v-*ros* or v-*myc* was not affected by either of the transdominant suppressors, suggesting that endogenous AP-1 function is not essential for their transforming activity. It is interesting that the nuclear oncogene v-*myc* and the gene encoding the transmembrane tyrosine kinase v-Ros show clear differences in their induction levels of AP-1 components. v-*myc* induced no change in the endogenous AP-1 components, indicating that the v-*myc* transformation pathway is independent of AP-1. v-*ros*-transformed cells have elevated levels of c-Jun, although these enhancements are not essential for cellular transformation. Since v-*ros* encodes a transmembrane tyrosine kinase and is a homolog of the *sevenless* gene, it might be expected to influence the *ras* and *raf* signalling pathways. Therefore, we think that there is another pathway that alone is sufficient for the *ros* transformation.

Although in this study we did not address the phosphorylation status of the c-Jun protein in vivo, several reports have indicated that phosphorylation of amino-terminal serines 63 and 73 of c-Jun accompanies the transformation of murine cells induced by v-*src*, activated c-Ha-*ras*, and activated *raf*-1, and it was also shown that phosphorylation is important for rat fibroblast transformation induced by high-level expressions of both c-*jun* and activated c-Ha-*ras* (2, 3, 34). Two lines of evidence, however, indicate that these findings are not directly

applicable to the chicken cell system. First, the N-terminal sites are apparently constitutively phosphorylated even in resting CEF and are only modestly affected by mitogenic stimulation. Second, a c-Jun mutant in which the N-terminal phosphorylation site is changed from a serine to an alanine residue retains the full activity to transform CEF as a single gene (24).

Hyperphosphorylation of Fra-2 proteins, involving a mobility change, was detected in v-*src*-, c-Ha-*ras*-, and ND-*raf*-transformed CEF. We previously reported similar phosphorylation of Fra-2 immediately after growth stimulation (39). In CEF, both nonphosphorylated (40-kDa) and phosphorylated (41- to 46-kDa) Fra-2 can be coimmunoprecipitated with c-Jun, and the resultant heterodimers seem to have equal specific DNA-binding activity. The 40-kDa Fra-2 protein synthesized in reticulocyte lysates also retains efficient dimer-forming activity with c-Jun and binding activity to AP-1 sites. Although we would expect that phosphorylation contributes to the enhancement of transactivation activity, the effect of phosphorylation on transcriptional control, as well as the protein kinases responsible for Fra-2 phosphorylation, remains to be established.

We would like to point out that these two transdominant mutants have the particular advantage that they are downstream in the signal transduction pathways (2), and both exhibit the blocking of cellular transformation caused by a wide range of oncogenes. Since supFos-1 and supJun-1 have only a modest effect on normal growth, we are now introducing these genes into primary cells to look for a differentiation process that involves endogenous AP-1 activation.

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