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High-level Expression of Human c-*jun* Gene Causes Cellular Transformation of Chicken Embryo Fibroblasts

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To analyze the transforming activity of c-*jun*, a Rous sarcoma virus (RSV) variant that carries human c-*jun* instead of v-*src* (JH1) was constructed. After infection onto chicken embryo fibroblasts (CEF), JH1 formed foci with a titer comparable to that of wild-type RSV, and the infected cells grew in soft agar, indicating that the human c-*jun* gene has transforming potential, like the v-*jun* gene. The expression of Fra-2, one of the recently isolated Fos-related antigens, but not Fos was detected in both JH1-infected CEF and CEF infected with the control retrovirus vector (DS3). Gel shift analysis using nuclear extracts from DS3-infected CEF revealed that the Fra-2/Jun complex contributes to the basal level of AP-1 DNA binding activity. A similar activity was detected in JH1-infected CEF, but these cells have an additional AP-1 binding activity derived from Jun homodimers that seems to play important roles in the cellular transformation.

Key words: Cellular transformation — c-*jun* gene — *fra*-2 gene — Transcription factor AP-1 — Retrovirus vector

When serum-starved fibroblasts are growth-stimulated by serum, TPA and many other extracellular signals, protooncogenes, c-*fos*[1] and c-*jun*,[2] as well as related genes such as *fra*-1,[3] *fra*-2,[4] *fos* B[5] and *jun* B,[6] are induced promptly and transiently. These gene products, having DNA binding domains composed of the leucine zipper motif,[7] are components of the mammalian transcriptional activator protein 1 (AP-1)[8,9] and seem to play a pivotal role in the transcriptional regulation of normal cell growth. Constitutive expression of one of these genes, c-*fos* (mouse), however, was shown to disturb the normal growth regulation and to cause cellular transformation of established fibroblast cell lines[10] or primary fibroblasts.[11] Furthermore, high-level expression of the *fra*-2 (chicken) gene causes cellular transformation of chicken embryo fibroblasts (CEF).[4]

The leucine zipper motif defines a new class of DNA-binding proteins that interact with DNA as a dimeric complex. In the case of c-*jun* or related genes, their gene products themselves can bind as a homodimer to the AP-1 recognition site (5′–TGAC/GTCA-3′, the AP-1 site[8]), but with relatively low apparent affinity.[12] In the presence of Fos, the Fos/Jun heterodimer is preferred and it has an increased affinity for AP-1 sites.[12–14] Fos and related antigens, however, can not form homodimers and are thus not able to bind to AP-1 sites. Mutational analyses of v-Fos protein showed that heterodimer formation between Fos and the endogenous c-Jun (or Jun-related proteins) is essential for the transforming potential of Fos (ref. 15, and our unpublished results).

The oncogene v-*jun* was originally identified in the genome of avian sarcoma virus 17 as a fusion protein with *gag*, p65$^{gag\text{-}jun}$.[16] v-Jun with or without the *gag* sequences is capable of transforming CEF.[17] The cellular counterpart, c-*jun* was cloned from chicken,[18] mouse[19] and human[20] and shown to be highly conserved. v-Jun has a large deletion composed of 27 amino acid residues at its N-terminal region.

In this report, we have addressed the question of whether the c-*jun* gene has transforming activity like its viral counterpart, v-*jun*. Using an avian retrovirus vector system, we show here that the human c-*jun* gene has transforming activity when it is expressed at high levels in CEF. Our result will be compared with the report by Schütte *et al.*,[21] who used primary rat embryo cells to test the transforming potential of the c-*jun* gene. We further analyze the protein components that are responsible for AP-1 binding activity in the transformed cells and show that Jun homodimer is probably the major component involved in the cellular transformation by this protooncogene.

MATERIALS AND METHODS

Plasmid construction For the construction of a retrovirus vector carrying c-*jun*, the 1.1 kb *Pma*CI-*Eco*T14I fragment containing the entire human c-*jun* coding region[20] was isolated from the human c-*jun* chimeric clone between genomic DNA and cDNA, pHJ (kind gift from Dr. R. Tjian). The fragment was blunt-ended by Klenow fragment, ligated to *Bam*HI linkers and cloned

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into the *Bgl*II site of pDS3 (containing the 3′-half of the proviral structure of the control vector)[24] in the sense orientation to generate pJH1.

To express human c-*jun* in *E. coli*, we used the improved T7 polymerase expression system developed by Studier *et al.*[22] The 1.1 kb c-*jun* fragment was ligated with *Bam*HI linkers as described above and inserted into the *Bam*HI site of pET3a in the sense orientation to generate pEJH.

Growth conditions and transfection procedure Primary and secondary CEF preparation, cell culture conditions and virus infection were essentially as described previously.[11, 23–25] For the virus titer assay, cells were maintained under soft agar containing MEM medium supplemented with 5% calf serum, 1% DMSO and 10% tryptose phosphate broth.

pDS3 (which contains the 3′-half of the proviral structure of the control vector without oncogene),[24] pN4 (with the v-*src* gene),[24] pFM4 (with the c-*fos* (mouse) gene)[11] and pJH1 (with the c-*jun* (human) gene) were completely digested with *Sal*I. The *Sal*I digests were ligated with a *Sal*I-digest of pREP-B (which contains the 5′-half of the proviral DNA structure)[26] to produce intact proviral DNA structures.[24] The ligated DNAs (total 2 μg) were introduced into CEF (60 mm plate) by the calcium phosphate DNA transfection method. About 5 days after transfection of these ligated DNAs, each culture was almost entirely infected with the recombinant virus that belongs to subgroup B as judged by challenging the transfected culture with RSV SRB. Each culture produced high titers of replication-competent viruses (approximately 10^7 infectious particles/ml). In some experiments, pREP[24] was used instead of pREP-B, but the recovered subgroup A virus gave essentially the same result as the corresponding subgroup B virus.

Protein analysis Logarithmically growing cells were labeled with Tran^{35}S (ICN Pharmaceuticals) for 1 h as described previously.[11, 25] Preparation of total cell lysates under denaturing and non-denaturing conditions was described previously.[4] Cell lysates were immunoprecipitated with the specific antiserum and analyzed by 10% SDS polyacrylamide gel electrophoresis. To obtain anti-Jun antiserum, *Escherichia coli* BL21 (DE3) carrying pEJH and pLysS simultaneously was grown in LB broth at 37°C to an $A_{600\,nm}$ of 0.4 for the preparation of the antigen. The cells were treated with 0.5 m*M* IPTG and further incubated for 2 h. Crude Jun protein was isolated from *E. coli* as inclusion bodies, further purified by SDS-polyacrylamide gel electrophoresis and used to immunize rabbits as described previously.[4, 25] Rabbit polyclonal antibodies raised against Fos peptide 1[25] and Fra-2 peptide 1 and peptide 2[4] were described previously. All the immunoprecipitation experiments in this study were performed under conditions of antiserum excess, because less than 3% of the antigen was detected after reimmunoprecipitation by the same antiserum.

Preparation of nuclear extract and gel shift analysis To prepare the DNA probe for gel shift analysis, two oligonucleotides encoding the AP-1 site of FSE2[27];

5′-TCGACTATTAAAAACATGACTCAGAGGAAAAC-3′
3′-GATAATTTTTGTACTGAGTCTCCTTTTGAGCT-5′

were synthesized, annealed (designated as FSE2-AP1) and inserted into the *Sal*I site of pUC118 to generate pFSE2. The 62bp dsDNA fragment was isolated after digesting pFSE2 with both *Hin*dIII and *Bam*HI and used as the DNA probe. Another set of oligonucleotides;

5′-TCGACTATTAAAAACAATACTCAGAGGAAAC-3′
3′-GATAATTTTTGTTATGAGTCTCCTTTGAGCT-5′

with mutations in the FSE2-AP1 sequence (designated as FSE2-MAP1, bold letters indicate the substituted nucleotides) was synthesized, and used for the competition experiment.

Nuclear extract was prepared essentially as described previously except that $MgCl_2$ was removed from buffer A.[28] From six plates (90 mm) of CEF infected with DS3 or JH1, 0.2–0.5 mg of nuclear extract protein was isolated and suspended in 0.3 ml of buffer C solution. Each nuclear fraction (2.0 μg protein) was mixed with the binding buffer (final 10 m*M* Tris HCl (pH 7.5), 50 m*M* NaCl, 5% glycerol, 5 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 1 m*M* EDTA) and 0.5 μg of poly(dI-dC)·poly(dI-dC) and incubated at room temperature for 15 min.[14] Then, 0.2 ng (10,000 cpm) of the ^{32}P-end-labeled probe was added to the reaction mixture. After further incubation for 15 min, DNA-protein complexes were analyzed by electrophoresis on a 5% polyacrylamide gel (TBE buffer) at room temperature for 1.5 h (15 V/cm). The gel was dried and visualized by autoradiography.

RESULTS

Transforming activity of the human c-*jun* gene CEF were transfected with pDS3 (which carries no oncogene) or pJH1 (which carries the human c-*jun* gene) after digestion with *Sal*I and subsequent ligation with the *Sal*I-digest of pREP-B. After 5 days post-transfection, the entire culture of CEF transfected with pJH1 assumed an elongated and sharp cellular morphology that was clearly distinct from that of CEF transfected with pDS3 (Fig. 1 A, B). The culture media were collected 5 days after transfection of pJH1 and used to infect fresh CEF. The virus induced foci composed of elongated cells (fusiform) (Fig. 1C). The titer of transforming virus in the JH1 virus stock (viruses are designated according to the names of plasmids, as summarized in Table I) was high and comparable to that of N4 (v-*src*-carrying virus) or

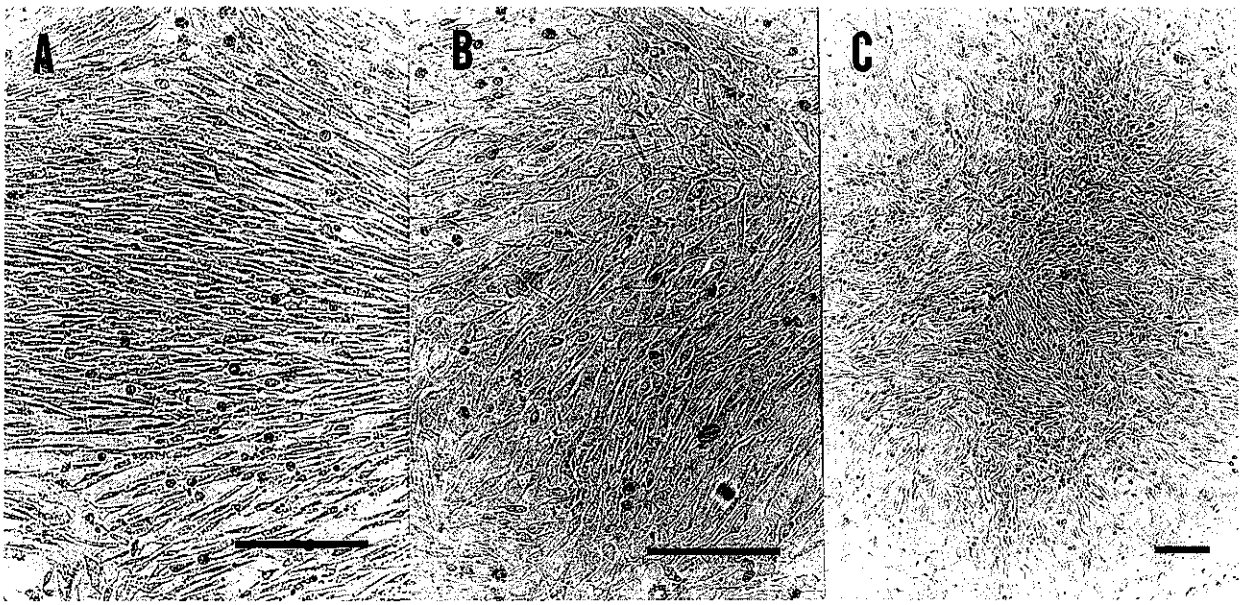

Fig. 1. Cellular and focal morphology of JH1-infected CEF. CEF were transfected with *Sal*I digest of pJH1 (A) or pDS3 (B) that was ligated with *Sal*I-cut pREP-B and kept for 6 days in liquid MEM medium. The JH1 recovered from the culture fluid was diluted, infected onto fresh CEF and kept under soft agar (C). The bar indicates 200 μm.

Table I. Titer of Transforming Virus Recovered 5 Days after Transfection

Plasmid[a)]	Virus recovered	Oncogene	Titer of transforming virus[b)]
pJH1	JH1	c-*jun*	8.1×10^6
pN4	N4	v-*src*	2.1×10^7
pFM4	FM4	c-*fos*	3.3×10^7
pDS3	DS3	none	not detectable

a) The plasmid was digested with *Sal*I and ligated with *Sal*I-cut pREP-B.
b) Focus forming unit (FFU)/ml.

FM4 (c-*fos*-carrying virus) stocks recovered by the same procedure (Table I). JH1-infected cells were able to grow in soft agar and formed visible colonies within 14 days at a similar efficiency to CEF infected with N4 (v-*src*-carrying virus) and FM4 (c-*fos*-carrying virus), although the colony size was a little smaller than that of FM4-infected CEF. From these results, we concluded that the human c-*jun* gene has transforming activity when expressed at a high level in CEF.

Protein analysis of DS3- and JH1-infected CEF To analyze the proteins expressed in JH1- and DS3-infected CEF as well as uninfected CEF, cells were labeled with ^{35}S-methionine and lysed under non-denaturing conditions. Since the protein analysis of the uninfected CEF

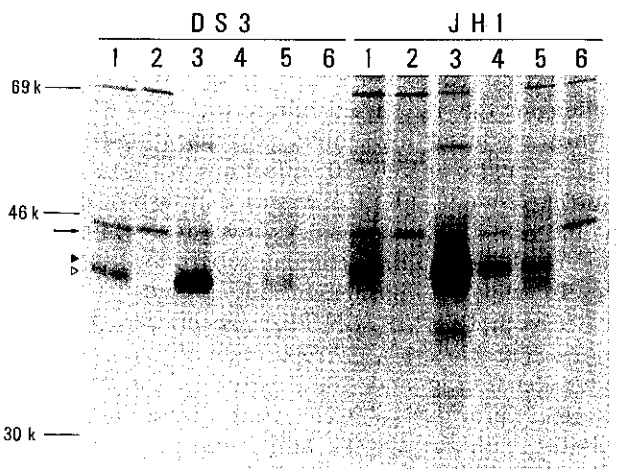

Fig. 2. Protein analysis of DS3- and JH1-infected CEF. DS3- and JH1-infected CEF were grown logarithmically, and labeled with ^{35}S-methionine for 1 h. Total cell lysates were prepared under non-denaturing conditions. The lysates were immunoprecipitated with anti-Fos pep1 (lane 1), anti-Fos pep1 preabsorbed by the antigen peptide (lane 2), anti-Jun (lane 3), anti-Jun preabsorbed and competed with the total Jun protein expressed in *E. coli* (lane 4) and anti-Fra-2 pep2 (lane 5), anti-Fra-2 pep2 preabsorbed by the antigen peptide (lane 6). The lines on the left indicate the comigrated molecular weight markers. The arrow indicates the 44k band, and open and closed triangles indicate the 40k and 41–42k bands, respectively.

gave essentially the same result as that of DS3-infected CEF (data not shown), only the results obtained with DS3-infected CEF will be presented.

When the cell lysates were immunoprecipitated with anti-Jun antiserum, a 40k band was detected in DS3-infected CEF while a broad band ranging from 41 to 42k and a 40k band were detected in JH1-infected CEF (Fig. 2 lane 3). These bands were significantly decreased when the anti-Jun antiserum was preabsorbed and competed with the antigen (Jun purified from *E. coli*) (Fig. 2 lane 4). This result indicates that the 40k band corresponds to the endogenous chicken c-Jun and that the broad band (41–42k) contains the overexpressed exogenous human c-Jun. Such a difference in the apparent molecular weight of the mammalian and avian c-*jun* gene product had been observed in SDS-polyacrylamide gel previously[11] and was also consistent with the DNA sequence analysis of the cloned human and chicken genes.[18, 20]

Since the cell lysates were prepared under non-denaturing conditions, Jun-associated proteins might be coimmunoprecipitated with anti-Jun antiserum. A candidate protein is a 44k band that is detectable in both DS3- and JH1-infected cells, because this protein band disappeared when the anti-Jun antiserum was preabsorbed and competed with the antigen. Furthermore when cell lysates were prepared under denaturing conditions, the 44k band was not detected (data not shown).

For the further identification of the Jun-associated proteins, we next analyzed the expression of the endogenous c-*fos* and *fra*-2 genes, because Fos and Fra-2 were previously shown to coimmunoprecipitate with the same anti-Jun antiserum from the cell extract.[4] The anti-Fos pep1 antiserum was previously shown to recognize the 62k band of c-Fos and the 44k and 46k bands of Fra-2.[4] The 46k protein was only detectable immediately after CEF were growth-stimulated, and was suggested to be a more highly modified (probably by phosphorylation) form of Fra-2 compared to the 44k protein.[4] When cell lysates were immunoprecipitated with this anti-Fos pep1 antiserum, a faint band of 44k was detectable in both DS3- and JH1-infected CEF (Fig. 2 lane 1). The expression of c-Fos protein, however, was not detectable in these cells. Immunoprecipitation by the anti-Fra-2 specific antisera (anti-Fra-2 pep2 and pep1) that does not cross-react with c-Fos clearly confirmed that this 44k protein is the less modified form of Fra-2 (Fig. 2 lane 5 and our unpublished results). Anti-Fra-2 pep2 and anti-Fos pep1 coimmunoprecipitated endogenous c-Jun (40k) from DS3-infected CEF. Exogenous c-Jun (41–42k) as well as a small amount of endogenous c-Jun (40k) was coimmunoprecipitated by these two sera from JH1-

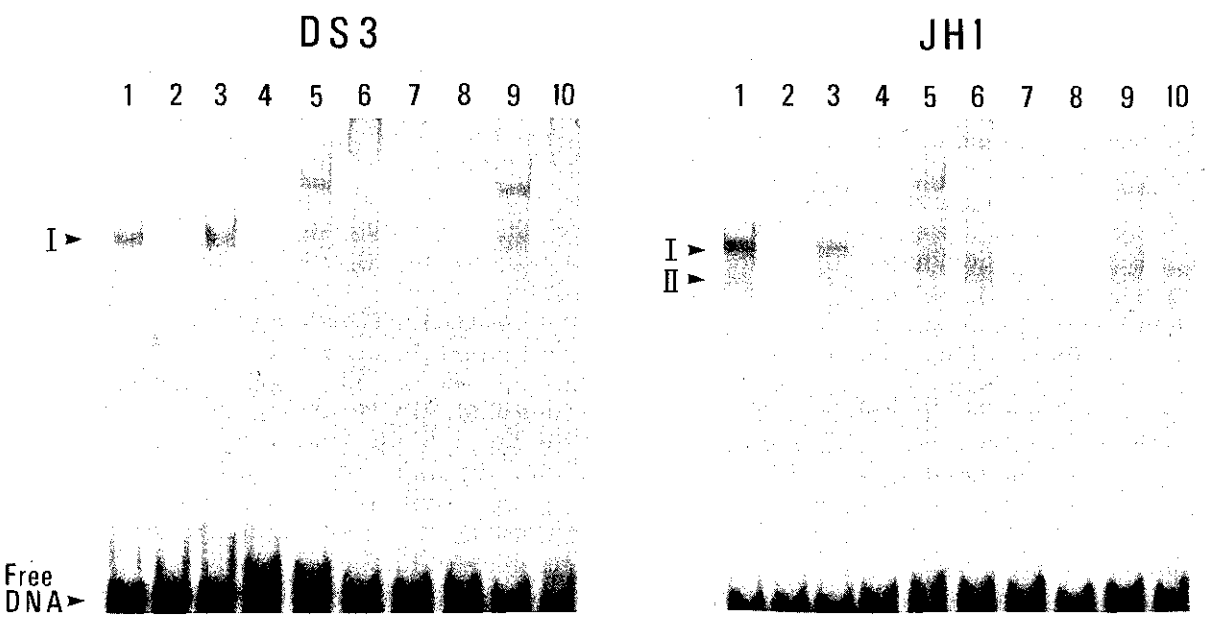

Fig. 3. Gel shift analysis of the nuclear extracts from DS3- and JH1-infected CEF. The nuclear extracts were mixed with ^{32}P-DNA probe (0.2 ng) having the AP-1 sequence in the absence (lane 1) or presence of 20 ng of FSE2-AP1 (lane 2) or FSE2-MAP1 (lane 3). In lanes 4–10, nuclear extracts were treated with anti-Jun (lane 4), anti-Fos pep1 (lanes 5, 7 and 9) or anti-Fra-2 pep1 (lanes 6, 8 and 10) before mixing with the labeled probe in the absence (lanes 4, 5 and 6) or presence of FSE2-AP1 (lanes 7 and 8) or FSE2-MAP1 (lanes 9 and 10).

infected CEF (Fig. 2 lanes 1 and 5). These results indicate that endogenous Fra-2 forms a stable complex with c-Jun in both DS3- and JH1-infected CEF. c-Jun that does not form a stable complex with Fra-2 as well as other Fos-related gene products detected by anti-Fos pep1 is expected to be more abundant in JH1-infected CEF than in DS3-infected CEF.

DNA binding activity in the DS3- and JH1-infected CEF

To detect specific AP-1 DNA binding activity, nuclear fractions were prepared from DS3- and JH1-infected CEF. The nuclear extracts were mixed with ^{32}P-labeled DNA probe containing the AP-1 site from the aP2 gene (FSE2), and analyzed by gel shift assay (Fig. 3). Only a single band (band I) was detected in the nuclear extract of DS3-infected CEF, but one additional band (band II) was also detected in that of JH1-infected CEF (Fig. 3 lane 1). These two bands are formed by protein components that specifically bind to the AP-1 site, because they disappeared when an excess of the non-labeled AP-1 sequence (FSE2-AP1) was added, and because they were not competed out by the mutated version (FSE2-MAP1) (Fig. 3 lanes 2 and 3). Band I was slightly more intense in JH1-infected CEF than in DS3-infected CEF.

To determine which protein complexes were responsible for the formation of band I and band II, nuclear fractions were treated with several antisera before incubation with the DNA probe (Fig. 3 lanes 4–10). Band I, from both DS3- and JH1-infected cells, was completely abolished by anti-Jun antibodies, indicating that this DNA-protein complex includes c-Jun (Fig. 3 lane 4). Band I was also sensitive to anti-Fos pep1 or anti-Fra-2 pep1 treatment (Fig. 3 lanes 5 and 6); the entire band I disappeared upon such treatment and a new band appeared that migrated much more slowly than band I. Such a specific retardation of the DNA-protein complex band by the specific serum would be observed when the antibody bound-protein retains specific DNA binding activity. The new band was further shown to be competed out by FSE2-AP1 (lanes 7 and 8) and not by FSE2-MAP1 (lanes 9 and 10). These results indicate that band I represents a c-Jun/Fra-2 heterodimeric complex.

Band II, which was detected only in the extract of JH1-infected CEF, was sensitive to anti-Jun antibodies while this band was almost unaffected by anti-Fra-2 pep1 or anti-Fos pep1. This result suggests that band II is formed specifically by the Jun homodimers.

DISCUSSION

In this report, we show that an RSV variant (JH1) carrying the human c-*jun* gene causes cellular transformation of CEF. We previously reported that transforming virus could be generated at a low frequency by mutations in such a non-transforming virus as NY501[24] (c-*src*-carrying virus). But JH1 does not require mutation for transforming activity, because the entire cell culture exhibited clear morphological changes 5 days after transfection.

Schütte and coworkers previously reported that c-*jun* was able to transform primary rat embryonic cells in cooperation with an activated c-Ha-*ras* gene, while c-*jun* alone failed to transform them.[21] Their result obtained with the murine system is in clear contrast to our observation that avian primary fibroblasts are transformed by the c-*jun* gene as a single gene. This might indicate that CEF are more sensitive for focus formation by this protooncogene, as was reported previously for the c-*fos* gene.[11] Schütte and coworkers further reported that an established rat cell line, Rat-1a could be transformed by the c-*jun* gene. But the morphological changes were subtle, occurring only in these cell lines with the highest c-*jun* expression.

To analyze the molecular mechanisms of cellular transformation induced by high-level expression of the c-*jun* gene, we examined the expression level of Fos and related antigens that are expected to function as efficient transcriptional regulators by forming stable complexes with c-Jun. In JH1-infected CEF, Fra-2 was synthesized at a basal level, while the expression of c-Fos was undetectable. The expression levels of these proteins in JH1-infected CEF showed no significant differences from those in DS3-infected CEF or in uninfected CEF, indicating that high-level expression of c-*jun* does not affect the expression of the *fos* and *fra*-2 genes. Coimmunoprecipitation experiments using three kinds of antisera clearly indicated that the endogenous Fra-2 forms a stable complex with c-Jun in both JH1- and DS3-infected CEF.

The band of c-Jun protein that is coimmunoprecipitated with Fra-2 by anti-Fra-2 antisera, is rather heavily labeled compared with Fra-2 (Fig. 2 lane 5). This might be partly explained by the higher methionine content of c-Jun[18] than that of Fra-2[4] (7 residues versus 4, including the first methionine) and also by the relative instability of c-Jun compared with Fra-2 (our unpublished observation). It is also possible that Fra-2 is not exclusively present as the 44k protein but is also present as an unmodified form or several differently modified forms that have a broad range of molecular weight, forming faint and smeared bands in gels.

A basal level of AP-1 binding activity (band I) was detected in DS3- and JH1-infected CEF (Fig. 3) as well as uninfected CEF (our unpublished observation). This activity apparently results from a Fra-2/Jun complex. In JH1-infected CEF, however, there is another protein complex that possesses additional AP-1 binding activity (band II). Since this protein complex contains Jun but does not include Fos and Fra-2, it is probably composed of Jun homodimers. Therefore, Jun homodimers are

expected to be mainly responsible for the cellular transformation by this protooncogene. A detailed description of all the protein components in bands I and II, as well as a careful analysis of their actual target genes and their regulatory effects will be necessary for the further understanding of the transforming mechanism of the *jun* protooncogene.

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