The EMBO Journal vol.11 no.2 pp.653-665, 1992

# Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity

**Steven Xanthoudakis and Tom Curran[1]**

Department of Molecular Oncology and Virology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110, USA

[1]Corresponding author

Communicated by E.Wagner

**Fos and Jun form a heterodimeric complex that regulates gene transcription by binding to the activator protein-1 (AP-1) DNA sequence motif. Previously, we demonstrated that the DNA-binding activity of Fos and Jun is regulated *in vitro* by a novel redox (reduction-oxidation) mechanism. Reduction of a conserved cysteine (cys) residue in the DNA-binding domains of Fos and Jun by chemical reducing agents or by a nuclear redox factor stimulates DNA-binding activity. Here, we describe purification and characterization of a 37 kDa protein (Ref-1) corresponding to the redox factor. Although Ref-1 does not bind to the AP-1 site in association with Fos and Jun, it partially copurifies with a subset of AP-1 proteins. Purified Ref-1 protein stimulates AP-1 DNA-binding activity through the conserved Cys residues in Fos and Jun, but it does not alter the DNA-binding specificity of Fos and Jun. Ref-1 may represent a novel redox component of the signal transduction processes that regulate eukaryotic gene expression.**

*Key words:* Fos/Jun/oncogenes/reduction-oxidation/transcription factor

## Introduction

Cell growth and differentiation are controlled, to a large extent, through the selective regulation of gene expression by extracellular signals. In the past several years, a large number of sequence-specific DNA-binding proteins have been identified that function as regulators of gene transcription. However, little is known about the events that determine target gene specificity and activation by transcription factors. This is of particular concern for the protein products of gene families that bind to similar or identical DNA sequence motifs. Given the complexity of the eukaryotic genome, it is likely that several distinct mechanisms operate in concert to dictate the recognition of a single gene control element by a specific transcription factor complex.

The cellular proto-oncogenes c-*fos* and c-*jun* have provided a useful paradigm for the investigation of stimulus-evoked alterations in gene expression. Like the cellular homologues of other retroviral oncogenes, c-*fos* and c-*jun* play key roles in the signal transduction processes that govern cell growth (for a review see Reddy *et al.*, 1988). Their protein products, Fos and Jun, are expressed transiently in a variety of cell types after treatment with mitogenic, differentiation-inducing or depolarizing stimuli (for review see Morgan and Curran, 1991). They are thought to function in coupling short-term signals, elicited at the cell surface, to long-term changes in cellular phenotype by regulating expression of specific target genes. Fos and Jun form a heterodimeric complex that binds to transcriptional control elements containing activator protein-1 (AP-1) binding sites and regulates gene expression (Curran and Franza, 1988). Protein dimerization occurs through a coiled-coil interaction involving leucine zipper domains in each protein (Kouzarides and Ziff, 1988; Landschulz *et al.*, 1988; Gentz *et al.*, 1989; O'Shea *et al.*, 1989; Schuermann *et al.*, 1989; Turner and Tjian, 1989). Consequently, a bipartite DNA-binding domain is formed by juxtaposition of regions rich in basic amino acids of each protein which are located adjacent to the leucine zipper.

Both the leucine zipper and the DNA-binding domains are conserved among a number of *fos*- and *jun*-related genes (for review see Kerppola and Curran, 1991a). These, and presumably other as yet unidentified proteins, collectively comprise the mammalian transcription factor AP-1. Heterodimer formation among members of the Fos, Jun and ATF/CREB families of transcription factors generates a diverse array of protein complexes with overlapping DNA-binding specificities but distinct transcriptional properties (Franza *et al.*, 1988; Nakabeppu and Nathans 1988; Rauscher *et al.*, 1988; Chiu *et al.*, 1989; Cohen *et al.*, 1989; Hirai *et al.*, 1989; Ryder *et al.*, 1989; Schutte *et al.*, 1989; Zerial *et al.*, 1989; Benbrook and Jones, 1990; Macgregor *et al.*, 1990; Matsui *et al.*, 1990; Nishina *et al.*, 1990; Hai and Curran, 1991; Nakabeppu *et al.*, 1991). The mechanisms that regulate assembly, targeting and functional specificity of the different AP-1 complexes are currently unclear, although differential expression of family members (Cohen and Curran, 1988; Bartel *et al.*, 1989; Hirai *et al.*, 1989; Nakabeppu and Nathans, 1991), interactions with unrelated transcription factors (Diamond *et al.*, 1990; Gaub *et al.*, 1990; Jonat *et al.*, 1990; Owen *et al.*, 1990; Schule *et al.*, 1990a, b; Yang-Yen *et al.*, 1990), conformational alterations (Patel *et al.*, 1990; Kerppola and Curran, 1991b) and altered DNA-binding specifities of heterodimers (Ryseck and Bravo, 1991; Hai and Curran, 1991) are all likely to play roles.

Superimposed upon these mechanisms is the important influence of post-translational modification. The levels of AP-1 DNA-binding activity and transcriptional responses from AP-1 elements can be increased in the absence of *de novo* protein synthesis, presumably through modification of pre-existing AP-1 proteins (Angel *et al.*, 1987; Chiu *et al.*, 1987; Welham *et al.*, 1990). In one case, increased AP-1 DNA-binding activity in response to TPA treatment has been attributed to dephosphorylation of Jun (Boyle *et al.*, 1991). However, this cannot be a general mechanism as many cell stimuli provoke an increase in phosphorylation of Fos and Jun (Curran and Morgan, 1985; Barber and Verma, 1987; Abate *et al.*, in press).

Recently, we identified an unusual mechanism involving

© Oxford University Press

reduction–oxidation (redox) that modulates AP-1 DNA-binding activity *in vitro* (Abate *et al.*, 1990a,b). Redox regulation is mediated by a conserved cysteine residue located in the DNA-binding domains of Fos and Jun that is flanked by basic amino acids (Abate *et al.*, 1990a). Fos and Jun can be converted to an inactive state by chemical oxidation or modification of this residue (Abate *et al.*, 1990a,c). In solution, the proteins adopt and remain in an inactive state even in the presence of 1 mM DTT (dithiothreitol). Inactivation is not caused by intra- or intermolecular disulfide bond formation but by conversion (presumably oxidation) of the cys (Fos-C154 and Jun-C272) to an inactive state (Abate *et al.*, 1990a). Conversely, the DNA-binding activity of Fos and Jun can be enhanced by mutation of Cys to Ser (Fos-C154S and Jun-C262S) or by treatment with high concentrations of reducing agents (Abate *et al.*, 1990a).

Two major lines of evidence suggest that redox regulation plays an important physiological role *in vivo*. Firstly, the v-*jun* oncogene contains a naturally occurring mutation of Cys to Ser at this site (Maki *et al.*, 1987). This substitution enhances the transforming potential of the c-*jun* oncogene (P.Vogt, USC Medical School, Los Angeles, personal communication). Similarly, conversion of C154 to S in Fos increases its ability to induce cellular transformation (H.Iba, University of Tokyo, personal communication). Thus, the oncogenic activity of Fos and Jun may be increased by deregulation of redox control. Secondly, a cellular nuclear protein can activate the DNA-binding activity of Fos and Jun in the absence of high levels of reducing agents (Abate *et al.*, 1990b). The activity of this nuclear factor can be increased in the presence of thioredoxin, thioredoxin reductase and NADPH, implying that it may participate in a redox cycle (Abate *et al.*, 1990a).

To characterize the role of redox control in transcription factor function and in signal transduction, it is necessary to isolate and reconstitute the components involved. Here, we present the purification and characterization of a 37 kDa protein (Ref-1) from HeLa nuclear extracts that corresponds to the AP-1 redox factor. Although Ref-1 co-purifies through several steps with a subset of AP-1 proteins, it is antigenically distinct from Fos and Jun and it does not bind to DNA in association with Fos and Jun. Purified Ref-1 stimulates the DNA-binding activity of Fos–Jun heterodimers and HeLa cell AP-1 proteins. This is the first isolation of a redox factor capable of regulating transcription factor function. These results imply that redox signaling could contribute to the selective control of gene expression by environmental cues.

## Results

### *Assay for Ref-1 activity*

The presence of Ref-1 activity in HeLa nuclear extracts was monitored by gel-shift assays as described previously (Abate *et al.*, 1990c). At low concentrations of reducing agents (<0.2 mM DTT) and in the absence of nuclear extract, a diminished level of AP-1 DNA-binding activity was detected using the truncated polypeptides Fos118-211 and Jun225-234 or full-length Fos and Jun (Figure 1A). In contrast, addition of 3 μg of nuclear extract or 10 mM DTT to the binding reaction significantly stimulated both exogenous and endogenous AP-1 DNA-binding activity. As shown in Figure 1A, the DNA–protein complex generated by Fos118-211 and Jun225-334 migrated more rapidly on native gels than the complex generated by the endogenous AP-1 proteins present in HeLa cell extracts. An additional low level protein–DNA complex was detected when full-length Fos and Jun were treated with the nuclear extract. Although this complex co-migrated with the Fos118-211–Jun225-334 heterodimer, it represented a non-specific protein–DNA interaction and was not detected in DNA-binding assays using more purified preparations of nuclear extract. Because Fos118-211 and Jun225-334 contain an intact leucine zipper domain and basic region, they interact with the AP-1 binding site in a manner that is characteristic of the full-length proteins (Abate *et al.*, 1990a,b). Thus, to distinguish Ref-1 mediated stimulation of AP-1 DNA-binding activity from endogenous AP-1 activity, Fos118-211 and Jun225-234 were used as substrates to monitor Ref-1 activity during purification.

### *Purification of Ref-1*

A preliminary investigation of the properties of Ref-1 on a selection of columns was used to derive the purification scheme outlined in Figure 1B. In all chromatographic steps, protein elution was monitored by continuous UV (280 nm) absorption. As a first step, ~450 mg of crude nuclear extract was applied to a 40 ml heparin–Sepharose column and eluted using a linear KCl gradient (Figure 2A). Ref-1 activity eluted after the major protein peak between 0.4 M and 0.5 M KCl, with a 26.5% recovery of the total activity and a 2.4-fold increase in the specific activity (Table I). As illustrated in Figure 2A, this step was also useful in separating Ref-1 from the majority of endogenous AP-1 DNA-binding activity, which migrated more slowly in the gel.

The active fractions from the heparin–Sepharose column were dialyzed against nuclear dialysis buffer (NDB) containing 0.1 M KCl and loaded onto a 5 ml DNA–cellulose column. Ref-1 activity was recovered in the flow-through fraction, while most of the endogenous AP-1 activity, that carried over from the previous column, was retained on DNA–cellulose (data not shown). This fractionation step resulted in an additional 1.7-fold increase in specific activity and an overall increase in total activity relative to the pooled sample from heparin–Sepharose. The increase in total activity, albeit modest, may reflect the removal of proteins that interfere with or inhibit Ref-1 function in the less pure protein preparations.

The next step in the purification process involved fractionation by blue Sepharose chromatography (Figure 2B). This affinity matrix has been successfully used to isolate a number of enzymes requiring dinucleotide co-factors, including reductases (Solomonson, 1975; Westbrook and Jarabak, 1978). The flow-through fraction from the DNA–cellulose column was applied directly onto a 7.5 ml blue Sepharose column and eluted with a linear 0.1–1.0 M KCl gradient. Ref-1 activity co-eluted with the main protein peak between 0.5 and 0.6 M KCl. Pooled fractions were dialyzed against phosphate buffer (NDB-P) containing 50 mM NaCl and further fractionated by two successive rounds of cation exchange chromatography on a mono S column (Figure 2C and D). In both mono S steps, Ref-1 activity eluted from the column between 0.7 and 0.8 M NaCl. After the second round of purification, the specific activity of Ref-1 increased ~10-fold, relative to the starting material. At this stage several candidate proteins were

identified that co-eluted with the stimulatory activity as determined by SDS–polyacrylamide gel electrophoresis (data not shown). To determine which protein(s) represented Ref-1 activity, the active mono S fractions were subsequently analyzed by Superose 12 and Superdex 75 gel filtration chromatography.

Ref-1 activity eluted from the Superose 12 column with an apparent molecular weight of 40 kDa (Figure 2E). Similarly, SDS–polyacrylamide gel analysis of the Superose 12 fractions identified a single major candidate protein of 37 kDa that co-purified with Ref-1 activity (Figure 2F). The specific activity of Ref-1 recovered from the Superose 12 column was ~5-fold higher than that in the previous mono S fractions. Greater protein resolution and an additional 3-fold increase in specific activity was achieved with a final fractionation over the Superdex 75 column (Figure 3). Once again, elution of the 37 kDa protein from Superdex 75 correlated directly with the enhanced level of DNA-binding activity. Finally, when thioredoxin was included in the DNA-binding assay, AP-1 DNA-binding activity was stimulated an additional 3 to 4-fold (Figure 3). Thioredoxin could act, at least in part, to regenerate Ref-1 activity that was lost through extensive fractionation. However, thioredoxin was unable to stimulate DNA-binding activity in the absence of Ref-1. Thus, the overall purification of Ref-1 in the presence of thioredoxin was estimated to be approximately 700-fold (Table I). These results are in agreement with previous experiments that demonstrated thioredoxin-mediated enhancement of Ref-1 activity in crude nuclear extracts (Abate *et al.*, 1990a).

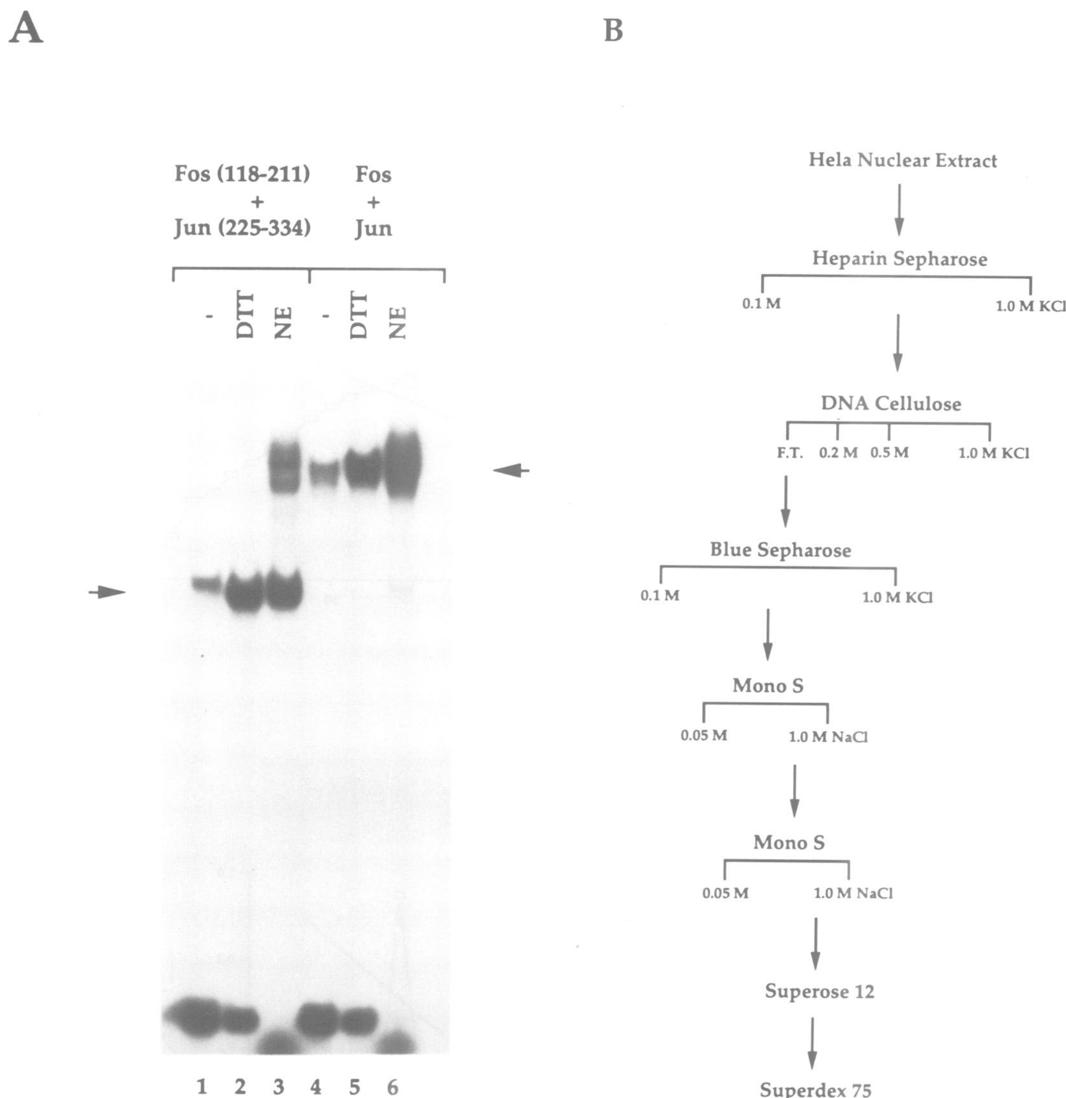
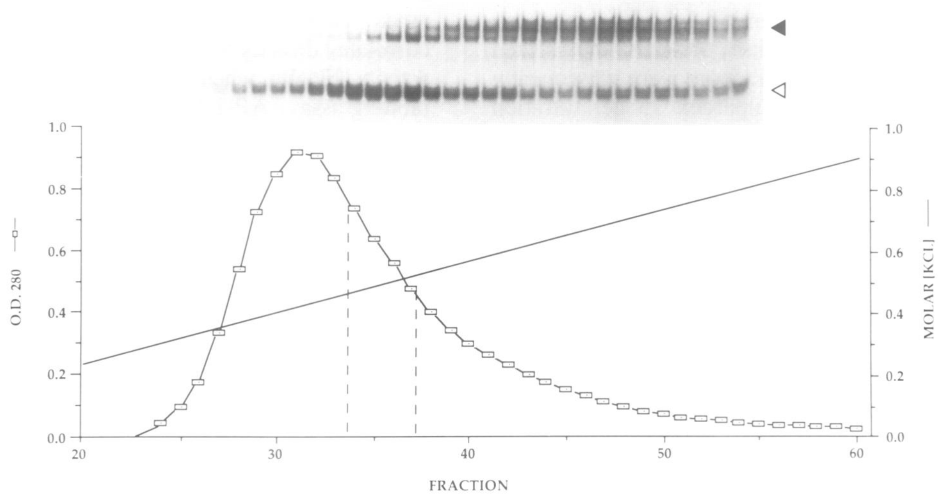
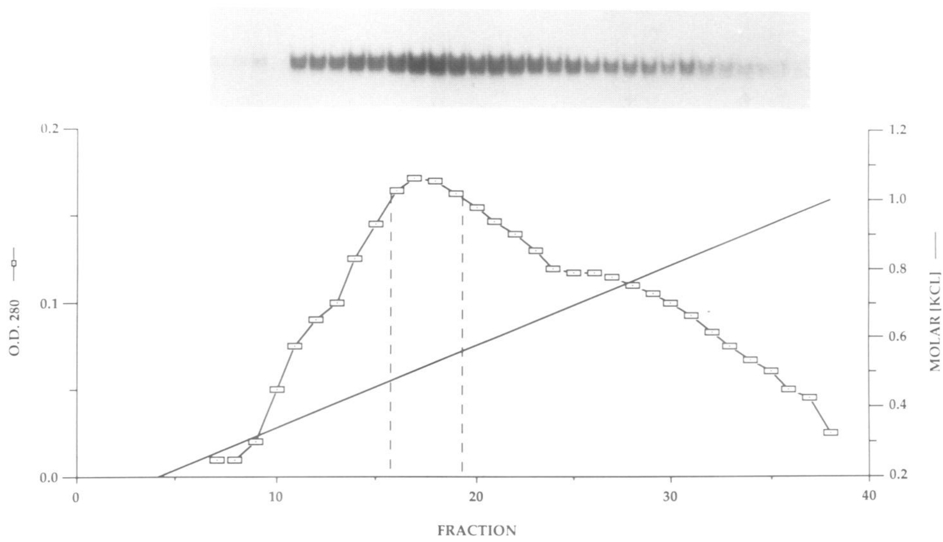



**Fig. 1.** (**A**) Binding of Fos and Jun polypeptides to DNA is stimulated by a nuclear factor. Truncated (lanes 1 and 3) and full-length (lanes 4 and 6) Fos and Jun were incubated at 37°C for 15 min in the presence of either NDB (lanes 1 and 4), 10 mM DTT (lanes 2 and 5) or 3 $\mu$g of crude HeLa cell nuclear extract (lanes 3 and 6). The proteins were then assayed for binding to a 25 bp $^{32}$P-labeled AP-1 oligonucleotide (0.2 ng) following preincubation with 1 $\mu$g of poly(dI–dC) for 5 min at room temperature. Protein–DNA complexes were resolved on a 4.5% native Tris–glycine gel and visualized by autoradiography of the dried gel. The arrows indicate the position of the protein–DNA complex. Complexes in lane 3 that comigrate with those formed using the full-length bacterial Fos and Jun represent endogenous HeLa cell AP-1 DNA binding activity. (**B**) Purification scheme for Ref-1. The strategy used for fractionation of Ref-1 from HeLa cell nuclear extracts is shown. The numbers shown indicate the KCl or NaCl gradient range used to elute Ref-1 activity.

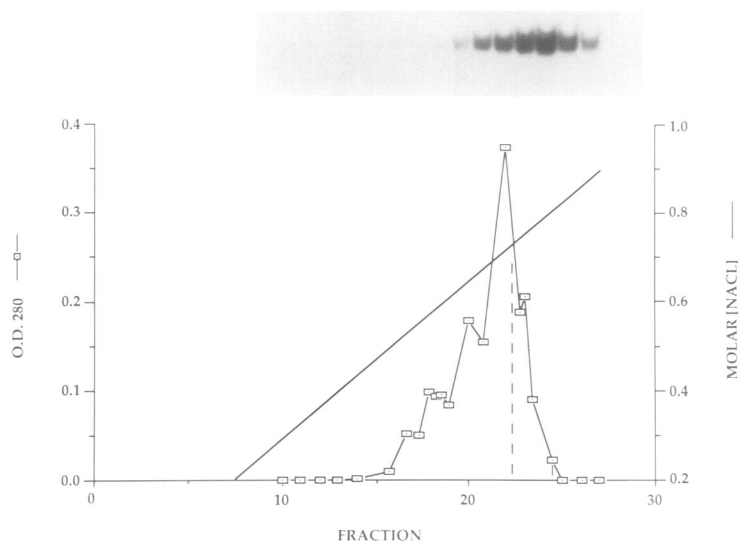
A




B




C



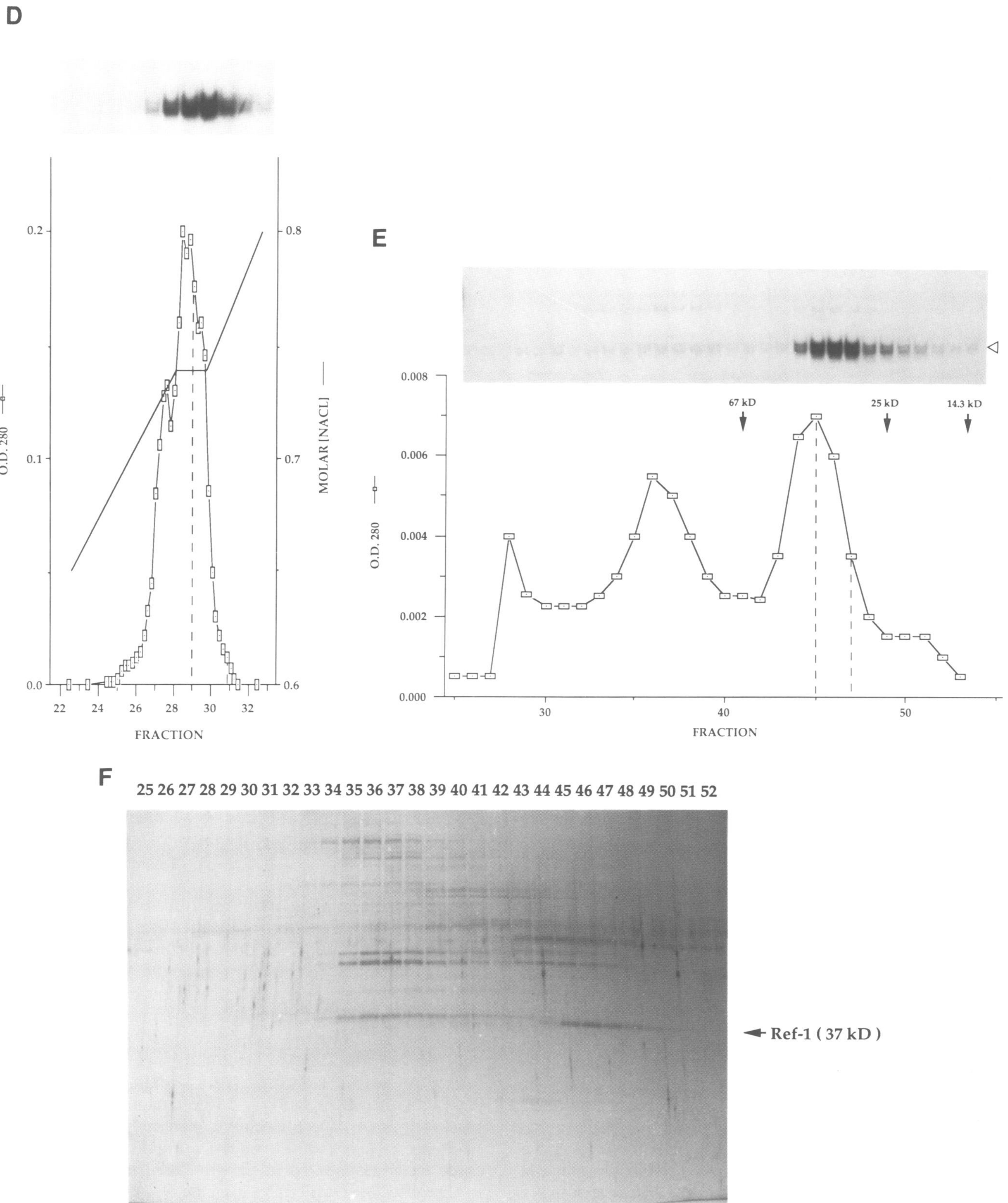



**Fig. 2.** Purification of Ref-1. The protein elution (O.D. 280 nm) and corresponding gel retardation profile for each column is shown: (**A**) heparin–Sepharose, (**B**) blue Sepharose, (**C**) mono S (first round), (**D**) mono S (second round), (**E**) Superose 12 gel filtration. The molar range of salt used to elute each columm is indicated to the right of each profile. The open and filled arrows in panels A (heparin–Sepharose) and E (Superose 12) indicate the position of exogenous and endogenous AP-1 complexes, respectively. The area encompassed between the dashed lines in each profile represents the pooled peak fractions containing Ref-1 activity. The molecular weight calibration standards (kDa) used for gel filtration are described in Materials and methods. (**F**) 5 $\mu$l of different protein fractions (#25–52) eluted from the Superose 12 column were resolved on a 9% SDS–polyacrylamide gel and visualized by silver staining (Bio-Rad). The position of the 37 kDa Ref-1 protein is indicated.

Table 1. Purification of Ref-1

Specific activity * (axis 0–5)

| | Nuc. Ext. | Hep. Seph. | DNA Cell. | Blue Seph. | Mono S-1 | Mono S-2 | Superose 12 | Superdex 75 |
|---|---|---|---|---|---|---|---|---|
| Volume (ml) | 60 | 16 | 22 | 6.0 | 2.5 | 0.2 | 0.9 | 1.2 |
| Total protein (mg) | 462 | 50.4 | 33.4 | 2.8 | 1.7 | 0.102 | 9.0 (μg) | 0.96 (μg) |
| Total binding activity ** | 11504 | 3044 | 3417 | 370 | 250 | 26 | 12.7 | 4.2 |
| Yield (%) | 100 | 26.5 | 29.7 | 3.2 | 2.2 | 0.23 | 0.11 | .04 |
| Purification (fold) | 1 | 2.4 | 4.1 | 5.3 | 5.9 | 10.2 | 56.6 | 176.8 (707)[1] |

* Specific activity is defined as the nanogram amount of bound AP-1 oligonucleotide probe per microgram of protein.
** Binding activity was calculated by multiplying the total amount of protein (μg) by the specific activity.
[1] Estimated fold purification in the presence of thioredoxin

### *Ref-1 co-purifies with Fos- and Jun-related proteins*

To exclude the possibility that Ref-1 represented an AP-1 protein, fractions from different stages of the purification process were examined by immunoblot analysis using antibodies raised against Fos and Jun (Curran *et al.*, 1985; Cohen and Curran, 1990) (Figure 4). These antibodies failed to recognize Ref-1 implying that the protein is antigenically distinct from either Fos or Jun. Interestingly, despite our efforts to separate Ref-1 activity from endogenous AP-1 activity by heparin–Sepharose and DNA–cellulose chromatography, several Fos- and Jun-related antigens were detected in the Ref-1 containing fractions from the second mono S column (Figure 4, lanes 13 and 20). Fos- and Jun-related antigens were not detected in the Superose 12 fraction (Figure 4, lanes 14 and 21). However, this could be attributed to the limiting quantities of protein analyzed, since ~15-fold less protein was assayed relative to other fractions. Consistent with this notion, prolonged exposure of the autoradiogram revealed the presence of a low level of the Fos-related antigens in the Superose 12 fraction (data not shown). This experiment demonstrates that a subset of AP-1 proteins co-purify with Ref-1 through several chromatographic steps. These proteins represent only a few of the many that comprise HeLa cell AP-1 activity (Rauscher *et al.*, 1988). It is possible that they bind with low affinity to Ref-1 and therefore co-purify through several steps. The sizes of the Fos-related antigens (50–60 kDa) detected in this experiment are consistent with those identified in earlier studies (Franza *et al.*, 1988; Rauscher *et al.*, 1988). However, the low molecular weight Jun-related protein (~33 kDa) identified here has not been reported previously. It is unlikely that this protein represents a proteolytic product of a higher molecular weight species, given that immunoblot analysis of crude nuclear extracts containing exogenous Jun, consistently detected Jun of the expected molecular weight (data not shown).

### *Characterization of purified Ref-1 activity*

To characterize the effect of purified Ref-1 on the DNA-binding activity of Fos and Jun, protein–DNA complexes were examined by footprinting analysis. A 154 bp fragment from the $HMTII_A$ gene containing a single AP-1 site was used to determine the methylation interference and DNase I protection patterns generated with Fos–Jun and Fos118-211–Jun225-334 heterodimers in the presence of either 10 mM DTT or Ref-1. As shown in Figure 5, Ref-1 does not alter the specificity of the interaction of Fos and Jun with DNA. The footprinting patterns generated in the presence of either DTT or Ref-1 are indistinguishable for both the truncated and full-length Fos–Jun proteins.

The effect of purified Ref-1 on the DNA-binding activity of endogenous AP-1 proteins was analyzed by the gel-shift assay. Partially purified AP-1 proteins from HeLa cell nuclear extracts depleted of Ref-1 activity, were obtained by heparin–Sepharose chromatography (Figure 2A). Removal of DTT by dialysis caused a marked but reversible reduction in the DNA-binding activity of these proteins (Figure 6A, lane 2). DNA-binding activity could be restored to a high level by the addition of 10 mM DTT or purified Ref-1 to the binding reaction (Figure 6A, lanes 3 and 4).

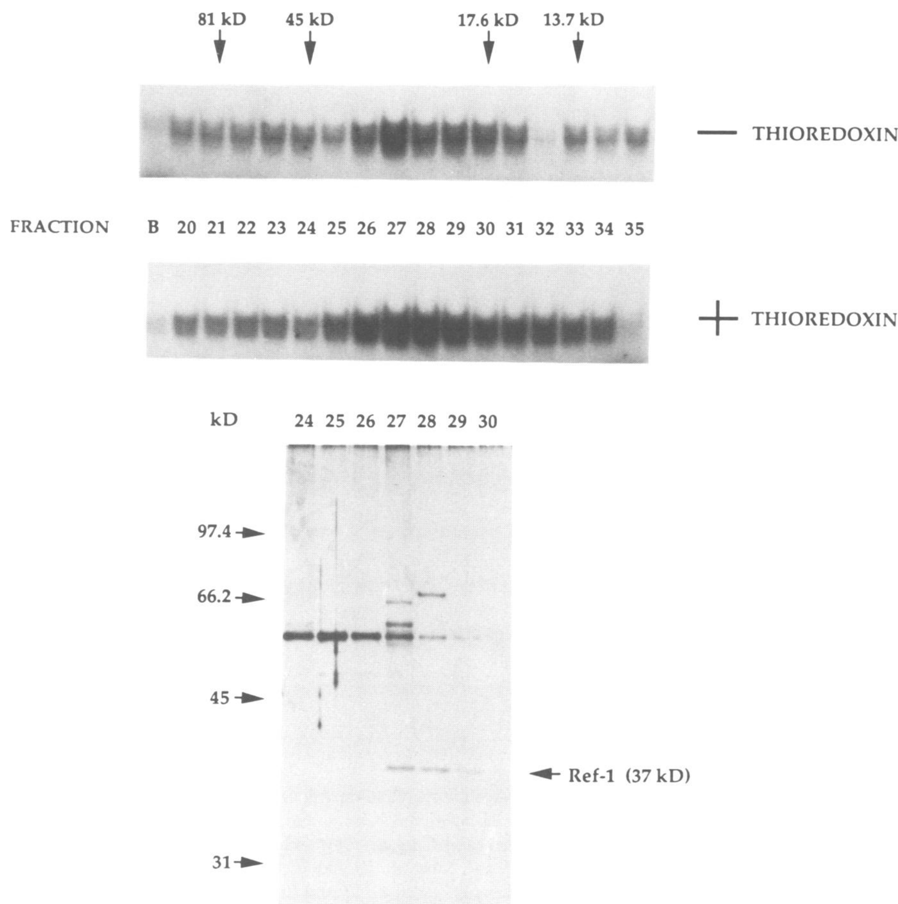



**Fig. 3.** Superdex 75 purification of Ref-1. (**Above**) Superdex 75 gel filtration fractions were assayed for Ref-1 activity by gel retardation analysis. The assay was performed in the absence (−) or presence (+) of thioredoxin/thioredoxin reductase/NADPH as described in Materials and methods. In the first lane (B), NDB buffer was assayed in the absence of any Superdex protein fraction. The molecular weight calibration standards (kDa) used for gel filtration are indicated above the gel. (**Below**) Superdex 75 fractions containing Ref-1 activity were analyzed by silver staining after resolution on a 9% SDS−polyacrylamide gel. The position of Ref-1 is indicated by an arrow. The molecular weight (kDa) protein standards (Bio-Rad) include: phosphorylase B (rabbit muscle), 97.4; bovine serum albumin, 66.2; ovalbumin, 45; bovine carbonic anhydrase, 31.

However, in the absence of endogenous AP-1 proteins, Ref-1 by itself did not exhibit AP-1 DNA-binding activity (Figure 6A, lane 5). Thus, there were insufficient amounts of any co-purifying Fos- or Jun-related proteins in the preparation to detect by gel-shift assay. In this, and in other experiments, we noted that Ref-1 was more potent than DTT at stimulating AP-1 DNA-binding activity. Furthermore, Ref-1 stimulated AP-1 DNA-binding activity to a level exceeding that seen with the undialyzed AP-1 sample, suggesting that AP-1 proteins isolated from HeLa cells by standard procedures are in a partially inactive state.

Previously, we established that mutation of the conserved cysteine residue in the basic region of Fos and Jun to serine, generated proteins that exhibited enhanced levels of DNA binding in the absence of DTT or nuclear extract. These alterations increase DNA-binding activity and therefore represent gain-of-function mutations (Abate *et al.*, 1990a). Figure 6B shows that high concentrations of DTT (10 mM) failed to increase the DNA-binding activity of F(C1-S) + J(C1-S) heterodimers (Figure 6B, lane 2). Similarly, the DNA-binding activity of these proteins was not stimulated further by Ref-1 (Figure 6B, lane 3), indicating that Ref-1 acts through the same cysteine residues previously shown to mediate redox regulation of Fos−Jun DNA-binding activity.

Although the presence of the 37 kDa protein in our most pure fractions corresponded to Ref-1 activity, the possibility remained that the activity could be conferred by undetectable levels of a contaminant. Therefore, to confirm the identity of Ref-1, the 37 kDa protein was transferred onto a polyvinylide difluoride membrane (PDVF) and the corresponding band was excised and used to derive 20 amino acids of N-terminal sequence (Matsudaira *et al.*, 1987). DNA fragments obtained by mixed-primer PCR amplification of the deduced 5′ coding sequences (Gubler *et al.*, 1991) were used to probe a Jurkat cDNA library (Ruben *et al.*, 1991) and isolate a putative cDNA clone encoding human Ref-1. Details of the cloning strategy and sequence analysis of the *ref-1* cDNA will be described elsewhere (Xanthoudakis *et al.*, in preparation). The cDNA clone was expressed as a hexahistidine fusion protein in *E.coli* and purified by nickel chelate chromatography as described for Fos and Jun (Abate *et al.*, 1990c). The recombinant protein of ~37 kDa was resolubilized and tested for Ref-1 activity (Figure 7). This protein stimulated Fos−Jun DNA binding activity in an analogous fashion to purified Ref-1 from HeLa cells. In

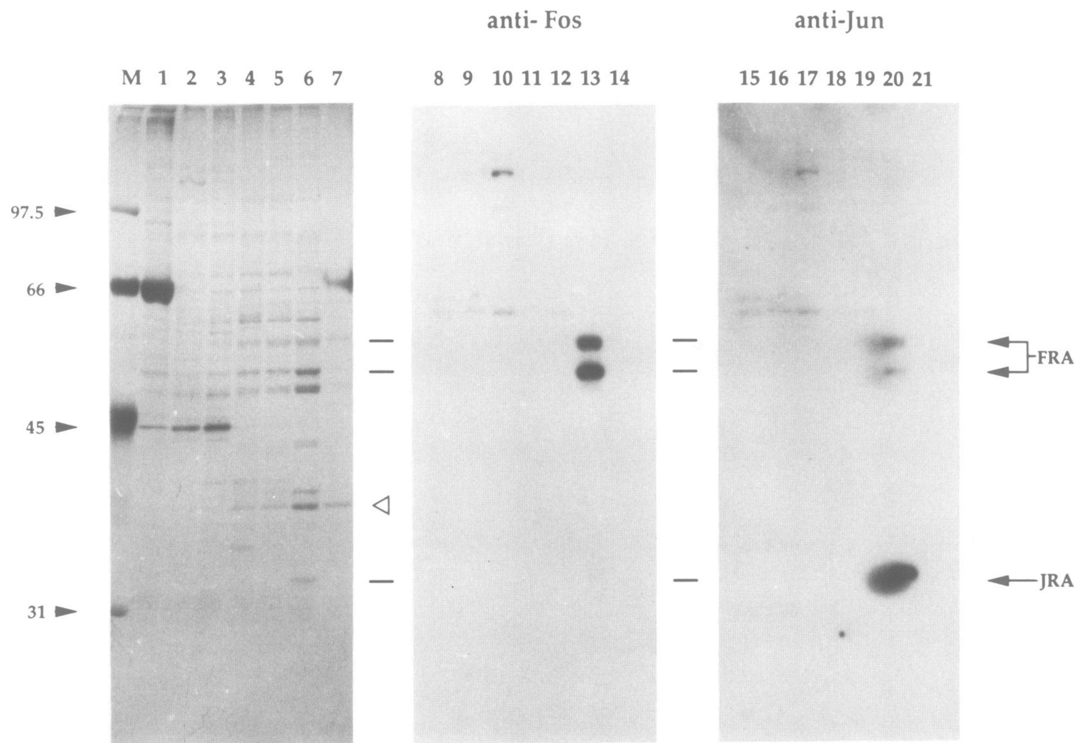



**Fig. 4.** Western analysis of chromatographic fractions containing Ref-1 activity. Column fractions were resolved in duplicate on a 9% SDS−polyacrylamide gel. Half of the gel was used to visualize proteins by silver staining and the other half examined by immunoblot analysis using anti-Fos antibodies (Curran *et al.*, 1985) and anti-Jun antibodies (Cohen and Curran, 1990). Crude HeLa nuclear extract, lanes 1, 8 and 15; heparin−Sepharose (1.5 μg), lanes 2, 9 and 16; DNA−cellulose (1.5 μg), lanes 3, 10 and 17; blue Sepharose (1.5 μg), lanes 4, 11 and 18; mono S (first round) (1.5 μg), lanes 5, 12 and 19; mono S (second round) (1.5 μg), lanes 6, 13 and 20; Superose 12, lanes 7, 14 and 21 (0.1 μg). The positions of Fos-related antigens (FRA), Jun-related antigens (JRA) and Ref-1 (open arrow) are indicated. The molecular weight (kDa) of the protein standards used (lane M) are listed in the legend to Figure 3.

contrast, control extracts prepared in an identical manner from untransformed *E.coli* or cells transformed with an expression vector lacking a cDNA insert (pDS56), were unable to stimulate DNA-binding activity. These data confirm the identity of the purified 37 kDa protein as Ref-1, the redox factor that stimulates Fos−Jun DNA-binding activity.

## Discussion

The unique biochemical attributes of Cys residues are often essential for functional and structural properties of proteins (Cooper, 1983). Indeed, their presence and relative distribution are often used to define characteristic protein domains such as the immunoglobulin repeat (Fearson *et al.*, 1990; and references therein) or the zinc finger (Evans and Hollenberg, 1988; Struhl, 1989). For these reasons, Cys residues are often conserved across phylogenetic boundaries and among gene families. A highly conserved Cys residue, flanked by one or two basic amino aicds, is present in the DNA-binding domain of the four *fos* family members, the three *jun* family members and at least four members of the ATF/CREB family of transcription factors (reviewed by Kerppola and Curran, 1991a). However, this residue does not participate in disulfide bond formation between Fos and Jun *in vivo* and it is not part of a zinc finger motif. In the proposed models of protein−DNA complexes involving basic region−leucine zipper proteins, the Cys is in close proximity to DNA (Vinson *et al.*, 1989; O'Neil *et al.*, 1990; Kerppola and Curran, 1991b). Recently, we demonstrated that the *in vitro* DNA-binding activity of Fos and Jun was regulated by a unique redox mechanism involving this residue (Abate *et al.*, 1990a). The regulatory cysteine acts as a 'sulfhydryl switch' that reversibly modulates binding of Fos and Jun to the AP-1 site. The exact chemical nature of the redox change in Fos and Jun has yet to be defined, but it is clear that oxidation of the Cys is not permissive for DNA binding, while reduction to a sulfhydryl state promotes the protein−DNA interaction. Thus, Cys residues flanked by basic amino acids define a novel Cys motif involved in the regulation of DNA-binding activity.

The role of redox switching in regulating transcription factor function has been questioned because of the generally accepted view that the intracellular environment is reducing (Meister and Anderson, 1983; Holmgren, 1985; 1989). However, very little is known about the redox environment of the nucleus. Indeed, even under glutathione buffering conditions, oxidized sulfhydryls have been shown to exist (Cappel and Gilbert, 1988). Moreover, the oxidation constant ($K_{ox}$) of a sulfhydryl group is influenced by its local environment (Walters and Gilbert, 1986; Clancey and Gilbert, 1987) and is subject to change caused by alterations in protein conformation. We have shown that the conserved Cys in Fos and Jun is not reduced in the presence of 1 mM DTT, presumably because of its highly basic micro-environment (Abate *et al.*, 1990a).

### *Ref-1 is the redox regulator of AP-1 DNA-binding activity*

In this study we have identified and purified a nuclear factor, Ref-1, that mediates redox regulation of AP-1 DNA-binding

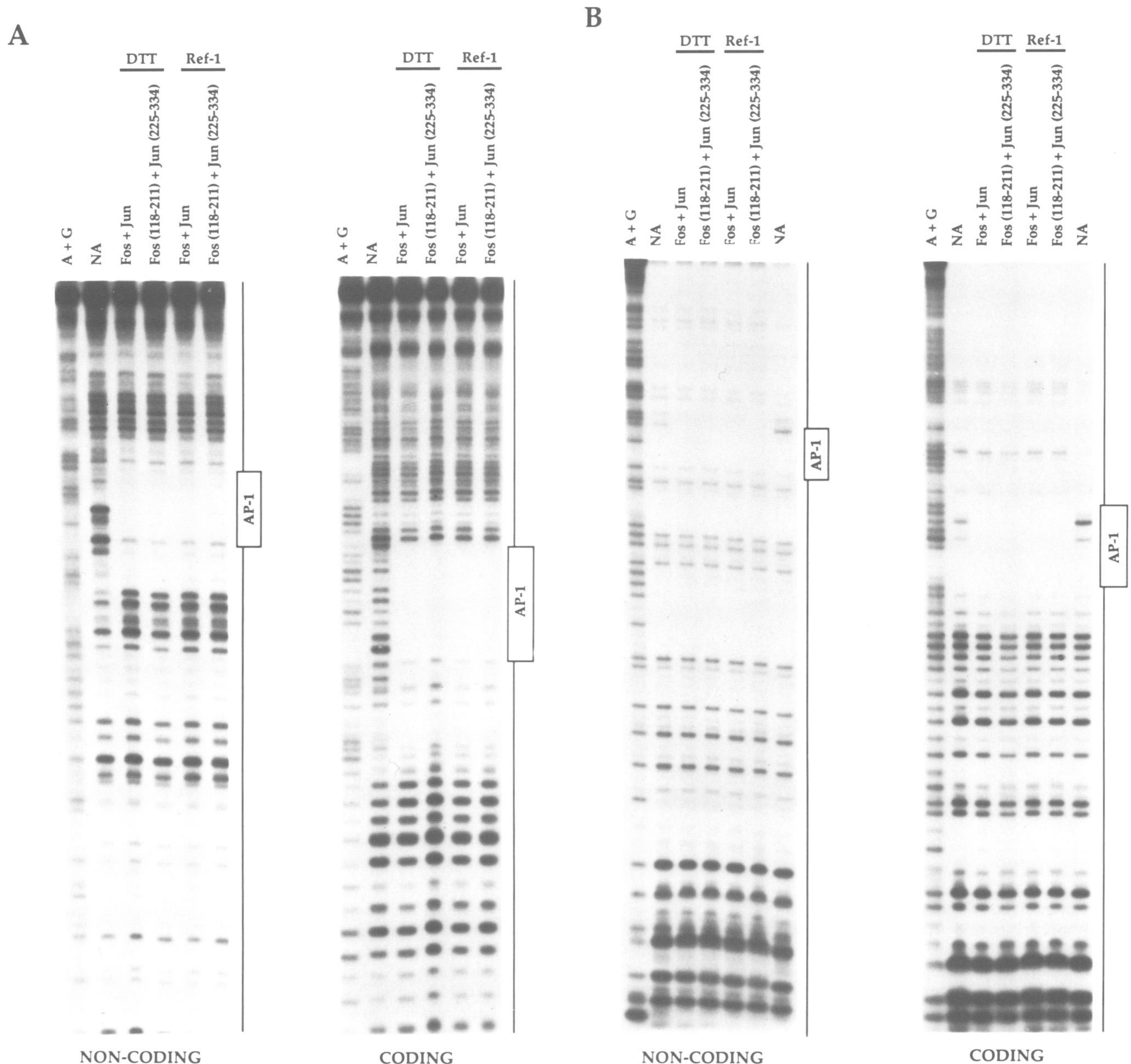



**Fig. 5.** Effect of Ref-1 on Fos-Jun interactions with DNA. (**A**) DNase I footprinting and (**B**) methylation interference analysis of Fos–Jun binding to DNA. Full-length and truncated Fos–Jun heterodimers were assayed for binding to a 154 bp [$^{32}$P]HMTII$_A$ promoter fragment (coding and non-coding strands) in the presence of 10 mM DTT or purified Ref-1 protein (5 $\mu$l of the pooled Superdex 75 fractions) as described in Materials and methods. The region of protection over the HMTII$_A$ AP-1 site is indicated by the boxed diagram. Lane A+G represents the adenine/guanine sequencing ladder. NA = no addition.

activity. This factor co-purifies with some of the components of HeLa cell AP-1 activity through several fractionation steps. Ref-1 does not alter the DNA footprinting pattern of Fos–Jun heterodimers or cause a supershift of the protein–DNA complex in gel-shift assays. Furthermore, UV cross-linking studies failed to detect qualitative changes in the pattern of AP-1 bound proteins in the presence or absence of crude nuclear extracts containing Ref-1 (Abate *et al.*, 1990b). These data suggest that Ref-1 is not a stable component of the Fos–Jun–DNA complex, but they do not exclude the possibility that there is a low affinity association in the absence of DNA.

We noted that the stimulatory activity of Ref-1 was diminished following extensive fractionation. However, Ref-1 activity could be regenerated by treatment with thioredoxin, suggesting that this factor may participate in a redox cycle by acting as an electron donor for AP-1 proteins. It is possible that the AP-1 redox signaling pathway comprises several components that are responsible for reducing Ref-1. Whether thioredoxin itself acts to regenerate active Ref-1 *in vivo* is not known, but it is clear that thioredoxin alone, as well as glutathione and glutathione transferase, failed to substitute for Ref-1 activity *in vitro* (S.Xanthoudakis, unpublished observations).

### Role of redox regulation in AP-1 signal transduction

A number of observations suggest that redox regulation of AP-1 DNA-binding activity may be important *in vivo*. Firstly, redox control of Fos–Jun DNA-binding activity is circumvented by mutation of the cysteine to serine in the KCR motif. This mutation is present in the transforming

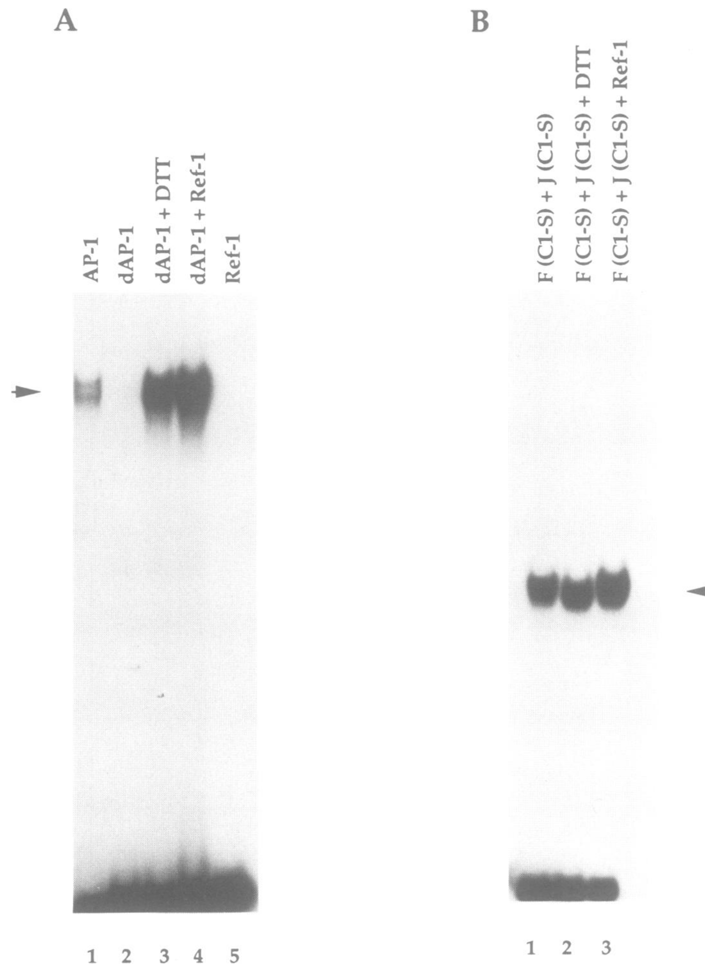



**Fig. 6.** Stimulation of endogenous AP-1 DNA-binding activity by Ref-1. (**A**) Partially purified HeLa cell AP-1 DNA-binding activity was assayed for binding to a $^{32}$P-labeled AP-1 oligonucleotide by gel retardation analysis after various treatments: no treatment, lane 1; dialysis against NDB buffer without DTT (dAP-1), lane 2; dAP-1 with the addition of 10 mM DTT, lane 3; dAP-1 with the addition of purified Ref-1 protein (5 $\mu$l of the pooled Superdex 75 fractions), lane 4. In lanes 3 and 4 DTT and Ref-1 were added during the DNA-binding reaction. In lane 5, Ref-1 was added alone in the absence of partially purified AP-1 DNA-binding activity. (**B**) Ref-1 acts via a conserved cysteine residue in the DNA-binding domain of Fos and Jun. Fos and Jun proteins containing a cysteine to serine substitution in the basic region (Abate *et al.*, 1990a) were assayed for AP-1 DNA binding activity upon the addition of NDB buffer without DTT, lane 1; NDB buffer with 10 mM DTT, lane 2; purified Ref-1 protein (5 $\mu$l of the pooled Superdex 75 fractions), lane 3.

v-Jun protein (Maki *et al.*, 1987) and results in Ref-1-independent AP-1 DNA-binding activity when it is introduced into c-*fos* and c-*jun* (Abate *et al.*, 1991a). Significantly, Fos and Jun proteins containing the Cys → Ser substitution display enhanced transforming potential when expressed *in vivo* (H.Iba, University of Tokyo and P.Vogt, USC Medical School, Los Angeles, personal communications), suggesting that the reactive Cys residue may be important in regulating the oncogenic potential of Fos and Jun.

In normal cells redox regulation may serve to selectively regulate the DNA-binding activity of subsets of AP-1 molecules in response to specific signals or during different stages of cell growth and differentiation. Activation/inactivation of selected AP-1 proteins could occur through co-localization of AP-1 dimers with nuclear regulatory redox factors such as Ref-1. For example, other non-histone redox enzymes have been shown specifically to co-localize with mRNA splicing factors in interchromatic regions of the cell nucleus (Bennett *et al.*, 1986), and histone as well as high mobility group (HMG) proteins containing exposed sulfhydryl groups are preferentially associated with transcriptionally active chromatin (Einck and Bustin, 1985; Chen and Allfrey, 1987; Walker *et al.*, 1990).

Ref-1-mediated reduction of Fos and Jun may also function to restore or maintain their DNA-binding activity following the oxidative burst that can accompany induction of immediate early gene expression. For example, potent stimulators of c-*fos* transcription, such as phorbol esters, create a pro-oxidant state within the cell and result in depletion of a number of enzymes and low molecular weight oxygen scavenging molecules that normally protect against oxidative stress (Cerutti, 1985). Similarly, PMA (phorbol 12-myristate 13-acetate) induces the formation of superoxide anions in various cell types (Christiansen, 1988; Gennaro *et al.*, 1986; Rosen and Freeman, 1984). Under these conditions, the activity of Fos would be dependent upon the ability of cellular factors, including Ref-1, to function in a homeostatic capacity and maintain the reduced active form of the protein.

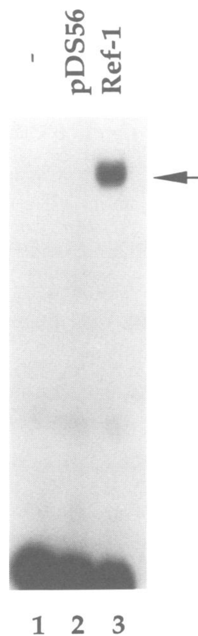



Fig. 7. Stimulation Fos–Jun DNA-binding activity by recombinant Ref-1. Fos118-211–Jun225-334 heterodimers were assayed for DNA-binding to a $^{32}$P-labeled AP-1 oligonucleotide following incubation with 0.2 $\mu$g of purified bacterial extract (Abate *et al.*, 1990c) prepared from untransformed *E.coli* cells (lane 1), *E.coli* cells transformed with a pDS56 expresion vector lacking a cDNA insert (lane 2), and *E.coli* cells transformed with a pDS56 expression vector containing a cDNA insert encoding Ref-1 (lane 3). DNA-binding to the AP-1 oligonucleotide was monitored using the gel retardation assay as described in Materials and methods. The position of the protein–DNA complex is indicated by an arrow.

### *Redox regulation, signal transduction and control of gene expression*

Several independent lines of evidence suggest that redox control may function as a general mechanism in signal transduction and gene regulation. Nitric oxide has been found to be a versatile second messenger in brain, acting to stimulate guanylyl cyclase activity (Crossin, 1991). ATL-derived factor (ADF), a thioredoxin-like protein with autocrine properties, is released by cells infected with the HTLV-I virus (Tagaya *et al.*, 1989; Wakasugi *et al.*, 1990). Moreover, thioredoxin can convert the glucocorticoid receptor to its steroid binding conformation (Grippo *et al.*, 1985) and it has been identified as a mediator of growth inhibitory signals in HeLa cells (Deiss and Kimichi, 1991).

In bacteria, the product of the *oxy*R gene activates transcription of a number of inducible genes in response to oxidative stress (Storz *et al.*, 1990). The ability of *oxy*R to activate target gene expression is dependent on its redox state and only the oxidized form of the protein is capable of stimulating transcription. Similarly, the iron-responsive element binding protein (IRE-BP), which co-ordinately regulates expression of ferritin and transferin receptor mRNA, is reversibly converted between low and high affinity binding forms through redox changes in the protein (Klausner and Harford, 1989; Hentze *et al.*, 1989). The DNA-binding activity of the glucocorticoid and progesterone receptors is dependent on their redox state and only the reduced form of these proteins interacts with DNA (Silva and Cidlowski, 1989; Peleg *et al.*, 1989). A requirement for reduced thiol groups has also been reported for the DNA-binding activity of TFIIIC (Cromlish and Roeder, 1989), ISGF3 (Levy *et al.*, 1989) and more recently NF-$\kappa$B (Toledano and Leonard, 1991). NF-$\kappa$B DNA-binding activity, like that of Fos and Jun, is sensitive to sulfhydryl modifying agents and is stimulated by thiol compounds. Indeed, preliminary experiments indicate that Ref-1 may stimulate the DNA-binding activity of several other transcription factors including NF-$\kappa$B (S.Xanthoudakis, unpublished observations). Reactive oyxgen intermediates have been implicated in activation of NF-$\kappa$B. In lymphoid cells, dissociation of NF-$\kappa$B from its cytosolic inhibitor protein, I$\kappa$B, is stimulated by agents that induce oxidative stress (Schreck *et al.*, 1991). Activation of NF-$\kappa$B by hydrogen peroxide, PMA or TNF is inhibited by thiol compounds that scavenge oxygen radicals, suggesting that reactive oxygen intermediates can act as common effector molecules in multiple signal transduction pathways (Staal *et al.*, 1990; Schreck *et al.*, 1991).

Finally, several studies have shown that expression of c-*fos* and c-*jun* is induced by oxidative stress (Crawford *et al.*, 1988; Shibanuma *et al.*, 1988; Devary *et al.*, 1991). Treatment of HeLa cells with hydrogen peroxide stimulates AP-1 DNA-binding activity, albeit modestly (Devary *et al.*, 1991). The relationship between oxidative stress, transcriptional induction of *fos* and *jun*, and Ref-1 stimulation of AP-1 DNA-binding activity is not clear at present. However, characterization of the gene encoding Ref-1 should provide insight into the redox control of gene expression. Analysis of the cloned gene *in vivo* will help elucidate the role of Ref-1 in the signal transduction pathways involving AP-1 and perhaps other transcription factors. The availability of purified recombinant Ref-1 will also permit a determination of the nature of the redox states of Fos and Jun active and inactive for DNA binding.

## Materials and methods

### *Preparation of recombinant proteins expressed in bacteria*

Fos, Fos118-211, F(C1-S), Jun, Jun225-334, J(C1-S) and Ref-1 were expressed as hexahistidine fusion proteins and purified by nickel-chelate chromatography as previously described (Abate *et al.*, 1990a,c). Protein preparations were extensively dialyzed against buffer A containing 50 mM sodium phosphate, pH 7.3, 1 mM EDTA, 1 mM DTT and 5% glycerol.

### *Nuclear extracts and DNA-binding assays*

Nuclear extracts were prepared from 60 l (152 g) of HeLa cells from suspension culture according to the procedure of Dignam *et al.* (1983) as modified by Briggs *et al.* (1986). Final extracts (10 mg/ml) were aliquoted, frozen on dry ice and stored in liquid nitrogen. DNA-binding activity was monitored by the gel retardation assay (Garner and Rezvin, 1981). DNA-binding assays were performed using a 25 bp $^{32}$P-labeled oligonucleotide containing the HMTII$_A$ AP-1 binding site (Abate *et al.*, 1990a). Fractionated nuclear extracts (1–3 $\mu$l) containing Ref-1 activity or recombinant Ref-1 from *E.coli* (0.2 $\mu$g) were preincubated with 1 ng of Fos and Jun at 37°C for 15 min in the presence of 0.1–0.2 mM DTT prior to the addition of 1 $\mu$g poly (dI–dC) and 0.2 ng of labeled oligonucleotide, as indicated in the figure legends. Protein–DNA complexes were resolved on 4.5% native Tris–glycine (pH 8.5) gels, dried and visualized by autoradiography. When used in DNA-binding reactions, the *E.coli* thioredoxin cocktail was added to a final concentration of 5.0 $\mu$M thioredoxin, 0.2 mM NADPH, pH 7.5 and 0.1 $\mu$M thioredoxin reductase (American Diagnostica).

DNase I footprinting and methylation interference assays were performed using a 154 bp fragment of the HMTII$_A$ gene containing a single AP-1 site as described (Abate *et al.*, 1990a). For methylation interference assays, the DNA was partially methylated using dimethylsulfate as described by the manufacturer (NEN). Protein–DNA complexes were resolved by electrophoresis and transferred on to DEAE (NA45) paper (Schleicher and

Schuell) overnight at 100 mA in 0.5 × TBE. DNA bands were identified by autoradiography and eluted from the DEAE paper in 1 M NaCl, 20 mM Tris, pH 8.0, 0.1 mM EDTA for 1 h at 55°C. The DNA was concentrated by ethanol precipitation, cleaved with piperidine and the DNA fragments were resolved on a 10% polyacrylamide−7 M urea sequencing gel.

### Purification of Ref-1

Protein purification was performed at 4°C and whenever possible samples were kept on ice. All chromatographic steps with the exception of DNA−cellulose fractionation were performed on a Waters 650 FPLC system. HeLa nuclear extract (450 mg) was loaded onto a 40 ml heparin−Sepharose column and washed with two column volumes of nuclear dialysis (NDB) buffer (50 mM Tris, pH 7.3, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 0.01% NP-40 and 10% glycerol). The bound protein was eluted with a 180 ml linear gradient of 0.1−1.0 M KCl in NDB buffer. In this and all subsequent chromatographic steps protein concentration was monitored by continuous UV absorption at 280 nm. The active Ref-1 fractions from the heparin−Sepharose column (as assayed by gel retardation analysis) were pooled, dialyzed against NDB buffer and loaded onto a 5 ml DNA−cellulose column equilibrated in the same buffer. The flow-through fraction containing most Ref-1 activity was directly applied to a 7.5 ml blue Sepharose column, washed with 5 column volumes of NDB buffer and eluted using a 53 ml linear gradient of 0.1−1.0 M KCl in NDB buffer. The fractions active for Ref-1 were dialyzed against NDB-P buffer (25 mM sodium phosphate, pH 7.3, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 0.01% NP-40 and 10% glycerol) and fractionated by two consecutive rounds of mono S chromatography. The pooled blue Sepharose fractions were applied to the mono S resin and the column was washed with 10 ml of NDB-P buffer. Ref-1 activity was eluted using either a 14 ml (first round) or 12 ml (second round) linear gradient of 0.05−1.0 M NaCl in NDB-P buffer. Fractions containing Ref-1 activity were pooled and dialyzed against NDB buffer before gel filtration chromatography on Superose 12 and Superdex 75 columns. The calibration standards used for gel filtration included: transferrin 81 kDa; bovine serum albumin, 68 kDa; ovalbumin, 45 kDa; chymotrypsinogen, 25 kDa; myoglobin, 17.6 kDa; lysozyme, 14.3 kDa; and ribonuclease A, 13.6 kDa. For the determination of Ref-1 specific activity, protein−DNA complexes were excised from the dried gel and the amount of radioactivity present in the complex was quantified by scintillation counting. Heparin−Sepharose fractions #47 and #48 were used as a source of partially purified endogenous AP-1 DNA-binding activity in Figure 6.

The protein composition of different column fractions was analyzed on 9% SDS polyacrylamide gels stained with Bio-Rad silver reagent or Coomassie Blue. The protein concentration of pooled fractions from individual columns was estimated using the method of Bradford (Bio-Rad).

### Immunoblot analysis

Affinity purified polyclonal rabbit antibodies directed against amino acids 127−152 of Fos have been described previously (Curran *et al.*, 1985). The Jun antibody is a whole rabbit antiserum raised against purified Jun produced in *E.coli* (Cohen and Curran, 1990). For immunoblot analysis, protein samples from different chromatography steps were resolved on a 9% SDS−polyacrylamide gel in duplicate; one half of the gel was stained with Bio-Rad silver reagent to visualize the total protein content and the proteins in the remaining half of the gel were electrotransferred (50 mA constant) onto a nitrocellulose filter (Schleicher and Schuell) for 18 h at 4°C. Following transfer, the filter was incubated for 3 h at room temperature in blocking buffer (10 mM imidazole, pH 7.0, 10 mM $MgCl_2$, 1.0 mM $CaCl_2$, 10 mM KCl, 5% BSA, 0.3% Tween-20, 0.02% sodium azide). Antibody incubation was carried out at room temperature for 2 h in blocking buffer containing anti-Fos antibodies. After hybridization, the filter was washed four times at room temperature (30 min intervals) with a 500 ml solution of phosphate buffered saline (PBS) containing 0.2% Triton X-100. Incubation with [$^{125}$I]protein A (20 μCi; 30 mCi/mg, Amersham) was carried out in 50 ml of PBS buffer containing 5% BSA for 1 h at room temperature. Unbound [$^{125}$I]protein A was rinsed from the filter at room temperature with four washes (30 min intervals) using a 250 ml solution of PBS containing 0.02% Triton X-100. The filter used for detection of Fos antigens was subsequently used to detect Jun antigens following preincubation in blocking buffer and incubation with anti-Jun antibodies as described above.

## Acknowledgements

We thank P.Familletti and D.Mueller of Hoffmann-La-Roche Inc. for providing HeLa cells and D.Nuss and members of the Curran Laboratory for suggestions and critical reading of the manuscript. S.X. is supported, in part, by a Medical Research Council of Canada Fellowship Award.

## References

Abate,C., Patel,L., Rauscher III,F.J. and Curran,T. (1990a) *Science*, **249**, 1157−1161.
Abate,C., Luk,D. and Curran,T. (1990b) *Cell Growth Differ.*, **1**, 455−462.
Abate,C., Luk,D,. Gentz,R., Rauscher III,F.J. and Curran,T. (1990c) *Proc. Natl. Acad. Sci. USA*, **87**, 1032−1036.
Angel,P., Imagawa,M., Chiu,R., Stein,B., Imbra,R.J., Rahmsdorf,H.J., Jonat,C., Herrlich,P. and Karin,M. (1987) *Cell*, **49**, 729−739.
Barber,J.R. and Verma,I.M. (1987) *Mol. Cell. Biol.*, **7**, 2201−2211.
Bartel,D.P., Sheng,M., Lau,L.F. and Greenberg,M. (1989) *Genes Dev.*, **3**, 304−313.
Benbrook,D.M. and Jones,N.C. (1990) *Oncogenes*, **5**, 295−302.
Bennett,C.F., Spector,D.L. and Yoeman,L.C. (1986) *J. Cell Biol.*, **102**, 600−609.
Boyle,W.J., Smeal,T., Defize,L.H.K., Angel,P., Woodgett,J.R., Karin,M. and Hunter,T. (1991) *Cell*, **64**, 573−584.
Briggs,M.R,, Kadanaga,J.T., Bell,S.D. and Tjian,R. (1986) *Science*, **234**, 47−52.
Cappel,R.E. and Gilbert,H.F. (1988) *J. Biol. Chem.*, **263**, 12204−12212.
Ceruti,P.A. (1985) *Science*, **227**, 375−381.
Chen,T.A. and Allfrey,V.G. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 5252−5256.
Chiu,R., Angel,P. and Karin,M. (1987) *Nature*, **329**, 648−651.
Chiu,R., Angel,P. and Karin,M. (1989) *Cell*, **59**, 979−986.
Christiansen,N.O. (1988) *FEBS Lett.*, **2**, 195−198.
Clancey,C.J. and Gilbert,H.F. (1987) *J. Biol. Chem*, **262**, 13545−13549.
Cohen,D.R. and Curran,T. (1988) *Mol. Cell. Biol*, **8**, 2063−2069.
Cohen,D.R. and Curran,T. (1990) *Oncogene*, **5**, 929−939.
Cohen,D.R., Ferreira,P.C.P., Gentz,R., Franza Jr,B.R. and Curran,T. (1989) *Genes Dev.*, **3**, 173−184.
Cooper,A.J.L. (1983) *Annu. Rev. Biochem.*, **52**, 187−222.
Crawford,D., Zbinden,I., Amstad,P. and Cerutti,P. (1988) *Oncogene*, **3**, 27−32.
Cromlish,J.A. and Roeder,R.G. (1989) *J. Biol. Chem.*, **264**, 18100−18109.
Crossin,K.L. (1991) *Trends Biochem. Sci.*, **16**, 81−82.
Curran,T. and Franza Jr,B.R. (1988) *Cell*, **55**, 395−397.
Curran,T. and Morgan,J.I. (1985) *Science*, **229**, 1265−1268.
Curran,T., Van Beveren,C., Ling,N. and Verma,I.M. (1985) *Mol. Cell. Biol.*, **5**, 167−172.
Deiss,L.P. and Kimichi,A. (1991) *Science*, **252**, 117−120.
Devary,Y., Gottlieb,R.A., Lau,L.F. and Karin,M. (1991) *Mol. Cell. Biol.*, **11**, 2804−2811.
Diamond,M.I., Miner,J.N., Yoshinago,S.K. and Yamamoto,K.R. (1990) *Science*, **249**, 1266−1272.
Dignam,J.D., Lebowitz,R.M. and Roeder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475−1489.
Einck,L. and Bustin,M. (1985) *Exp. Cell Res.*, **156**, 295−310.
Evans,R.M. and Hollenberg,S.M. (1988) *Cell*, **52**, 1−3.
Fearon,F.R., Cho,K.K., Negro,J.M., Kern,S.E., Simons,J.W., Ruppert,J.M., Hamilton,S.R., Presinger,A.C., Thomas,G., Kinzler,K.W. and Vogelstein,B. (1990) *Science*, **247**, 49−56.
Franza, Jr,B.R., Rauscher III,F.J., Josephs,S.F. and Curran,T. (1988) *Science*, **239**, 1150−1153.
Garner,M.M. and Revzin,A. (1981) *Nucleic Acids Res.*, **9**, 3047−3060.
Gaub,M.-P., Bellard,M., Scheuer,I., Chambon,P. and Sassone-Corsi,P. (1990) *Cell*, **43**, 1267−1276.
Gennaro,R., Florio,C. and Romeo,D. (1986) *Biochem. Biophys, Res. Commun.*, **134**, 305−312.
Gentz,R., Rauscher III,F.J., Abate,C. and Curran,T. (1989) *Science*, **243**, 1695−1699.
Grippo,J.F., Holmgren,A. and Pratt,W.B. (1985) *J. Biol. Chem.*, **260**, 93−97.
Gubler,U., Chua,A.O., Schoenhaut,D.S., Dwyer,C., McComas,W., Motyka,R., Nabavi,N., Wolitzky,A.G., Quinn,P.M., Familletti,P.C. and Gately,M.K. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 4143−4147.
Hai,T. and Curran,T. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 3720−3724.
Hentze,M.W., Rouault,T.A., Harford,J.B. and Klauser,R.D. (1989) *Science*, **244**, 357−359.
Hirai,S.-I., Ryseck,R.-P., Mechta,F., Bravo,R. and Yaniv,M. (1989) *EMBO J.*, **8**, 1433−1439.
Holmgren,A. (1985) *Annu. Rev. Biochem.*, **54**, 237−271.
Holmgren,A. (1989) *J. Biol. Chem.*, **264**, 13963−13966.
Jonat,C., Rahmsdorf,H.J., Park,K.-K., Cato,A.C.B., Gebel,S., Ponta,H. and Herrlich,P. (1990) *Cell*, **62**, 1189−1204.
Kerppola,T.K. and Curran,T. (1991a) *Curr. Opin. Struct. Biol.*, **1**, 71−79.
Kerppola,T.K. and Curran,T. (1991b) *Cell*, **66**, 317−326.
Klausner,R.D. and Harford,J.B. (1989) *Science*, **246**, 870−872.

Kouzarides,J.T. and Ziff,E. (1988) *Nature*, **366**, 646–651.
Landschulz,W.H., Johnson,P.F. and McKnight,S.L. (1988) *Science*, **240**, 1759–1766.
Levy,D.E., Kessler,D.S., Pine,R. and Darnell, Jr, J.E. (1989) *Genes Dev.*, **3**, 1362–1371.
Macgregor,P.F., Abate,C. and Curran,T. (1990) *Oncogene*, **5**, 451–458.
Maki,Y., Bos,T.J., Davis,C., Starbuck,M. and Vogt,P.K. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 2828–2852.
Matsudaira,P. (1987) *J. Biol. Chem.*, **262**, 10035–10038.
Matsui,M., Nomura,N. and Ishizaki,R. (1990) *Oncogene*, **5**, 249–255.
Meister,A. and Anderson,M.E. (1983) *Annu. Rev. Biochem.*, **52**, 711–760.
Morgan,J. and Curran,T. (1991) *Annu. Rev. Neurosci.*, **14**, 421–451.
Nakabeppu,Y. and Nathans,D. (1991) *Cell*, **64**, 751–759.
Nakabeppu,Y., Ryder,K. and Nathans,D. (1988) *Cell*, **55**, 907–915.
Nishina,H., Sato,H., Suzuki,T., Sato,N. and Iba,H. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 3619–3623.
O'Neil,K.T., Hoess,R.H. and DeGrado,W.F. (1990) *Science*, **249**, 774–778.
O'Shea,E.K., Rutkowski,R. and Kim,P.S. (1989) *Science*, **243**, 538–542.
Owen,T.A. *et al.* (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9990–9994.
Patel,L., Abate,C. and Curran,T. (1990) *Nature*, **347**, 572–575.
Peleg,S., Schrader,W.T. and O'Malley,B.W. (1989) *Biochemistry*, **28**, 7373–7379.
Rauscher III,F.J., Voulalas,P.J., Franza,Jr,B.R. and Curran,T. (1988) *Genes Dev.*, **2**, 1687–1699.
Reddy,E.P., Skalka,A.M. and Curran,T. (1988) *The Oncogene Handbook*. Elsevier, Amsterdam.
Rosen,G.M. and Freeman,B.A. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7269–7273.
Ruben,S.M., Dillon,P.,J., Schreck,R., Henkel,T., Chen.C.-H., Maher,M., Baeuerle,P.A. and Rosen,D.A. (1991) *Science*, **251**, 1490–1493.
Ryder,K., Lanahan,A., Perez-Albuerne,E. and Nathans,D. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 1500–1503.
Ryseck,R.-P. and Bravo,R. (1991) *Oncogene*, **6**, 533–542.
Schreck,R., Rieber,P. and Baeuerle,P.A. (1991) *EMBO J.*, **10**, 2247–2258.
Schuermann,M., Neuberg,M., Hunter,J.B., Jenuwein,T. Ryseck,R.-P., Bravo,R. and Muller,R. (1989) *Cell*, **56**, 507–516.
Schule,R., Umesono,K., Mangelsdorf,D.J., Bolado,J., Pike,J.W. and Evans,R.M. (1990a) *Cell*, **61**, 497–504.
Schule,R., Rangarajan,P., Kliewer,S., Ransone.L.J., Bolado,J., Yang,N., Verma,I.M. and Evans,R.M. (1990b) *Cell*, **62**, 1217–1226.
Schutte,J., Viallet,J., Nau,M., Segal,S., Fedorko,J. and Minna,J. (1989) *Cell*, **59**, 987–997.
Shibanuma,M., Kourki,T. and Nose,K. (1988) *Oncogene*, **3**, 17–21.
Silva,C.M. and Cidlowski,J.A. (1989) *J. Biol. Chem.*, **264**, 6638–6647.
Solomonson,L.P. (1975) *Plant Physiol.*, **56**, 853–855.
Staal,F.J.T., Roederer,M., Herzenberg,L.A. and Herzenberg,L.A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9943–9947.
Storz,G., Tartaglia,L.A. and Ames,B.N. (1990) *Science*, **248**, 189–194.
Struhl,K. (1989) *Trends Biochem. Sci.*, **14**, 137–140.
Tagaya,Y., Maeda,Y., Mitsui,A., Kondo,N., Matsui,H., Hamuro,J., Brown,N., Arai,K.-I., Yokota,T., Wakasugi,H. and Yodoi,J. (1989) *EMBO J.*, **8**, 757–764.
Toledano,M.B. and Leonard,W.J. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 4328–4332.
Turner,R. and Tjian,R. (1989) *Science*, **243**, 1689–1694.
Vinson,C.R., Sigler,P.B. and McKnight,S.L. (1989) *Science*, **246**, 911–916.
Wakasugi,N., Tagaya,Y., Wakasugi,H., Mitsui,A., Maeda,M., Yodoi,J. and Tursz,T. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 8282–8286.
Walker,J., Chen,T.A., Sterner,R., Berger,M., Winston,F. and Allfrey,V.G. (1990) *J. Biol. Chem.*, **265**, 5736–5746.
Walters,D.W. and Gilbert,H.F. (1986) *J. Biol. Chem.*, **261**, 13135–13143.
Welham,M.J., Wyke,J.A., Lang,A. and Wyke,A.W. (1990) *Oncogene*, **5**, 161–169.
Westbrook,C. and Jarabak,J. (1978) *Arch. Biochem. Biophys.*, **185**, 429–442.
Yang-Yen,H.F., Chambard,J.-C., Sun,Y.L., Smeal,T., Schmidt,T.J., Drouin,J. and Karin,M. (1990) *Cell*, **62**, 1205–1215.
Zerial,M., Toschi,L., Ryseck,R.-P., Schuermann,M., Muller,R. and Bravo,R. (1989) *EMBO J.*, **8**, 805–813.

*Received on September 19, 1991; revised on October 5, 1991*