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Redox activation of Fos-Jun DNA binding activity is mediated by a DNA repair enzyme

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The DNA binding activity of Fos and Jun is regulated *in vitro* by a post-translational mechanism involving reduction-oxidation. Redox regulation occurs through a conserved cysteine residue located in the DNA binding domain of Fos and Jun. Reduction of this residue by chemical reducing agents or by a ubiquitous nuclear redox factor (Ref-1) recently purified from Hela cells, stimulates AP-1 DNA binding activity *in vitro*, whereas oxidation or chemical modification of the cysteine has an inhibitory effect on DNA binding activity. Here we demonstrate that the protein product of the *ref*-1 gene stimulates the DNA binding activity of Fos-Jun heterodimers, Jun-Jun homodimers and Hela cell AP-1 proteins as well as that of several other transcription factors including NF-κB, Myb and members of the ATF/CREB family. Furthermore, immunodepletion analysis indicates that Ref-1 is the major AP-1 redox activity in Hela nuclear extracts. Interestingly, Ref-1 is a bifunctional protein; it also possesses an apurinic/apyrimidinic (AP) endonuclease DNA repair activity. However, the redox and DNA repair activities of Ref-1 can, in part, be distinguished biochemically. This study suggests a novel link between transcription factor regulation, oxidative signalling and DNA repair processes in higher eukaryotes.

Key words: AP endonuclease/Fos/Jun/reduction-oxidation/transcription factor

Introduction

Neoplasia results from a breakdown of the mechanisms responsible for normal growth and development. Insights into the molecular basis of cancer have been gained through the genetic and biochemical analyses of cellular proto-oncogenes, the progenitors of retroviral transforming genes. Proto-oncogenes appear to function in the transmission of inter- and intracellular information through several signal transduction pathways and have been divided into classes based on the functional activities of the protein products that they encode. These include growth factors, cell surface receptors, G-proteins and protein kinases (for reviews see Reddy *et al.*, 1988; Cantley *et al.*, 1991; Hunter, 1991). A subset of proto-oncogenes, exemplified by c-*fos* and c-*jun*, encode nuclear transcription factors that regulate gene expression (Curran and Franza, 1988; Abate and Curran, 1990). In many cell types, c-*fos* and c-*jun* are expressed at low basal levels, but they can be induced rapidly and transiently by a variety of extracellular stimuli. They are thought to function in coupling short-term signals elicited at the cell surface to long term changes in cellular phenotype by modulating the expression of specific target genes (for a review see Morgan and Curran, 1991). c-*fos* and c-*jun* are members of a multigene family that has been implicated in a number of signal transduction cascades associated with growth, differentiation, neuronal excitation and cellular stress (Holbrook and Fornace, 1991; Morgan and Curran, 1991). The proteins encoded by this family and members of the related ATF/CREB family form an array of heterodimeric complexes (Franza *et al.*, 1988; Nakabeppu *et al.*, 1988; Rauscher *et al.*, 1988; Chiu *et al.*, 1989; Cohen *et al.*, 1989; Hirai *et al.*, 1989; Ryder *et al.*, 1989; Schütte *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 and Nathans, 1991; Ryseck and Bravo, 1991) via a coiled-coil structure termed the leucine zipper (Kouzarides and Ziff, 1988; Landschulz *et al.*, 1988; Rauscher *et al.*, 1988; Gentz *et al.*, 1989; O'Shea *et al.*, 1989; Schuermann *et al.*, 1989; Turner and Tjian, 1989). These complexes bind to regulatory elements containing the activator protein-1 (AP-1) and the related cyclic AMP responsive (CRE) motifs (for a review see Kerppola and Curran, 1991). DNA binding is mediated by a highly conserved domain that is rich in basic amino acids, located adjacent to the leucine zipper. Fos does not form homodimers and consequently fails to bind DNA by itself. In contrast, Jun can bind to DNA as a homodimer, but does so with lower affinity than Fos-Jun heterodimers (Halazonetis *et al.*, 1988; Nakabeppu *et al.*, 1988; Rauscher *et al.*, 1988).

Induction of oncogenesis by c-*fos* and c-*jun* is primarily a consequence of deregulation of gene expression. Continuous expression of c-*fos* (Miller *et al.*, 1984; Lee *et al.*, 1988) or c-*jun* (Bos *et al.*, 1990; Curran and Vogt, 1991; Okuno *et al.*, 1991; Suzuki *et al.*, 1991) can result in transformation of fibroblasts in culture and the induction of tumors in animals (Rüther *et al.*, 1987, 1989; Wang *et al.*, 1991). This does not require overexpression of the oncogene because the endogenous counterparts can be induced, albeit transiently, to levels exceeding those in transformed cells. The critical feature is that expression of the oncogene, unlike that of the proto-oncogene, cannot be switched off.

Several mutations have occurred during the genesis of the three independent viral isolates of the *fos* (Van Beveren *et al.*, 1983, 1984; Nishizawa *et al.*, 1987) and *jun* (Maki *et al.*, 1987) oncogenes that significantly enhance their ability to induce transformation (Forrest and Curran, 1992). A

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common effect of some of these mutations is that they reduce post-translational modification of the encoded product. C-terminal truncations in v-*fos* have reduced the phosphorylation of its protein product (Curran *et al.*, 1984, 1985) and two point mutations in v-*jun* affect sites of phosphorylation (Boyle *et al.*, 1991) and redox regulation *in vitro* (Abate *et al.*, 1990c). Post-translational modification of the endogenous c-Fos and c-Jun proteins in response to extracellular stimuli has been implicated in the control of AP-1 function (Curran and Morgan, 1985; Barber and Verma, 1987; Müller *et al.*, 1987; Franza *et al.*, 1988; Boyle *et al.*, 1991; Pulverer *et al.*, 1991). Thus, the oncogenic forms, v-Fos and v-Jun may have enhanced transforming activity because they have escaped these regulatory constraints. The naturally occurring mutations in v-Fos and v-Jun have helped to pinpoint the sites of critical post-translational modification. Phosphorylation of the C-terminus of Fos has been invoked as a mechanism responsible for repression of SRE-mediated transcriptional activation (Guis *et al.*, 1990; Ofir *et al.*, 1990). In v-Jun, Ser243 has been mutated to phenylalanine (Maki *et al.*, 1987). Dephosphorylation of Ser243 in response to phorbol ester treatment of Hela cells has been suggested to enhance the DNA binding activity of Jun (Boyle *et al.*, 1991).

In addition to phosphorylation, an unusual post-translational modification involving reduction–oxidation (redox) also regulates the DNA binding activity of Fos and Jun *in vitro* (Abate *et al.*, 1990a,b,c; Xanthoudakis and Curran, 1992). Redox regulation of AP-1 DNA binding activity is mediated by a conserved cysteine residue, which lies in the DNA binding domain of the proteins. (Abate *et al.*, 1990c). This cysteine residue, which is mutated to a serine in v-Jun, is flanked by basic amino acids and is conserved in all of the Fos- and Jun-related proteins (Kerppola and Curran, 1991), including those identified in *Drosophila* (Perkins *et al.*, 1990) and in several of the ATF/CREB proteins. Substitution of Cys154 in Fos and Cys272 in Jun with a serine residue results in increased DNA binding activity and a loss of redox control (Abate *et al.*, 1990c). These cysteine → serine mutations enhance the transforming activity of both c-*fos* (H.Okuno, A.Akahori, H.Sato, T.Curran and H.Iba, in preparation) and c-*jun* (P.Vogt, personal communication). The exact mechanism responsible for redox control of DNA binding activity is not yet clearly understood. In the absence of high concentrations of reducing agents, the critical cysteine residue is converted to a state that is not permissive for DNA binding, but does not involve the formation of a disulfide bond (Abate *et al.*, 1990c). However, chemical reduction of the cysteine residue to its sulfhydryl state with DTT or 2-mercaptoethanol stimulates DNA binding activity dramatically. The cysteine residue is in close contact with DNA; treatment with *N*-ethylmaleimide, but not other classes of sulfhydryl modifying agents inhibits DNA binding activity, whereas the bound form of the protein is protected from modification (Abate *et al.*, 1990c; Patel *et al.*, 1990), Thus, the v-*jun* product may have evaded an oxidative regulatory mechanism.

Redox stimulation of Fos and Jun DNA binding activity can be catalysed by a ubiquitous protein that is present in mammalian nuclear extracts (Abate *et al.*, 1990a,b,c) To understand the redox control of transcription factor function further, we have characterized the nuclear activity, designated redox factor-1 (Ref-1). It has been purified to near homogeneity from HeLa cell nuclear extracts and shown to correspond to a 37 kDa protein (Xanthoudakis and Curran, 1992). Purified Ref-1 stimulates the DNA binding activity of recombinant Fos and Jun as well as that of endogenous AP-1 proteins purified from HeLa cells. It does not bind DNA in association with Fos and Jun, although it partially copurifies with some of the components of HeLa cell AP-1 activity. Interestingly, Ref-1 itself is subject to redox control and its activity can be augmented by thioredoxin, suggesting that a redox cascade could be involved in AP-1 regulation (Abate *et al.*, 1990c; Xanthoudakis and Curran, 1992).

Here we report the cloning and characterization of the human *ref*-1 cDNA. The protein product of *ref*-1, expressed and purified from *Escherichia coli*, stimulates the DNA binding activity of AP-1 proteins and as well as other transcription factors. Nucleotide sequence analysis has revealed that in addition to its redox activity, Ref-1 is identical to an apurinic/apyrimidinic (AP) endonuclease enzyme involved in DNA repair. The nuclease activity of Ref-1 is specific for DNA templates that are damaged by a variety of agents (e.g. hydrogen peroxide), some of which induce expression of c-*fos* and c-*jun*. Ref-1 may represent a novel component of the signal transduction processes that regulate eukaryotic gene expression in response to cellular stress.

Results

cDNA cloning and nucleotide sequence analysis of Ref-1

The cloning strategy used to isolate the Ref-1 cDNA is illustrated in Figure 1A. Briefly, N-terminal amino acid sequence analysis was performed on purified Ref-1 (70 pmol) that had been resolved by SDS–PAGE and transferred onto a polyvinylide difluoride (PVDF) membrane. The sequence of the first 20 amino acids was determined as shown in Figure 1A. A short DNA fragment was generated from HeLa cell poly(A)$^+$ RNA by mixed primer PCR amplification of the deduced 5′ coding region and verified by DNA sequencing. The DNA fragment was radiolabelled and used to screen a human cDNA library derived from Jurkat cells (Ruben *et al.*, 1991). Six positive cDNA clones containing *ref*-1 N-terminal coding sequences were isolated and the complete nucleotide sequence of the largest cDNA clone was determined (Figure 2). This cDNA spans 1441 nucleotides and contains a single open reading frame with a predicted coding potential for a protein of 318 amino acids. The calculated molecular mass of 35.5 kDa agrees well with the apparent molecular mass of cell-derived Ref-1 (37 kDa) estimated using gel filtration and SDS–PAGE. Analysis of the primary amino acid sequence indicates that Ref-1 is rich in basic amino acids (Arg/Lys, 14.5%; pI = 8.12) and has a highly hydrophilic character in the amino terminal portion of the protein. A consensus site homology search revealed no sequence motifs characteristic of other reductases. However, the protein contains a number of potential casein kinase II and protein kinase C phosphorylation sites, as well as putative targeting sequences for nuclear translocation (residues 2–7, 24–27, 31–35) (Roberts, 1989; Silver, 1991). Using the *ref*-1 cDNA as a probe, a single mRNA species of 1.6 kb was detected by Northern analysis of poly(A)$^+$ RNA prepared from HeLa cells (Figure 1B). An mRNA transcript of similar size was also detected in several other human and rodent cell lines

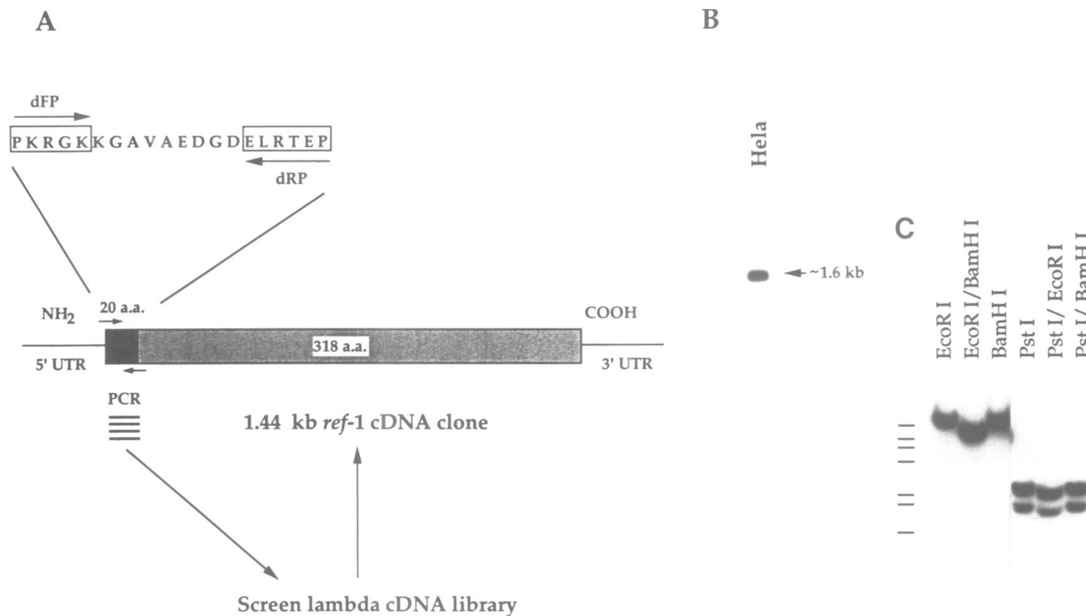

Fig. 1. cDNA cloning of *ref*-1. (**A**) The twenty amino acid region of the Ref-1 N-terminal sequence obtained by microsequence analysis is indicated. The boxed regions indicate residues used for designing the forward and reverse degenerate PCR primers (dFP/dRP). The stippled and shaded boxes represent the entire 318 amino acid *ref*-1 open reading frame. The 5′ and 3′ untranslated regions (UTRs) of the *ref*-1 cDNA are drawn as a solid line. (**B**) *Ref*-1 encodes a 1.6 kb mRNA. *Ref*-1 mRNA was examined by Northern blot analysis HeLa cell poly(A)$^+$ RNA (1.5 μg) was separated on an agarose–formaldehyde gel, transferred onto nitrocellulose and hybridized with the 1.44 kb ^{32}P-labelled *ref*-1 cDNA probe (clone 4.1). (**C**) Southern blot analysis of human genomic DNA. Genomic DNA (10 μg) isolated from HeLa cells was digested with *Eco*RI, *Bam*HI, *Pst*I and different combinations thereof; the resulting fragments were resolved by agarose gel electrophoresis and transferred to a nylon membrane. The filter was probed with the ^{32}P-labelled *ref*-1 cDNA (clone 4-1), washed and autoradiographed. The position of the DNA markers (kb) is indicated by the bars (top to bottom: 23, 9.4, 6.6, 4.4, 2.3, 2.0 and 1.35).

(data not shown). Thus, the cloned *ref*-1 cDNA encodes most of the mature mRNA sequence.

To determine the copy number of the genomic *ref*-1 gene and examine the presence of related sequences, Southern blot analysis was performed on genomic DNA isolated from HeLa cells (Figure 1C). *Eco*RI and *Bam*HI, neither of which cleave the ref-1 cDNA, generated DNA fragments >23 kb, whereas *Pst*I which cleaves the *ref*-1 cDNA once, generated two fragments of 3 kb and 2.2 kb. These data indicate that the human *ref*-1 gene is contained within a region of no more than 5.2 kb. Since hybridization at reduced stringencies failed to detect additional bands (data not shown) we conclude that human Ref-1 is encoded by a single gene.

Expression and purification of functional cloned Ref-1

Previously, we demonstrated that neither Ref-1 nor a related activity was present in *E.coli* cell extracts (Abate *et al.*, 1990a). Therefore to obtain a source of recombinant Ref-1 (rRef-1), the 318 amino acid open reading frame was subcloned into the pDS56 expression vector and over-produced in *E.coli* as a hexahistidine fusion protein. rRef-1 was purified to near homogeneity by nickel chelate chromatography from bacterial extracts and analysed by SDS–PAGE (Figure 3A). Taking into account the contribution of the fused histidine residues, the apparent molecular mass of rRef-1 produced in *E.coli* was consistent with the estimated molecular mass of Ref-1 isolated from HeLa nuclear extracts (37 kDa) (Xanthoudakis and Curran, 1992). In addition, *in vitro* transcription of the cloned *ref*-1 cDNA followed by translation in both reticulocyte and wheatgerm lysates yielded a polypeptide of the expected size (data not shown).

rRef-1 purified from *E.coli* was assayed for its ability to stimulate the DNA binding activity of purified recombinant Fos and Jun, and AP-1 proteins purified from HeLa cells (Figure 3B). In the presence of low concentrations of DTT (0.25 mM), rRef-1 stimulated DNA binding of Fos–Jun heterodimers and Jun–Jun homodimers, but not Fos alone, to the AP-1 oligonucleotide (Figure 3B, lanes 1–8). Similarly, rRef-1 augmented the DNA binding activity of AP-1 proteins purified from HeLa cell extracts that were first inactivated by removal of DTT through dialysis (Figure 3B, compare lanes 9–12). rRef-1 failed to interact with the AP-1 oligonucleotide in the absence of Fos and Jun (Figure 3B, lane 13). In this and other experiments, we noted that rRef-1 was consistently more effective than DTT at stimulating AP-1 DNA binding activity. rRef-1 enhanced the DNA binding activity of dialysed HeLa-derived AP-1 proteins to a level exceeding that of the non-dialysed AP-1 sample, suggesting that a fraction of AP-1 proteins, isolated from HeLa cells by standard procedures, exists in an inactive state.

We previously showed that oxidation of Ref-1 significantly diminishes its ability to stimulate AP-1 DNA binding activity (Abate *et al.*, 1990c; Xanthoudakis and Curran, 1992). However, thioredoxin can regenerate the stimulatory activity of oxidized Ref-1, suggesting that Ref-1 may participate in a redox cycle by acting as an electron donor for AP-1 proteins. To determine whether thioredoxin could affect the activity *E.coli*-derived Ref-1, an aliquot of rRef-1 protein was dialysed for 24 h against phosphate buffer in the absence of reducing agents. This procedure leads to oxidation and loss of rRef-1 activity (Figure 3C). Following this treatment rRef-1 had only a modest effect on the interaction of Fos and Jun with DNA (Figure 3C, lane 2). However, in the presence of thioredoxin, a significant fraction of rRef-1

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CGTTAGGAGGAGCTAGGCTGCCATCGGGCCGGTGCAGATACGGGGTTGCTCTTTTGCTCA  60
TAAGAGGGGCTTCGCTGGCAGTCTGAACGGCAAGCTTGAGTCAGGACCCTTAATTAAGAT  120
CCTCAATTGGCTGGAGGGCAGATCTCGCGAGTAGGGCAACGCGGTAAAAATATTGCTTCG  180

                                               M  P  K  R  G  K
GTGGGTGACGCGGTACAGCTGCCCAAGGGCGTTCGTAACGGGAATGCCGAAGCGTGGGAA  240

 K  G  A  V  A  E  D  G  D  E  L  R  T  E  P  E  A  K  K  S
AAAGGAGCGGTGGCGGAAGACGGGGATGAGCTCAGGACAGAGCCAGAGGCCAAGAAGAG  300

 K  T  A  A  K  K  N  D  K  E  A  A  G  E  G  P  A  L  Y  E
TAAGACGGCCGCAAAGAAAAATGACAAAGAGGCAGCAGGAGAGGGCCCAGCCCTGTATGA  360

 D  P  P  D  Q  K  T  S  P  S  G  K  P  A  T  L  K  I  C  S
GGACCCCCCAGATCAGAAAACCTCACCCAGTGGCAAACCTGCCACACTCAAGATCTGCTC  420

 W  N  V  D  G  L  R  A  W  I  K  K  K  G  L  D  W  V  K  E
TTGGAATGTGGATGGGCTTCGAGCCTGGATTAAGAAGAAAGGATTAGATTGGGTAAAGGA  480

 E  A  P  D  I  L  C  L  Q  E  T  K  C  S  E  N  K  L  P  A
AGAAGCCCCAGATATACTGTGCCTTCAAGAGACCAAATGTTCAGAGAACAAACTACCAGC  540

 E  L  Q  E  L  P  G  L  S  H  Q  Y  W  S  A  P  S  D  K  E
TGAACTTCAGGAGCTGCCTGGACTCTCTCATCAATACTGGTCAGCTCCTTCGGACAAGGA  600

 G  Y  S  G  V  G  L  L  S  R  Q  C  P  L  K  V  S  Y  G  I
AGGGTACAGTGGCGTGGGCCTGCTTTCCCGCCAGTGCCCACTCAAAGTTTCTTACGGCAT  660

 G  D  E  E  H  D  Q  E  G  R  V  I  V  A  E  F  D  S  F  V
AGGCGACGAGGAGCATGATCAGGAAGGCCGGGTGATTGTGGCTGAATTTGACTCGTTTGT  720

 L  V  T  A  Y  V  P  N  A  G  R  G  L  V  R  L  E  Y  R  Q
GCTGGTAACAGCATATGTACCTAATGCAGGCCGAGGTCTGGTACGACTGGAGTACCGGCA  780

 R  W  D  E  A  F  R  K  F  L  K  G  L  A  S  R  K  P  L  V
GCGCTGGGATGAAGCCTTTCGCAAGTTCCTGAAGGGCCTGGCTTCCCGAAAGCCCCTTGT  840

 L  C  G  D  L  N  V  A  H  E  E  I  D  L  R  N  P  K  G  N
GCTGTGTGGAGACCTCAATGTGGCACATGAAGAAATTGACCTTCGCAACCCCAAGGGGAA  900

 K  K  N  A  G  F  T  P  Q  E  R  Q  G  F  G  E  L  L  Q  A
CAAAAAGAATGCTGGCTTCACGCCACAAGAGCGCCAAGGCTTCGGGGAATTACTGCAGGC  960

 V  P  L  A  D  S  F  R  H  L  Y  P  N  T  P  Y  A  Y  T  F
TGTCCCACTGGCTGACAGCTTTAGGCACCTCTACCCCAACACACCCTATGCCTACACCTT  1020

 W  T  Y  M  M  N  A  R  S  K  N  V  G  W  R  L  D  Y  F  L
TTGGACTTATATGATGAATGCTCGATCCAAGAATGTTGGTTGGCGCCTTGATTACTTTTT  1080

 L  S  H  S  L  L  P  A  L  C  D  S  K  I  R  S  K  A  L  G
GTTGTCCCACTCTCTGTTACCTGCATTGTGTGACAGCAAGATCCGTTCCAAGGCCCTCGG  1140

 S  D  H  C  P  I  T  L  Y  L  A  L  *
CAGTGATCACTGTCCTATCACCCTATACCTAGCACTGTAACACCACCCCTAAATCACTTT  1200

GAGCCTGGGAAATAAGCCCCCTCAACTACCATTCCTTCTTTAAACACTCTTCAGAGAAAT  1260
CTGCATTCTATTTCTCATGTATAAAACTAGGAATCCTCCAACCAGGCTCCTGTGATAGAG  1320
TTCTTTTAAGCCCAAGATTTTTTATTTGAGGGTTTTTTGTTTTTTAAAAAAAAATTGAAC  1380
AAAGACTACTAATGACTTTGTTTGAATTATCCACATGAAAATAAAGAGCCATAGTTTCAA  1440
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Fig. 2. Nucleotide and amino acid sequences of human *ref*-1. The 1441 nucleotide human *ref*-1 cDNA sequence and the predicted protein coding region are illustrated. The partial N-terminal amino acid sequence obtained by microsequence analysis and the putative polyadenylation signal sequence located in the 3′ untranslated region are underlined.

stimulatory activity was restored (Figure 3C, lane 4), but thioredoxin alone was unable to enhance AP-1 DNA binding activity (Figure 3C, lane 3). Taken together, these data demonstrate that the product of the cloned Ref-1 gene is functionally and biochemically indistinguishable from cell-derived Ref-1.

Ref-1 stimulates DNA binding activity of several transcription factors

Redox regulation of Fos–Jun DNA binding activity is mediated through a conserved cysteine residue present in the DNA binding domain, which is flanked by basic amino acids (KCR) (Abate *et al.*, 1990c). This tri-amino acid motif is conserved among all of the described Fos- and Jun-related proteins and in several of the ATF/CREB bZIP proteins (Figure 4). A similar motif is present in the DNA binding domain of Myb (KQCR) and p65-NF-κB (KICR), both of which bind DNA in a redox-dependent manner (E.P.Reddy and P.Saikumar, personal communication; Toledano and Leonard, 1991). In Myb, the cysteine-containing motif is located within the basic domain of repeat 2, a region that shares homology with the DNA binding domain of Fos and Jun (Carr and Mott, 1991). To determine whether rRef-1 could stimulate the DNA binding activity of these and other transcription factors, gel retardation assays were performed using purified recombinant proteins (Figure 5). All reactions to which rRef-1 or control extract was added contained 0.25 mM DTT, contributed by the buffer in which the proteins were stored. Extract from bacterial cells, which contained a pDS56 expression vector lacking the Ref-1 cDNA insert, was purified in parallel with the rRef-1-containing extract and used as a negative control. Each panel of assays also included a positive control in which 10 mM DTT was added to the DNA binding reaction in the absence of rRef-1.

Purified bacterial extracts containing rRef-1 stimulated the DNA binding activity of Fos–Jun heterodimers and Jun–Jun homodimers, but not Fos alone (Figure 5). A stimulatory effect relative to the control extract (pDS56) was also observed with CREB, ATF-1, ATF-2, NF-κB and Myb. In contrast, the DNA binding activity of the glucocorticoid receptor peptide remained unchanged following the different treatments, indicating that redox effect was specific for a subset of transcription factors. In these experiments CREB interacted with the CRE oligonucleotide with higher affinity than ATF-1 or ATF-2. When CREB was assayed in the reaction mixture at final concentration of 0.3 μM the requirement for DTT or rRef-1 was reduced, although some stimulatory effect was still observed. At roughly the same concentration the DNA binding activity of ATF-1 and ATF-2 was highly dependent on the presence of a reducing source. On the other hand, rRef-1/DTT-dependent binding of CREB was clearly evident when CREB was diluted an additional 10-fold, suggesting that rRef-1 can enhance the reduced DNA binding activity of CREB observed at lower protein concentrations. Similar observations have been made previously with Fos and Jun (Abate *et al.*, 1990b). We noted that the effect of reducing agents was most obvious on high affinity Fos–Jun–DNA interactions that predominate at lower concentrations of the proteins. Overloading the binding assay with excess protein abrogated this effect by driving a low affinity protein–DNA interaction. Finally, rRef-1 was more effective than DTT at stimulating the DNA binding activity of the Fos, Jun and Myb proteins, whereas DTT was more efficient with the ATF and NF-κB proteins. This could reflect differences in the microenvironment of the cysteine residue, which may influence its accessibility to different reagents as well as its reactivity (pKa).

Ref-1 is a nuclear protein

To determine the subcellular location of Ref-1, Cos cells were transiently transfected with a Ref-1 expression vector and analysed by indirect immunofluorescence using antisera raised against a purified preparation of rRef-1. The specificity of the antibody was confirmed by its ability to immunoprecipitate Ref-1 synthesized in HeLa cells as well as programmed rabbit reticulocyte lysates (data not shown). As shown in Figure 6A, Ref-1 localized to the nucleus in transfected Cos cells. Confocal imaging analysis indicated that staining was stronger around the inside of the nuclear membrane (Figure 6B). A low level of background nuclear

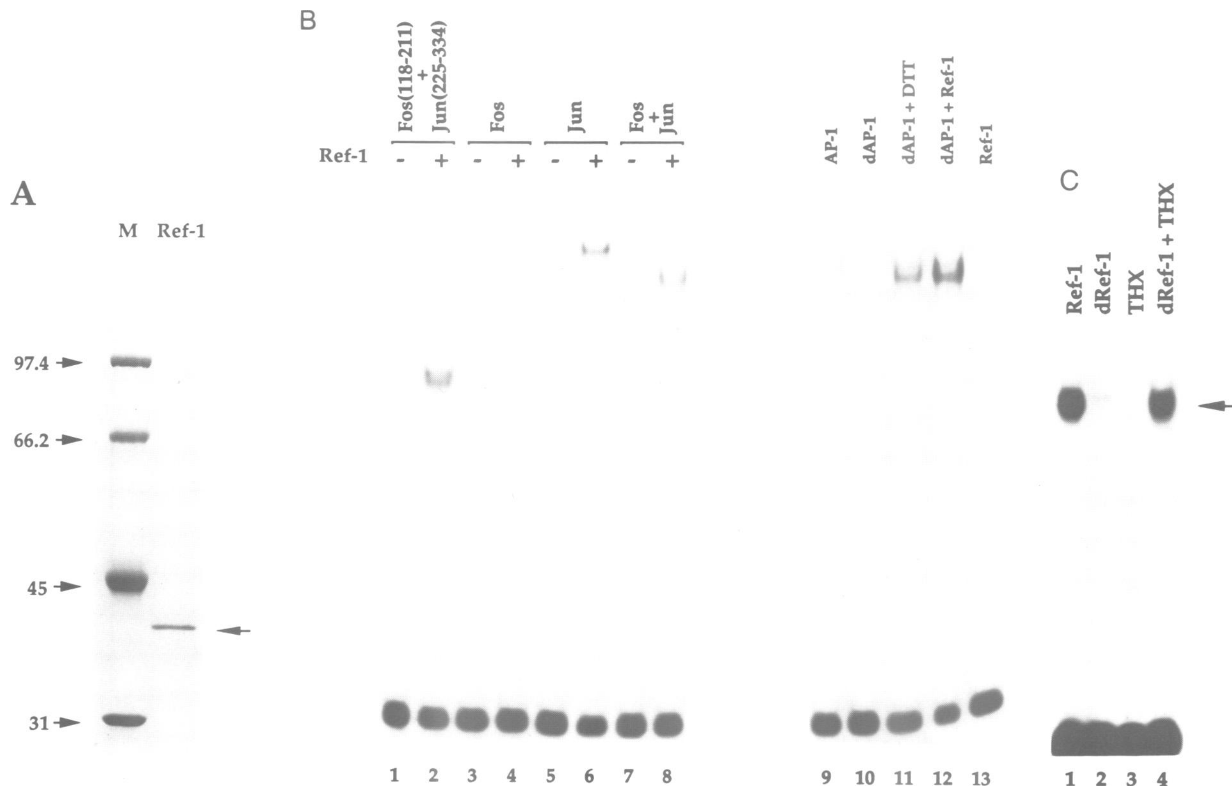

Fig. 3. Purification and functional analysis of recombinant Ref-1. (**A**) SDS–PAGE of purified Ref-1 expressed in *E.coli*. 1 μg of *E.coli*-produced Ref-1 was resolved on a 9% denaturing SDS–PAGE following purification by nickel chelate chromatography. Protein bands were visualized by Coomassie blue staining. (**B**) Stimulation of endogenous and exogenous AP-1 DNA binding activity by recombinant Ref-1 (rRef-1). rRef-1 (0.2 μg) was assayed for stimulation of AP-1 DNA binding activity using full-length and truncated Fos and Jun proteins (0.1 μM) or partially purified HeLa cell AP-1 activity (2 μg) (Xanthoudakis and Curran, 1992) as substrates. Fos118-211 + Jun225-334, lanes 1 and 2; Fos, lanes 3 and 4; Jun, lanes 5 and 6; Fos + Jun, lanes 7 and 8. Lanes 9–13 show rRef-1 activity on endogenous AP-1 proteins purified from HeLa cells. The DNA binding activity of HeLa cell AP-1 proteins was assayed after different treatments: no treatment, lane 1; dialysis against nuclear extract buffer without DTT (dAP-1), lane 2; dAP-1 with the addition of 10 mM DTT; dAP-1 with the addition rRef-1 protein (0.2 μg). In lanes 3 and 4, DTT and rRef-1 were added during the DNA binding reaction. In lane 5, rRef-1 was assayed alone in the absence of HeLa cell AP-1 proteins. DNA binding to the 25 bp ^{32}P-labelled AP-1 oligonucleotide was monitored by the gel retardation assay as described in Materials and methods. (–) addition of phosphate buffer without rRef-1; (+) addition of rRef-1. (**C**) Activation of Ref-1 redox activity by thioredoxin. rRef-1 protein (0.2 μg) was dialysed (dRef-1) for 24 h at 4°C against 50 mM sodium phosphate buffer, pH 7.3 and assayed for stimulation of Fos–Jun DNA binding activity as outlined above. Untreated (undialysed) rRef-1, lane 1; dialysed rRef-1, lanes 2 and 4. A mixture of bacterial thioredoxin/NADPH/thioredoxin reductase was added to the DNA binding assays in lanes 3 and 4 (see Materials and methods).

staining was also detected in non-transfected cells with the Ref-1 antibody. A similar background level of staining was observed in cells transfected with the vector sequences alone (data not shown). This probably reflected the presence of endogenous Ref-1 or a related antigen. Nuclear staining was not detected in cells treated with preimmune sera or with Ref-1 antibodies that had been pre-absorbed with an excess of the rRef-1 protein (data not shown). Thus, Ref-1 appears to be a nuclear protein, which is consistent with the presence of nuclear localization sequences in its N-terminal domain and with earlier studies indicating that the majority of the cellular redox activity was present in nuclear extracts (Abate *et al.*, 1990a).

Ref-1 is the major AP-1 redox activity in HeLa nuclear extracts

The ability of the Ref-1 antisera to recognize Ref-1 suggested that the antibody could be used to immunodeplete Ref-1 from HeLa nuclear extracts. A highly purified preparation of Ref-1 antibody was obtained by rRef-1 affinity chromatography on a rRef-1–agarose column and examined for its ability to inhibit Ref-1-mediated stimulation of AP-1 DNA binding activity. Increasing amounts of affinity purified Ref-1 antibody were pre-incubated with rRef-1 or HeLa nuclear extract and immune complexes were cleared by immunoprecipitation under non-denaturing conditions. The depleted extracts were assayed for Ref-1 activity using purified recombinant Fos and Jun proteins as substrates (Figure 6C). Incubation of the Ref-1 antibody with either rRef-1 (Figure 6C, lanes 2–4) and or HeLa nuclear extract (Figure 6C, lanes 6–8) resulted in a dose-dependent inhibition of AP-1 DNA binding activity. However, DTT-mediated stimulation of DNA binding activity was not affected by the addition of anti-Ref-1 antibody, suggesting that the observed inhibition was not due to a direct effect of the antibody on Fos and Jun (Figure 6C, lanes 9 and 10). At the highest Ref-1 antibody concentration examined, >75% inhibition was observed relative to the control samples to which an equivalent amount of purified total rabbit immunoglobulin was added (Figure 6C, compare lanes 1 and

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Fos     RRERNKMAAAKCRNRRRELT
FosB    RRERNKLAAAKCRNRRRELT
Fra1    RRERNKLAAAKCRNRRKELT
Fra2    RRERNKLAAAKCRNRRRELT

Jun     KRMRNRIAASKCRKRKLERI
JunB    KRLRNRLAATKCRKRKLERI
JunD    KRLRNRIAASKCRKRKLERI

CREB    RLMKNREAARECRRKKKEYV
ATF1    RLMKNRE-ARECRRKKKEYV
ATF2    FLERNRAAASRCRQKRKVWV
ATF3    RRERNKIAAAKCRNKKKEKT
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Fig. 4. Sequence alignment of the basic motif in several leucine zipper-containing transcription factors. Amino acid sequences corresponding to the basic region in the DNA binding domain of different Fos, Jun and ATF/CREB family members are aligned. The position of the conserved cysteine residue and flanking amino acids are indicated by the box.

4, 5 and 8). The magnitude of this inhibition demonstrates that Ref-1 is the major AP-1 redox activity in HeLa nuclear extracts.

Ref-1 is a DNA repair enzyme

A search of the Genbank and NBRF data bases revealed a significant degree of sequence similarity between Ref-1 and several DNA repair enzymes known to specifically cleave apurinic/apyrimidinic (AP) sites in DNA (Figure 7). Ref-1 is apparently identical to a recently cloned human AP endonuclease activity, designated HAP1 (human AP endonuclease 1) (Robson and Hickson, 1991) and APE (AP endonuclease) (Demple *et al.*, 1991). In addition to a class II AP endonuclease activity, the bovine homolog of HAP1/Ref-1 is also capable of removing blocking groups from the 3′ terminus of DNA strand breaks induced by oxidative damage (Robson *et al.*, 1991). Ref-1 also shares a striking degree of sequence similarity with *Drosophila* Rrp1 AP endonuclease (Sander *et al.*, 1991) and also two bacterial DNA repair enzymes, exonuclease A (exoA) of *Streptococcus pneumoniae* (Puyet *et al.*, 1989) and *exo*III of *E.coli* (Saporito *et al.*, 1988). The latter enzyme constitutes the majority of class II AP endonuclease activity in *E.coli*. A sequence comparison of the different Ref-1-related proteins is shown in Figure 7. Ref-1 is identical to HAP1 with the exception of a single amino acid difference at position 237 [Ref-1 (Arg237) versus HAP1 (Ala237)]. An arginine residue is present at position 237 in APE, Rrp1, exoA and exoIII. The homology to Rrp1 is restricted to the C-terminal portion of the *Drosophila* protein. Ref-1 shares 50% identity and 68% similarity with Rrp1 across this region. Excluding the N-terminal 62 amino acids of Ref-1, which encompass the putative nuclear translocation sequences, the similarity with exoA and exoIII extends throughout the entire Ref-1 coding region. Ref-1 shares 40% and 28% identity with exoA and exoIII, respectively. The amino acid sequence similarity shared among these three proteins is ~59% across the conserved regions. Thus, Ref-1 is identical to mammalian enzymes involved in DNA repair processes and is distantly related to bacterial exonucleases.

Comparison of the Ref-1 redox and AP endonuclease activities

To examine the AP endonuclease activity of rRef-1, bacterial plasmid DNA was acid-treated to generate an appropriate apurinic DNA substrate. Heat treatment of supercoiled plasmid DNA at low pH (as described in Materials and methods) introduces apurinic sites (Kane and Linn, 1981). AP endonuclease activity can then be assayed by monitoring enzyme-mediated conversion of supercoiled acid-depurinated DNA to open circular forms on agarose gels. The acid-depurinated plasmid preparation used in these experiments contained a mixture of supercoiled (SC) and open circular (OC) DNA (Figure 8A). Incubation of AP plasmid DNA with 0.1 μg of rRef-1 resulted in complete conversion of the supercoiled DNA to open circles (Figure 8A, lane 5). In contrast, rRef-1 failed to cleave untreated plasmid DNA (Figure 8A, lane 7). AP endonuclease activity was not detected using control proteins (Figure 8A, lane 2), purified extracts prepared from untransformed bacterial cells (Figure 8A, lane 3) or purified extracts from cells transformed with

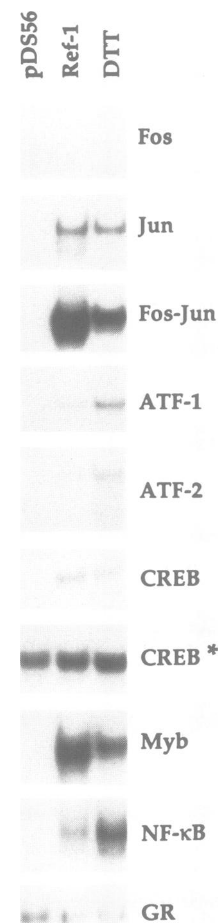

Fig. 5. Ref-1 stimulates the DNA binding activity of different transcription factors. Recombinant proteins: Fos (0.1 μM), Jun (0.1 μM), Fos−Jun (0.1 μM), ATF-1 (0.25 μM), ATF-2 (0.25 μM), CREB (0.3 μM), CREB* (0.03 μM), T-Myb (0.03 μM), NF-κB (p65) (2.2 μM) and the glucocorticoid receptor (GR) (0.73 μM) were assayed for DNA binding activity following incubation with purified bacterial extract from pDS56-transformed M15 cells: 0.2 μg, lane 1; rRef-1 (0.2 μg), lane 2; or 10 mM DTT, lane 3. Carrier BSA protein was included in the reactions to help stabilize the recombinant proteins and normalize for differences in protein concentration. DNA binding activities were analysed by the gel retardation assay using ^{32}P-labelled oligonucleotide probes (AP-1, CRE, NF-κB, Myb, GRE) containing the appropriate recognition sequence. The amino acid end-points for each protein are specified in Materials and methods.

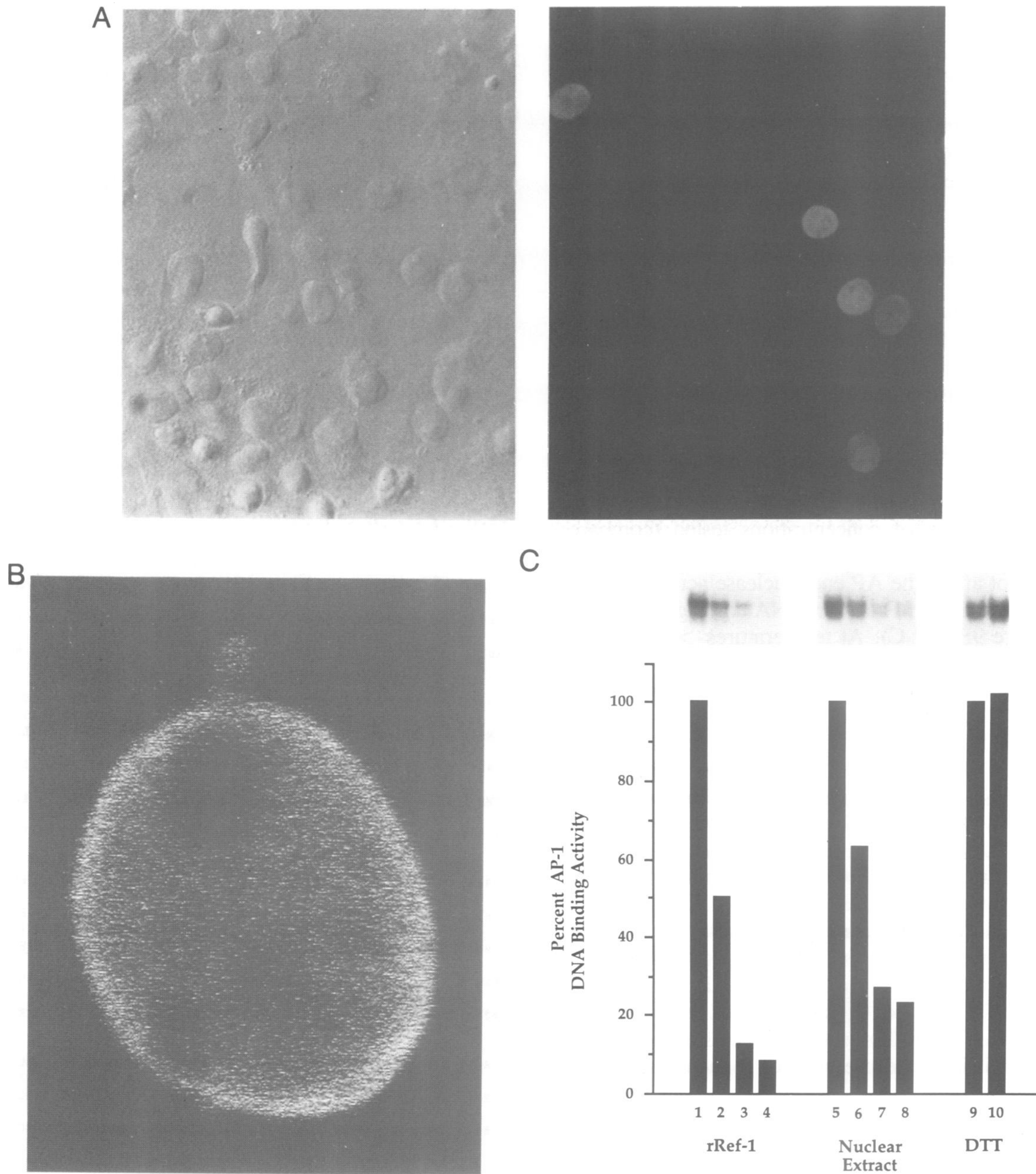

Fig. 6. Immunological analysis of Ref-1: nuclear localization and immunodepletion from the HeLa nuclear extracts. (A) Cos cells were transfected with 5 μg of a mammalian Ref-1 expression vector (BL-soCMVINPA-Ref1). 48 h after transfection, the cells were fixed and analysed by indirect immunofluorescence using as 1:1000 dilution of polyclonal Ref-1 antisera raised against *E.coli*-purified Ref-1 protein. The secondary antibody was a fluorescein-conjugated goat anti-rabbit IgG. Stained cells from a representative field were examined by phase contrast (left panel) and fluorescence (right panel) microscopy (63 × magnification). (B) Confocal imaging of fluorescence in the nucleus of a transfected Cos cell expressing Ref-1. The image displayed is an optical cross-section through the middle of a single nucleus (63 × magnification; 4.0 × zoom); (C) Inhibition of AP-1 redox activity by immunodepletion of Ref-1 from HeLa nuclear extracts. rRef-1 (0.1 μg) (lanes 1–4), HeLa nuclear extract (4 μg) (lanes 5–8) and bovine serum albumin (5 μg) (lanes 9 and 10) were incubated for 20 min at room temperature with 0.75 μg of protein A-purified total rabbit immunoglobulin (lanes 1, 4 and 9) or with 0.15 μg (lanes 2 and 6). 0.45 μg (lanes 3 and 7) and 0.75 μg (lanes 4, 8 and 10) of affinity-purified polyclonal Ref-1 antibody. BSA was added to each sample to bring the total amount of protein in the assay to 5 μg. Immune complexes were cleared by immunoprecipitation using 15 ml of a 10% fixed *Staphylococcus aureus* cell suspension (Pansorbin cells, Calibiochem) prepared in gel-shift binding buffer containing 25 μg/ml BSA (Xanthoudakis and Curran, 1992). The cleared supernatant was assayed for AP-1 redox activity by gel retardation analysis using 0.1 μM of Fos133-207 and Jun252-326 (as described in Materials and methods). In lanes 9 and 10, DTT was added to each sample at a final concentration of 10 mM. Protein–DNA complex formation was quantitated by excising the radiolabelled band from the gel and measuring radioactivity by scintillation counting.

he pDS56 expression vector lacking the *ref*-1 cDNA insert Figure 8A, lane 4).

The lack of Ref-1 activity on untreated DNA indicates that ts ability to stimulate Fos–Jun DNA binding activity is not elated to an effect on the AP-1 oligonucleotide. Indeed, *n vitro* analyses of Fos–Jun interactions with the AP-1 site indicate that the stimulatory effect of Ref-1 is not a consequence of modifications introduced into the DNA (Xanthoudakis and Curran, 1992 and S.Xanthoudakis, unpublished data). Moreover, given the nucleotide specificity of the protein–DNA interaction, it is highly unlikely that abasic positions in the AP-1 recognition sequence, which

might be recognized by Ref-1, would be tolerated (Gartenberg *et al.*, 1990).

In light of its sequence similarity to bacterial endo exonucleases, rRef-1 was tested for exonuclease activity on single and double stranded end-labelled DNA substrates. In contrast to ExoIII, rRef-1 exhibited no detectable exonuclease activity against either substrate at concentrations as high as 50 μg/ml (data not shown). Similarly, rRef-1 lacked the strand-transferase activity reported to be associated with the *Drosophila* Rrp1 protein (data not shown) (Sander *et al.*, 1991).

To compare the two functional properties of Ref-1, redox activation of DNA binding activity and AP endonuclease activity, we examined these activities at different temperatures and also tested the DTT requirement of the nuclease activity (Figure 8). In contrast to the AP-1 redox activity, which is inactivated following dialysis against buffers that do not contain DTT, the AP endonuclease activity of rRef-1 remained active in the absence of DTT across a broad range of concentrations tested (compare Figures 3C and 9A). Furthermore, pretreatment of rRef-1 at 45°C did not affect the AP endonuclease activity, but it reduced the activation of Fos−Jun DNA binding activity by ~30% (Figure 9B and C). At temperatures >45°C both activities were inactivated, although some residual AP-1 stimulatory activity was still observed at even higher temperatures. The temperature optima for these activities was determined by carrying out the reactions between 0 and 37°C (Figures 9B and C). To ensure the appropriate formation of Fos−Jun heterodimers, Fos and Jun were pre-associated at 37°C prior to incubation with rRef-1. AP endonuclease activity remained unchanged across this temperature range, whereas Fos−Jun DNA binding activity was reduced at temperatures <37°C. Although these activities are difficult to compare because of inherent differences in the assays, our results suggest that the biochemical requirements for the redox and AP endonuclease activities of Ref-1 may be, in part, distinct. In particular, dialysed (oxidized) rRef-1 is just as active as a nuclease in the absence as it is in the presence of reducing agents.

Discussion

Relationships among oxidative stress, DNA repair, redox and AP-1 activity

Cellular organisms have evolved a number of adaptive responses to cope with environmental stress. These responses are mediated by a set of inducible genes whose products confer protection against cellular damage. In bacteria, DNA damage caused by mutagenic agents leads to the rapid induction of specific regulons, each controlling multiple genes. The type of stress inflicted usually determines the spectrum of genes that are induced. For instance, activation of the SOS regulon occurs in response to UV irradiation and causes a number of genetic and phenotypic alterations that enhance the capacity for DNA repair (Walker, 1985). In addition, the OxyR and SoxRS regulons are induced in response to oxidative stress. These loci encode transcription factors that respond to changes in the oxidative state of the cell (Demple and Amabile-Cuevas, 1991). Generally, a cellular pro-oxidant state is characterized by the accumulation of active oxygen in the form of superoxides and other reactive oxygen intermediates (Cerutti, 1985; Halliwell and Gutteridge, 1990). This can occur indirectly; for example, ionizing radiation causes ubiquitous oxidative damage to DNA and other cellular macromolecules and DNA damaging drugs such as bleomycin, paraquat and peroxides, generate

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Ref1     1 MPKRGKKGAV AEDGDELRTE PEAKKSKTAA KKNDKEAAGE GPALYEDPPD 50
HAP1     1 MPKRGKKGAV AEDGDELRTE PEAKKSKTAA KKNDKEAAGE GPALYEDPPD 50
RRP1   346 KPAKGAKKAP VKAEDVEDIE EAAEESKPAA GAKKAAAKAE EPDVDEESGS 395
EXO A      .......... .......... .......... .......... ..........
EXOIII     .......... .......... .......... .......... ..........

Ref1    51 QKTSPSGKPA T......... .......... .LKICSWNVD GL........ 72
HAP1    51 QKTSPSGKPA T......... .......... .LKICSWNVD GL........ 72
RRP1   396 KTTKKAKKAE TKTTVTLDKD AFALPADKEF NLKICSWNVA GL........ 437
EXO A    1 .......... .......... .......... .MKLISWNID SLNAALTSDS 20
EXOIII   1 .......... .......... .......... .MKFVSFNIN GL........ 12

Ref1    73 .AAWIKKKGL DWVKEEAPDI LCLQET.... KCSENKLP.A ELQEL.PGLS 115
HAP1    73 .AAWIKKKGL DWVKEEAPDI LCLQET.... KCSENKLP.A ELQEL.PGLS 115
RRP1   438 .AAWLKKDGL QLIDLEEPDI FCLQET.... KCANDQLP.E EVTRL.PGYH 480
EXO A   21 AAAKLSQEVL QTLVAENADI IAIQETKLSA KGPTKKHV.E ILEELFPGYE 69
EXOIII  13 .AAA.PHQLE AIVEKHQPDV IGLQET.... KVHDDMFPLE EVAKL..GYN 54

Ref1   116 HQYWSAP.SD KEGYSGVGLL .SRQCPLKVS YGI..GDEEH DQEGRVIVAE 161
HAP1   116 HQYWSAP.SD KEGYSGVGLL .SRQCPLKVS YGI..GDEEH DQEGRVIVAE 161
RRP1   481 P.YWLCMP.G ..GYAGVAIY .SKIMPIHVE YGI..GNEEF DDVGRMITAE 523
EXO A   70 NTWRSSQEPA AKGYAGTMFL YKKELTPTIS FPEIGAPSTM DLEGRIITLE 119
EXOIII  55 VFYH.....G QKGHYGVALL .TKETPIAVR RGFPGDDEEA QA..RIIMAE 96

Ref1   162 FDSFV....L VTAYVPNAG. .AGLVRLEYR QRWDEAFRKF LK.GLASRKP 204
HAP1   162 FDSFV....L VTAYVPNAG. .AGLVRLEYR QRWDEAFRKF LK.GLASRKP 204
RRP1   524 YEKFY....L INVYVPNSG. .AKLVNLEPR MAWEKLFQAY VK.KLDALKP 566
EXO A  120 FDAFF....V TQVYTPNAG. .DGLKRLEER QVWDAKYAEY LA.ELDKEKP 162
EXOIII  97 IPSLLGNVTV INGYFPQGES RDHPIKFPAK AQFYQNLQNY LETELKRDNP 146

Ref1   205 LVLCGDLNVA HEEIDLRNPK GNKK...... NAGFTPQERQ GFGELLQAVP 248
HAP1   205 LVLCGDLNVA HEEIDLRNPK GNKK...... NAGFTPQERQ GFGELLQAVP 248
RRP1   567 VVICGDMNVS HMPIDLENPK NNTK...... NAGFTQEERD KMTELL.GLG 609
EXO A  163 VLATGDYNVA HNEIDLANPA SNRR...... SPGFTDEERA GFTNLL.ATG 205
EXOIII 147 VLIMGDMNIS PTDLDIGIGE ENRKAWLATG KCSFLPEERE WMDRLM.SWG 195

Ref1   249 LADSFRHLYP NTPYAYTFWT YM.MNARSKN VGWRLDYFLL SHSL....LP 293
HAP1   249 LADSFRHLYP NTPYAYTFWT YM.MNARSKN VGWRLDYFLL SHSL....LP 293
RRP1   610 FVDTFRHLYP DRKGAYTFWT YM.ANARARN VGWRLDYCLV SERF....VP 654
EXO A  206 FTDTFRHVHG DVPERYTWWA QRSKTSKINN TGWRIDYWLT SNRI....AD 251
EXOIII 196 LVDTFRHANP QTADRFSWFD YRSK.GFDDN RGLRIDLLLA SQPLAECCVE 244

Ref1   294 ALCDSKIRSK ALGSDHCPIT LYLAL 318
HAP1   294 ALCDSKIRSK ALGSDHCPIT LYLAL 318
RRP1   655 KVVEHEIRSQ CLGSDHCPIT IFFNI 679
EXO A  252 KVTKSDMIDS GARQDHTPIV LEIDL 276
EXOIII 245 TGIDYEIRSM EKPSDHAPVW ATFAR 269
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Fig. 7. Sequence comparison between Ref-1 and DNA repair enzymes in *Drosophila* and bacteria. An amino acid alignment of Ref-1, HAP1, *Drosophila* Rrp1, *E.coli* exonuclease III (ExoIII) and *S.pneumoniae* exonuclease A (Exo A) is shown. The bars indicate amino acid positions that are conserved relative to the Ref-1 coding sequence. Sequence alignments were generated using the Genetics Computer Group (GCG) Bestfit and Pileup protein analysis software packages.

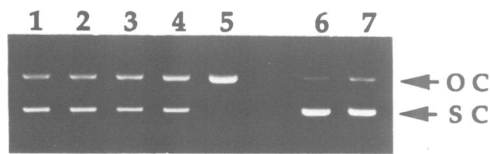

Fig. 8. Ref-1 exhibits AP endonuclease activity. Acid-depurinated plasmid DNA (0.5 μg) was incubated for 15 min at 37°C alone (lane 1) or with 0.1 μg of either BSA (lane 2), purified bacterial extract from M15 cells (lane 3), purified bacterial extract from pDS56-transformed M15 cells (lane 4) and rRef-1 (lane 5). Untreated plasmid DNA was incubated alone for 15 min at 37°C (lane 6) or with 0.1 μg of rRef-1 (lane 7). Following incubation the reactions were chilled on ice, the DNA products were then resolved on a 1% agarose gel containing ethidium bromide and subsequently visualized under a UV trans-illuminator. Supercoiled DNA (SC), open circular DNA (OC).

apurinic (AP) sites in DNA via the production of reactive oxygen species (Cerutti, 1985). It is interesting to note that activation of the SoxR regulon results in induction of endonuclease IV (Greenberg *et al.*, 1990). This enzyme contributes to the AP endonuclease activity present in *E.coli* although it has little sequence similarity to Ref-1 (Cunningham *et al.*, 1986).

The situation in mammalian cells is less well characterized. DNA-damaging agents such as oxidative stress and UV irradiation have been shown to induce expression of cellular immediate-early genes (Holbrook and Fornace, 1991), in particular c-*fos* and c-*jun* (Crawford *et al.*, 1988; Shibanuma *et al.*, 1988; Stein *et al.*, 1989; Devary *et al.*, 1991). Indeed, treatment of cells with phorbol esters, which induce AP-1 transcriptional activity, generates a pro-oxidant state (Cerutti, 1985). Although tumor promoters have been linked to alterations in AP-1 activity through both modification of pre-existing Fos and Jun (Angel *et al.*, 1987, 1988; Chiu *et al.*, 1987) and through increased transcription of c-*fos* and c-*jun* (Morgan and Curran, 1991), these effects may not be a direct consequence of protein kinase C kinase activation. In fact, enhancement of Jun function in TPA-treated HeLa cells has been linked to dephosphorylation (Boyle *et al.*, 1991).

The induction of AP-1 DNA binding activity which accompanies treatment of cells with DNA damaging agents such as UV or hydrogen peroxide, does not require *de novo* protein synthesis, suggesting that post-translational modification may be involved (Stein *et al.*, 1989; Devary *et al.*, 1991). Here we demonstrate that redox control of the *in vitro* DNA binding activity of several transcription factors can be mediated by an enzyme involved in DNA repair. Immunodepletion experiments using Ref-1 antibodies demonstrate that Ref-1 is the major AP-1 redox activity in HeLa nuclear extracts, although other factors may contribute to the regulation. The exact nature of the connections among the processes of redox regulation, DNA repair and transcription control, is not yet clear. Direct oxidation results in a loss of Fos–Jun DNA binding activity *in vitro*. We speculate that while transcriptional activation of Fos and Jun may be necessary to maintain an extended DNA repair response, post-translational activation of latent AP-1 proteins by factors such as Ref-1 would ensure their immediate availability and allow for rapid activation of target genes. Alternatively, since DNA repair is an ongoing process in mammalian cells, Ref-1 may act to maintain reduced AP-1

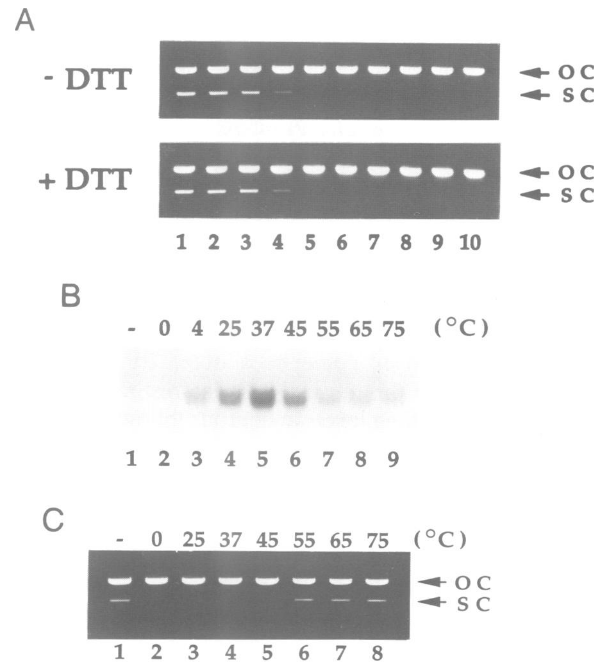

Fig. 9. Comparison of Ref-1 redox and AP endonuclease activities. (**A**) DTT dependence of the Ref-1 AP endonuclease activity. rRef-1 was dialysed against buffer without DTT as described in Figure 3C and then assayed for AP endonuclease activity on acid-depurinated plasmid DNA (0.5 μg) in the absence and presence of 10 mM DTT at different concentrations (μg/ml): no rRef-1, lane 1; 0.025, lane 2; 0.05, lane 3; 0.1, lane 4; 0.25, lane 5; 0.5, lane 6; 1.25, lane 7; 2.5, lane 8; 5.0, lane 9; 10.0, lane 10. (**B**) The effect of temperature on Ref-1 redox activity. Fos118-211 and Jun225-334 (0.1 μM) were assayed for AP-1 DNA binding activity following incubation with rRef-1 (5 μg/ml) at different temperatures as indicated (lanes 1–5). Prior to incubation with rRef-1, Fos118-211 and Jun225-334 were preassociated for 15 min at 37°C to ensure the formation of heterodimers. In lanes 6–9, rRef-1 was preheated for 30 min at the indicated temperatures and then assayed for AP-1 redox activity in the presence of Fos118-211 and Jun225-334 at 37°C. (**C**) The effect of temperature on Ref-1 AP endonuclease activity. rRef-1 (5 μg/ml) was assayed for AP endonuclease activity on acid-depurinated plasmid DNA (0.5 μg) at different temperatures (lanes 1–4). In lanes 5–9, rRef-1 was preheated for 30 min at the indicated temperatures and then assayed for AP endonuclease activity. Details of the gel retardation and AP endonuclease assays are described in Materials and methods. Supercoiled DNA (SC), open circular DNA (OC).

proteins under the prooxidant conditions generated through normal oxidative metabolism or in response to stimulation by tumor promoters and other DNA damaging agents. Both possibilities are consistent with the presence of Ref-1 activity in unstimulated nuclear extracts.

Regardless of its basal activity, Ref-1 may itself be under post-translational control. Treatment of cells with oxidizing agents causes a homeostatic response involving increased synthesis and activity of anti-oxidants and reducing enzymes such as thioredoxin and glutathione (Meister and Anderson, 1983; Holmgren, 1984, 1985, 1989). Here we show that Ref-1 is stimulated by thioredoxin/thioredoxin reductase/NADPH mixtures; therefore it is likely to be sensitive to the redox state of the cell. We have observed a similar enhancing effect of thioredoxin on Ref-1 activity present in nuclear extracts (Abate *et al.*, 1990c). This may account for the finding that a fraction of the AP-1 proteins present in HeLa nuclear extracts exist in an oxidized state. We are

now investigating the regulation and activity of Ref-1 in response to oxidative stress.

Although Fos and Jun have been linked to DNA repair (Stein *et al.*, 1989; Devary *et al.*, 1991) and replication processes (Wasylyk *et al.*, 1990; Murakami *et al.*, 1991), and Fos is expressed at its highest basal level continuously in skin cells (Smeyne *et al.*, 1992), the site of most oxidative and UV damage repair, it is clear that these proteins are expressed in many cell types and circumstances not associated with oxidative stress (Morgan and Curran, 1991). However, it is at least conceivable that regulatory mechanisms, originally evolved to combat the effects of environmental stress, could be adopted for use in other circumstances. In this regard, it is interesting to note that Fos is induced to high levels in neurons following excitation or during ischemia (Morgan and Curran, 1991; Smeyne *et al.*, 1992) and that nitric oxide has been implicated in signal transduction processes in neurons (Crossin, 1991).

Ref-1 redox enzyme versus nuclease

Here we demonstrate that the product of the cloned *ref*-1 gene catalyses two apparently distinct reactions. First, it stimulates the DNA binding activity of endogenous and exogenous AP-1 proteins in a manner that is characteristic of purified Ref-1 obtained from HeLa cells (Xanthoudakis and Curran, 1992). Secondly, although Ref-1 was purified and cloned on the basis of its AP-1 redox activity, it also functions as a class II AP endonuclease DNA repair enzyme. These two properties appear to be somewhat distinct. Ref-1 would not be unique in this regard as several bifunctional enzymes have been characterized. For example, the 37 kDa subunit of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase also possesses a DNA repair activity (uracil DNA glycosylase) (Meyer-Seigler *et al.*, 1991). Another glycolytic enzyme, lactate dehydrogenase-5, has been reported to be identical to a helix destabilizing protein (Williams *et al.*, 1985). Furthermore, the epidermal growth factor receptor exhibits partial DNA topoisomerase II activity (Mroczkowski *et al.*, 1984). Biochemical characterization of the redox and AP endonuclease activities of Ref-1 has revealed some distinguishing characteristics. The most significant of these is that only reduced Ref-1 is capable of stimulating Fos–Jun DNA binding activity, whereas either reduced or oxidized Ref-1 functions as an endonuclease. However, a direct comparison of these activities is difficult using the available assays. Monitoring AP-1 DNA binding activity by gel-shift assays gives only an indirect measure of Ref-1 redox activity. While Ref-1 may efficiently reduce Fos and Jun, other factors in addition to reduction may influence the DNA binding activity of the proteins. Thus, at present, the precise relationship between the two functions is not clear. Preliminary mutagenesis studies indicate that the C-terminus of Ref-1 is required for both the endonuclease and redox functions (unpublished observations).

Class II AP endonucleases cleave the phosphodiester bond 5′ to apurinic/apyrimidinic sites in double-stranded DNA (Levin and Demple, 1990). The redox reaction catalysed by Ref-1 is not yet defined; therefore it may be premature to compare the two reactions. The reduced form of the regulatory cysteine residue in Fos and Jun is a sulfhydryl (Abate *et al.*, 1990c). The problem is that the presumptive oxidized state that inhibits DNA binding activity has not yet been identified. This state occurs spontaneously, even in the presence of 1 mM DTT and it is not a disulfide bond (Abate *et al.*, 1990c). It has been proposed that the oxidized cysteine residue in Fos and Jun is in the form of a reversible sulfenic acid derivative (Abate *et al.*, 1990c) as suggested for the OxyR protein (Storz *et al.*, 1990). OxyR is involved in the oxidative stress response in *E.coli*. It encodes a transcription factor whose DNA binding capacity is altered depending on whether it is reduced or oxidized (Storz *et al.*, 1990). One possibility is that a nuclear protein capable of catalysing cleavage of a phosphate–oxygen bond could be adapted to also catalyse reduction of a sulphur–oxygen bond. However, until the presumptive oxidized state of Cys154 in Fos and Cys272 in Jun is identified, this possibility will remain untested.

Redox: general mechanisms of control of other transcription factors

Reduction or oxidation could provide a general mechanism for post-translational control of transcription factor function in an analogous fashion to phosphorylation. Changes in the redox state of several transcription factors including OxyR (Storz *et al.*, 1990), NF-κB (Tolando and Leonard, 1991). TFIIIC (Cromlish and Roeder, 1989), ISGF3 (Levy *et al.*, 1989), EBV-Zta (P.M.Lieberman, personal communication), HPV-E2 (P.M.Howley, personal communication) and various steroid receptors (Grippo *et al.*, 1985; Peleg *et al.*, 1989; Silva and Cidlowsky, 1989) have been shown to influence their DNA binding activity. Furthermore, the histone and high mobility group protein component of transcriptionally active nucleosomes preferentially contains exposed sylfhydryl groups (Einck and Bustin, 1985; Chen and Allfrey, 1987; Walker *et al.*, 1990). Cytoplasmic activation of NF-κB occurs through active oxygen signalling (Staal *et al.*, 1990; Schreck *et al.*, 1991). Redox regulation has also been implicated in the translational control of ferritin expression (Hentze *et al.*, 1989; Klausner and Harford, 1989) and in metabolic pathways involved in membrane transport (Ruppersberg *et al.*, 1991), neuronal signalling (Crossin, 1991), cell activation (Wakasugi *et al.*, 1990) and growth inhibition (Deiss and Kimchi, 1991). Thus, there is a substantial body of circumstantial evidence implicating reduction/oxidation processes as mechanisms for signal transduction. Ref-1 may be a common component of these pathways. Its redox activity is mediated through a conserved cysteine basic amino acid motif (KCR) that is present in all of the Fos- and Jun-related proteins identified to date (Figure 4). However, we also found that Ref-1 could stimulate the DNA binding activity of NF-κB, Myb and several members of the ATF/CREB family. Each of these factors has cysteine residues close to basic amino acids that are candidate targets of Ref-1. NF-κB DNA binding activity, like that of Fos and Jun, is sensitive to alkylating or sulfhydryl modifying agents and oxidation reversibly inactivates NF-κB *in vitro* (Toledano and Leonard, 1991). In the case of Myb, we have recently found that a short peptide consisting of repeats 2 and 3 is sufficient to mediate the redox effect (unpublished observations). A single cysteine residue flanked by basic amino acids is located within repeat 2 of Myb in a region that shares homology with the DNA binding domain of Fos and Jun (Carr and Mott, 1991). Thus, our findings support the notion that redox regulation by Ref-1 may be a common mechanism for the control of transcription factor function.

As mentioned above, redox processes also appear to be involved in NF-κB signal transduction. Active oxygen has been implicated as a common intermediate in the cytoplasmic

activation of NF-κB by phorbol esters, tumor necrosis factor and hydrogen peroxide (Staal *et al.*, 1990; Schreck *et al.*, 1991). However, hydrogen peroxide fails to stimulate dissociation of inactive NF-κB complexes in soluble extracts, indicating that activation by peroxides occurs indirectly. In addition, NF-κB DNA binding activity, like AP-1 DNA-binding activity, is also increased in soluble extracts from UV-treated cells and is stimulated by reducing agents *in vitro* (Stein *et al.*, 1989; Toledano and Leonard, 1991). Together with the data indicating that Ref-1 stimulates the DNA binding activity of p65NF-κB, these findings suggest some overlap between the AP-1 and NF-κB regulatory pathways. At first sight, it is paradoxical that both oxidative and reducing signals activate NF-κB, albeit in different ways. However, it is well known that redox systems are tightly regulated homeostatic cycles and that every oxidizing reaction has a corresponding reducing counterpart. These systems are highly complex and very little is known about their activity in the nucleus. Here we present the identity of one enzyme that may be involved in redox control of transcription factor function as a first step in unraveling these complexities.

Materials and methods

Cell culture and transfections

Hela, Jurkat and Cos cells were grown in RPMI or DMEM supplemented with 10% fetal calf serum, glutamine and antibiotics. Cos cells were seeded at a density of 2.5×10^5 cells/35 mm plate one day prior to transfection. Each plate of cells was transfected with 5 μg of the Ref-1 expression vector (BL-soCMVINPA-Ref1) or the control plasmid (BL-soCMVINPA) using the DEAE-dextran–cloroquine method (Dillon *et al.*, 1991). Cells were harvested for indirect immunofluorescence analysis at 48 h post-transfection.

Protein sequencing

Purified Ref-1 from Hela cells (70 pmol) was resolved by electrophoresis on a polyacrylamide gel and electroblotted onto a PVDF membrane (Millipore, Milford, MA) (Matsudaira, 1987). Sequence analysis of the immobilized protein was performed using an ABI gas phase sequencer model 470A (Applied Biosystems, Foster City, CA) equipped with an ABI blot cartridge (Hewick *et al.*, 1981). Phenylthiohydantoin (PTH) amino acid derivatives were identified 'on-line' with a 120 PTH amino acid analyser (Hunkapiller *et al.*, 1986).

cDNA cloning of Ref-1

Degenerate PCR probes were derived from the 20 amino acid N-terminal sequence of Ref-1 (PKRGKKGAVAEDGDELRTEP). The first five amino acids were used to derive the nucleotide sequence of the forward primer and the last six amino acids were used to derive the nucleotide sequence of the reverse primer. The degeneracy of the forward and reverse primers was 1/256 and 1/512, respectively: PKRGK (aa 2-6) degenerate forward primer (dFP), 5'-GAGAGAATTCCCNAAA/GC/AGNGGNAA-3'; ELRTEP (aa 15-20) degenerate reverse primer (dRP), 5'-GAGA-GAATTCGGTTCNGTNCG/TNAG/AT/CTC-3'.

The *ref*-1 N-terminal DNA coding sequence was obtained by mixed primer PCR amplification of Hela cDNA and confirmed by DNA sequencing. HeLa cell poly(A)$^+$ RNA and hexamer primed cDNA were prepared as previously described (Gubler *et al.*, 1991). The resulting N-terminal *ref*-1 PCR amplicon was digested with *Eco*RI to generate 5' overhangs, subcloned into a Bluescript vector (Stratagene) to generate plasmid pRef-1NT and the *ref*-1 insert was verified by DNA sequencing. The N-terminal *Ref*-1 DNA fragment was excised from plasmid pRef-1NT, gel purified, end-labelled with Klenow and [α-^{32}P]dNTPs (Amersham International plc), and used to screen a λZAP Jurkat cDNA library (Ruben *et al.*, 1991) for full-length *Ref*-1 cDNA clones. Approximately 2.5×10^5 duplicate plaques were analysed in the primary screening as described (Cohen and Curran, 1988). Eighteen positive cDNA clones were isolated and subjected to two additional rounds of screening and plaque purification. Six of the original eighteen clones remained positive after tertiary screening and were subjected to further analysis. Bacterial plasmid sequences containing *Ref*-1 cDNA inserts were rescued from phage particles and isolated according to the manufacturers recommendations (Stratagene). The authenticity of all six clones was confirmed by PCR amplification of the N-terminal cDNA coding sequences using the forward and reverse *Ref*-1 degenerate oligonucleotides as described above. The nucleotide sequence (both strands) of the *Ref*-1 clone containing the largest cDNA insert (clone 4-1, 1441 bp) was determined. Homology searches were performed using the Genbank data base and GCG sequence analysis software packages.

RNA analysis

HeLa cell poly(A)$^+$ RNA was prepared from $\sim 10^7$ cells as previously described (Gubler *et al.*, 1991). Northern blot hybridization was performed using the human 1.44 kb cloned *ref*-1 cDNA (Gubler *et al.*, 1991). The radiolabelled DNA probe was prepared by the random priming method (Amersham International plc) using [α-^{32}P]dCTP (3000 Ci/mmol) and Klenow enzyme.

Isolation of genomic DNA and Southern blot analysis

Human genomic DNA was isolated from Hela cells. Cells were harvested, washed once with PBS and resuspended in lysis buffer (100 mM NaCl, 10 mM Tris–HCl pH 8.0, 1 mM EDTA, 10 μg/ml Proteinase K, 100 μg/ml RNase A and 0.5% SDS). The lysate was incubated overnight at 37°C and extracted several times with phenol–chloroform. High molecular weight DNA was precipitated with 2 vol of 100% ethanol, spooled out with a pasteur pipet and dissolved in TE (10 mM Tris–HCl pH 8.0 and 1 mM EDTA). Southern blot analysis was carried out on 10 μg genomic DNA digested with different restriction enzymes. DNA digests were resolved by electrophoresis on 0.8% TBE–agarose gels that were subsequently treated in 0.25 N HCl for 10 min, denatured and neutralized for 30 min each and finally capillarily blotted onto nylon membranes (Hybond-N, Amersham International plc) in 20 × SSC. Pre-hybridization was performed in a solution containing 50% Formamide, 6 × SSPE, 1% SDS, 5 × Denhardt's and 100 μg/ml denatured salmon sperm DNA at 37°C for 2 h. The *ref*-1 cDNA probe was labelled by the random priming method (Oligolabeling Kit, Pharmacia) to a specific activity of 1×10^9 c.p.m./μg and added to the pre-hybridization solution at 2×10^6 c.p.m./ml. Hybridization was carried out at 37°C for 16–20 h. The hybridized membrane was washed twice in 2 × SSC–0.1% SDS at room temperature for 15 min and once in 0.1 × SSC–0.1% SDS at 50°C for 30 min. Radioactive bands were visualized by autoradiography of the membrane at −80°C using intensifying screens.

Purification of recombinant proteins from E.coli

Recombinant Fos, Fos118-211, Jun, Jun224-234 and Ref-1 were expressed as histidine fusion proteins in *E.coli* (strain M15) and purified by nickel chelate chromatography as previously described (Abate *et al.*, 1990b). Control bacterial extracts from untransformed M15 cells or M15 cells transformed with only the pDS56 expression vector were purified in parallel. All protein preparations were extensively dialysed against buffer containing 50 mM sodium phosphate, pH 7.3, 1 mM EDTA, 1 mM DTT and 5% glycerol and stored in liquid nitrogen. The purified proteins were analysed on polyacrylamide gels and visualized by coomassie blue staining (Bio-Rad). Protein concentrations were determined using the Bio-Rad assay kit.

DNA binding assays

E.coli purified Ref-1 or control bacterial extracts (0.1–0.2 μg) were preincubated in binding buffer with either recombinant Fos and Jun (0.1 μM), ATF-1 (aa 1–232) (0.25 μM), ATF-2 (aa 350–505; 0.25 μM), CREB (aa 1–327; 0.03–0.3 μM), T-Myb (aa 1–396; 0.03 μM), p65NF-κB (aa 1–309; 2.2 μM), or glucocorticoid receptor (GR, aa 440–533; 0.73 μM) at 37°C for 15 min prior to the addition of 1 μg poly dI:dC–dI:dC (5 min incubation at 25°C) and 0.2 ng of the appropriate [α-^{32}P]labeled oligonucleotide probe (15 min incubation at 25°C) as described (Abate *et al.*, 1990d). DNA binding activity was monitored by the gel retardation assay (Garner and Revzin, 1981). DNA–protein complexes were resolved on 4.5% native Tris–glycine (pH 8.5) gels, dried and visualized by autoradiography. When used in the DNA binding assays, the *E.coli* thioredoxin cocktail was added to the reaction at a final concentration of 5.0 μM thioredoxin, 0.2 mM NADPH, pH 7.5 and 0.1 μM thioredoxin reductase (American Diagnostics). For immunodepletion experiments rRef-1 (0.1 μg) and Hela nuclear extract (4 μg) was preincubated with protein A-purified total rabbit immunoglobulin or affinity purified Ref-1 antibody (2.2) for 30 min at room temperature. Immune complexes were cleared by immunoprecipitation using 15 μl of a 10% Pansorbin cell suspension (Calbiochem) prepared in gel shift binding buffer containing 25 μg/ml BSA (Xanthoudakis and Curran, 1992) as previously described (Cohen *et al.*, 1989). Cleared supernatants were transferred to fresh tubes and assayed for stimulation of AP-1 DNA binding activity (as described above) using *E.coli* expressed FPLC purified Fos133-207 and Jun252-326 proteins (0.1 μM) lacking a polyhistidine tag (a gift from M.Glover and S.Harrison).

Hela nuclear extracts were prepared as previously described (Xanthoudakis and Curran, 1992). The AP-1, NF-κB and Myb oligonucleotides used in this study have been described (AP-1, Abate *et al.*, 1990c; NF-κB, Ruben *et al.*, 1991; Myb, Saikumar *et al.*, 1990. The CRE (5'-TC GACAATGAC-GTCAGCAT-3') and GRE (5'-CTAGACAGAACATCATGTTCTGAG-3') oligonucleotides were synthesized on an Applied Biosystems DNA Synthesizer and gel purified before annealing to the complementary strand.

AP endonuclease and exonuclease assays

AP endonuclease assays were performed as described (Ikeda *et al.*, 1991). A mixture of supercoiled and open circular Bluescript SK − DNA (50 μg) was acid-depurinated by incubation with 3 vol of 50 mM sodium citrate pH 3.5 for 15 min at 60°C. The DNA preparation was chilled on ice and dialysed overnight at 4°C against 50 mM Tris−HCl, pH 7.4. The AP endonuclease assay mixture (20 μl) contained 10 mM Tris−HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, 0.01% NP-40, 1−2 ml of purified recombinant Ref-1 protein (0.5−200 ng) and 0.5 μg of acid-depurinated plasmid DNA. The reaction mixture was incubated at 37°C for 15 min, chilled on ice and then immediately analysed by electrophoresis on a 1% TAE−agarose gel. For Ref-1 exonuclease assays, the reaction mixture was the same except that either the single-stranded (50 nucleotides) or double-stranded (373 bp) [γ-^{32}P]labeled DNAs were substituted as substrates. The DNA products were analysed by electrophoresis on 6% TBE−polyacrylamide gels and visualized by autoradiography of the dried gels.

Affinity purification of Ref-1 antisera

Six milliliters of rabbit polyclonal Ref-1 antisera were fractionated over a 2 ml protein A−agarose column according to the Manufacturer's instructions (Bio-Rad). Ten milliliters of antibody eluate (3 mg/ml) from the protein A column were dialysed against PBS overnight at 4°C and purified over the Ref-1 affinity column. The rRef-1-conjugated affinity column was prepared with Bio-Rad Affi-Gel 10 using 1 mg of *E.coli*-purified recombinant Ref-1 protein per ml of gel as recommended by the manufacturer. Affinity purification was performed as previously described (Curran *et al.*, 1985).

Indirect immunofluorescence and confocal microscopy

Transfected and untransfected Cos cell cultures were analysed by indirect immunofluorescence as described previously (Curran *et al.*, 1985) using rabbit preimmune or polyclonal Ref-1 antisera (1:1000 dilution). Preabsorption of the Ref-1 antisera was achieved by pre-incubation of the purified antibody preparation with 10 μg/ml of Ref-1 N-terminal peptide (aa 2−21) for 30 min at room temperature. Confocal imaging of fluorescent nuclei was performed using the Bio-Rad MRC-600 confocal laser scanning system.

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References

Abate,C. and Curran,T. (1990) *Semin. Cancer Biol.*, **1**, 19−26.

Abate,C., Luk,D. and Curran,T. (1990a) *Cell Growth Differ.*, **1**, 455−462.

Abate,C., Luk,D., Gentz,R., Rauscher,F.J.III and Curran,T. (1990b) *Proc. Natl. Acad. Sci. USA*, **87**, 1032−1036.

Abate,C., Patel,L., Rauscher,F.J.III and Curran,T. (1990c) *Science*, **249**, 1157−1161.

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.

Angel,P., Hattori,K., Smeal,T. and Karin,M. (1988) *Cell*, **55**, 875−885.

Barber,J.R. and Verma,I.M. (1987) *Mol. Cell. Biol.*, **7**, 2201−2211.

Benbrook,D.M. and Jones,N.C. (1990) *Oncogene*, **5**, 295−302.

Bos,T.J., Monteclaro,F.S., Mitsunobu,F., Ball,A.R.Jr, Cang,C.H.W., Nishimura,T. and Vogt,P.K. (1990) *Genes Dev.*, **4**, 1677−1687.

Boyle,W.J., Smeal,T., Defize,L.H.K., Angel,P., Woodgett,J.R., Karin,M. and Hunter,T. (1991) *Cell*, **64**, 573−584.

Cantley,L.C., Auger,K.R., Carpenter,C., Duckworth,B., Graziani,A., Kapeller,R. and Soltoff,S. (1991) *Cell*, **64**, 281−302.

Carr,M.D. and Mott,R.F. (1991) *FEBS Lett.*, **282**, 293−294.

Cerutti,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., Imagawa,M., Imbra,R.J., Bokhoven,J.R. and Karin,M. (1987) *Nature*, **329**, 648−651.

Chiu,R., Angel,P. and Karin,M. (1989) *Cell*, **59**, 979−986.

Cohen,D.R., Ferreira,P.C.P., Gentz,R., Franza,B.R.,Jr, and Curran,T. (1989) *Genes Dev.*, **3**, 173−184.

Cohen,D.R. and Curran,T. (1988) *Mol. Cell. Biol.*, **8**, 2063−2069.

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.

Cunningham,R.P., Saporito,S.M., Spitzer,S.G. and Weiss,B. (1986) *J. Bacteriol.*, **168**, 1120−1127.

Curran,T. and Morgan,J.I. (1985) *Science*, **229**, 1265−1268.

Curran,T. and Franza,B.R.Jr (1988) *Cell*, **55**, 395−397.

Curran,T. and Vogt,P.K. (1992) In Yamamoto,K.R. and McKnight,S. (eds), Transcriptional Regulation, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, in press.

Curran,T., Miller,A.D., Zokas,L. and Verma,I.M. (1984) *Cell*, **36**, 259−268.

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.

Demple,B. and Amabile-Cuevas,C.F. (1991) *Cell*, **67**, 837−839.

Demple,B., Herman,T. and Chen,D.S. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 11450−11454.

Devary,Y., Gottlieb,R.A., Lau,L.F. and Karin,M. (1991) *Mol. Cell. Biol.*, **11**, 2804−2811.

Dillon,P.J., Nelbock,P., Perkins,A. and Rosen,C.A. (1991) *Mol. Cell. Biol.*, **65**, 445−449.

Einck,L. and Bustin,M. (1985) *Exp. Cell Res.*, **156**, 295−310.

Forrest,D. and Curran,T. (1992) *Curr. Opin. Genet. Dev.*, **2**, 19−27.

Franza,B.R. Jr, Rauscher,F.J.,III, Josephs,S.F. and Curran,T. (1988) *Science*, **239**, 1150−1153.

Garner,M.M. and Revzin,A. (1981) *Nucleic Acids Res.*, **9**, 3047−3060.

Gartenberg,M.R., Ampe,C., Steitz,T.A. and Crothers,D.M. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 6034−6038.

Gentz,R., Rauscher,F.J.,III, Abate,C. and Curran,T. (1989) *Science*, **243**, 1695−1699.

Greenberg,J.T., Monach,P., Chou,J.H., Josephy,P.D. and Demple,B. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 6181−6185.

Grippo,J.F., Holmgren,A. and Pratt,W.B. (1985) *J. Biol. Chem.*, **260**, 93−97.

Gubler,U. *et al.* (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 4143−4147.

Guis,D., Cao,X., Rauscher,F.J.,III, Cohen,D.R., Curran,T. and Sukhatme,V.P. (1990) *Mol. Cell. Biol.*, **10**, 4243−4255.

Hai,T. and Curran,T. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 3720−3724.

Halazonetis,T.D., Georgopoulos,K., Greenberg,M.E. and Leder,P. (1988) *Cell*, **55**, 917−924.

Halliwell,B. and Gutteridge,J.C.M. (1990) *Methods Enzymol.*, **186**, 1−86.

Hentze,M.W., Rouault,T.A., Harford,J.B. and Klauser,R.D. (1989) *Science*, **244**, 357−359.

Hewick,R.M., Hunkapiller,M.W., Hood,L. and Dreyer,W.J. (1981) *J. Biol. Chem.*, **256**, 7990−7997.

Hirai,S.-I., Ryseck,R.-P., Mechta,F., Bravo,R. and Yaniv,M. (1989) *EMBO J.*, **8**, 1433−1439.

Holbrook,N.J. and Fornace,A.J. Jr. (1991) *New Biologist*, **3**, 825−833.

Holmgren,A. (1984) *Methods Enzymol.*, **107**, 295−351.

Holmgren,A. (1985) *Annu. Rev. Biochem.*, **54**, 237−271.

Holmgren,A. (1989) *J. Biol. Chem.*, **264**, 13963−13966.

Hunkapiller,M.W., Granlund-Moyer,K. and Whiteley,N.W. (1986) In Shively,J.E. (ed.), *Methods of Protein Characterization*. The Humana Press, Clifton, NJ, pp. 315−327.

Hunter,T. (1991) *Cell*, **64**, 249−270.

Ikeda,S., Seki,S., Watanabe,S., Hatsushika,M. and Tsutsui,K. (1991) *Anal. Biochem.*, **192**, 96−103.

Kane,C.M. and Linn,S. (1981) *J. Biol. Chem.*, **256**, 3405−3414.

Kerppola,T.K. and Curran,T. (1991) *Curr. Opin. Struct. Biol.*, **1**, 71−79.

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.

Lee,W.M.F., Lin,C. and Curran,T. (1988) *Mol. Cell. Biol.*, **8**, 5521–5527.
Levin,J.D. and Demple,B. (1990) *Nucleic Acids Res.*, **18**, 5069–5075.
Levy,D.E., Kessler,D.S., Pine,R. and Darnell,J.E. Jr. (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.
Meyer-Seigler,K., Mauro,D.J., Seal,G., Wurzer,J., De Riel,J.K. and Sirover,M.A. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 8460–8464.
Miller,A.D., Curran,T. and Verma,I.M. (1984) *Cell*, **36**, 259–268.
Morgan,J. and Curran,T. (1991) *Annu. Rev. Neurosci.*, **14**, 421–451.
Mroczkowski,B., Mosig,G. and Cohen,S. (1984) *Nature*, **309**, 270–273.
Murakami,Y., Satake,M., Yamaguchi-Iwai,Y., Sakai,M., Muramatsu,M. and Ito,Y. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 3947–3951.
Müller,R., Bravo,R., Müller,D., Kurz,C. and Renz,M. (1987) *Oncogene Res.*, **2**, 19–32.
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.
Nishizawa,M., Goto,N. and Kawai,S. (1987) *J. Virol.*, **61**, 3733.
O'Shea,E.K., Rutkowski,R. and Kim,P.S. (1989) *Science*, **243**, 538–542.
Ofir,R., Dwarki,V.J., Rashid,D. and Verma,I.M. (1990) *Nature*, **348**, 80–82.
Okuno,H., Suzuki,T., Yoshida,T., Hashimoto,Y., Curran,T. and Iba,H. (1991) *Oncogene*, **6**, 1491–1497.
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.
Perkins,K.K., Admon,A., Patel,N. and Tjian,R. (1990) *Genes Dev.*, **4**, 822–834.
Pulverer,B., Kyriakis,J.M., Avruch,J., Nikolakaki,E. and Woodgett,J.R. (1991) *Nature*, **353**, 670–674.
Puyet,A., Greenberg,B. and Lacks,S.A. (1989) *J. Bacteriol.*, **171**, 2278–2286.
Rauscher,F.J.,III, Voulalas,P.J., Franza,B.R. Jr and Curran,T. (1988) *Genes Dev.*, **2**, 1687–1699.
Reddy,E.P., Skalka,A.M. and Curran,T. (1988) *The Oncogene Handbook.* Elsevier, Amsterdam.
Roberts,B. (1989) *Biochim. Biophys. Acta*, **1008**, 263–280.
Robson,C.N. and Hickson,I.D. (1991) *Nucleic Acids Res.*, **19**, 5519–5523.
Robson,C.N., Milne,A.M., Pappin,D.J.C. and Hickson,I.D. (1991) *Nucleic Acids Res.*, **19**, 1087–1092.
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.
Ruppersberg,J.P., Stocker,M., Pongs,O., Heinemann,S.H., Frank,R. and Koenen,M. (1991) *Nature*, **352**, 711–714.
Rüther,U., Garber,C., Komitowski,D., Müller,R. and Wagner,E.F. (1987) *Nature*, **325**, 412–416.
Rüther,U., Komitowski,D., Schubert,F.R. and Wagner,E.F. (1989) *Oncogene*, **4**, 861–865.
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.
Saikumar,P., Murali,R. and Reddy,E.P. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 8452–8456.
Sander,M., Lowenhaupt,K., Lane,W.S. and Rich,A. (1991) *Nucleic Acids Res.*, **19**, 4523–4529.
Saporito,S.M., Smith-White,B.J. and Cunningham,R.P. (1988) *J. Bacteriol*, **170**, 4542–4547.
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 Müller,R. (1989) *Cell*, **56**, 507–516.
Schütte,J., Viallet,J., Nau,M., Segal,S., Fedorko,J. and Minna,J. (1989) *Cell*, **59**, 987–997.
Shibanuma,M., Kuroki,T. and Nose,K. (1988) *Oncogene*, **3**, 17–21.
Silva,C.M. and Cidlowski,J.A. (1989) *J. Biol. Chem.*, **264**, 6638–6647.
Silver,P.A. (1991) *Cell*, **64**, 489–497.
Smeyne,R.J., Schilling,K., Robertson,L., Luk,D., Oberdick,J., Curran,T. and Morgan,J.I. (1992) *Neuron*, **8**, 13–23.
Staal,F.J.T., Roederer,M., Herzenberg,L.A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9943–9947.
Stein,B., Rahmsdorf,H.J., Steffen,A., Litfin,M. and Herrlich,P. (1989) *Mol. Cell Biol.*, **9**, 5169–5181.
Storz,G., Tartaglia,L.A. and Ames,B.N. (1990) *Science*, **248**, 189–194.
Suzuki,T., Hashimoto,Y., Okuno,H., Sato,H., Nishina,H. and Iba,H. (1991) *Jap. Cancer Res.*, **82**, 58–64.
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.
Van Beveren,C., Van Straaten,F., Curran,T., Müller,R. and Verma,I.M. (1983) *Cell*, **32**, 1241–1255.
Van Beveren,C., Enami,S., Curran,T. and Verma,I.M. (1984) *Virology*, **135**, 229–243.
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,G.C. (1985) *Annu. Rev. Biochem.*, **54**, 425–457.
Walker,J., Chen,T.A., Sterner,R., Berger,M., Winston,F. and Allfrey,V.G. (1990) *J. Biol. Chem.*, **265**, 5736–5746.
Wang,Z.-Q., Grigoriadis,A.E., Mohle-Steinlein,U. and Wagner,E.F. (1991) *EMBO J.*, **10**, 2437–2450.
Wasylyk,C., Schneikert,J. and Wasylyk,B. (1990) *Oncogene*, **5**, 1055–1058.
Williams,K.R., Reddigari,S. and Patel,G.L. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5260–5264.
Xanthoudakis,S. and Curran,T. (1992) *EMBO J.*, **11**, 653–665.
Zerial,M., Toschi,L., Ryseck,R.-P., Schuermann,M., Müller,R. and Bravo,R. (1989) *EMBO J.*, **8**, 805–813.

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