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Heterodimer formation of cJun and ATF-2 is responsible for induction of *c-jun* by the 243 amino acid adenovirus E1A protein

Hans van Dam[1,3], Monique Duyndam[1], Robbert Rottier[1], Anne Bosch[1], Lydia de Vries-Smits[1], Peter Herrlich[2], Alt Zantema[1], Peter Angel[2] and Alex J.van der Eb[1]

[1]Laboratory for Molecular Carcinogenesis, Sylvius Laboratories, University of Leiden, 2300 RA Leiden, The Netherlands and [2]Kernforschungszentrum Karlsruhe, Institut für Genetik und Toxikologie, D-7500 Karlsruhe 1, Germany

[3]Present address: Institut für Genetik und Toxikologie, Kernforschungszentrum Karlsruhe, Institut für Genetik und Toxikologie, D-7500 Karlsruhe 1, Germany

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The adenovirus E1A proteins differentially regulate AP-1-responsive genes. Collagenase and stromelysin are repressed by E1A, whereas the expression of *c-jun* is elevated. Inhibition of collagenase has been found to be exerted through the consensus AP-1 binding site TGAGTCA. Here we show that the distal AP-1 binding site in the *c-jun* promoter, the jun2TRE (TTACCTCA), is the decisive element of this promoter in mediating the positive response to the 243 amino acid E1A product. *In vitro* binding studies revealed that, in contrast to the consensus AP-1 site which is preferentially targeted by dimers composed of the Jun and Fos families, the jun2TRE binds heterodimers composed of cJun and ATF-2(-like) proteins. Since stimulation of *c-jun* transcription is a function of the transforming domain of E1A encoded by conserved region 1, cJun–ATF-2 may be one of the effector factors involved in transformation. The data further suggest that E1A can distinguish between cJun–cJun and cJun–ATF-2 in imposing opposite states of activity.

Key words: ATF-2/*c-jun*/243R-E1A/transcription/transformation

Introduction

Oncogenic transformation by human adenoviruses (Ad) is dependent on the functionality of the proteins encoded by their early region 1A (E1A). In the case of Ad serotype 5, these proteins [289 and 243 amino acid (aa) residues], exhibit a multitude of activities, several of which are linked to their transforming function. For example, the E1A polypeptides bind a set of cellular proteins implicated in cell cycle regulation, including p105-RB, p58-cyclin A and p33-cdk2 (Egan *et al.*, 1988; Whyte *et al.*, 1989; Giordano *et al.*, 1991; Tsai *et al.*, 1991), and are able to induce DNA synthesis in growth-arrested cells (Stabel *et al.*, 1985; Howe *et al.*, 1990). The 243 and 289 aa E1A products, which will be abbreviated here as 243R and 289R, contain all the functions of E1A that are essential for transformation. They differ only in an internal stretch of 46 residues unique for the 289R protein, which is one of the regions conserved among the adenovirus serotypes (conserved domain 3). This region carries a strong transcriptional transactivation domain, which is essential for efficient virus reproduction in permissive cells, but is not required for transformation (for a review see Flint and Shenk, 1989). Despite the absence of a strong transactivation domain, the 243 aa form must obviously also influence gene expression to achieve transformation, which has been amply documented (e.g. Lillie *et al.*, 1986, 1987; Schneider *et al.*, 1987; Zerler *et al.*, 1987; van Dam *et al.*, 1989, 1990; Offringa *et al.*, 1990).

Cellular genes are turned on or off by 243R-E1A, predominantly at the level of transcription. The type of interaction with gene-specific transcription factors has yet been addressed with only two examples: E2F/DRTF1 and AP-1. E1A stimulates the activity of E2F/DRTF1, probably by releasing it from its interaction with p105-RB (Bagchi *et al.*, 1990; Bandara and La Thangue, 1991; Chellapan *et al.*, 1991). This would thus be a function of the E1A domain interacting with p105-RB, which is mainly exerted by conserved domain 2 (Egan *et al.*, 1988; Whyte *et al.*, 1989). The interaction with AP-1, on the other hand, is exerted by conserved domain 1 (Offringa *et al.*, 1990; van Dam *et al.*, 1990).

AP-1 activity is a function of a number of distinct dimeric protein complexes consisting of members of the Jun and Fos families, as well as specific members of the ATF/CREB families, e.g. cJun, JunB, JunD, cFos, FosB, Fra-1, Fra-2 and ATF-2/CRE-BP1. Depending on the composition of the dimer, different sequence elements are preferentially recognized. cJun–cJun homodimers and cJun–cFos heterodimers bind to the AP-1 consensus sequence $T^{G}_{T}A^{C}_{G}TCA$, while ATF-2 homodimers and cJun–ATF-2 heterodimers prefer the ATF/CREB consensus sequence $T^{G}_{T}ACGTCA$ (Benbrook and Jones, 1990; Ivashkiv *et al.*, 1990; Hai and Curran, 1991). E1A interacts with the AP-1 family in a complicated fashion. It represses the activities of the promoters of the stromelysin and collagenase genes through the TPA-responsive element (TRE) by inhibiting transactivation by cJun/cJun or cJun/cFos (Offringa *et al.*, 1990). In contrast, the activity of the *c-jun* promoter, which is also controlled by AP-1 binding sites (Angel *et al.*, 1988b; Stein *et al.*, 1992), is not repressed by E1A. Rather, *c-jun* transcription is strongly enhanced (van Dam *et al.*, 1990; de Groot *et al.*, 1991; Kitabayashi *et al.*, 1991) suggesting that E1A can distinguish between different members of the AP-1 family. Interestingly, *c-jun* transcription driven by experimental overexpression of cJun, such that cJun homodimers are forced onto the AP-1 binding sites, is again repressed by E1A (van Dam *et al.*, 1990).

In this paper, we define the mechanism of 243R-E1A-induced *c-jun* transcription. The *c-jun* promoter carries two relevant similar AP-1 binding sites. The distal element at

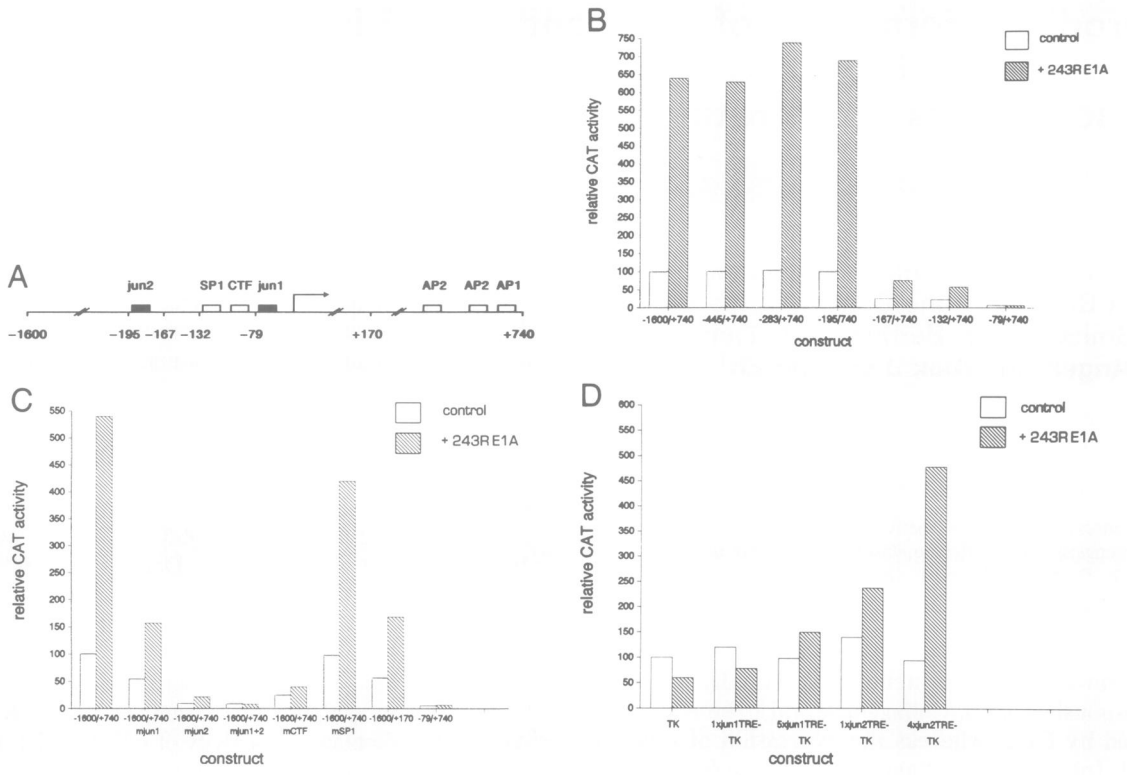

Fig. 1. Delimitation of *c-jun* promoter sequences required for transactivation by 243R-E1A. (**A**) Map of the human *c-jun* promoter region showing the locations of the transcription factor binding sites identified thus far. (**B–D**) HeLa tk$^-$ cells were transiently transfected via the DEAE–dextran method with 6 μg of the indicated *c-jun*–CAT or TK–CAT constructs together with 8 μg of either pRSVneo (control) or pRSV-JF12, which expresses only the 243R-Ad5E1A protein. After 40 h, cells were harvested, protein extracts were prepared and CAT activity was determined. The values are the mean of at least three independent experiments in which the indicated constructs were tested in parallel. Because of variation of transfection efficiency between different experiments, basal promoter activity is represented in arbitrary units (−1600/+740 or TK = 100). Standard deviations were <20% in all cases.

position −190 is required for E1A induction of *c-jun* and can act as an E1A-responsive element in a minimal promoter. *In vitro*, the element binds complexes containing cJun and ATF-2, in contrast to the collTRE. Thus, the actual effect of E1A on a cJun-containing transcription factor, inhibition or activation, appears to be determined by the second component of the dimer. Since analysis of cJun mutants has revealed an inverse correlation between cJun-dependent transformation and enhanced activation of collagenase in chicken embryo fibroblasts (Havarstein *et al.*, 1992; M.Hartl and P.K.Vogt, personal communication), constitutive activation of cJun–ATF-2, rather than cJun–cJun or cJun–cFos, may be one of the events crucial for cell transformation.

Results

The distal AP-1 binding site in the c-jun promoter plays a decisive role in the E1A response

We have shown previously that maximal activation of the human *c-jun* promoter by E1A in HeLa tk$^-$ cells requires the jun(1)TRE, located at position −71 (Angel *et al.*, 1988b), as well as other sequences in the *c-jun* promoter region, either between positions −1600 and −132 or positions +170 and +740 (van Dam *et al.*, 1990; see Figure 1A). To identify the crucial elements, we first analysed a set of promoter mutants that were progressively deleted from the 5′ terminus. These were cotransfected with an E1A expression vector that only expresses 243R-E1A to exclude induction of promoter activity by the promiscuous transactivating function of 289R-E1A-specific conserved region 3. As evident from Figure 1B, deletion of the sequences up to position −195 did not significantly affect the activation by E1A, but further deletion up to position −167 strongly reduced both basal promoter activity and the inducibility by 243R-E1A. In this region, an AP-1 binding site is present between positions −190 and −183 (TTACCTCA), designated jun2TRE as it can confer TPA inducibility to heterologous promoters (Stein *et al.*, 1992). Like the jun1TRE (TGACATCA) and the consensus binding site for ATF/CREB (TGACGTCA), the jun2TRE is an 8 base pair motif. To verify whether this second junTRE was indeed involved in the E1A-dependent induction of *c-jun*–CAT, we tested constructs in which the jun1TRE and/or the jun2TRE were eliminated by site-directed mutagenesis (Figure 1C). Deletion of the jun2TRE severely reduced, but only mutation of both TREs completely eliminated induction by E1A. Thus, both junTREs are relevant for E1A induction, with the jun2TRE being the dominant E1A-responsive element.

Although the jun1- and jun2TRE were thus found to be essential for the induction of *c-jun* by E1A within the complete promoter, a single jun1TRE was found not to be E1A inducible under the same experimental conditions, neither in the −79/+170, −132/+170 and −79/+740 promoters, nor when linked to a minimal tata promoter (van Dam *et al.*, 1990; Figure 1B). An explanation could be that the jun1TRE can function as an E1A-inducible element only

in the presence of a second junTRE or other transcription factor binding sites. To test this possibility and to examine whether the jun2TRE requires cooperation with a second element, we assayed constructs with one or multiple copies of the jun1- or jun2TRE linked to the −105/+51 Herpes simplex virus thymidine kinase (TK) promoter for 243R-E1A inducibility. In addition to a tata element, this promoter contains two SP1 and one CTF binding sites. As shown in Figure 1D, the 1xjun1TRE−TK construct behaves similarly as the TK promoter, which is weakly repressed by 243R-E1A, whereas the 1xjun2TRE−TK clone is slightly stimulated. The activity of the 4xjun2TRE−TK construct is increased ~5-fold by E1A, whereas the 5xjun1TRE-TK construct is weakly stimulated. The observation that the jun2TRE can act as an E1A-inducible element independent of the *c-jun* promoter context, was further confirmed by the finding that five copies of the jun2TRE were also able to confer 243R-E1A inducibility to a minimal tata promoter (data not shown). These data suggest that the factors binding to the jun2TRE act more independently or are activated by E1A more strongly than those binding to the jun1TRE.

Although the results described above point to the jun2TRE as the decisive element in the positive response of the *c-jun* promoter to E1A, it is clear that the jun2TRE function is enhanced by other elements. Within its natural context, the sequence between +170 and +740 appears to contribute (van Dam *et al.*, 1990, Figure 1C). Furthermore, the region between positions −132 and −79 contributes to the E1A response, since the −167/+740 and −132/+740 promoter constructs are still weakly induced, in contrast to the −79/+740 construct (Figure 1B). Like the +170/+740 region, the −132/−79 region can contribute to the induction only in the presence of the junTREs, since the −1600/+740 mjun1+2 and −79/+740 clones were not induced by E1A. To identify the contributing elements in the −132/−79 region, we analysed complete promoter constructs in which either the SP1 site or the CTF site (see Figure 1A) were eliminated. As shown in Figure 1C, mutation of the CTF element strongly reduced the response to E1A, whereas the SP1 site appears to play only a minor role. Also visible is that deletion of the +170/+740 region has a less strong effect than mutation of the CTF site.

In conclusion, these results demonstrate that the jun1TRE and jun2TRE cooperate to mediate the full positive response of the c-jun promoter to 243R-E1A. The jun2TRE appears to predominate since only this element can efficiently confer E1A inducibility to a heterologous promoter. The CTF element and the +170/+740 region are also required for a maximal response in the context of the complete promoter. These sequences are not functional in the absence of the junTREs. One may envisage the formation of a multi-protein complex that interacts efficiently with the transcription-initiation factors.

Induction of the jun2TRE by E1A, but not by cAMP, in NIH3T3 cells

In our previous studies, we have demonstrated that the collagenase AP-1 site (collTRE) and the junTREs not only show differences in their response to E1A, but also to treatment with UV light and phorbol esters (van Dam *et al.*, 1990; Stein *et al.*, 1992). This might indicate that the junTREs, which appear to be more related to the 8 bp cyclic AMP-responsive element (CRE) than to the 7 bp collTRE,

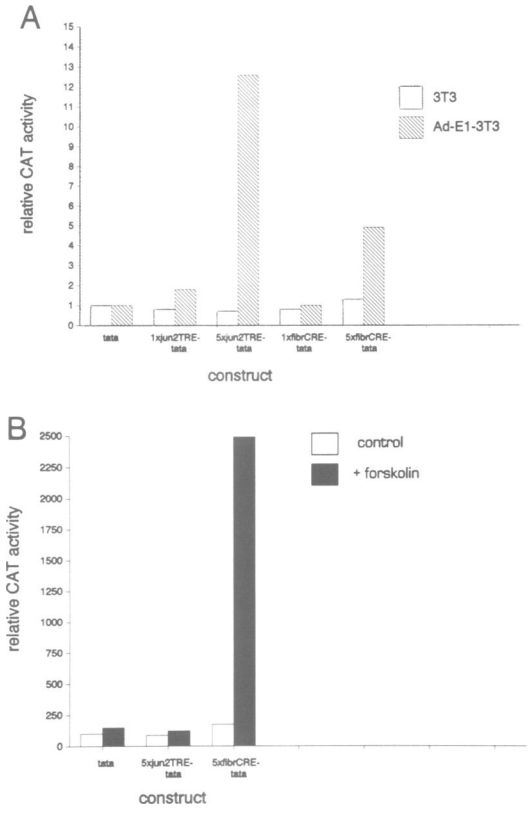

Fig. 2. Enhanced activity of the jun2TRE by E1A in NIH3T3 cells. (**A**) Untransformed and adenovirus-transformed NIH3T3 cells (Ad5E1 clone XhoC1) were transiently transfected via the $CaPO_4$ method with 6 μg of the indicated synthetic tata promoter constructs and 6 μg of carrier plasmid pSP64. Forty hours later, cells were harvested and analysed for CAT activity. The values are the mean of two independent experiments. For both cell types, CAT activity is represented in arbitrary units (tata = 1.0). (**B**) NIH3T3 cells were transiently transfected as described in A. After removal of the precipitate, the cells were incubated for 16 h in medium containing 0.5% fetal calf serum. Then 20 μM forskolin was added when indicated and after an additional 24 h cells were harvested and analysed for CAT activity.

are regulated by a different set of AP-1/ATF family members than the collTRE. Using extracts from HeLa cells, we found the factors binding to the collTRE and junTREs *in vitro* to differ in their relative affinities for these binding sites (Stein *et al.*, 1992). To examine protein binding to these elements in the absence or presence of E1A, we have chosen a cell type of which stable E1A transformants were available. We used NIH3T3 cells as these cells respond efficiently to increased cAMP levels, which enabled us to investigate the putative differences between the jun2TRE and the classical CRE (TGACGTCA) with respect to cAMP inducibility as well.

Like in HeLa tk^- cells, the complete *c-jun* promoter is stimulated upon cotransfection with a 243R-E1A expression vector in NIH3T3 cells, and both junTREs are required. In E1A-transformed 3T3 cells, the endogenous *c-jun* mRNA levels are increased ~5-fold (data not shown). In these stable E1A transformants, the activity of reporter constructs carrying copies of the fibronectin CRE are much less induced by E1A transformation than constructs carrying the jun2TRE. In contrast, treatment of NIH3T3 cells with forskolin, which increases cAMP levels via activation of adenylate cyclase, does not affect the activity of the

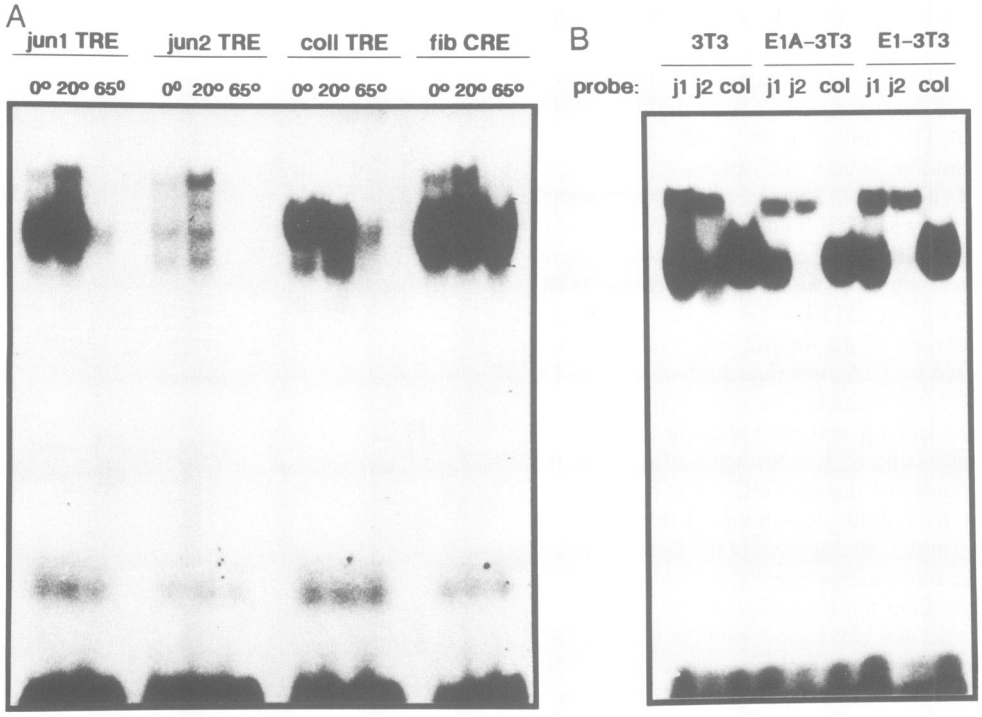

Fig. 3. Different protein complex formation with the jun1TRE, jun2TRE, collTRE and fibrCRE *in vitro*. (**A**) 15 μg of untreated (lanes 0°C and 20°C) or heat-treated (lane 65°C) extracts of NIH3T3 cells were incubated with the indicated radioactively labelled DNA elements for 30 min, either on ice (lane 0°C) or at room temperature (lanes 20°C and 65°C). The resulting DNA–protein complexes were resolved on 25 cm long native gels. Free probe is visible at the lower part of the gel. (**B**) 15 μg of extracts from untransformed, Ad5E1A-transformed (clone RC3) and Ad5E1-transformed (clone XhoC1) NIH3T3 cells were incubated with radioactively labelled jun1TRE (j1), jun2TRE (j2) or collTRE (col) and analysed by gel retardation. Free probe is just visible at the bottom of the gel.

5xjun2TRE but, as expected, strongly induces the 5xfibrCRE enhancer (Figure 2).

To verify the effect of E1A transformation on the activity of the jun2TRE and fibrCRE in a different cell system, we also analysed the jun2TRE and fibrCRE-driven tata constructs in untransformed and E1A-transformed normal rat kidney (NRK) cells. The results obtained were similar to those obtained in 3T3 cells (data not shown). In summary, these data suggest that junTREs, fibrCRE and collTRE all bind different members of the AP-1/ATF family.

Different proteins bind to the junTREs, collTRE and CRE in vitro

Since both core sequence and neighbouring bases influence protein binding to AP-1 and ATF sites (Ryseck and Bravo, 1991), we synthesized oligonucleotides containing the elements under study with at least four flanking bases on either site. To analyse factor binding to the jun1TRE, jun2TRE, collTRE and fibrCRE *in vitro*, gel retardation analysis was performed with extracts of untransformed 3T3 cells. As shown in Figure 3A, the patterns obtained with these elements are clearly different. Most strikingly, the complex binding to the consensus AP-1 binding site, the collTRE, does not or only very weakly binds to the jun2TRE. Moreover, both the jun1TRE, the jun2TRE and the fibrCRE bind a low-mobility complex which represents the main binding activity to the jun2TRE, but which does not bind to the collTRE. This low-mobility complex forms inefficiently at 0°C. Since the pattern obtained for the jun1TRE strongly resembled that obtained for the fibrCRE, we analysed whether the binding activity on the jun1TRE was resistant to heat treatment, as has been described for the CRE (Hurst *et al.*, 1990). In contrast to the fast-migrating activity on the CRE, almost all of the binding activity to the junTREs and collTRE turned out to be heat sensitive (Figure 3A, lanes 65°C) indicating that, except for the low-mobility complex, different factors are involved.

A comparison of retarded patterns obtained with the jun1TRE, jun2TRE and collTRE in untransformed and E1A-transformed 3T3 cells did not reveal major differences, except that the low-mobility complex was the only complex found to bind to the jun2TRE in the E1A transformants (Figure 3B). This suggests that also *in vivo* the low-mobility complex represents the factor that mediates the activation of the jun2TRE by E1A.

The jun2TRE binds heterodimers composed of ATF-2 and cJun

The suggestion that the low-mobility complex binding to the jun2TRE also binds to the fibrCRE and jun1TRE was confirmed by cross-competition analysis (data not shown). Therefore, this complex was likely to be composed of members of the ATF/AP-1 (super)family. However, since the binding activity to the jun2TRE is heat sensitive (see above), it is unlikely to include homo- and/or heterodimers of CREB and ATF-1, the heat-resistant factors that constitute the main binding activity to the CRE (Hurst *et al.*, 1990, 1991). The fact that the jun2TRE, in contrast to the CRE, is not inducible by cAMP (Figure 2B) also argues against CREB being a physiological jun2TRE-binding protein. Another member of the ATF family, ATF-2 (also known as CRE-BP1), appeared to be a more likely candidate: although predominantly known as a target for transactivation by 289R-E1A via conserved region 3, it can, under

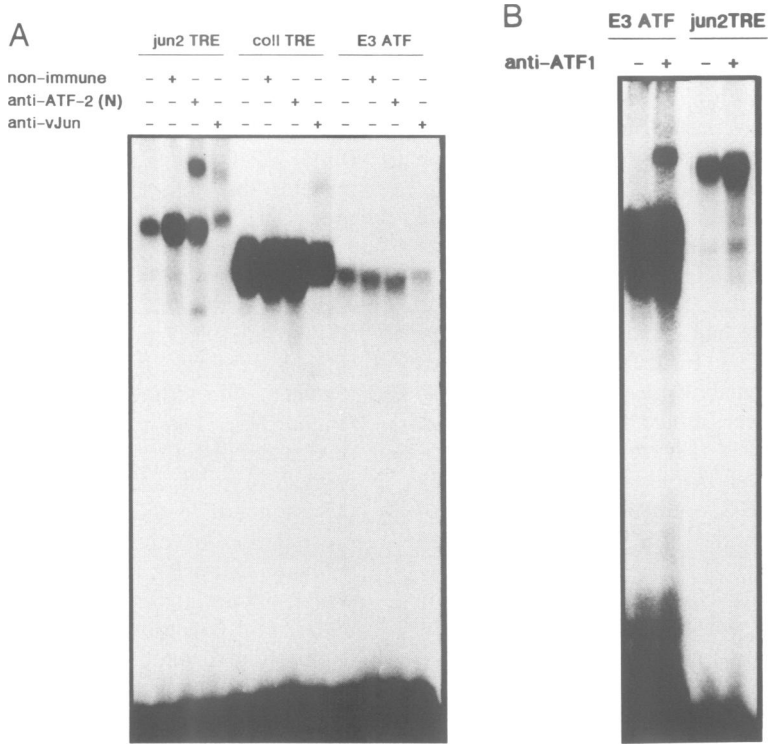

Fig. 4. Presence of cJun and ATF-2 in the protein complex binding to the jun2TRE. (**A,B**) Extract from Ad5E1A-transformed NIH3T3 cells (clone RC1) was incubated for 2 h on ice in the presence or absence of the indicated antibodies (see Materials and methods). After addition of the indicated radioactively labelled DNA elements and 30 min incubation at room temperature, the resulting DNA–protein complexes were resolved on native gels. (The antibodies themselves do not exhibit any binding activity; not shown).

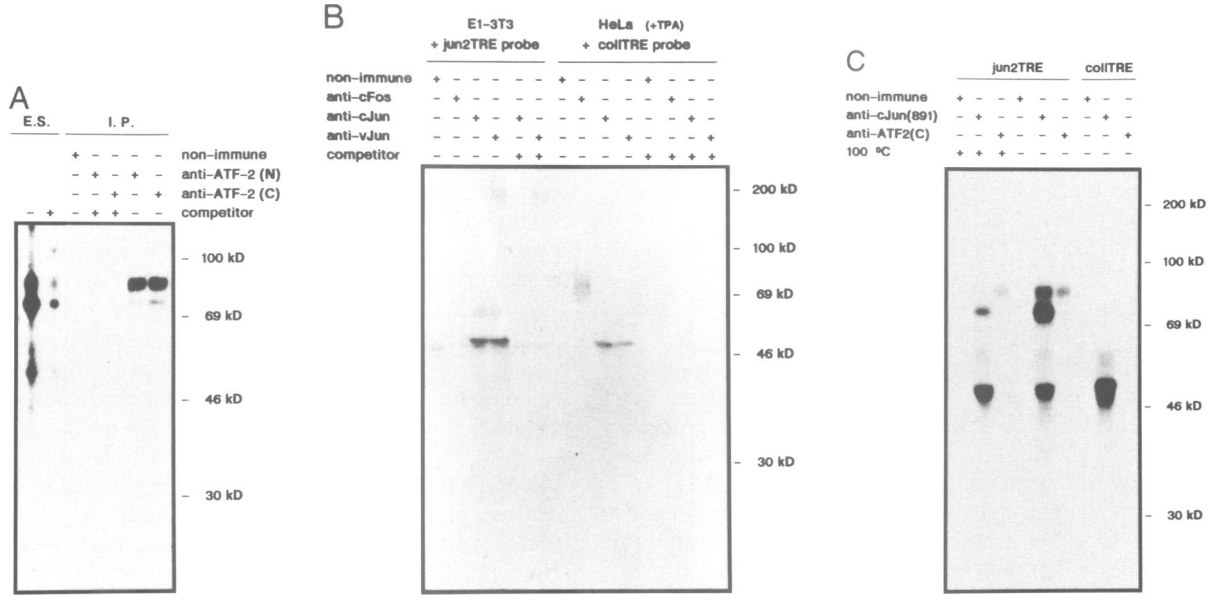

Fig. 5. Immunoprecipitation of ATF-2 and cJun after UV cross-linking to the jun2TRE. (**A**) 20 μg of extract from Ad5E1-transformed NIH3T3 cells (clone XhoC1) were incubated with a radioactively labelled jun2TRE probe in the presence or absence of a 20-fold excess of non-labelled homologous competitor and subsequently irradiated with UV (see Materials and methods). The resulting DNA–protein complexes were resolved on a native gel, excised and submitted to SDS–gel electrophoresis (ES: excised shift). For immunoprecipitations (IP), the binding mixtures were diluted in RIPA buffer directly after UV irradiation and incubated with the indicated antibodies (see Materials and methods). For each precipitation, 50 μg of cell extract were used. (**B**) Immunoprecipitation of proteins cross-linked to the jun2TRE and collTRE, performed similarly as described in A, except that for the collTRE probe 10 μg of extract from HeLa tk^- cells stimulated for 4 h with TPA, were used. (**C**) Extract of AdE1-transformed 3T3 cells was diluted in E1A* buffer (see Materials and methods) after UV cross-linking. In the case of the jun2TRE, half of the extract was boiled for 5 min prior to the addition of the antibodies.

certain conditions, also be stimulated by 243R–E1A, but is not inducible by cAMP (Liu and Green, 1990; Flint and Jones, 1991; Maekawa *et al.*, 1991). Therefore, we examined the effect on the jun2TRE-bound protein complex of an ATF-2-specific antibody that is able to 'supershift' ATF-2-containing DNA–protein complexes in gel retarda-

tion analyses (N.Jones, unpublished results). Since cJun can form heterodimers with ATF-2 (Benbrook and Jones, 1990; Ivashkiv *et al.*, 1990; Hai and Curran, 1991), we also used an antibody that recognizes cJun. As controls we analysed the effects of these antibodies on the binding activity to the collTRE and the minimal ATF site present in the Ad2E3 promoter, and included an antibody specific for ATF-1.

As shown in Figure 4A, the antibodies against ATF-2 and Jun both supershift the jun2TRE-bound complex formed with an extract of E1A-transformed 3T3 cells, whereas only the Jun antibody supershifts the collTRE-bound complex. Neither of these antibodies had an effect on the protein binding obtained with the E3ATF site. (Also, on an 8 times longer exposure no extra bands were visible; not shown.) The ATF-1 antibody, however, shifted the E3ATF complex, but not the jun2TRE complex (Figure 4B). These experiments thus strongly suggest that the jun2TRE complex contains both cJun and ATF-2, and that the composition of the complexes formed on the jun2TRE, collTRE and AdE3ATF site is different.

To confirm the presence of cJun and ATF-2 epitopes by a second line of evidence, we radioactively labelled the jun2TRE and collTRE-binding proteins by covalent cross-linking to the DNA probes with UV light. Subsequently, immunoprecipitations were performed with different antibodies. As can be seen in Figure 5A, both the ATF-2(N) antibody, raised against a peptide located in the N-terminal 100 amino acids, and the ATF-2(C) antibody, raised against a C-terminal peptide (see Materials and methods), recognize two proteins specifically cross-linked to the jun2TRE probe with apparent mol. wts of ~75 and 85 kDa. (Note that cross-linking increases the molecular weight by at least 5 kDa.) At the leftmost two lanes (ES), two cross-linked proteins with similar molecular weights can be detected when the DNA–protein complex is first separated on a non-denaturing gel and the gel slice containing the retarded complex is subsequently run on a denaturing gel. The 75 kDa protein is recognized less efficiently by the antibodies than the 85 kDa protein, as it shows up weakly in the immunoprecipitations, but is cross-linked very efficiently. It might, therefore, be a modified version of ATF-2 or an ATF-2-related protein. In addition to the 75 and 85 kDa proteins, the excised jun2TRE complex also contains a cross-linked protein with an apparent mol. wt of ~50 kDa. (Note that all three proteins are cross-linked much less efficiently in the presence of a 20-fold excess of competitor probe.) To investigate whether this 50 kDa protein is cJun, as was suggested by the supershift shown in Figure 4A, we performed immunoprecipitations both with the antibody used in Figure 4A and a second antibody raised against the C-terminal peptide of cJun (see Materials and methods). As can be seen in Figure 5B, both antibodies indeed recognize a protein of 50 kDa specifically cross-linked to the jun2TRE in E1A-transformed 3T3 cells. Moreover, a protein of similar size is also detected after cross-linking to the collTRE, both with extracts from TPA-treated HeLa cells and E1A-transformed 3T3 cells (see also Figure 5C). This 50 kDa protein cannot be JunB or JunD since the anti-cJun and anti-cJun (891) antibodies used in Figure 5B and C are raised against regions that are unique for cJun. Moreover, anti-cJun (891) was found by immunofluorescence to react only with cJun, but not with JunB or JunD (using transiently transfected F9 cells; not shown). Figure 5B also shows that the proteins cross-linked to the jun2TRE cannot be precipitated by a cFos-specific antibody, in contrast to one of the proteins cross-linked to the collTRE.

The immunoprecipitations shown in Figure 5A and 5B were performed in a stringent buffer containing 0.1% SDS, 0.1% NP40 and 0.1% deoxycholate to ensure that only proteins that interact directly with the antibodies were precipitated. Under these conditions, the dimerization between cJun and cFos is disrupted (data not shown). However, in a mild buffer containing only 0.1% Triton X-100 as detergent, two jun2TRE cross-linked proteins of 75 and 85 kDa, migrating at the same positions as the proteins recognized by the ATF-2 antibodies, are precipitated together with cJun by the cJun-specific antibody 891 (Figure 5C). The larger two proteins are unlikely to be recognized directly by the 891 antibody since boiling of the lysate prior to the addition of the antibody strongly reduced the amount of 75 and 85 kDa proteins without affecting the precipitation of cJun itself. Moreover, a second cJun-antibody, anti-pep1, raised against a different region of cJun, was also found to coprecipitate the 75 and 85 kDa proteins under mild conditions (data not shown). These data thus demonstrate that both cJun and ATF-2 directly interact with the jun2TRE probe and are present in the same complex, presumably as heterodimers. (Note that in a given dimer only one of the partners is covalently linked to the labelled probe since the cross-linking efficiency is rather low).

Discussion

In this paper, we delimit the 243R-E1A-responsive elements in the human *c-jun* promoter. These elements correspond to the previously identified TPA-responsive elements in this promoter, the junTREs, which resemble both 'classical' AP-1 binding sites and ATF/CREB sites. The more decisive role in the response to E1A can be attributed to the distal TRE, the jun2TRE. Mutation of this element in the complete promoter strongly reduces E1A inducibility and it is efficiently activated by E1A in a heterologous promoter when present as a multimer, in contrast to the proximal TRE in the *c-jun* promoter, the jun1TRE. The jun1TRE contributes, but does not respond significantly to E1A by itself. We furthermore show that in the *c-jun* promoter the two TREs cooperate with additional transcription factor binding sites, including the CTF element, in mediating the maximal E1A response. Recently, the induction of *c-jun* during differentiation of F9 embryonal carcinoma (EC) cells by retinoic acid or adenovirus infection has also been found to require multiple promoter elements. Kitabayashi *et al.* (1992) identified five motifs in the rat *c-jun* promoter, designated retinoic acid- and E1A-responsive elements (REREs), one of which overlapped with the jun1TRE. Interestingly, the jun2TRE also overlaps with one of these REREs, suggesting that the jun2TRE plays a role in the regulation of *c-jun* during differentiation as well, again in combination with other elements.

In vitro binding studies revealed that the jun2TRE binds protein complexes which contain both cJun and ATF-2 or ATF-2-related proteins. Since *in vitro* translated cJun and ATF-2 have been shown to form heterodimers (Benbrook and Jones, 1990; Ivashkiv *et al.*, 1990; Hai and Curran, 1991), the jun2TRE-binding complexes most likely represent such heterodimers. The two ATF-2 anti-peptide

antibodies used in this study recognize both a 75 and an 85 kDa protein covalently cross-linked to a jun2TRE probe, of which the 75 kDa protein appears to be precipitated rather inefficiently. These proteins may be encoded by two different mRNAs, as multiple ATF-2/CRE-BP1(-like) cDNA clones have been described with differences in size and sequence (Maekawa *et al.*, 1989; Gaire *et al.*, 1990; Ivashkiv *et al.*, 1990; Georgopoulus *et al.*, 1992). Some of these differences appear to be due to alternative splicing, but two of the cDNA clones, ATF-a and ATF-δa, seem to correspond to an ATF-2-related gene. It is also possible that a subset of the ATF-2 proteins in the cell are modified post-translationally and therefore migrate with different electrophoretic mobility and interact less efficiently with the antibodies.

Using transient transfection experiments, cJun–ATF-2 heterodimers have been reported to exhibit only a very low basal transactivating activity, in contrast to cJun homodimers (Benbrook and Jones, 1990). Consistent with these findings, we found that the jun2TRE fails to increase the activity of a heterologous promoter significantly in the absence of E1A (e.g. compare the basal activities of the TK and jun2TRE–TK constructs in Figure 1D). Since both cJun homodimers and cJun–ATF-2 heterodimers interact directly with the *c-jun* promoter (Angel *et al.*, 1988b; this paper), this difference in activity is particularly interesting with respect to the autoregulation of cJun. As we and others reported previously, the cJun protein appears to be implicated both in positive and negative autoregulation (Angel *et al.*, 1988b, van Dam *et al.*, 1990; Castellazi *et al.*, 1990). These opposite effects may thus be determined by the second component of the dimer and involve the replacement of inactive factors by active ones, and vice versa.

The stimulation of the jun2TRE by E1A may be mediated by either cJun, or ATF-2, or both proteins. ATF-2 is already known as a target for transactivation by the 289R-E1A-specific conserved region 3, either when fused to the GAL4 protein or to the cMyb protein (Liu and Green, 1990; Flint and Jones, 1991; Maekawa *et al.*, 1991). In the latter case, it was also found to be stimulated by 243R, albeit only weakly. Transactivation of ATF-2 by 243R may be stronger when ATF-2 is dimerized to cJun. In addition, the extent of stimulation may depend on the promoter context, as is the case in the *c-jun* promoter.

Dimerization to ATF-2 has been found to alter the DNA-binding specificity of cJun, causing it to bind preferentially to the 8 bp CRE motif (Benbrook and Jones, 1990; Ivaskiv *et al.*, 1990; Hai and Curran, 1991). The data presented here indicate that the differences in the binding specificities of cJun–cJun and cJun–cFos, on the one hand, and cJun–ATF-2, on the other, can account for the opposite regulation of the 7 bp collagenase TRE and the 8 bp jun2TRE by E1A. Although cJun homodimers bind only with a low affinity to the jun2TRE, constructs driven by one or multiple jun2TREs can be induced strongly by experimental overexpression of cJun. As already described for the jun1TRE (van Dam *et al.*, 1990), we found cJun homodimer-induced activity of the jun2TRE to be repressed by E1A (H.van Dam, unpublished results). Apparently, E1A can distinguish between cJun–cJun and cJun–ATF-2 in imposing opposite states of activity. We have tried to compare the E1A-dependent regulation of cJun–ATF-2, cJun–cJun, and ATF-2–ATF-2 via transient cotransfection into F9 cells, as these cells do not express endogenous cJun. However, these attempts were hampered by the presence of an as yet uncharacterized ATF activity, absent in HeLa, 3T3 or NRK cells, which shows high affinity for the jun2TRE and which is also inducible by 243R-E1A (H.van Dam, unpublished results).

Since the opposite effects of 243R-E1A on consensus 7 bp AP-1 binding sites, as present in the collagenase promoter, and 8 bp AP-1/ATF motifs, as found in the *c-jun* promoter, both depend on the transforming domain of E1A encoded by conserved region 1 (Offringa *et al.*, 1990; van Dam *et al.*, 1990), this differential regulation may play a crucial role in E1A-induced transformation. The increased levels of cJun protein in E1A-transformed cells apparently lead to increased expression of only a specific subset of cJun-inducible genes, i.e. the ones regulated by jun2TRE-like motifs. cJun is believed to have a key function in the regulation of cell proliferation and differentiation because it mediates responses to a multitude of extra- and intracellular stimuli (Angel and Karin, 1991). Moreover, it can function as a transforming protein itself when overexpressed or when specific amino acid residues are mutated, and also appears to be essential for transformation by the *ras* oncogene (Bos *et al.*, 1990; Alani *et al.*, 1991; Lloyd *et al.*, 1991). However, the cJun-inducible genes that are directly responsible for the establishment of the transformed phenotype are as yet unknown. Importantly, by analysis of *v-jun* and *c-jun* mutants in chicken embryo fibroblasts, Vogt and collaborators found an inverse correlation between transformation and transcriptional activation of collagenase and stromelysin, which are both regulated via consensus AP-1 binding sites (Havarstein *et al.*, 1992; M.Hartl and P.K.Vogt, personal communication). Another example of a correlation between transformation and inhibition of collTRE activity is provided by dexamethasone, which has been found to support polyomavirus-dependent transformation (Martens *et al.*, 1988). Like E1A, dexamethasone downregulates the expression of collagenase and stromelysin via inhibition of cJun/cFos, but induces *c-jun* expression (Offringa *et al.*, 1990; Jonat *et al.*, 1990). cJun-dependent genes involved in the establishment of a transformed phenotype may thus, at least in some cell types, not be regulated by cJun–cJun or cJun–cFos via consensus AP-1 sites, but by cJun–ATF-2 via junTRE- and CRE-like elements. Identification of target genes relevant for *c-jun*-dependent transformation may thus help to unravel the role of cJun in the regulation of growth control and, in addition, to explain transformation by the adenovirus E1A genes.

Materials and methods

Plasmid constructs

The *c-jun* promoter–CAT constructs −1600/+740, −1600/+740 mjun1 (previously described as −1600/+740 mTRE), −132/+740 and −1600/+170 have been described elsewhere (Angel *et al.*, 1988b; van Dam *et al.*, 1990; Stein *et al.*, 1992). −1600/+740 mjun2 and −1600/+740 mjun1+2 were obtained by replacing the *Sac*I inserts of −1600/+740 mjun2 and −1600/+170 mjun1+2 (Stein *et al.*, 1992) by the corresponding fragment of −1600/+740. −283/+740, −195/+740, −167/+740 and −79/+740 were derived similarly from −283/+170 (Stein *et al.*, 1992) and other corresponding '+170' clones. −445/+740 was generated by cloning the −445/−132 *Sma*I–*Nco*I fragment of −1600/+740 into a *Hin*dIII (filled-in)–*Nco*I vector derived from −1600/+740. −1600/+740 mCTF and −1600/+740 mSp1 were obtained by first converting −132/+170 mSP1 and −132/+170 mCTF (I.Herr and P.Angel, unpublished data) in their −1600/+170 derivatives by exchanging the *Nco*I fragments, and by subsequently replacing the *Stu*I inserts by the correspon-

ding fragment of −132/+740. [In the mSP1 construct, the −120 CGGGCCCGCCC −110 sequence has been replaced by −120 AATTCCTGCAG −110; in the mCTF construct the −91 CCAAT −87 sequence has been replaced by −91 ATATC −87. The mutations introduced in the mjun1 and mjun2 constructs are: −191 ATTACCTCATC −181 into TGGACCTCGAG and −71 TGACAT −66 into ATCCAC (Angel *et al.*, 1988b; Stein *et al.*, 1992)].

pRSV-JF12, pRSV-neo, pTK-CAT (identical to pBLCAT4) and tata-CAT have been described previously (Jonat *et al.*, 1990; Offringa *et al.*, 1990). The other TK-CAT and tata-CAT plasmids, containing single or multiple copies of the fibronectin CRE, jun1TRE or jun2TRE, were constructed by ligation of the corresponding monomeric or multimeric double-stranded oligonucleotides (see below) into *Hin*dIII/*Bam*HI or *Bam*HI linearized vector plasmid.

Cell lines and cell culture

HeLa tk$^-$ and NIH3T3 cell clones have been described previously (Angel *et al.*, 1987; Schönthal *et al.*, 1988). Ad5E1- and Ad5E1A-transformed NIH3T3 derivatives were kindly provided by A.G.Jochemsen and were obtained by transfection with plasmids p5E1B-neo (containing the Ad5E1B promoter region in front of the coding region of the neomycin resistance gene; A.G.Jochemsen, unpublished results) and p5XhoI-C (Bernards *et al.*, 1982) or pRSV-5E1A (Offringa *et al.*, 1990) using the calcium phosphate method and selection for G418 resistance. These cells express high levels of E1A protein, as examined by immunofluorescence. All cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics.

Transient transfection and analysis of gene expression

Transfection of HeLa tk$^-$ cells and NIH3T3 cells, using the DEAE−dextran or calcium phosphate method, followed by chloramphenicol acetyltransferase (CAT) assays, were performed as described (Angel *et al.*, 1987). To accurately quantify low amounts of CAT activity, up to 1.2 mg of total protein extract was used per reaction. Prior to the incubation with substrate, the extract was incubated for 5′ at 65°C and denatured proteins were removed by centrifugation. CAT activity was quantified by liquid scintillation counting of excised sections of thin-layer plates and expressed as percent conversion of [^{14}C]chloramphenicol to its acetylated derivatives.

Cell extracts and gel retardation

Cell extracts were prepared from rapidly growing subconfluent cultures from which medium had been changed 1 day prior to isolation. Cells were washed twice with ice-cold phosphate-buffered saline (PBS), harvested with a rubber policeman and centrifuged at 4°C. Cell pellets were resuspended in 1 vol. of ice-cold lysis buffer [20 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulphonic acid)-KOH (pH 7.9), 600 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM phenyl methyl sulphonyl fluoride (PMSF), 0.5 mM trypsin inhibitor, and phosphatase inhibitors (50 mM NaF and 1 mM Na_3VO_4)], incubated on ice for 30 min and centrifuged at 10 000 *g*. The supernatant was subsequently diluted with 1 vol. of buffer II ('lysis' buffer containing 20% glycerol instead of KCl).

Gel retardation assays were performed as described elsewhere (Stein *et al.*, 1989), using double-stranded oligonucleotides as probes obtained by annealing of 5′ AGCTGGGGTGACATCATGGG 3′ and 5′ GATCCCCATGATGTCACCCC 3′ (jun1TRE: *c-jun*, position −75 to −60), AGCTAGCATTACCTCATCCC 3′ and 5′ GATCGGGATGAGGTAATGCT 3′ (jun2TRE: *c-jun*, position −194 to −179), 5′ AGCTCCCGTGACGTCACCCG 3′ and 5′ GATCCGGGTGACGTCACGGG 3′ (fibrCRE: fibronectin, position −177 to −162), 5′ GATCCGGCTTTCGTCACAGG 3′ and 5′ AGCTCCTGTGACGAAAGCCG 3′ (E3ATF:AdE3, positions −65 to −50) and 5′ AGCT AGCATGAGTCAGACAC 3′ and 5′ AGCTGTGTCTGACTCATGCT 3′ (collTRE: collagenase, position −76 to −61). In most of the experiments, 5 mM $MgCl_2$ was added to the incubation buffer, which was found to stabilize the specific DNA−protein complex formed at the jun2TRE. Unless indicated otherwise, 5 μg of whole cell extract were incubated with 10 fmol of ^{32}P-labelled probe for 30 min at room temperature, after which DNA−protein complexes were separated on 4% Tris−glycine gels (Buratowski *et al.*, 1989). Competition experiments were performed by pre-incubating the extracts with the unlabelled competitor for 5 min. To examine the effect of heat treatment, extracts were incubated for 10 min at 65°C, after which denatured proteins were removed by centrifugation. To test the effect of specific antibodies 2 μl of antiserum (see below) were added to the incubation mixture on ice 2 h prior to the addition of labelled probe.

UV cross-linking and immunoprecipitation

To synthesize the jun2TRE probe, a 32-base oligonucleotide template, containing the sequence between −202 and −171 of the human *c-jun* gene, was annealed to an oligonucleotide primer complementary to the final (3′) 12 bases of the template and filled in with [^{32}P]dATP, [^{32}P]dCTP, dGTP and 50% dTTP:50% bromo-dUTP (Sigma) by DNA polymerase, Klenow fragment. The collTRE probe was obtained similarly, using a 32-base oligonucleotide containing the sequence between −76 and −61 of the human collagenase gene. After incubation with protein extract, the reaction mixtures were irradiated (on ice) for 20 min with UV (254 nm, 80 kJ/m^2). For immunoprecipitation studies, reaction mixtures were diluted at least 4-fold in RIPA buffer [10 mM Hepes (pH 7.9), 150 mM NaCl, 0.1% sodium deoxycholate (DOC), 0.1% NP40, 0.1% sodium dodecyl sulphate (SDS), 0.5 mM PMSF, 0.5 mM trypsin inhibitor] or E1A* buffer [50 mM Tris (pH 7.5), 5 mM EDTA, 125 mM NaCl, 0.1% Triton, 0.5 mM PMSF, 0.5 mM trypsin inhibitor] and incubated with antibodies coupled to protein A−Sepharose beads for at least 3 h. To analyse the proteins cross-linked to the jun2TRE probe directly, reaction mixtures were separated on a non-denaturing gel after UV irradiation, DNA−protein complexes were visualized by autoradiography ('wet exposure') and the gel slices corresponding to the jun2TRE shift were cut out and boiled in 40 μl of SDS sample buffer. Labelled proteins were analysed on 10% SDS−polyacrylamide gels.

Rabbit polyclonal antibodies: anti-ATF-1, raised against purified ATF-1 from HeLa cells (Hurst *et al.*, 1990, 1991), anti-ATF-2 (N), raised against amino acids 85−97 of ATF-2/CRE-BP1, anti-ATF-2 (C), raised against amino acids 490−505 of ATF-2/CRE-BP1, all kindly provided by N.C.Jones. Anti-vJun, raised against the complete trypE-vJun protein (Angel *et al.*, 1988a), kindly provided by H.J.Rahmsdorf, anti-cJun (891), raised against amino acids 47−59 of human cJun, kindly provided by A.Darling, anti-cFos (Ab-2), raised against amino acids 4−17 of human cFos, obtained from Oncogene Science.

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