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Interference between pathway-specific transcription factors: glucocorticoids antagonize phorbol ester-induced AP-1 activity without altering AP-1 site occupation *in vivo*

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Phorbol esters stimulate and glucocorticoid hormones down-regulate a variety of promoters such as that of the collagenase gene through the transcription factor AP-1 (Fos/Jun). We now show by genomic footprinting of the collagenase promoter that phorbol ester treatment of cells results in the binding of AP-1 to its cognate DNA binding site *in vivo*. The DNA – protein contacts obtained in living cells are also found *in vitro* using cloned DNA and purified AP-1. Although *in vitro* synthesized glucocorticoid receptor can disturb the DNA binding of Jun homodimers, it does not interfere with the binding of Fos – Jun heterodimers or of purified AP-1 *in vitro*. Consistently, fully inhibitory doses of glucocorticoid hormone cause no change in apparent occupation of the AP-1 binding site *in vivo*. The hormone receptor acts without itself binding to DNA.

Key words: AP-1 binding site/collagenase transcription/glucocorticoid repression/*in vivo* footprinting/phorbol ester induction

Introduction

The transcription of AP-1 (Fos/Jun) dependent genes and the activity of AP-1 can be induced by phorbol ester tumor promoters (Angel *et al.*, 1987a,b; Boyle *et al.*, 1991) and inhibited by glucocorticoid hormones (Mordacq and Linzer, 1989; Diamond *et al.*, 1990; Jonat *et al.*, 1990; Lucibello *et al.*, 1990; Schüle *et al.*, 1990; Yang-Yen *et al.*, 1990), retinoic acid (Schüle *et al.*, 1991), thyroid hormones (Zhang *et al.*, 1991) and the adenovirus E1a protein (Offringa *et al.*, 1988, 1990). The 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) responsive element (TRE) (Angel *et al.*, 1987b) of the human collagenase gene is located between positions −73 and −65 with respect to the transcriptional start site, initially identified by deletion analysis of the collagenase promoter and by *in vitro* DNase I footprinting (Angel *et al.*, 1987a,b). It binds the transcription factor AP-1 (Angel *et al.*, 1987b). Transcriptional repression of the collagenase gene by glucocorticoid hormones appears to be mediated by an interaction between the glucocorticoid receptor (GR) and AP-1 (Jonat *et al.*, 1990; Yang-Yen *et al.*, 1990; Touray *et al.*, 1991). To investigate the mechanism of interference between AP-1 and the GR *in vivo*, we performed genomic footprinting experiments in normal diploid human skin fibroblasts under conditions of phorbol ester induction and glucocorticoid-mediated repression of collagenase transcription. The experiments supply evidence for the phorbol ester-induced DNA binding of AP-1 *in vivo* and for an indirect mechanism of AP-1 inhibition by the steroid hormone receptor involving protein – protein interaction.

Results and discussion

Genomic dimethyl sulfate (DMS) footprinting analyses were performed in untreated human normal skin fibroblasts and in cells treated with either the glucocorticoid dexamethasone, with the phorbol ester TPA, or with a combination of TPA and dexamethasone. The pattern of DMS reactivity of guanines in the collagenase 5′-flanking region was analyzed by the ligation-mediated polymerase chain reaction (PCR) technique (Mueller and Wold, 1989). Figure 1A shows an example of the *in vivo* footprinting analyses. In this case the analysis was performed at 6 h after addition of the agents. Low basal level DMS protections and hyper-reactivities of guanine residues were detected in untreated cells at the TRE sequence within box 2 (Figure 1A, compare lane n, 'naked' DNA, with lane C, non-treated cells). TPA treatment increased these protections and hyper-reactivities at the TRE indicating phorbol ester-induced protein binding to the TRE *in vivo* (Figure 1A; quantifications in Figure 1B). In order to confirm that the DMS reactivity pattern observed *in vivo* at the TRE is due to the presence of bound AP-1, we performed *in vitro* methylation experiments using cloned collagenase 5′-flanking sequences and affinity purified AP-1 (Figure 2). The DMS protections and hyper-reactivities observed at guanines of the TRE sequence are almost identical to those detected in living cells (Figure 2A) suggesting that the TPA-induced changes in DMS reactivity at guanines within the TRE seen *in vivo* are caused by interaction of AP-1 (or a closely related factor) with its DNA binding site.

Interestingly, TPA also induced changes in the DMS reactivity pattern within several other protein binding sites downstream (box 1) and upstream (boxes 3 – 5) of the TRE (Figure 1A). These boxes have previously been identified as binding sites of proteins by *in vitro* DNase I footprinting (Angel *et al.*, 1987b). One of these boxes (box 3) contains a PEA3-like motif which can bind the transcription factor PEA3 (Gutman and Wasylyk, 1990); the proteins binding to the other sites are as yet unknown. The TPA-induced *in vivo* occupation of protein binding sites upstream of the TRE is consistent with the finding that, although the TRE is absolutely required for TPA induction, full TPA responsiveness of the collagenase gene depends on additional sequences upstream of the TRE (Angel *et al.*, 1987a).

Inhibiting doses of dexamethasone added simultaneously with TPA did not change the pattern of protection and hyper-reactivity at the AP-1 binding site induced by TPA (Figure 1A and B) with the exception of the guanine at position −71 which was marginally less well protected after dexamethasone treatment. Induction by TPA and repression of collagenase transcription by dexamethasone was confirmed

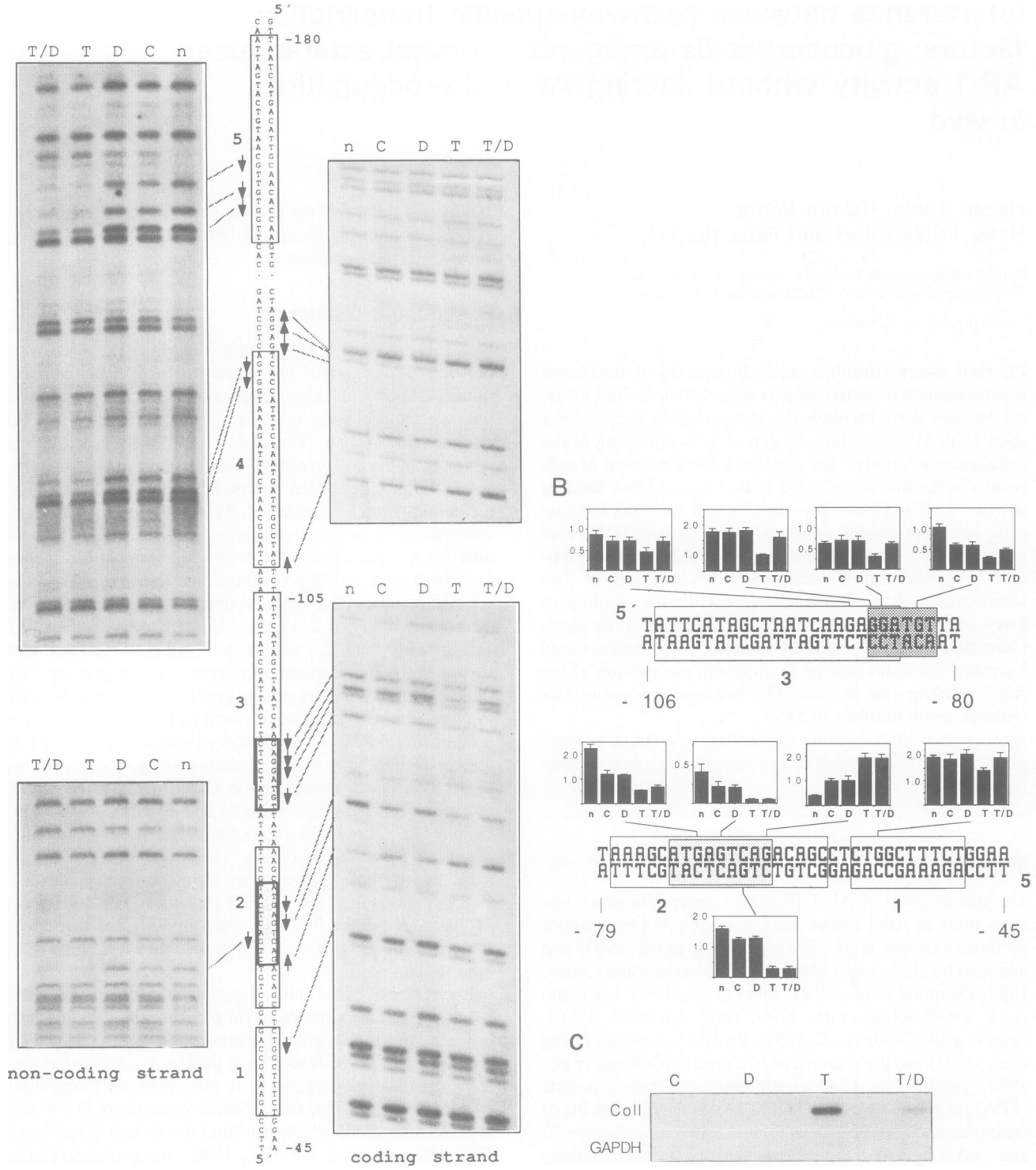

Fig. 1. *In vivo* DMS reactivity of the collagenase 5′-flanking region in human diploid skin fibroblasts. (**A**) Guanine ladders for the coding and non-coding strand between 180 and 45 bp upstream of the transcriptional start site. n, *in vitro* methylated protein-free ('naked') genomic DNA (always shown next to the sequence, Angel *et al.*, 1987a); C, DNA from untreated cells; D, DNA from cells treated with dexamethasone (10^{-7} M for 6 h); T, DNA from TPA-treated cells (100 ng/ml for 6 h); T/D, DNA from cells treated with TPA and dexamethasone simultaneously (for 6 h); boxes 1–5, protein binding sites previously identified by *in vitro* DNase I footprinting (Angel *et al.*, 1987b); bold box within box 2, TRE sequence (Angel *et al.*, 1987b); bold box overlapping box 3, PEA3 recognition sequence (Gutman and Wasylyk, 1990); arrowheads pointing upward and downward indicate hyper-reactivity to and protection from DMS, respectively. (**B**) Quantitative analysis of DMS reactivity in boxes 1–3. Only the DMS reactivity of those guanines that are affected significantly is shown. The bars represent means of at least three independent experiments. Standard errors of the means are indicated above bars. (**C**) Nuclear run-on analysis of gene transcription of cells treated in parallel with those used for one of the genomic footprinting analyses. Coll, collagenase gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase gene.

by nuclear run-on analysis using nuclei from parallel cultures of cells (Figure 1C).

The apparent DNA binding properties of AP-1 *in vivo* and the TRE occupation were identical when the order of addition of TPA and dexamethasone was changed. Dexamethasone applied 30 min prior to TPA could not prevent AP-1 binding

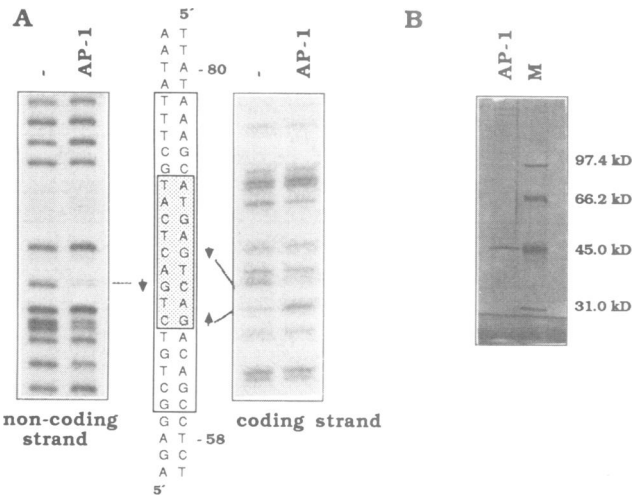

Fig. 2. Changes in DMS reactivity at the TRE following binding of AP-1 *in vitro*. (**A**) Guanine ladders obtained for the coding and non-coding strands. −, without protein; AP-1, with affinity-purified AP-1. Symbols as in Figure 1A. (**B**) Silver-staining of the AP-1 fraction used in A after SDS−PAGE. M, protein molecular weight markers, sizes are indicated in kDa; AP-1, affinity purified AP-1 fraction.

to the TRE although repressing transcription (Figure 3A, lanes 2−4 and 9−11; Figure 3B). Moreover, dexamethasone added 5.5 h after TPA, equivalent to 30 min prior to DMS footprinting, inhibited collagenase transcription, again without dislodging AP-1 from its binding site (Figure 3A, lanes 1 and 3, 10 and 12; Figure 3B). Thus, the GR represses collagenase transcription without affecting DNA binding of AP-1, irrespective of its activation prior to that of AP-1, or after AP-1 had already bound to DNA.

Surprisingly, dexamethasone caused some partial reversions of protections within the PEA3 recognition sequence (Gutman and Wasylyk, 1990) in box 3 (nucleotide positions −83, −86, −87 and −89) and within box 1 (−56) (Figure 1A and B). Since these boxes can be deleted without abolishing inhibition by dexamethasone (Jonat *et al.*, 1990; Schüle *et al.*, 1990), these changes in DNA−protein interaction do not seem to be required for repression of collagenase transcription. They may reflect steric hindrance in protein binding to these sites caused by GR association with AP-1 bound to the neighboring TRE.

The experiments thus show, that *in vivo*, phorbol esters induce the binding of AP-1 to its cognate DNA binding site and that the inhibitory action of glucocorticoids does not alter DNA−protein contacts within the TRE while blocking transactivation. The latter suggests that there is no competition of GR for AP-1 binding *in vivo*. The data are consistent with our previous observation that *in vitro* AP-1 binding activity is not reduced in extracts from dexamethasone-treated cells (Jonat *et al.*, 1990). A complementary result was obtained in gel retardation experiments using a TRE oligonucleotide and *in vitro* synthesized Fos/Jun in the presence or in the absence of *in vitro* synthesized GR (Figure 4). The *in vitro* synthesized GR forms a specific complex with an oligonucleotide probe comprising the palindromic glucocorticoid response element (GRE) (Figure 4B, lanes 2−4) indicating that a functional GR (at least with respect to DNA binding activity) had been produced *in vitro*. Fos and Jun form the heterodimeric complex of AP-1 which is the transcription factor activating the collagenase promoter (Chiu *et al.*, 1988; Schönthal *et al.*, 1988). The specific gel retardation complex formed with the TRE oligonucleotide (Figure 4C, see competitions in lanes 11 and 12) is not affected by increasing amounts of GR (maximum amount shown in Figure 4C, lanes 5 and 6). Purified AP-1 forms a complex of the same mobility and its formation is also not disturbed by GR (lanes 7 and 8). GR itself does not bind to the TRE (lane 2). However, in the presence of GR there was no further retardation of the Fos/Jun and AP-1 complexes detectable as one could expect from an association of GR with Fos/Jun and purified AP-1. Because the complexes seem to be very unstable *in vitro* in the absence of chemical cross-linking agents (Diamond *et al.*, 1990; Yang-Yen *et al.*, 1990), the lack of a supershift is probably due to the disruption of GR association during gel resolution. Interestingly, Jun homodimers form a specific complex (see competitions in lanes 9 and 10) of slightly lower mobility and this complex is partially disrupted by GR (Figure 4C, lanes 3 and 4). The complex formed by Jun homodimers is known to be considerably less stable than the heterodimeric Fos/Jun complex (Rauscher *et al.*, 1988; Smeal *et al.*, 1989). Thus, the Jun homodimers are disrupted by GR interaction *in vitro* and perhaps also *in vivo*, which might be due to conformational constraints imposed by GR interaction. These data are in agreement with a similar report on the behavior in the presence of GR of complexes formed by a bacterially expressed truncated Jun protein (Yang-Yen *et al.*, 1990).

Taking into account (i) the absence of any change in TRE occupation *in vivo*, (ii) the lack of interference of GR with binding to the TRE by the heterodimeric Fos/Jun *in vitro*, and (iii) the co-precipitation of Fos/Jun and GR described previously (Diamond *et al.*, 1990; Jonat *et al.*, 1990; Yang-Yen *et al.*, 1990; Touray *et al.*, 1991), the following possibilities can be envisaged as mechanisms for the functional interference between two *bona fide* transcription factors. (a) GR modifies AP-1; (b) GR titrates out an AP-1 restricted activator (squelching); (c) GR prevents Fos/Jun from DNA binding, thus allowing a non-transactivating member of the AP-1 family (which would have to establish the same DNA−protein contacts) to occupy the TRE; (d) GR blocks the transactivating potential of AP-1 (but not its binding to DNA) by direct protein−protein interaction.

While modification remains an option (so far we have been unable to detect by two-dimensional peptide mapping any modification in phosphorylation or anything else after hormone treatment), the squelching is made unlikely by detection of mutants in the GR (Cato,A.C.B., Besenbeck,B., Nebl,G., Ponta,H. and Herrlich,P., in preparation) that disturb the negative action without interfering with the inherent transactivating potential of the mutant transcription factor. Furthermore, mutants of GR that lack transactivating domains, still repress (Jonat *et al.*, 1990; Schüle *et al.*, 1990; Yang-Yen *et al.*, 1990; A.C.B.Cato *et al.*, in preparation). The prevention of Fos/Jun binding to DNA by GR interaction and the subsequent occupation of the TRE by a non-transactivating AP-1 subtype cannot be totally excluded. However, at least *in vitro*, DNA binding of Fos/Jun (synthesized *in vitro*) and also of purified AP-1 seems unimpaired by GR (see Figure 4). Thus, we suggest that GR interferes with the transactivating potential of AP-1 by protein−protein interactions without abolishing its DNA binding capability. This mechanism may not be the only type of mutual interference between a steroid hormone receptor

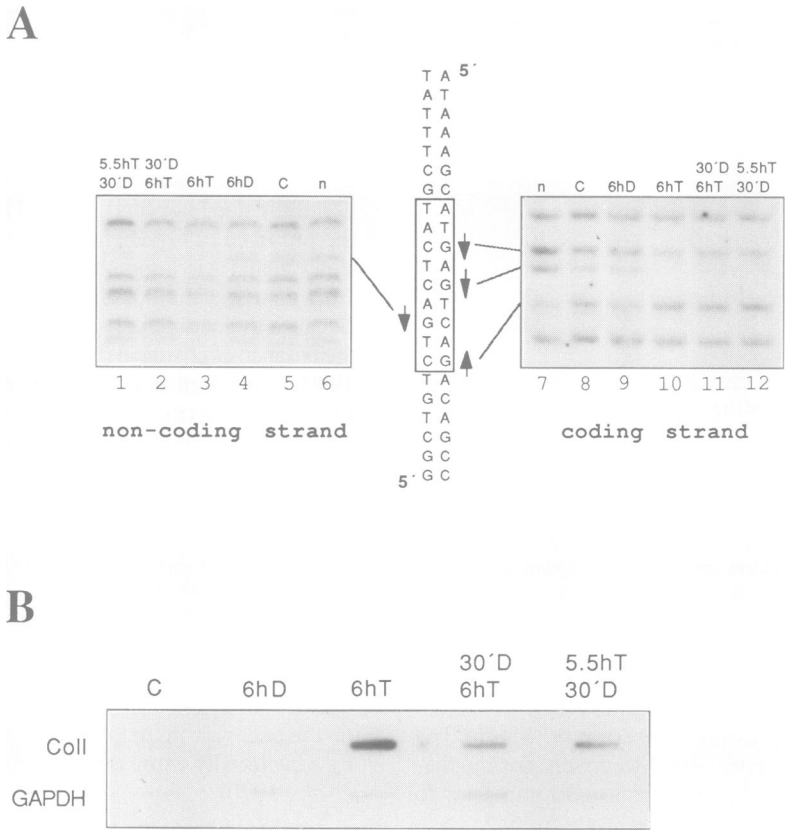

Fig. 3. (**A**) *In vivo* DMS reactivity of the AP-1 binding site within the collagenase 5′-flanking region in human normal skin fibroblasts after treatment with TPA and/or dexamethasone as indicated. n, 'naked' (protein-free) genomic DNA methylated *in vitro*; C, DNA from untreated cells; 6hD, DNA from cells treated with dexamethasone for 6 h (10^{-7} M); 6hT, DNA from cells treated with TPA for 6 h (100 ng/ml); 30′D 6hT, DNA from cells pretreated with dexamethasone for 30 min before TPA was added for 6 h (total dexamethasone treatment was for 6.5 h); 5.5hT 30′D, DNA from cells treated with TPA for 5.5 h prior to the addition of dexamethasone for 30 min (total TPA treatment was for 6 h). Upward pointing arrowhead, hyper-reactivity; downward pointing arrowheads, DMS protection. (**B**) Nuclear run-on analysis of transcription using nuclei from cells treated in parallel to the cells used for the genomic footprinting analysis shown in (A). Coll, collagenase gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase gene.

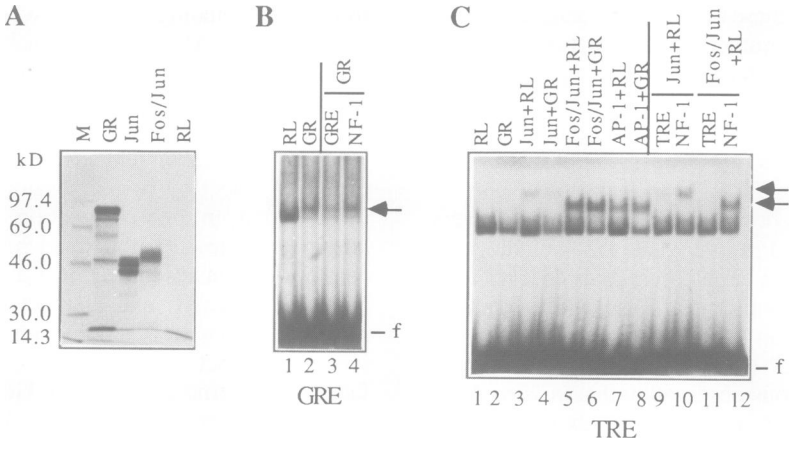

Fig. 4. Effect of glucocorticoid receptor on DNA binding of *in vitro* synthesized AP-1 components and purified AP-1 *in vitro*. (**A**) SDS−gel electrophoresis of *in vitro* synthesized protein components used in the gel retardation assays (see B and C). M, protein molecular weight markers (sizes are indicated in kDa); RL, reticulocyte lysate; GR, Jun, RLs programmed with GR and *jun* RNA, respectively; Jun/Fos, RL progammed with *fos* and *jun* RNA. (**B**) Gel retardation analysis of *in vitro* synthesized GR using a radioactively labeled GRE oligonucleotide probe. RL, unprogrammed reticulocyte lysate (4 μl, lane 1); GR, reticulocyte lysate programmed with GR RNA (4 μl, lane 2); lanes 3 and 4 show reactions containing a 25-fold molar excess of either unlabeled GRE oligonucleotide or of an unrelated oligonucleotide containing an NF-1 recognition sequence. (**C**) Gel retardation assay using a radioactively labeled TRE oligonucleotide probe and *in vitro* translated Jun (2 μl, lanes 3 and 4), cotranslated Fos/Jun (2 μl, lanes 5 and 6) or affinity-purified AP-1 (lanes 7 and 8) in the presence of 4 μl of either unprogrammed reticulocyte lysate (RL) or GR−RNA-programmed reticulocyte lysate (GR). Lanes 1 and 2 show binding of 4 μl of RL or GR alone. Lanes 9−12 show competition experiments using a 25-fold molar excess of either unlabeled TRE oligonucleotide or the NF-1 oligonucleotide. Arrowheads indicate specific bands; f, free oligonucleotide probe.

and AP-1 or AP-1 related transcription factors. It has been suggested that in certain promoters DNA binding sites overlap (Akerblom *et al.*, 1988; Mordacq and Linzer, 1989; Diamond *et al.*, 1990), thus permitting a GR or an AP-1 bound state of the promoter. In this case, repression by glucocorticoids would work only if the GR could bind DNA in an inhibitory conformation. The mutual exclusion of AP-1 and GR dependent pathways appears to be extremely important for the cell, such that perhaps more than one mechanism exists to control their interference.

Materials and methods

Cell culture

Normal human skin fibroblasts (CRL 1497, American Type Culture Collection) and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. Prior to treatment with TPA (to a final concentration of 100 ng/ml) or dexamethasone (final concentration 10^{-7} M), CRL 1497 cells were grown in DMEM/0.5% FCS for 48 h.

Genomic footprinting

The cell monolayers were washed with phosphate buffered saline (PBS) and treated with 0.5% DMS in DMEM/10 mM HEPES pH 7.5 at room temperature (RT) for 2 min. After washing the cells twice with ice-cold PBS, genomic DNA was prepared as described (Mueller *et al.*, 1988). *In vitro* methylation of protein-free genomic DNA (1 min at RT) and piperidine cleavage of DNA was performed as described by Maxam and Gilbert (1980). Selective amplification of the cleavage products by ligation-mediated PCR was done according to Mueller and Wold (1989) using specific oligonucleotide primers.

Primers for the coding strand were: (1) 5′-TCCCAGCCTCTTGCTGCTCCAATAT-3′; (2) 5′-GCTGCTCCAATATCCCAGCTAGGAA-3′; (3) 5′-CTCCAATATCCCAGCTAGGAAGCTCCC-3′; primers for the non-coding strand were: (1) 5′-ACCACTGTTTACATGGCAGAGTGTG-3′; (2) 5′-ATGGCAGAGTGTGTCTCCTTCGCAC-3′; (3) 5′-CAGAGTGTGTCTCCTTCGCACACATCT-3′. PCRs were performed in a Coy TempCycler by 19 cycles consisting of 1 min at 95°C, 2 min at 62.5°C and 3 min at 75°C each. After adding end-labeled primer (3), denaturation for 2 min at 95°C and annealing for 2 min at 65.5°C, a single extension step of 10 min at 75°C was performed. Amplification products were resolved on a 6% standard sequencing gel (Maxam and Gilbert, 1980) and autoradiographed. Autoradiograms were scanned with a LKB Ultroscan XL laser densitometer and peak integrals were calculated for each guanine band. To correct for minor variations in sample loading, values were normalized to guanine bands that are outside of protein binding sites.

Nuclear run-on analysis

Nuclear run-on analysis was performed as described previously (König *et al.*, 1989) using nuclei prepared from one 14.5 cm tissue culture plate each. For preparation of nuclei, cells were washed with ice-cold PBS, pelleted by centrifugation and resuspended in 40 vol ice-cold lysis buffer (10 mM Tris−HCl pH 7.4, 3 mM $CaCl_2$, 2 mM $MgCl_2$). Cells were pelleted again and resuspended in 1 ml lysis buffer. After adding the same volume of lysis buffer containing 1% Nonidet P-40, cells were transferred to a dounce homogenizer and treated by 10−15 strokes with an S-pestle until nuclei appeared free of membrane components. DNA probes used for hybridization were a 2 kb *Sma*I−*Hin*dIII fragment of the plasmid pCllase 1 (collagenase) (Angel *et al.*, 1987a) and a 1.3 kb *Pst*I fragment of the plasmid pGAPDH-13 (GAPDH) (Fort *et al.*, 1985).

In vitro methylation studies

AP-1 was purified from TPA-treated (100 ng/ml for 2 h) HeLa cells as described elsewhere (Angel *et al.*, 1987b) except that the heparin−agarose step was omitted. A *Hin*dIII−*Xho*I fragment of the plasmid −517/+63CAT (Angel *et al.*, 1987a) spanning collagenase gene sequences from positions −517 (*Hin*dIII) to +63 (*Xho*I) was labeled at the *Xho*I site using T4 polynucleotide kinase and [γ-^{32}P]ATP (non-coding strand) or by fill-in reaction using Klenov fragment of *Escherichia coli* DNA polymerase I and [α-^{32}P]dCTP (coding strand). The labeled fragment (30 000 c.p.m.) was incubated with ~5 ng of affinity-purified AP-1 in a volume of 30 μl containing 20 mM HEPES pH 7.9, 20% glycerol, 100 mM KCl, 2 mM $MgCl_2$, 0.2 mM EDTA and 0.5 mM DTT. After 45 min at room temperature, 4 μl of 5% DMS were added and the reactions were stopped after a 2 min incubation at room temperature by addition of 90 μl of 400 mM sodium acetate pH 6.9, 140 mM β-mercaptoethanol and 270 μg/ml yeast transfer RNA. The reactions were extracted by phenol/chloroform/isoamylalcohol and ethanol precipitated. Piperidine cleavage was performed according to Maxam and Gilbert (1980). Piperidine was removed by ethanol precipitation and fragments were resolved on a 6% sequencing gel (Maxam and Gilbert, 1980).

In vitro transcription and in vitro translation

In vitro transcriptions using bacteriophage T7 and SP6 RNA polymerases were performed according to Struhl (1987) using appropriate linearized DNA templates containing human GR [2.5 kb *Kpn*I−*Dra*I fragment of pRShGRα (Giguere *et al.*, 1986)] and human c-*jun* cDNA sequences [1.4 kb *Hin*dIII−*Not*I fragment of pRSV-cJ (Angel *et al.*, 1988)], respectively, in pBluescript II SK (Stratagene); c-*fos* RNA was transcribed from the plasmid pGEMfos3 (Rivera *et al.*, 1990). *In vitro* translations were performed in nuclease-treated rabbit reticulocyte lysate in the presence of [^{35}S]methionine according to the instructions of the manufacturer (Promega). SDS−gel electrophoresis of *in vitro* translation products was done in a 10% SDS−polyacrylamide gel (Laemmli, 1970).

Gel retardation assay

DNA binding reactions were performed in 25 μl incubations containing 20% glycerol, 20 mM HEPES pH 7.9, 20 mM KCl, 2 mM $MgCl_2$, 2 mM spermidine, 2.5 mM EDTA, 1 mg/ml bovine serum albumin and 10^{-7} M dexamethasone. Protein components were incubated at 25°C for 20 min in the presence of 0.5 μg poly(dI-dC) (and competitor oligonucleotide where indicated) prior to the addition of 0.2 ng of radioactively labeled oligonucleotide probes. After incubation at 20°C for 10 min the reactions were resolved by non-denaturing 4% PAGE in 1 × TBE (90 mM Tris base, 90 mM boric acid, 2.5 mM EDTA) at RT for 2 h (at 20 mA) and the gels were dried and autoradiographed. The TRE probe was obtained by annealing the following two oligonucleotides: 5′-GATCGAGCATGAGTCAGACAC-3′; 5′-GATCGTGTCTGACTCATGCTC-3′. The GRE probe was generated from the oligonucleotides 5′-AGCTTAGAACACAGTGTTCTCTAGAG-3′ and 5′-GATCCTCTAGAGAACACTGTGTTCTA-3′. Radioactive end-labeling was performed using T4 polynucleotide kinase and [γ-^{32}P]ATP. The NF-1 oligonucleotide was generated by annealing the two oligonucleotide sequences 5′-AGCTTTTGGAATCTATCCAAGTC-3′ and 5′-GATCCGACTTGGATAGATTCCAAA-3′, derived from a sequence of the mouse mammary tumor virus (MMTV) long terminal repeat.

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