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QUARTERLY

# Interference between DNA binding activities of AP-1 and GR transcription factors in rat thymocytes undergoing dexamethasone-induced apoptosis*

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**The early molecular events of glucocorticoid-induced apoptosis have been investigated by studying glucocorticoid receptor levels, as well as binding activities to GRE and AP-1 sequences, using nuclear extracts from dexamethasone (Dex)-treated rat thymocytes. When the time-course of glucocorticoid-receptor complexes in nuclei of thymocytes was evaluated by binding studies using the tritiated ligand, we found that nuclear accumulation of radioactive complexes occurred in the first hour of incubation, and was followed by a progressive decline. This trend was confirmed by immunoblotting of nuclear proteins using a monoclonal anti-glucocorticoid receptor antibody. When the kinetics of binding activity to AP-1 and GRE sequences were studied, using nuclear extracts prepared from Dex-treated thymocytes in gel shift assays, we found peaks at 1 and 2 h after Dex treatment, and a return to basal levels in the following hours. Binding specificity was proved by competition studies using non-radioactive sequences, including mutated AP-1. Unexpectedly, however, protein binding to GRE was better competed for by AP-1 sequence than by GRE itself. Data obtained using the super gel shift assay suggested that AP-1/Jun can be responsible for the high affinity for the GRE sequence. Thus, we report here for the first time that an interference between AP-1 and GR in the binding to DNA consensus sequences — previously described in other biological systems — also occurs during apoptosis induced by glucocorticoids in lymphoid cells.**

Apoptosis has been observed in cells of different origin under physiological conditions, as well as after a number of experimental manipulations including glucocorticoid treatment (reviewed in [1]). Apoptotic death appears to have an active role in different functions of the immune system, including the killing of target cells by natural killer cells and cytotoxic T-lymphocytes [2], the deletion of aged neutrophils during the inflammatory response [3] and graft-*versus*-host reactions [4]. Moreover, apoptosis may represent a fundamental mode of

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**Abbreviations:** CRE, cAMP responsive element; Dex, dexamethasone; DTT, dithiothreitol; GR, glucocorticoid receptor; GRE, glucocorticoid responsive element.

selectively deleting autoreactive immune cells [5].

Glucocorticoid-induced apoptosis in rat thymocytes is a classical model used to study the cellular and molecular events of this type of cell death [5]. This model can also help to explain important physiological events which occur in the thymus, such as negative and positive selection, which are apparently modulated by glucocorticoid hormones [6]. However, despite intense investigation the scenario is far from clear. The response of lymphoid cells to glucocorticoid hormones is believed to be triggered by binding of the steroid to its intracellular glucocorticoid receptor (GR) [7]. The GR seems to be a link in the transduction of the signal for apoptosis since: i) lymphocytes with mutant GR fail to die in response to glucocorticoids [8]; ii) only the glucocorticoid class of steroids induces apoptosis and glucocorticoid antagonists competitively inhibit this response [9].

Receptors for steroid hormones belong to a large family which includes receptors for thyroid hormones, retinoic acid and hydroxylated vitamin D metabolites [10]. They differ markedly from membrane receptors in that they are intracellular and appear to act primarily through regulation of DNA transcription brought about by binding of activated (ligand-bound) receptors to their responsive elements in the promoter region of target genes [11]. Several receptors for steroid hormones bind to a common consensus sequence, originally designed as glucocorticoid responsive element (GRE). Similarly to many transcriptional regulators, the receptors can stimulate gene expression in some contexts and repress expression in others [10]. This raises very interesting questions about the mechanisms by which a single product can either enhance or repress transcription. In pathways which are regulated by direct activation of transcription factors, e.g., the activation of steroid hormone receptor by their ligands, the explanation could be the crosstalk between different transcription factors. Regulatory interactions between AP-1 and steroid hormone receptors have been described (reviewed in [12]). AP-1 is a transcription factor composed of the protein products of c-*fos* and c-*jun* families of proto-oncogenes which can form several types of different heterodimers and homodimers, and can be induced by a variety of physiological and non-physiological agents [13]. At least two hypotheses to explain the mutual interference between AP-1 and steroid receptors have been put forward. In the first case, mutually exclusive binding of the transcription factor AP-1 and members of the steroid hormone receptor family due to overlapping of *cis*-acting elements has been postulated. Indeed, overlapping binding sequences for both transcription factors have been described in the genes coding for mouse proliferin, rat α-fetoprotein, human α-subunit of chorionic gonadotropin and human osteocalcin (reviewed in [12]). As an alternative to mutually exclusive binding to DNA, the steroid hormone receptor could interfere with AP-1 mediated transcriptional activation *via* protein-protein interaction. Examples of such a mechanism are the regulation of the expression of rat stromelysin [14] and collagenase genes [15]. Nevertheless, the molecular basis for the multiple regulatory interactions between Fos/Jun and nuclear hormone receptors at different promoters and in different cell types is unknown. Moreover, to our knowledge, no data are available on possible interactions between AP-1 and GR in another phenomenon induced by glucocorticoids, i.e. apoptosis.

Previously, we have shown that the synthetic glucocorticoid hormone, dexamethasone (Dex) induced binding activity to the AP-1 consensus sequence in rat thymocytes undergoing apoptosis [16, 17]. The kinetics of the process, monitored by means of the gel-shift assay, showed very early and transient binding activity to the AP-1 consensus sequence. In the present study we investigated the DNA-binding activities of nuclear extracts from Dex-treated rat thymocytes to GRE and AP-1 consensus sequence. We demonstrated that an interference between AP-1 and GR occurs during Dex-induced apoptosis of lymphoid cells, as AP-1 shows high binding activity not only to the AP-1 site but also to the GRE consensus.

## MATERIALS AND METHODS

***Materials.*** [6,7-$^3$H(N)]Dex ([$^3$H]Dex) (44.7 Ci/mmol) was from DuPont NEN. $^{125}$I-Labelled anti-mouse immunoglobulin (from sheep), Hyperfilm-MP, Hyperfilm-$^3$H, and Hybond-C extra were purchased from Amersham International (Buckinghamshire, England). Mouse

monoclonal anti-glucocorticoid receptor antibody (BuGR2) was obtained from Affinity BioReagents, Inc. Serum from normal mice was the source of non-specific antibodies. Mouse monoclonal anti-c-Fos and rabbit polyclonal anti-c-Jun/AP-1 were from Santa Cruz Biotechnology. Prestained molecular mass markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and non-radioactive Dex were from Sigma Chemical Co. All other reagents were of analytical grade.

*Cells.* Suckling rats of the Sprague-Dawley strain were sacrificed by decapitation and the thymus was rapidly excised. Thymocytes, prepared as previously described [18], were resuspended at a concentration of $5 \times 10^6$ cells/ml in RPMI 1640 medium supplemented with 10% foetal calf serum and incubated at 37°C in the presence of $10^{-7}$ M Dex.

***Preparation of nuclear extracts.*** Nuclear extracts were typically obtained from $2 \times 10^7$ cells. Thymocytes were washed three times by resuspension in 1 ml of 20 mM phosphate buffer, pH 7.4, 0.15 M NaCl (PBS buffer), and centrifugation for 8 min at $600 \times g$. The cells were lysed by resuspending them in 20 mM Tris/HCl, pH 7.5, containing 10 mM NaCl, 1.5 $MgCl_2$, 3% (w/v) digitonin followed by 30 min incubation with occasional vortexing, as previously described [19]. In some experiments cell resuspension was done in 10 mM Hepes, pH 7.9, 10 mM KCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride and 10 mg/ml aprotinin. After a 20 min incubation at 2°C with occasional vortexing, Nonidet P-40 was added to the sample at a 0.4% (v/v) final concentration, as previously described [20]. Using these two methods of cell lysis we did not observe any differences in DNA binding activities to AP-1 or to GRE consensus sequences. Lysates were centrifuged for 30 s in an Eppendorf microfuge and the nuclear precipitates were resuspended in 20 mM Hepes, pH 7.9, 0.4 M NaCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride and 10 mg/ml aprotinin. After a 15 min incubation at 2°C with vortexing, samples were centrifuged for 5 min at 14000 × *g*, to obtain the supernatants, which represented the nuclear extracts and were stored at –70°C. DNA was measured by the diphenylamine test, using calf thymus DNA as the standard [21]. The protein content of nuclear extracts was estimated according to Bradford [22].

***Measurement of glucocorticoid receptor by binding studies.*** Suspensions of thymocytes in RPMI 1640 containing 10% foetal calf serum were incubated at 37°C with $10^{-7}$ M [$^3$H]Dex, in the presence or in the absence of a 200-fold molar excess of cold competitor. At the indicated times after hormone addition, aliquots of cells suspensions were collected and cells were recovered by centrifugation for 8 min at $600 \times g$, washed three times with PBS buffer, and processed to obtain nuclear extracts, as described above. Samples were then incubated for 7 min at 2°C with a dextran-coated charcoal pellet [23] to remove free steroid, and aliquots of these extracts were used for determination of radioactivity. Specific glucocorticoid binding was determined by subtracting unspecific binding (with competitor) from total binding (without competitor).

***Measurement of glucocorticoid receptor by immunoblotting.*** Nuclear extracts were brought to 2% sodium dodecyl sulfate, 1.5 mM EDTA, 5% β-mercaptoethanol and were treated for 5 min at 100°C, before being fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 8.5% separating gels, and a 3% stacking gel, according to Laemmli [24]. Proteins were electrophoretically transferred to Hybond-C extra and membranes were subjected to immunoblotting by the procedure of Bresnick *et al.* [25]. Detection of the antigen-antibody complexes was performed by a $^{125}$I-labelled secondary antibody, and exposing the membranes to Hyperfilm-$^3$H.

***Gel shift and super gel shift assay.*** The double-stranded oligonucleotides harboring the AP-1 (5'CTAGTGATGAGTCAGCCGGATC3'), GRE (5'GATCAGAACACAGTGTTCTCTA3'), SP-1 (5'GATCGATCGGGGCGGGGCGATC3'), and cAMP responsive element (CRE) (5'GATTGGCTGACGTCAGAGAGCT 3') sites were from a Stratagene "gel shift" kit. Mutated AP-1 and anti-Fos and anti-AP-1/Jun was purchased from Santa Cruz Biotechnology. AP-1 and GRE oligonucleotides were labelled with terminal transferase (Boehringer-Mannheim GmbH, Wien, Austria) and purified on Nick-columns purchased from Pharmacia. The reaction was carried out in 16 µl of mixture containing buffer from "gel shift" kit (Stratagene GmbH, Heidelberg, Germany), labelled probe (37.5 pg; about 20 000 c.p.m. in 1 µl), 3 µg of nuclear protein (3 µl) and 1–2 µl of competitor

if necessary. In super gel shift assay 1 μl of the proper antibody was incubated for 1 h at 0°C before addition of labelled oligonucleotide. After 20 min incubation at room temperature 1 μl of 0.1% bromophenol blue was added and samples were electrophoresed through a 4% polyacrylamide gel (30:1 cross-linked) at 20 mA in a cold room. After running (about 2 h) gels were dried and exposed overnight to Hyperfilm-MP, with intensifying screens.

## RESULTS

The kinetics of binding activity to AP-1 and GRE sequences using nuclear extracts prepared from Dex-treated thymocytes were evaluated. In extracts prepared from both untreated and Dex-treated cells, binding activities to GRE and AP-1 consensus sequences were demonstrated by the presence of retarded bands at the top of the gel, in comparison with free probe at the bottom (Fig. 1, panels A and B). In Dex-treated cells the induction of both GRE and AP-1 binding activity was observed. In untreated cells a very low basal level of GRE binding activity was observed during the entire time-course (0–8 h). The constant level of noninducible SP-1 transcription factor [16] observed in extracts from Dex-treated cells (Fig. 2) indicated that the changes in AP-1 and GRE activities were not due to trivial factors, such as differences in the protein content of our extracts.

In order to better evaluate the time-course of GRE and AP-1 binding activities, quantitative analyses were performed by densitometric

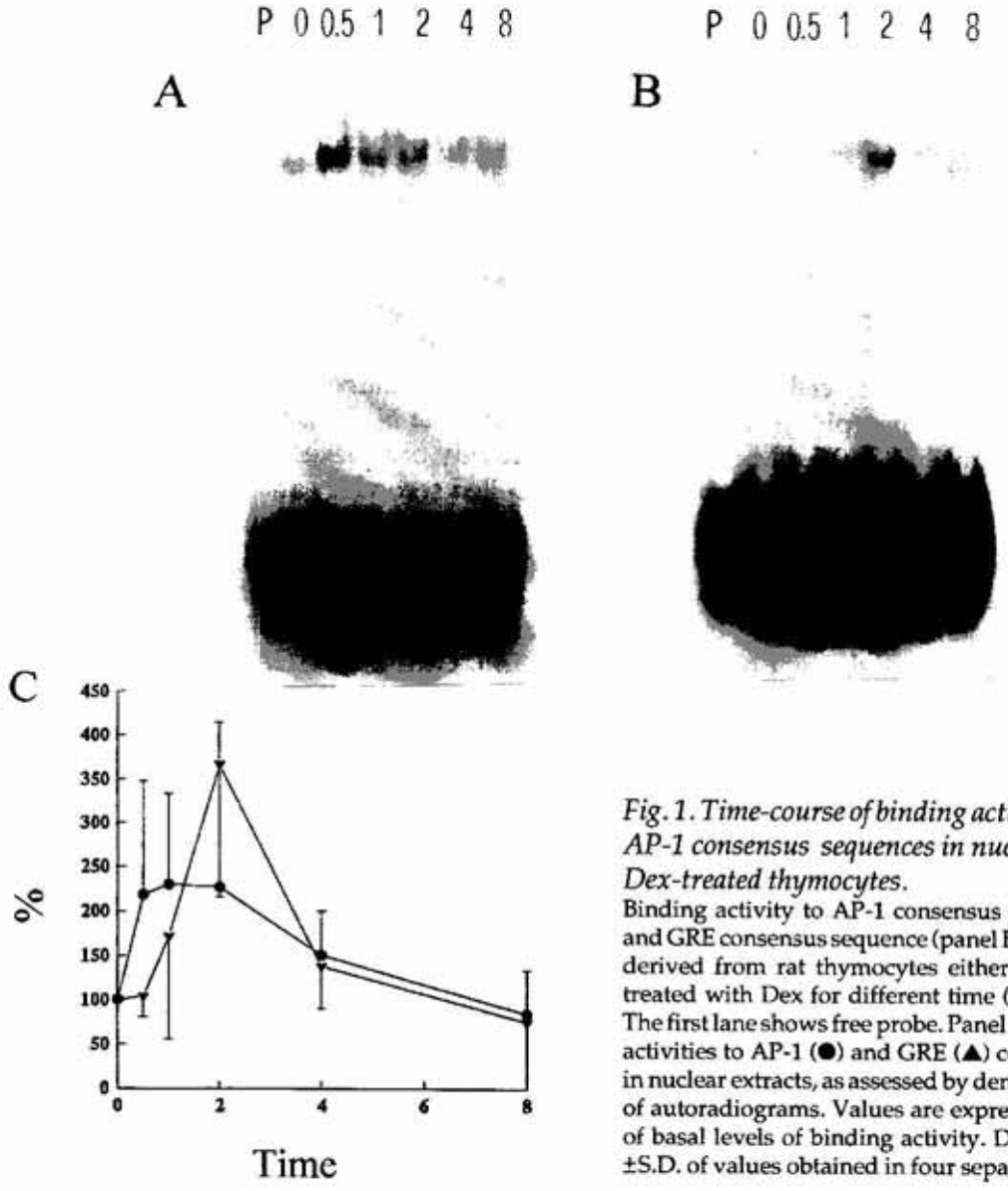


*Fig. 1. Time-course of binding activities to GRE and AP-1 consensus sequences in nuclear extracts from Dex-treated thymocytes.*

Binding activity to AP-1 consensus sequence (panel A) and GRE consensus sequence (panel B) in nuclear extracts derived from rat thymocytes either untreated (0 h) or treated with Dex for different time (in hours) is shown. The first lane shows free probe. Panel C: Levels of binding activities to AP-1 (●) and GRE (▲) consensus sequences in nuclear extracts, as assessed by densitometric scanning of autoradiograms. Values are expressed as percentages of basal levels of binding activity. Data represent mean ±S.D. of values obtained in four separate experiments.


*Fig. 2. Time-course of binding activities to SP-1 consensus sequences in nuclear extracts prepared from Dex-treated thymocytes.*
Cells were either untreated (0 h) or treated with Dex for different time (in hours), as indicated. The first lane shows free probe. 50-Fold excess of unlabelled SP-1 was used as a competitor (C) for 8 h extracts.

scanning of autoradiograms from four independent experiments (Fig. 1, panel C). The data we obtained confirmed that the increase of nuclear proteins capable of binding to AP-1 reached a maximum within 1 h of Dex treatment. When the same protein extracts were analyzed for their binding activity to the GRE consensus sequence, a sharp increase was observed 2 h after Dex treatment, returning to basal levels within the next 2–4 h. These data were unexpected, taking into account the early increase of binding activity to AP-1 consensus sequence observed after Dex treatment. A major variable to account for these results is the nuclear level of GR. Thus, experiments were performed to evaluate the level of GR in nuclear extracts of Dex-treated thymocytes. Thymocytes were incubated with [$^3$H]Dex, and the levels of GR complexes in nuclear extracts were measured. We found that accumulation of [$^3$H]Dex-receptor in thymocyte nuclei occurred in the first hour of incubation at 37°C, and was followed by a progressive decline (Fig. 3).

As shown in Fig. 4, the time-course of GR protein measured by immunoblotting using an anti-GR antibody [25], after fractionation of the extracts by SDS/PAGE, reproduced that found in binding studies.

Comparing the results of the time-course studies after Dex addition shown in Figs. 1, 3 and 4, it appeared that in nuclear extracts binding activity to GRE consensus sequence did not correlate with levels of GR, measured either as protein or as hormone-receptor complexes. Indeed, the highest levels of binding activity to GRE consensus sequence were detected 2 h

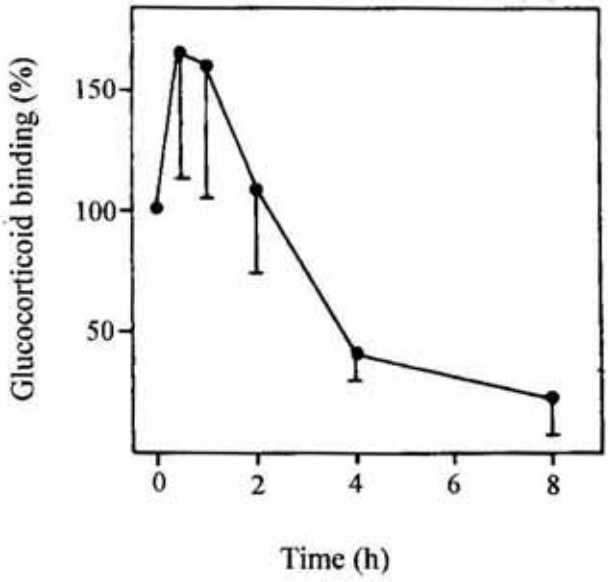


*Fig. 3. Time-course of glucocorticoid binding in nuclear extracts from Dex-treated thymocytes.*
Rat thymocytes were incubated at 37°C with $10^{-7}$M [$^3$H]Dex, in the presence or in the absence of a 200-fold molar excess of non-radioactive competitor. At the indicated times after Dex addition, aliquots were collected from cell suspensions and processed to measure specific Dex binding in nuclear extracts, which was normalized on the basis of the DNA content of the sample. Values are expressed as percentages of basal level of glucocorticoid binding and represent mean ±S.D. of data obtained in four–six separate experiments. Basal levels of glucocorticoid binding were measured in cell suspensions run in parallel and incubated with [$^3$H] Dex ± competitor for 2 h at 2°C.

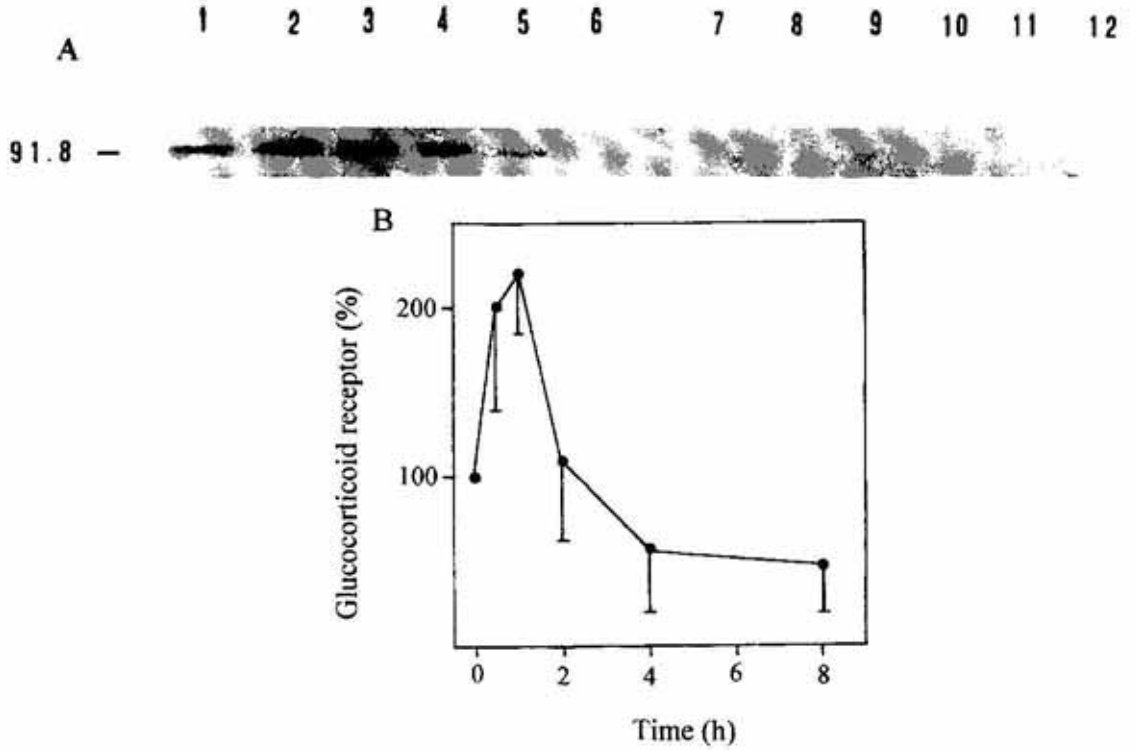


*Fig. 4. Time-course of glucocorticoid receptor levels in nuclear extracts from Dex-treated thymocytes.* Rat thymocytes were incubated with $10^{-7}$ Dex at 37°C. At the indicated times after Dex addition, aliquots were collected from cell suspension and processed to prepare nuclear extracts. Glucocorticoid receptor protein was measured by immunoblotting, as described under Material and Methods. Panel A: Autoradiogram obtained upon immunoblotting of nuclear extracts. Aliquots of cell suspensions were collected at 0 (lanes 1, 7), 0.5 (lanes 2, 8), 1 (lanes 3, 9), 2 (lanes 4, 10), 4 (lanes 5, 11) and 8 (lanes 6, 12) h after Dex and using either anti-glucocorticoid receptor antibody (lanes 1–6) or non-specific antibody (lanes 7–12). The position of the fructose-6-phosphate kinase marker (91.8 kDa) is indicated. Panel B: Level of glucocorticoid receptor protein in nuclear extracts. Measurements were performed by densitometric scanning of autoradiograms. Values are expressed as percentages of basal levels of glucocorticoid receptor protein, after they had been normalized on the basis of the DNA content of the sample. Data represent mean ±S.D. of values obtained in three separate experiments.

after steroid addition, but nuclear GR was found to peak at 0.5–1 h. This observation would imply that non-GR component(s) might contribute to binding activity to GRE consensus sequence of our nuclear extracts.

To investigate the nature of the component(s) involved in GRE binding, the specificity of binding to GRE and AP-1 consensus sequences was evaluated by competition studies. Thus, the specificity of the retarded bands obtained after incubation of nuclear extracts from Dex-treated thymocytes with $^{32}$P-labelled AP-1 and GRE oligonucleotides was tested (Fig. 5). The 2 h nuclear extracts were examined for GRE binding activity in the presence of unlabelled GRE or unlabelled AP-1 sequences, as competitors. As shown in Fig. 5A, the retarded bands were inhibited by a 50-fold molar excess of AP-1, but not by a 50-fold molar excess of GRE sequence. The competition in binding to the AP-1 consensus sequence was also studied, and the results showed that the retarded band was inhibited in a dose-dependent manner (10- and 50-fold excess) by unlabelled AP-1, but not by unlabelled mutated AP-1 (Figs. 5B and 5C), indicating the specificity of the binding activity to AP-1 consensus sequence. These data are reinforced by the fact that AP-1 binding was only slightly competed for by 50-fold excess of GRE and by 50-fold excess of CRE sequences (Fig. 5B). This last result is not completely unexpected as AP-1 and CRE consensus sequences differ only in one nucleotide, and a cross-interaction between these cognate transcription factors has been reported [26]. Further experiments revealed that only a great excess of unlabelled GRE was able to compete with $^{32}$P-labelled GRE oligonucleotide, using nuclear extracts from 2 h Dex-treated thymocytes. Figure 6 shows that the retarded band was inhibited by

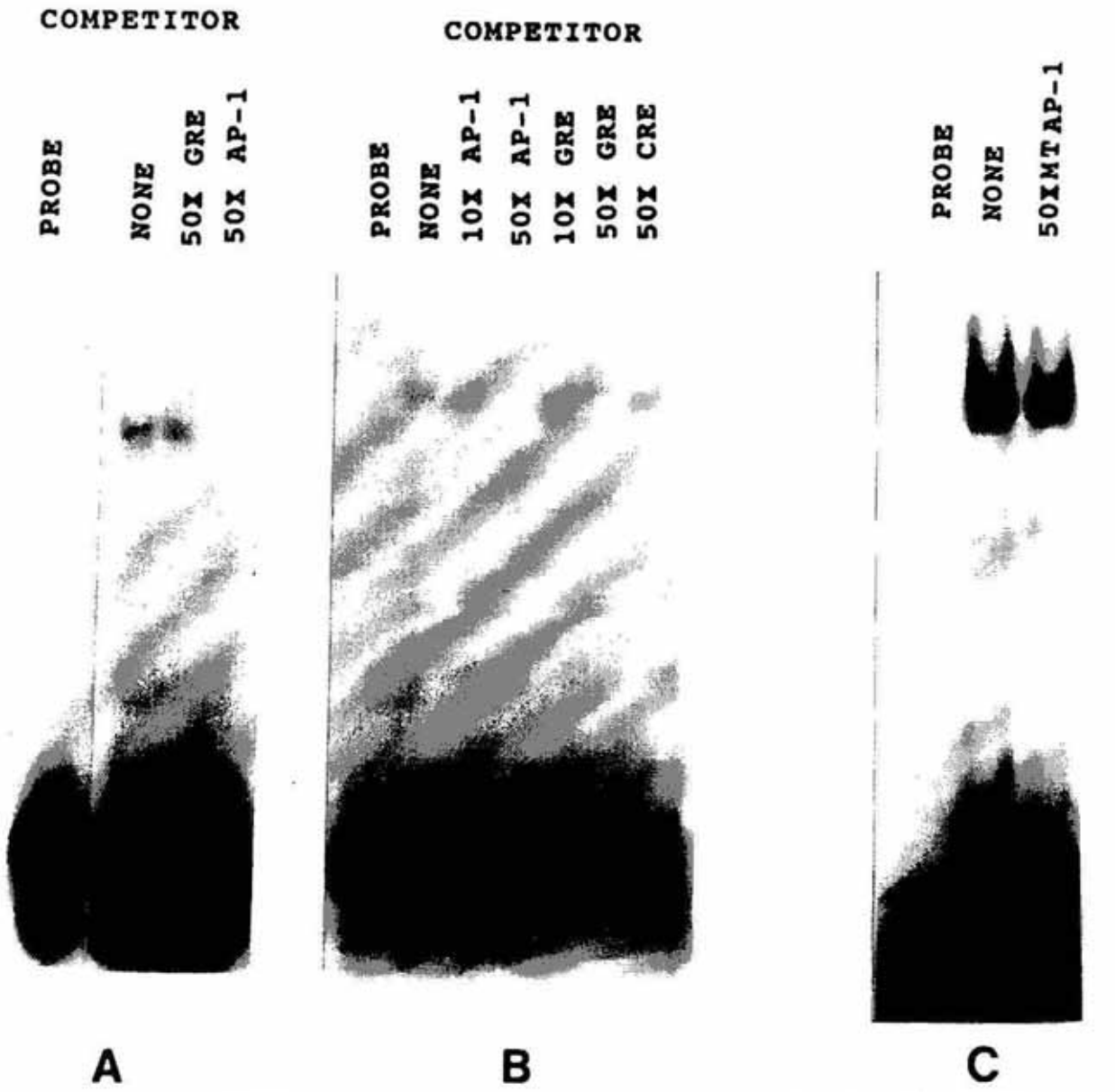


*Fig. 5. Competition of GRE (panel A) and AP-1 (panels B and C) binding activity by unlabelled oligonucleotides.*

Binding activity was measured using nuclear extracts derived from rat thymocytes treated with Dex for 2 h, 50-fold excess of unlabelled competitors was used as specified. MTAP-1, mutated AP-1.

both cold GRE and AP-1 oligonucleotides in a dose-dependent manner (10, 50, 200-fold excess). Nevertheless, the competition was much stronger in the case of AP-1 oligonucleotide (almost complete at a 50-fold excess) than in the case of GRE (still not complete at a 200-fold excess). Neither CRE nor SP-1 competed with GRE for GRE binding activity (data not shown).

On the whole, these data suggest that AP-1 protein complexes present in nuclear extracts from Dex-treated thymocytes have a high affinity not only for AP-1 but also for GRE sequence. This intriguing observation was confirmed by the super gel shift assay (Fig. 7). In our experiments anti-c-Fos antibody broadly reacting with all members of the Fos family and anti-c-Jun/AP-1 antibody reacting with DNA binding domain of all Jun proteins were used. A clear inhibition of GRE binding activity was observed in samples containing anti-c-Jun/AP-1 antibody, but not in samples containing anti-Fos antibody, proving the presence of c-Jun/ /AP-1 in the protein complex binding to GRE sequences.


*Fig. 6. Effect of increasing concentrations of non-radioactive GRE and AP-1 consensus sequences on GRE binding of nuclear extracts from Dex-treated thymocytes.*
Binding activity was measured using nuclear extracts derived from rat thymocytes treated with Dex for 2 h. GRE and AP-1 oligonucleotides were added as competitors in different concentration, as indicated.


*Fig. 7. Supershift analysis of the protein composition of binding activity to GRE sequences.*
Binding activity was measured using nuclear extracts derived from rat thymocytes treated with Dex for 2 h. Anti-c-Fos and anti-c-Jun/AP-1 antibodies were added as indicated.

## DISCUSSION

Apoptosis, like cell division, is a very complex biological process regulated by many genes such as p53, Rb, *bcl-2* family members, ICE, *crm*A and others [27]. Also the proto-oncogenes c-*myc*, c-*jun* and c-*fos*, which are known to be induced in cell proliferation, are activated in some cells undergoing apoptosis [18, 28].

We have shown for the first time that not only *c-fos* and *c-jun* gene expression, but also a direct AP-1 transcription factor activity was increased in rat thymocytes undergoing apoptosis after Dex-treatment [16, 17]. This observation was recently confirmed by others using different models of apoptosis [29–32].

Our studies were performed to investigate the possible relationship between AP-1 and GR during apoptosis. To this end, we analyzed the level of active GR(s) as well as AP-1 and GR binding activities to their specific sequences. Unexpectedly, nuclear levels of GRE binding activity (Fig. 1) did not coincide with those of glucocorticoid-receptor complexes (Fig. 3) and GR protein (Fig. 4). This asynchrony indicates that some other component(s) present in nu-

clear extracts might interact with GRE under our assay conditions, and poses the question of its (their) nature.

The competition studies we have performed on GRE binding activity could help in understanding this point. The finding that GRE binding activity was competed for by high concentrations of non-radioactive GRE (Fig. 6) could be explained by the presence in nuclear extracts of either unspecific binders or high levels of specific components. The latter interpretation is supported by the following data: i) GRE binding was detected in the presence of an excess of poly(dI:dC) and of a salt concentration higher than 0.15 M NaCl (see Material and Methods); ii) other oligonucleotides such as SP-1 and CRE (data not shown) did not compete with GRE sequence. Furthermore, GRE binding activity was efficiently competed for by an oligonucleotide harboring the AP-1 consensus sequence (Figs. 5A and 6), showing that the components interacting with GRE displayed an affinity for AP-1 sequence higher than that for GRE itself. The simplest explanation of these data is that *jun/fos* products which compose AP-1 might bind very efficiently not only to specific AP-1 sequence but also to GRE consensus sequences. Thus, the levels of GRE binding activity would be affected by both GR and AP-1 when GRE consensus sequence was used in the experiments. The super gel shift assay indicated that member(s) of the Jun family had high affinity to GRE sequence (Fig. 7). These data extend those obtained in competition experiments, and can be considered as a direct proof that the main protein(s) in this complex may be a member(s) of the Jun family. When AP-1 sequence was used, preliminary experiments, using anti-Fos and anti-Jun antibodies broadly reacting with all members of Fos and Jun families, showed that Fos/Jun heterodimer(s) is(are) responsible for AP-1 DNA binding activity (not shown). This is in agreement with previous data obtained by Northern analysis showing that both *c-fos* and *c-jun* are induced early in Dex-treated apoptotic thymocytes [21]. If these findings are considered together with the discrepancy of the time courses we detected for binding activities to GRE and AP-1 consensus sequence in nuclear extracts from DEX-treated thymocytes (Fig. 1), it can be speculated that our extracts derived from rat thymocytes at different time points after DEX treatment contain sets of AP-1 complexes with different capability of binding to AP-1 and/or GRE sequences. Our data, however, do not provide information regarding the molecular basis of the diversity among components displaying affinity for GRE and AP-1 oligonucleotides in our system. Moreover, it cannot be excluded that the diversity among AP-1 components suggested by the data of this study, might depend on distinct arrangements of the same entities, as a consequence of changes in their relative abundance induced by glucocorticoids. Alternatively, differences might be due to selected patterns of covalent modification of AP-1 components, following glucocorticoid treatment of thymocytes Studies using specific antibodies for different members of these families (i.e. Jun B, Jun D, c-Jun, Fra1, Fra2, c-Fos) are needed to clarify the composition of protein complexes able to bind to AP-1 and GRE sequences.

The critical importance of the interference between AP-1 and active GR in the binding to DNA consensus sequences for the apoptosis to occur is also indicated by data recently published by Helmberg *et al.* [33]. These authors found that apoptosis is still induced by glucocorticoids in lymphocytes transfected with an activation-deficient glucocorticoid receptor mutant gene which is as effective as the wild-type receptor in interference with AP-1 activity. Our results showing that AP-1 binds with higher affinity to GRE sequence than GR itself suggest that during the apoptosis of T cells another transcriptional repressive circuit can occur. Direct studies on this important point are needed.

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