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Negative Regulation of a Special, Double AP-1 Consensus Element in the Vimentin Promoter: Interference by the Retinoic Acid Receptor

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The growth-regulated vimentin gene contains a functional double AP-1 binding site formed by two nearly perfect inverted repeats. We present evidence for down-regulation of vimentin expression by the retinoic acid receptor (RAR) in two mesodermally derived cell types. By mutation analysis we prove that the double consensus element is responsible for this negative regulation. From in vitro protein-DNA interaction studies we conclude that AP-1 binding is inhibited at RAR amounts required for occupation of the cognate RAR binding site in nuclear extracts from 3T3 cells and differentiated embryonal carcinoma cells. Furthermore, we show that, unlike in other cases, *trans*-activation of the vimentin AP-1 enhancer element can occur in undifferentiated embryonal carcinoma cells, despite the low amount of Jun and Fos proteins present in these cells. Here, however, down-regulation by retinoic acid cannot be detected.

The cytoskeletal protein vimentin belongs to the group of intermediate filament proteins (IF). Each member of this group shows a more or less tissue-specific distribution pattern. Vimentin is characteristic for mesenchymal cells. Unlike other IF proteins, vimentin is also detectable during specific stages in embryonal development. Its expression is switched off in later stages and is replaced by a more specialized IF protein. For instance, during muscle cell differentiation vimentin is exchanged for the muscle-specific IF protein desmin, whereas in epithelial differentiation specific types of keratins are being switched on. Apart from its complex expression pattern during development, the vimentin gene is identified as a growth-regulated gene (Ferrari et al., 1986). This is believed to be due to the presence of an enhancer element to which a complex of proteins, named AP-1, can bind (Rittling et al., 1989; Pieper et al., 1992). AP-1 is mainly composed of the proto-oncogene products Fos and Jun (Curran and Franza, 1988).

The vitamin A metabolite, retinoic acid (RA), plays a major role in differentiation and patterning in a number of developmental systems. The effects of RA on gene transcription are believed to be mediated by the structurally related nuclear retinoic acid receptors (RAR), which fall into two different groups, the classic RARs (RAR α, β, and γ) and the more recently discovered retinoid X receptors (RXR α, β, and γ). All these receptors are encoded by distinct genes and belong to a larger family of DNA binding regulatory proteins, the steroid and thyroid hormone receptors (Petkovitch, 1992). The RARs and RXRs bind as homodimers or heterodimers to their target sequences, the retinoid response elements (RARE), included in regulatory regions of retinoid responsive genes. The DNA sequences required for the action of all members of the family of hormone receptors display varying degrees of homology (reviewed by Petkovitch, 1992; Zhang and Pfahl, 1993). Another important resemblance of these nuclear receptors is that apart from their positive effects on transcription, they are also involved in gene repression. This negative regulatory mechanism can take place via different kinds of interactions (reviewed by Schüle and Evans, 1991; Miner et al., 1991; Pfahl, 1993). For instance, for the osteocalcin and proliferin genes, repression is exerted through a combined response element, which serves simultaneously as a hormone receptor and an AP-1 recognition site (Schüle et al., 1990a; Diamond et al., 1990). A second inhibitory pathway by hormone receptors has been put forward. A functional AP-1 site in the 5′ regulatory region of certain genes seems to be directly responsible for the repressive effect. It has been proposed that Fos and Jun are able to interact with the hormone receptor in solution, which subsequently prevents the complex from DNA binding and gene activation (Schüle et al., 1990b; Nicholson et al., 1990; Yang-Yen et al., 1990; Jonat et al., 1990). Whereas this phenomenon has been well documented for the collagenase and stromelysin genes, the exact mechanism for this interaction is not clear yet. It has been reported that the leucine zipper regions of Fos, as well as Jun, play a major role (Shemshedini et al., 1991;

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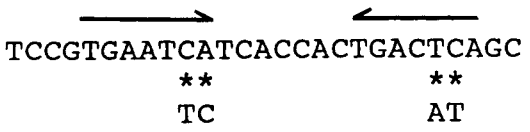


Fig. 1. Nucleotide sequence of the positive strand of the vimentin region encompassing the double AP-1 consensus element. The mutated bases are marked by asterisks.

Schüle et al., 1990b). Furthermore, mutation analysis of the hormone receptor protein indicates that regions involved in normal protein-protein or protein-DNA interactions are important for AP-1 inhibition (Kerppola et al., 1993; Schüle et al., 1991; Hudson et al., 1990).

Since the vimentin gene also contains an AP-1 enhancer element, down-regulation by for instance RA might be an important mechanism during development or differentiation of certain tissues. Previous reports indicated a negative as well as a positive effect exerted by RA on vimentin expression, depending on the cell type used (Rius and Aller, 1992; Leung et al., 1992; Järvinen, 1990; Wood et al., 1990). However, the elements involved in the vimentin promoter have not yet been investigated. In this report the role of the double AP-1 consensus element in the repression of gene transcription by RA has been analyzed by mutation analysis. For this purpose two different cell lines have been used, the NIH 3T3 cell line cotransfected with all three RARs and the mouse P19 embryonal carcinoma (EC) cell line in undifferentiated and differentiated states. The P19 cell line serves as a model system for cells sensitive to differential regulation of gene expression by administration of RA (McBurney et al., 1982).

MATERIALS AND METHODS

Recombinant plasmids

The wild-type vimentin reporter construct -3100vimcat and the deletion construct -602vimcat are derivatives of pSUPERCAT and have been described earlier (van de Klundert et al., 1992). The AP-1 mutant was constructed by subcloning a 2.3 kb *Xho*I fragment from the hamster vimentin gene (Quax et al., 1983) in pGem-7Zf(+) (Promega, Madison, WI). In this construct the double AP-1 consensus sequence was altered by in vitro mutagenesis (Fig. 1) and subsequently, the mutagenized 0.8 kb *Bgl*II/*Xho*I subfragment was exchanged with a corresponding fragment in the wild-type vimentin reporter construct, resulting in -3100[AP-1]vimcat. The mutagenized bases have been verified by sequence analysis. The tkcat[AP-1] construct is derived from pBLCAT2 and contains an oligonucleotide fragment, comprising the vimentin AP-1 element, shown in Figure 1 inserted in the downstream multiple cloning site. In this construct the cat gene is under control of the heterologous herpes simplex tk promoter (Luckow and Schütz, 1987). The RARα, RARβ, and RARγ expression plasmids have been kindly provided by Dr. P. Chambon. The RARα bacterial expression vector has been created by inserting a 1.8 kb hRARα$_1$ *Kpn*I/*Eco*RI fragment (Petkovich et al., 1987; Giguere et al., 1987) lacking the first 19 amino acids in pGEX3X. This system promotes expression and isolation of a glutathione-S-transferase (GST) fusion protein from bacterial lysates by affinity chromatography using glutathione agarose beads. The 3xRARE[tkcat] expression vector, which was used as a positive control for RA responsiveness, and the RSV c-jun expression vector were kindly provided by Dr. P. van der Saag and Dr. A. van der Eb, respectively.

Cell culture, DNA transfections, and CAT assays

3T3 cells have been cultured according to standard conditions in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Undifferentiated P19EC cells have been cultured in 1:1 DMEM/Ham's F12 medium containing 7.5% fetal bovine serum. Differentiated P19EC derivatives, designated P19EPI-7, P19END-2, and P19MES-1, were kindly provided by Dr. C. Mummery and represent subclones with mainly epidermal, endodermal, and mesodermal characteristics, respecively (Mummery et al., 1985, 1986). Test transfections have been carried out in medium containing charcoal-stripped fetal bovine serum. Since serum only contains RA concentrations up to 10^{-10}M and the results encountered were comparable with the CAT values obtained with standard serum, we continued our experiments under these standard conditions. Cells were plated into 8 cm Petri dishes in DMEM containing 5% fetal bovine serum for the NIH 3T3 cells and 1:1 DMEM/Ham's F12 with 5% fetal bovine serum for the P19EC and P19MES cells 48 hours before transfection. All cell types have been transfected at densities of approximately 25% via a calcium phosphate-mediated method described earlier (van de Klundert et al., 1992). Cultures are transfected by a mixture of CsCl-isolated DNA containing cat plasmid, internal control plasmids, pRSV-β-galactoside or pSV40-β-galactoside, and either RARα, β, and γ (NIH 3T3) or c-jun (P19EC) expression vectors. After the glycerol shock, DMEM (or DMEM/F12) containing 5% fetal bovine serum and 10^{-7} M *all-trans*-RA has been applied. Forty-eight hours post-transfection cells are harvested and transfection efficiencies are determined by monitoring β-galactoside levels. After correction for protein contents, cell lysates have been assayed for CAT expression by a CAT ELISA assay (Boehringer Mannheim, Germany).

Northern blot analysis

RNA isolation, Northern blotting, and hybridization have been performed as described previously (Pieper et al., 1992). Total RNA from P19EC, P19EPI, P19END, and P19MES cells, cultured under high and low serum conditions, has been isolated. As a vimentin probe, a hamster vimentin cDNA has been used. Relative RNA levels have been quantitated by densitometric scanning (Biorad, imaging densitometer, GS-670; Biorad, Richmond, CA).

Bacterial expression of RARα and preparation of nuclear extracts

Nuclear extracts from NIH 3T3, P19MES, and P19EC cells have been prepared by a modification of the method described by Dignam et al. (1983). Briefly cells are lysed in 10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EGTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Nuclei are isolated by centrifugation in lysis buffer containing 6.8% sucrose and extracted in 400 mM NaCl, containing 5%

glycerol. Nuclear proteins are concentrated by $(NH_4)_2SO_4$ precipitation (33% w/v). Dialysis takes place in 20 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, with 20% glycerol in the dialysis solution and 5% glycerol in the dialysis sample. After dialysis, extracts are quickly frozen in liquid nitrogen and stored in small samples at −70°C. A hRARα-GST fusion protein (GST-RARα) has been expressed in bacteria according to procedures previously described (Smith and Johnson, 1988). Briefly, bacteria are sonicated and the debris is pelleted. The supernatant is mixed with 1 ml of preswollen glutathione-agarose beads in 20 mM Hepes, pH 7.6, 100 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, containing 20% glycerol. With constant stirring for 2 hours at 4°C the beads are washed in the same buffer, subsequently proteins are eluted by reduced glutathione. Proteins from different elution steps are quickly frozen in small samples and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) to confirm their molecular weight. The same procedure has been applied to a construct without insert to obtain GST protein alone.

DNA binding assays

Electrophoretic mobility shift assays have been performed with two oligonucleotide fragments. The RARE oligonucleotide contains the RA response element of the RARβ promoter (coding strand: 5'GGGTTCACCGAAAGTTCACTCATCTTA3'). The AP-1/TRE oligonucleotide, which comprises the vimentin double consensus or TPA responsive elements (TRE), is shown in Figure 1. The double-stranded oligonucleotide probes contain *Hind*III and *Eco*RI overhangs, respectively, and have been end-labeled with ^{32}P, using Klenow large fragment polymerase. As a control the binding of GST-RARα fusion protein to its recognition sequence has been tested under the following conditions: 20 mM Tris, pH 8.0, 4 mM $MgCl_2$, 5 mM spermidine, 50 mM KCl, 0.03 mg/ml poly[d(I-C)], 2 mM DTT in 8% glycerol, and 0.1% NP40. Labeled DNA (2 ng) and approximately 1 μg of purified RAR fusion protein have been incubated on ice for 10 minutes and loaded onto a 6% native polyacrylamide gel prepared in 45 mM Tris-borate, pH 8.3, 1 mM EDTA (TBE). Nuclear extract of 3T3 cells has been preincubated with or without different concentrations of GST-RAR or GST protein for 10 minutes at 37°C, after which 3 ng of labeled AP-1 DNA has been supplied, following incubation for 15 minutes at 37°C. Subsequently, incubations with P19MES or P19EC nuclear extracts have been performed. The buffer conditions for the incubations with nuclear extracts are as indicated for the GST fusion protein alone, with minor modifications (10 mM Tris instead of 20 mM and 6% Ficoll instead of glycerol). Also higher amounts of poly[d(I-C)] are required (0.1 mg/ml). Samples have been electrophoresed under the same conditions.

RESULTS

Vimentin expression is differentially regulated in P19EC cells

We and others previously characterized the positive as well as negative regulation of the vimentin gene (Rittling et al., 1989; van de Klundert et al., 1992; Pieper et al., 1992). Vimentin expression is differentially regulated during development. In certain stages a switch occurs between vimentin and a more tissue-specific IF protein. For instance during differentiation of myoblasts into mature muscle cells vimentin is exchanged for desmin. P19EC cells can be induced to differentiate by different concentrations of RA or DMSO and thereby serve as a model system for the study of changes in gene expression during development. Cells differentiate into mainly ectodermal cells, like neural and neuromesodermal cells, by addition of 5×10^{-7} M RA. Administration of 10^{-9} M RA (or 0.5% DMSO) leads to predominantly endodermal cells and RA concentrations of 10^{-8} M (1% DMSO) stimulates the production of mesodermal cells, like skeletal muscle cells. By applying these procedures stably growing cell lines expressing ectodermal, endodermal, or mesodermal markers can be obtained (McBurney et al., 1982).

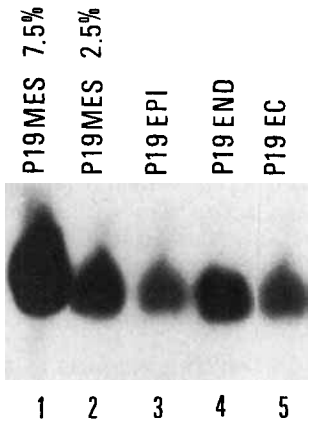


Fig. 2. Northern blot analysis of P19EC cells and differentiated derivatives. Approximately equal amounts of RNA have been electrophoresed. Blots are hybridized with a hamster vimentin cDNA probe.

Total RNA has been isolated from cell lines, named P19EPI, P19END, and P19MES respectively, representing all three alternate pathways and from the undifferentiated parent cell line P19EC. After blotting, filters have been hybridized with a hamster vimentin cDNA probe. Approximately equal amounts of RNA have been electrophoresed, as confirmed by hybridization with a ribosomal DNA probe (results not shown). P19EC cells and P19EPI cells contain only small amounts of vimentin mRNA (Fig. 2). The latter cells represent the neural cell populations which are devoid of vimentin when terminally differentiated. Endodermal cells, represented by the cell line P19END, express threefold higher levels of vimentin mRNA in comparison to ectodermal cells. Furthermore the P19MES cell line representing the progenitor cells for muscle differentiation has been cultured under high (7.5%) and low (2%) serum conditions (Doney et al., 1984). Terminal differentiation of muscle cells requires a conversion of culture conditions from high to low mitogen content. We have observed a 3.5-fold decrease in vimentin mRNA expression, under low serum conditions. Furthermore, expression levels are tenfold higher in P19MES cells cultured under 7.5% serum conditions, in comparison to undifferentiated P19EC cells (Fig. 2). Treatment of P19MES cells cultured under 2.5% serum

with 10^{-7} M RA for 48 hours results in a further 50% decrease of vimentin mRNA (results not shown). Since RA has been shown to induce muscle cell differentiation (Halevy and Lerman, 1993) we have decided to perform transient transfections in P19MES cells by treating these cells with RA. For comparison undifferentiated P19EC cells have also been used in our transient transfection experiments. The time between the addition of RA and harvest of the cells is not long enough for P19EC cells to differentiate. Moreover, the undifferentiated P19EC cells serve as a control for cells expressing *low amounts* of vimentin.

Analysis of 5′-flanking regions of the hamster vimentin gene

For transient transfections using vimentin-cat constructs three cell types have been used. The NIH 3T3 cell line has been chosen as a representative of cells from mesodermal origin, normally expressing vimentin, but containing low levels of RARs. P19EC cells differentiate upon RA addition and thus contain functional amounts of RARs. This is also true for the P19MES cells, which in contrast to the parent cell line, contain high amounts of vimentin. To study the effects of RA addition on vimentin-CAT expression, NIH 3T3 cells have been cotransfected with a mixture of RAR expression vectors (RARα, RARβ and RARγ, 1 μg each). Since we expected a low vimentin expression in P19EC cells, due to low AP-1 activity, an RSV c-jun expression vector has been cotransfected in increasing amounts (0, 2, and 4 μg). In all transfections the CAT activity of a construct with the SV40 promoter and enhancer (Promega) has been used as a positive control and all CAT values are presented as a percentage of the CAT activity of the positive control. Test transfections have been performed in NIH 3T3 and P19EC cells with a construct containing three copies of the RARE and the thymidine kinase promoter driving the cat gene. Very high CAT activities are obtained, under the same conditions applied to the vimentin-cat transfections. CAT activities after RA admission are 35-fold (P19EC) and 25-fold (3T3) higher than controls without RA (Fig. 3). The latter cells have been cotransfected with RAR expression vectors as mentioned earlier.

Previous reports have provided evidence for the presence of an AP-1 enhancer in the vimentin promoter located approximately 700 bp upstream from the transcription initiation site (Rittling et al., 1989; van de Klundert et al., 1992; Pieper et al., 1992). The element, which is very conserved between hamster and humans, is responsible for serum inducibility and TPA responsiveness. Since the element has been reported to be involved in steroid-mediated down-regulation of the collagenase and stromelysin genes (Schüle et al., 1991), we investigate here whether the AP-1 element might also be involved in vimentin down-regulation.

In Figure 4A the results from the transfections with four different vimentin-cat constructs in P19MES cells and NIH 3T3 cells are shown. Mutagenesis of the double AP-1 consensus sequence in the full length vimentin 5′-flanking region leads to sixfold lower amounts of CAT in P19MES cells and fourfold lower amounts in 3T3 cells. These results are comparable with the effect of deletion of all regulatory sequences up to -602 bp,

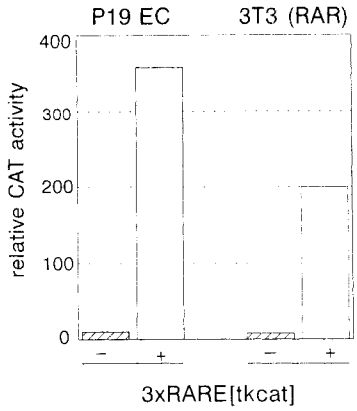


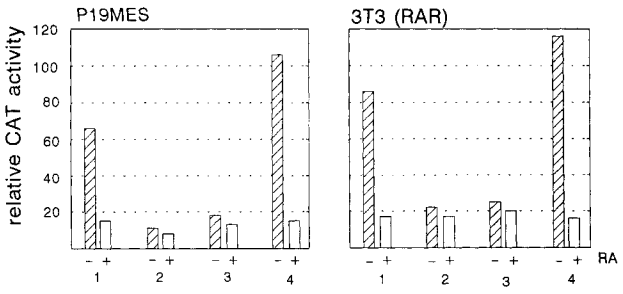
Fig. 3. Control transfection assays with a construct containing three RAR recognition sites. P19EC cells and NIH 3T3 cells are incubated without (−) or with (+) 10^{-7} M RA. CAT values are represented as relative amounts, compared to the activity of the SV40 promoter.

including the AP-1 element, as has been shown earlier for HeLa cells (van de Klundert et al., 1992). Addition of 10^{-7} M RA after transfection suppresses CAT activity of the wild-type vimentin promoter by more than fourfold in both cell types used. This effect is even more pronounced using the construct with the heterologous tk promoter and the vimentin AP-1 containing oligonucleotide. The effect of RA admission is very low (nonsignificant) for both constructs lacking the AP-1 enhancer element.

In P19EC cells the situation seems to be different. The relatively high CAT activity of the vimentin promoter constructs in P19EC cells (without cotransfection of c-jun) is a rather unexpected result (see Fig. 4B). Relative CAT activities (compared to the activity of the SV40-cat positive control) are approximately 40% in P19EC cells compared to almost 70% in P19MES cells. Comparison of the cat transfection results with the Northern blots reveals a more pronounced difference in vimentin mRNA abundance. This discrepancy probably arises from a difference in expression of the positive control in both cell types. Comparison of CAT values obtained by using the SV40 promoter construct in P19EC cells and P19MES cells reveals a four to fivefold lower activity in P19EC cells, corrected for transfection efficiency. Moreover, we would have expected a low activity of the vimentin promoter in P19EC cells considering the low amount of AP-1 activity in these cells (de Groot et al., 1990). It is striking that the construct containing the mutagenized AP-1 consensus sequence exhibits fourfold lower CAT activities compared to the wild-type promoter construct. This implies the *trans*-activation of the AP-1 enhancer element in these cells, despite the absence of Fos and Jun protein. CAT expression of the wild-type vimentin promoter and the AP-1 oligo-derived construct is up-regulated three to fourfold using the highest concentration (4 μg) of cotransfected jun expression plasmid. Here, we would have expected a much higher response too, in view of the results obtained with, for instance, the collagenase promoter (Angel et al., 1988; Chiu et al., 1988; Sassone-Corsi et al., 1988). Furthermore, administration of 10^{-7} M RA

A

	P19MES -	P19MES + RA	3T3 RAR -	3T3 RAR + RA
1. -3100vimcat	66	15	86	17
2. -3100[AP-1mut]vimcat	11	8	22	17
3. -602vimcat	18	13	25	20
4. tkcat[AP-1]	106	15	116	16



B

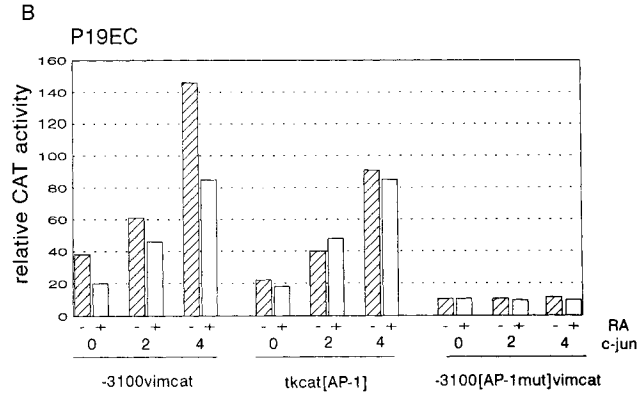


Fig. 4. **A:** Schematic representation of CAT activities using four different vimentin cat constructs and two different cell types. The wild-type hamster vimentin 5′-flanking region is represented by -3100vimcat. In -3100[AP-1mut]vimcat the double AP-1 consensus sequence is destroyed by site-directed mutagenesis and -602vimcat is a deletion construct lacking the AP-1 element. The last construct, tkcat[AP-1]CAT, is a reporter construct which contains the heterologous tk promoter and an oligonucleotide comprising the vimentin AP-1 element. NIH 3T3 cells are cotransfected with RARα, RARβ, and RARγ (1 μg each). Values are presented as relative amounts of the CAT activity of the positive control (SV40-cat) and are the result of three to four independent experiments. RA was added immediately after transfection (concentration 10^{-7} M) and cells were cultured in the presence of RA for 2 days. **B:** The bar diagram represents the results obtained with the P19EC cells. Cells are cotransfected with 0, 2, or 4 μg RSV c-jun expression vector. RA was admitted directly after transfection and cells were cultured in the presence of 10^{-7} M RA for 2 days post-transfection. CAT values are normalized for the activity of the positive control (SV40 CAT).

only leads to a small decrease in CAT activity (Fig. 4B). As expected, the CAT activity of the construct with the mutated AP-1 element is not affected by the cotransfection of c-jun expression vector. In conclusion, vimentin expression is down-regulated by addition of RA in P19MES and NIH 3T3 cells but not in P19EC cells. Unexpectedly, the AP-1 enhancer element in the vimentin promoter can be transactivated in P19EC cells, most probably due to the presence of binding proteins other than Fos or Jun.

In vitro DNA protein interaction studies

Binding of components of the AP-1 complex to the vimentin double consensus sequence has been shown earlier (Rittling et al., 1989; van de Klundert et al., 1992). To investigate whether RARα is able to diminish the binding activity of the AP-1 complex, we have expressed the protein as a GST-fusion product in *Escherichia coli*. For this purpose a partial cDNA fragment lacking the coding sequences for the first 19 aminoacids has been used. To confirm the binding of this bacterially expressed fusion protein to its cognate recognition sequence, we have end-labeled an oligonucleotide fragment containing a functional RARE and have incubated it with the affinity purified RARα fusion protein (Fig. 5A). A competition assay has been performed simultaneously with a 5 and 40-fold molar excess unlabeled fragment.

To investigate the inhibition of AP-1 complex binding by RARα, we have performed competition experiments with 3T3 nuclear extracts and the bacterially expressed fusion protein. Using electrophoretic mobility assays the binding activities of nuclear extract, to an end-labeled probe containing the AP-1 recognition sites or TREs (coding strand: 5′GGGTTCACCGAAAGTTCACTCATCTTA3′), with or without preincubation incubation with different amounts (0.5, 0.8, and 1.5 μg) of RARα fusion protein, have been tested (Fig. 5B). First, as a control the binding of bacterially expressed RARα fusion protein to the AP-1 consensus has been tested and found to be negligible (lane 8). Also as a control, GST protein alone has been incubated with 3T3 nuclear extract (lane 4) using the same amount of protein as in the lane with the highest concentration of RARα-GST. As shown in Figure 5B lanes 5, 6, and 7, the binding of AP-1 complex to its cognate binding sequence can be inhibited by addition of RAR fusion protein. This inhibition is concentration dependent, since higher amounts of RAR lead to a more faint band representing the retarded complex. The same amount of RAR fusion protein needed for occupancy of the RARE site leads to almost complete exclusion of AP-1 binding. This implies that the concentrations of RARα fusion protein used in these experiments lie within physiological range. Incubation of the AP-1/TRE probe from the vimentin promoter with a nuclear extract from P19MES cells causes a retarded complex resembling the complex revealed by 3T3 extract. The amount of shifted complex can also be diminished by incubation with GST-RARα. On the other hand when P19EC nuclear extract is used, the shifted complex migrates with a higher mobility and competition with GST-RARα does not result in a disappearance of the retarded band. This sustains the idea that the proteins binding to the double AP-1 consensus sequence in the vimentin promoter in P19EC cells are different from the ones in P19MES and 3T3 cells.

DISCUSSION

We report here that vimentin expression is inhibited following treatment of fibroblastic NIH 3T3 cells and mesodermally differentiated P19 cells with RA. Our results support the data obtained with the collagenase and stromelysin genes, where AP-1 elements were the main target of down-regulation by hormonal receptors

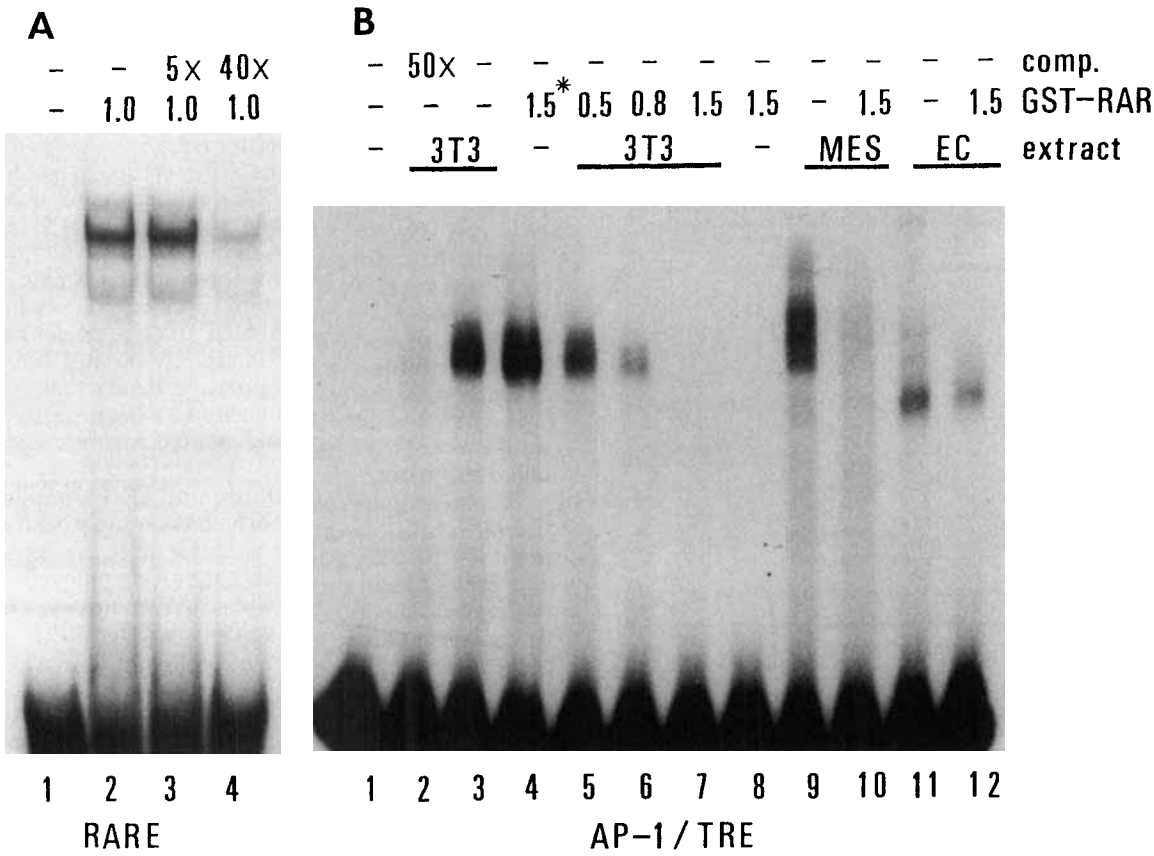


Fig. 5. **A:** Control gel mobility shift assay with an end-labeled RARE probe. The labeled DNA has been incubated in binding buffer either with 1 μg of a bacterially expressed RARα fusion protein (lane 2) or without any added protein (lane 1). Competition has been performed with a 5-fold and 40-fold molar excess of unlabeled DNA fragment (lanes 3, 4). **B:** Gel mobility shift assay with nuclear extracts and a bacterially expressed RARα GST-fusion protein incubated with an AP-1 consensus (TRE) probe are shown. Lane 1: Control without nuclear extract. Lanes 3, 2: 3T3 extract (2.5 μg) has been added to the probe and extract with 50-fold molar excess unlabeled competition DNA (comp.), respectively. Lane 4: Marked with an asterisk, 1.5 μg GST protein (mock) has been added together with 2.5 μg of 3T3 nuclear extract. Lanes 5–7: Increasing amounts (0.5, 0.8, and 1.5 μg) of GST-RARα (GST-RAR) in combination with 2.5 μg of 3T3 nuclear extract have been used. Lane 8: GST-RARα only has been incubated with the ^{32}P-labeled probe. Lanes 9–12: Shifted complexes obtained with 2.4 μg P19MES (MES) and P19EC (EC) nuclear extract. Lanes 10, 12: 1.5 μg GST-RARα has been added.

(Jonat et al., 1990; Lafyatis et al., 1990; Nicholson et al., 1990; Schüle et al., 1990b, 1991; Yang-Yen et al., 1990). We present additional evidence for the involvement of the AP-1 element, by mutation analysis of this sequence in the vimentin promoter, a special double consensus formed by two inverted repeats (Fig. 1). We conclude from our in vitro binding assays that the binding of AP-1 complex in nuclear extracts from P19MES and 3T3 cells can be blocked by addition of purified GST-RARα protein. The inhibition of binding of AP-1 by RAR is concentration dependent and no RA seems to be necessary in vitro. Furthermore the amount of RAR protein required for inhibition lies within physiological range because the same amount of protein is needed for occupation of its own recognition sequence. Using *P19EC cells, however*, a shifted complex with a higher mobility is obtained. Addition of the highest concentration of RARα fusion protein does not lead to full inhibition of AP-1 binding activity, in contrast to the effects of RAR on AP-1 binding in P19MES and 3T3 extracts.

The mechanism published so far to be responsible for this repression implies direct binding of Fos or Jun protein to the hormonal receptor in solution, thereby preventing the AP-1 complex from gene activation. There is, however, evidence from in vivo footprinting experiments, that the AP-1 site remains occupied during, for instance, dexamethasone repression by the glucocorticoid receptor of collagenase expression (König et al., 1992). An hypothesis to explain this phenomenon has been presented by Kerppola et al. (1993) who suggested that the glucocorticoid receptor causes a depletion by differential binding of Fos protein, resulting in a shift from Fos-Jun heterodimers to Jun-Jun homodimers. Since Jun-Jun homodimers are less potent transcriptional activators, this may lead to a lower promoter activity without reducing AP-1 *site occupancy*. Often both Jun and Fos (but in some cases only Jun) are reported to be the preferential target of a specific receptor, like in the case of the thyroid hormone receptor (Lopez et al., 1993).

Repression of transcription by RA of the vimentin promoter has only been found in the two mesodermally derived cell types tested. This implies a cell type-specific effect. The results obtained with the undifferenti-

ated P19EC cells point to a different mechanism, because RA addition does not decrease promoter activity significantly. Furthermore, we expected a very low activity of the vimentin promoter in these cells and therefore we performed cotransfection experiments with a c-jun expression vector. In the case of the collagenase promoter this has led to high increases in promoter activity (Angel et al., 1988; Chiu et al., 1988; Sassone-Corsi et al., 1988). Our results demonstrate that only a low amount of stimulation is obtained with this procedure. The main difference between the collagenase AP-1 and the vimentin AP-1 enhancer element is formed by its arrangement. In the collagenase promoter only one AP-1 element is present (Angel et al., 1987), whereas in the vimentin regulatory region the double AP-1 consensus sequence can be regarded as two inverted repeats forming a nearly perfect palindrome. Exactly the same arrangement has been published earlier for the rat glutathione transferase P gene (Okuda et al., 1990). Here, transfection of promoter constructs in F9EC cells also revealed rather high CAT activities, which could hardly be stimulated by addition of c-Jun or c-Fos. The only member of the Jun family present in significant amounts in undifferentiated F9 and P19EC cells is Jun D (de Groot et al., 1990). Therefore, it is tempting to speculate about a role for this particular protein in binding to the specific conformation of the two consensus sequences caused by the palindromic arrangement. The data obtained by the transient transfection assays are in accordance with the gel mobility shift analysis using P19EC nuclear extracts. In this case a faster migrating complex has been revealed which does not disappear by the highest concentration of RARα fusion protein. This also points to a different protein or protein complex binding in P19EC cells. This hypothesis is currently under investigation. The binding of Jun D instead of for instance c-jun, might also be an explanation for the lack of RA repression. Proof for this assumption can be gained from a study, describing inhibition of estrogen receptor activity by overexpression of c-Jun and c-Fos, but not Jun D protein (Doucas et al., 1991). More recently it has been shown that Jun D is not able to inhibit glucocorticoid receptor activity in contrast to c-Jun (Berkoflint et al., 1994). Furthermore, the region which is defined to be important for binding to the hormone receptor is not conserved in Jun D (Berger and Shaul, 1991). A question which remains is why overexpression of c-jun does not lead to higher vimentin expression. This can only be explained by assuming that activation of the double AP-1 consensus by Jun-Jun homodimers can hardly contribute to the amount of activation already caused by some other protein present in untransfected P19EC cells. Since RA addition is rather brief, Jun or Fos protein will not be induced (de Groot et al., 1990), so the fact that there is no inhibition detectable must imply that the RAR protein cannot prevent an AP-1-activating protein or protein complex from binding in undifferentiated P19EC cells. This strongly suggests that under the conditions described vimentin expression is not inhibited by the RAR.

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LITERATURE CITED

Angel, P., Baumann, J., Stein, B., Delius, H., Rahmsdorf, H.J., and Herrlich, P. (1987) 12-O-tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5′-flanking region. Mol. Cell. Biol., *7*:2256–2266.

Angel, P., Allegretto, E.A., Okino, S., Hattori, K., Boyle, W.J., Hunter, T., and Karin, M. (1988). Oncogene *jun* encodes a sequence-specific *trans*-activator similar to AP-1. Nature, *332*:166–171.

Berger, I., and Shaul, Y. (1991) Structure and function of human Jun-D. Oncogene, *6*:561–566.

Berkoflint, Y., Levkowitz, G., and Vardimon, L. (1994) Involvement of c-Jun in the control of glucocorticoid receptor transcriptional activity during development of chicken retinal tissue. EMBO J., *13*:646–654.

Chiu, R., Boyle, W.J., Meek, J., Smeal, T., Hunter, T., and Karin, M. (1988) The c-Fos protein interacts with c-jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell, *54*:541–552.

Curran, T., and Franza, B.R., Jr. (1988) Fos and Jun: The AP-1 connection. Cell, *55*:395–397.

Diamond, M.I., Miner, J.N., Yoshinaga, S.K., and Yamamoto, K.R. (1990) Transcription factor interactions: Selectors of positive or negative regulation from a single DNA element. Science, *249*:1266–1272.

Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res., *11*:1475–1489.

Doney, C., Kessel, M., and Gruss, P. (1984) An embryonal carcinoma cell line as a model system to study developmentally regulated genes during myogenesis. Cell. Differ., *15*:275–279.

Doucas, V., Spyrou, G., and Yaniv, M. (1991) Unregulated expression of c-Jun or c-Fos but not Jun D inhibits oestrogen receptor activity in human breast cancer derived cells. EMBO J., *10*:2237–2245.

Ferrari, S., Battini, R., Kaczmarek, L., Rittling, S., Calabretta, B., de Riel, J.K., Philiponis, V., Wei, J.-F., and Baserga, R. (1986) Coding sequence and growth regulation of the human vimentin gene. Mol. Cell. Biol., *6*:3614–3620.

Giguere, V., Ong, E.S., Segui, P., and Evans, R.M. (1987) Identification of a receptor for the morphogen retinoic acid. Nature, *330*:624–629.

Groot, de R.P., Schoorlemmer, J., van Genesen, S.T., and Kruijer, W. (1990) Differential expression of *jun* and *fos* genes during differentiation of mouse P19 embryonal carcinoma cells. Nucleic Acids Res., *18*:3195–3202.

Halevy, O., and Lerman, O. (1993) Retinoic acid induces adult muscle cell differentiation mediated by the retinioc acid receptor-α. J. Cell. Physiol., *154*:566–572.

Hudson, L.G., Santon, J.B., Glass, C.K., and Gill, G.N. (1990) Ligand-activated thyroid hormone and retinoic acid receptors inhibit growth factor receptor promoter expression. Cell, *62*:1165–1175.

Järvinen, M. (1990) Vimentin in human erythroleukemia (HEL) cells is modulated with differentiation inducers. Cell Biol. Int. Rep., *14*:199–209.

Jonat, C., Rahmsdorf, H.J., Park, K.K., Cato, A.C., Gebel, S., Ponta, H., and Herrlich, P. (1990) Antitumor promotion and antiinflammation: Down-modulation of AP-1/(Fos/Jun) activity by glucocorticoid hormone. Cell, *62*:1189–1204.

Kerppola, T.K., Luk, D., and Curran, T. (1993) Fos is a preferential target of glucocorticoid receptor inhibition of AP-1 activity in vitro. Mol. Cell. Biol., *13*:3782–3791.

Klundert van de, F.A.J.M., van Eldik, G.J., Pieper, F.R., Jansen, H.J., and Bloemendal, H. (1992) Identification of two silencers flanking an AP-1 enhancer in the vimentin promoter. Gene, *122*:337–343.

König, H., Ponta, H., Rahmsdorf, H.J., and Herrlich, P. (1992) Interference between pathway-specific transcription factors: Glucocorticoids antagonize phorbol ester-induced AP-1 activity without altering AP-1-site occupancy in vivo. EMBO J., *11:*2241–2246.

Lafyatis, R., Kim, S.-J., Angel, P., Roberts, A.B., Sporn, M.B., Karin, M., and Wilder, R.L. (1990) Interleukin-1 stimulates and *all-trans*-retinoic acid inhibits collagenase gene expression through its 5′ activator protein-1-binding site. Mol. Endocrinol., *4:*973–980.

Leung, M.-F., Sokoloski, J.A., and Sartorelli, A.C. (1992) Changes in microtubules, microtubule-associated proteins, and intermediate filaments during the differentiation of HL-60 leukemia cells. Cancer Res., *52:*949–954.

Lopez, G., Schaufele, F., Webb, P., Holloway, J.M., Baxter, J.D., and Kushner, P.J. (1993) Positive and negative modulation of Jun action by thyroid hormone receptor at a unique AP-1 site. Mol. Cell. Biol., *13:*3042–3049.

Luckow, B., and Schütz, G. (1987) CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. Nucleic Acids Res., *13:*5490.

McBurney, M.W., Jones-Villeneuve, E.M.V., Edwards, M.K.S., and Anderson, P.J. (1982) Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. Nature, *299:*165–167.

Miner, J.N., Diamond, M.I., and Yamamoto, K.R. (1991) Joints in the regulatory lattice: Composite regulation by steroid receptor-AP-1 complexes. Cell Growth Differ., *2:*525–530.

Mummery, C.L., Feijen, A., van der Saag, P.T., van den Brink, C.E., and de Laat, S.W. (1985) Clonal variants of differentiated P19 embryonal carcinoma cells exhibit epidermal growth factor receptor kinase activity. Dev. Biol., *109:*402–410.

Mummery, C.L., Feijen, A., Moolenaar, W.H., van de Brink, C.E., and de Laat, W.W. (1986) Establishment of a differentiated mesodermal line from P19 EC cells expressing functional PDGF and EGF receptor. Exp. Cell Res., *165:*229–242.

Nicholson, R.C., Mader, S., Nagpal, S., Leid, M., Rochette-Egly, C., and Chambon, P. (1990) Negative regulation of the rat stromelysin gene promoter by retinoic acid is mediated by an AP1 binding site. EMBO J., *9:*4443–4454.

Okuda, A., Imagawa, M., Sakai, M., and Muramatsu, M. (1990) Functional cooperativity between two TPA reponsive elements in undifferentiated F9 embryonic stem cells. EMBO J., *9:*1132–1135.

Petkovitch, M. (1992) Regulation of gene expression by vitamin A: The role of nuclear retinoic acid receptors. Annu. Rev. Nutr., *12:*443–471.

Petkovich, M., Brand, N.J., Krust, A., and Chambon, P. (1987) A human retinoic acid receptor which belong to the family of nuclear receptors. Nature, *330:*444–450.

Pfahl, M. (1993) Nuclear recptor/AP-1 interaction. Endocr. Rev., *14:*651–658.

Pieper, F.R., van de Klundert, F.A., Raats, J.M., Henderik, J.B., Schaart, G., Ramaekers, F.C., and Bloemendal, H. (1992) Regulation of vimentin expression in cultured epithelial cells. Eur. J. Biochem., *210:*509–519.

Quax, W., Vree Egberts, W., Hendriks, W., Quex-Jeuken, Y., and Bloemendal, H. (1983) The structure of the vimentin gene. Cell, *35:*215–223.

Rittling, S.R., Coutinho, L., Amram, T., and Kolbe, M. (1989) AP-1/*jun* binding sites mediate serum inducibility of the human vimentin promoter. Nucleic Acids Res., *17:*1619–1633.

Rius, C., and Aller,P. (1992) Vimentin expression as a late event in the in vitro differentiation of human promonocytic cells. J. Cell Sci., *101:*395–401.

Sassone-Corsi, P., Lamph, W.W., Kamps, M., and Verma, I.M. (1988) Fos-associated cellular P39 is related to nuclear transcription factor AP-1. Cell, *54:*553–560.

Schüle, R., and Evans, R.M. (1991) Cross-coupling of signal transduction pathways: Zinc finger meets leucine zipper. Trends Genet., *7:*377–381.

Schüle, R., Umesono, K., Mangelsdorf, D.J., Bolado, J., Pike, J.W., and Evans, R.M. (1990a) Jun-Fos and receptors for vitamins A and D recognize a common response element in the human osteocalcin gene. Cell, *61:*497–504.

Schüle, R., Rangarajan, P., Kliewer, S., Ransone, L.J., Bolado, J., Yang, N., Verma, I.M., and Evans, R.M. (1990b) Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. Cell, *62:*1217–1226.

Schüle, R., Rangarajan, P., Yang, N., Kliewer, S., Ransone, L.J., Bolado, J., Verma, I.M., and Evans, R.M. (1991) Retinoic acid is a negative regulator of AP-1-responsive genes. Proc. Natl. Acad. Sci. USA, *88:*6092–6096.

Shemshedini, L., Knauthe, R., Sassone-Corsi, P., Pornon, A., and Gronemeyer, H. (1991) Cell-specific inhibitory and stimulatory effects of Fos and Jun on transcriptional activation by nuclear receptors. EMBO J., *10:*3839–3849.

Smith, D.B., and Johnson, K.S. (1988) Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene, *67:*31–40.

Wood, W.R., Seftor, E.A., Lotan, D., Nakajima, M., Misiorowski, R.L., Seftor, R.E.B., Lotan, R., and Hendrix, M.J.C. (1990) Retinoic acid inhibits human melanoma tumor cell invasion. Anticancer Res., *10:*423–432.

Yang-Yen, H.-F., Chambard, J.C., Sun, Y.L., Smeal, T., Schmidt, T.J., Drouin, J., and Karin, M. (1990) Transcriptional interference between c-Jun and the glucocorticoid receptor: Mutual inhibition of DNA binding due to direct protein-protein interaction. Cell, *62:*1205–1215.

Zhang, X.-K., and Pfahl, M. (1993) Regulation of retinoid and thyroid hormone action through homo- and heterodimeric receptors. Trends Endocrinol. Metab., *4:*156–162.