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Negative regulation of the rat stromelysin gene promoter by retinoic acid is mediated by an AP1 binding site

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Stromelysin is a member of the metalloproteinase family which plays an important role in extracellular matrix remodelling during many normal and disease processes. We show here that in polyomavirus-transformed rat embryo fibroblast cells (PyT21), the transcription from the stromelysin gene is repressed by the vitamin A derivative retinoic acid (RA). Furthermore, expression vectors encoding the human RA receptors hRAR-α, hRAR-β and hRAR-γ repress chloramphenicol acetyltransferase (CAT) expression from stromelysin promoter–CAT gene expression vectors in RA-treated PyT21 and human HeLa cells, as determined by transient transfection assays. Through mutation and deletion analysis, we show that the RA dependent repression is mediated by a 25 bp region from nucleotide positions −72 to −48 of the rat stromelysin 5′-flanking DNA sequence. Further mutation analysis of this region indicates that the DNA sequence required for RA dependent repression co-localizes with an AP1 binding site which is essential for promoter activity. We show also that RA represses the transcriptional activity of a reporter gene containing a TPA responding AP1 binding site driving the HSV tk promoter. Thus the RAR–RA complex appears to repress transcription of the stromelysin gene by blocking activation by positive regulatory factors. However, we found no evidence supporting the possibility that the RA dependent repression could be due to RAR binding to the AP1 binding site or to the AP1 components c-*fos* and c-*jun*.

Key words: extracellular matrix/metalloproteinase/retinoic acid receptors/transcriptional repression/tumoral cells

Introduction

Retinoic acid (RA), retinol (vitamin A) and other retinoids are known to exert striking effects on cell proliferation and differentiation, to inhibit the growth of some malignant cells and to inhibit the invasiveness of metastatic cells (Lotan, 1980; Roberts and Sporn, 1984; Nakajima *et al.*, 1989). The cDNAs of several mRNAs which increase in abundance in cells treated with RA have been isolated (Wang *et al.*, 1985; Horton *et al.*, 1987; Chiocca *et al.*, 1988; LaRosa and Gudas, 1988a,b) and many of these mRNAs encode components of the extracellular matrix (ECM), such as fibronectin, laminin, and types III and IV collagen. The ECM forms a substrate for cell attachment and migration and directs cell form and function through ECM–cell interactions (Yamada, 1983; Dufour *et al.*, 1988; McDonald, 1989; Morrison-Graham and Weston, 1989; Perris and Bronner-Fraser, 1989). The ECM provides not only a substratum for migration, but is also a source of differentiative cues for developing cells. The ECM also forms a barrier which must be removed in order for tumour invasion or metastases to occur (Mullins and Rohrlich, 1983; Liotta, 1986; Tryggvason *et al.*, 1987).

A controlled rate of turnover of the ECM must accompany the changes which occur during development, as well as at times such as during uterine involution, or wound healing (Brenner *et al.*, 1989; Werb, 1989). Uncontrolled degradation of the ECM can have severe consequences, however, as in pathological conditions such as rheumatoid arthritis, or tumor invasion and metastases (Mullins and Rohrlich, 1983; Murphy and Reynolds, 1985; Liotta, 1986; Tryggvason *et al.*, 1987; Alexander and Werb, 1989). The remodelling of the ECM requires the action of extracellular proteolytic enzymes such as the metal dependent family of proteinases, the metalloproteinases (Tryggvason *et al.*, 1987; Alexander and Werb, 1989; Werb, 1989). The metalloproteinase family includes collagenase, an enzyme which is specific for degradation of interstitial collagens (types I, II and III) (Werb and Reynolds, 1975; Murphy *et al.*, 1982) and stromelysin, a broad spectrum enzyme which degrades fibronectin, collagens type III, IV and V, laminin, and proteoglycans (Galloway *et al.*, 1983; Chin *et al.*, 1985; Okada *et al.*, 1986; Nicholson *et al.*, 1989), as well as several other enzymes with a variety of substrate specificities (Werb, 1989; Nicholson *et al.*, 1989; Matrisian, 1990; and references therein). The ability to modulate the proteolytic activity of these metalloproteinases (Murphy *et al.*, 1985; Sanchez-Lopez *et al.*, 1988; and references therein), in addition to regulating the level of their gene transcription, suggests that these proteinases represent a critical control point in modifying the rate of ECM turnover.

Earlier work from this laboratory showed that an RNA (termed transin) originally identified as being overexpressed in polyoma virus transformed rat embryo fibroblasts, as compared with the non-transformed parental cells, is also inducible by the activated cellular oncogene Ha-*ras*, and by epidermal growth factor (EGF) (Matrisian *et al.*, 1985a). Transin RNA was shown to be more abundant in invasive mouse skin squamous cell carcinomas than in benign papillomas (Matrisian *et al.*, 1986a), and to be dramatically elevated in carcinomas with a high propensity for metastasis (Ostrowski *et al.*, 1988). We have recently shown that the protein encoded by the transin RNA is the rat equivalent of human stromelysins (Nicholson *et al.*, 1989).

Stromelysin and collagenase are often coordinately

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expressed and synthesized (Chin *et al.*, 1985; Frisch *et al.*, 1987; Sellers *et al.*, 1978), and indeed gene expression for both of these proteinases is induced by interleukin 1, tumor promoting phorbol esters (TPA), as well as by EGF (Mizel *et al.*, 1981; Chua *et al.*, 1985; Frisch and Ruley, 1987; Frisch *et al.*, 1987; Kerr *et al.*, 1988a; Saus *et al.*, 1988). In addition to studying the factors involved in stimulating expression of these metalloproteinases, it is equally important to examine the factors which inhibit the expression of these proteinases. Transforming growth factor $\beta 1$, the synthetic glucocorticoid dexamethasone, and retinoic acid are all known to repress expression of collagenase and stromelysin (Brinckerhoff *et al.*, 1986; Edwards *et al.*, 1987; Frisch and Ruley, 1987; Kerr *et al.*, 1988b; Quinones *et al.*, 1989; Sirum and Brinckerhoff, 1989). The recent characterization, in this laboratory and others, of cloned cDNAs encoding receptors for RA [RAR-α (Giguere *et al.*, 1987; Petkovich *et al.*, 1987), RAR-β (Brand *et al.*, 1988; de Thé *et al.*, 1987), RAR-γ (Krust *et al.*, 1989; Zelent *et al.*, 1989)] has indicated that the RARs are related to the steroid/thyroid hormone receptor superfamily (Green and Chambon, 1988; Evans, 1988). This suggested that the cellular effects of RA may occur as a result of the modulation of the expression of certain target genes by direct interactions of an RAR with target gene promoters. These cloned RARs have allowed us to address the question of what molecular mechanisms are involved in RA-mediated repression of metalloproteinase gene expression.

In this paper, we show that transcription from the stromelysin gene is repressed in the presence of RA via a process which is at least in part a primary transcriptional response, requires RA receptor, occurs at physiological RA concentrations, and is directed through a promoter element which is a binding site for the transcriptional activator protein, AP1.

Results

RA-mediated repression is a primary transcriptional response

The polyomavirus-transformed rat fibroblast cell line, PyT21, expresses the stromelysin gene (Matrisian *et al.*, 1985a) and, as shown in Figure 1A, the amount of stromelysin RNA was reduced by 85% after exposure of the cells to RA for 48 h. To determine if this decrease occurred at the level of transcription, nuclei were isolated from cells treated with RA for various periods of time, and the *in vivo* initiated RNA radioactively labelled during an *in vitro* nuclear RNA elongation assay (nuclear run-on) was detected by hybridization to immobilized cDNAs (Figure 1B). The amount of RNA hybridizing to the stromelysin (STR) cDNA decreased by 60% after 3 h of RA treatment and by 85% after 12 h. In contrast, the amount of RNA corresponding to genes encoding a structural protein, actin (ACT), or the metabolic enzyme lactate dehydrogenase (LDH), did not decrease significantly during these time periods. This repression of transcription of the stromelysin gene requires the continuous presence of RA since the transcriptional levels rose to the control level after removal of RA (data not shown). The amount of RNA hybridizing to each of these cDNAs decreased by 70–95% when 1 μg/ml α-amanitin (αa) was present in the RNA elongation reaction, confirming that the RNA detected

Fig. 1. (**A**) Steady-state analysis of rat stromelysin (STR) RNA levels in rat PyT21 cells before (–) and after (+) exposure to retinoic acid (RA). PyT21 cells were treated with 1×10^{-6} M RA (+) or ethanol (–) for 48 h in serum-free medium (Matrisian *et al.*, 1985a). The migration position of the rRNA markers (28S and 18S) were determined by ethidium bromide staining of the gel (not shown). (**B**) Transcription levels of the stromelysin gene as determined by nuclear run-on transcription assay after exposure of cells to RA for different times in serum-free medium. Autoradiography of a slot blot hybridization comparing *in vitro* synthesized ^{32}P-labelled RNA, using nuclei of cells treated with RA for 3 or 12 h periods, and hybridized to nitrocellulose bound cDNAs for stromelysin (STR), lactate dehydrogenase (LDH), actin (ACT), or to a control plasmid DNA (VEC). αa represents in the nuclear run-on assay 1 μg/ml α-amanitin. (**C**) The effect of cycloheximide (CY) on transcriptional repression by RA as determined by hybridization of ^{32}P-labelled RNA to cDNAs as in panel B. (–) or (+) indicates absence or presence of RA or CY in the culture medium. Treatment with RA (1×10^{-6} M) was for 4 h, and treatment with cycloheximide (1 μg/ml) was begun 1 h before addition of RA.

resulted from transcription by RNA polymerase B(II). Figure 1C shows that the repression of the stromelysin gene is at least in part a primary transcriptional response to RA, since the amount of stromelysin RNA transcribed was reduced after a 4 h exposure to RA even when protein synthesis had been inhibited with cycloheximide. Cycloheximide alone had less effect on transcription of the stromelysin gene, or on transcription of the actin or LDH genes either alone or in the presence of RA.

Requirement of RAR and of 5′-flanking DNA sequences

To determine if DNA sequences located in the 5′-flanking region of the rat stromelysin gene were involved in the RA dependent transcriptional response, DNA sequences from –1100 to +8 of the stromelysin gene (Matrisian *et al.*, 1986b) were linked upstream of a promoterless chloramphenicol acetyltransferase (CAT) gene to create the fusion plasmid STR–CAT. RA treatment of PyT21 cells transfected with STR–CAT (Figure 2A) showed an average of 50% of the CAT activity detected in the absence of RA. This CAT activity was decreased by (on average) 85%, 72% or 89% in the presence of RA when STR–CAT was co-transfected with a plasmid expressing the cDNA for either RAR-α, RAR-β or RAR-γ respectively (Figure 2A). In contrast, the rabbit β-globin promoter (GLOB) did not confer RA dependent regulation on linked CAT sequences even when GLOB-CAT was co-transfected with an RAR expression plasmid (Figure 2B). This indicates that RA represses stromelysin expression through 5′-flanking DNA sequences in a manner which requires RAR.

To delineate the region within the 5′-flanking DNA sequences of the stromelysin gene which is involved in the RA dependent transcriptional repression, we constructed additional CAT fusion plasmids which contain DNA sequences from positions +8 to –550 (550-CAT), or –121 (121-CAT) or –84 (84-CAT) of the stromelysin gene (Figure 3A). Figure 3B shows the effect of RA on CAT

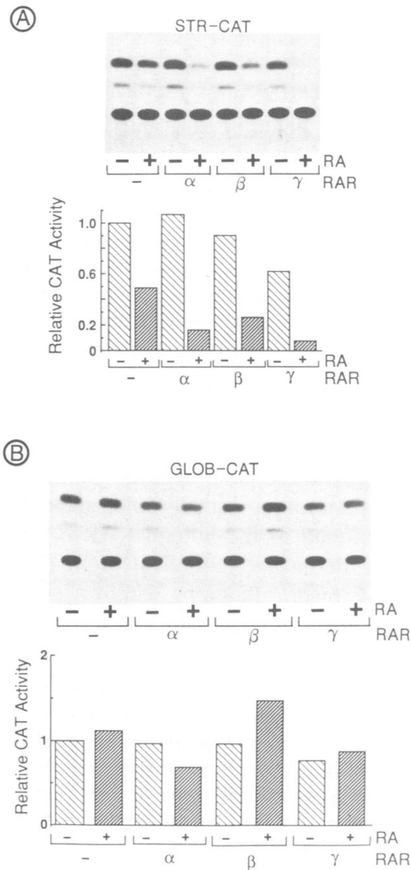

Fig. 2. RAR-mediated repression of stromelysin promoter activity in PyT21 cells. (**A**) STR−CAT (see Figure 3) was co-transfected into PyT21 cells with 1 μg of hRAR-α0 (α), hRAR-β0(β), hRAR-γ0(γ) or the parental expression vector pSG5 (−) in the presence (+) or absence of RA (Materials and methods). The averaged values (±15%) of CAT activities obtained from four independent transfections are presented in the histogram. (**B**) As in A except that the rabbit β-globin gene promoter linked to the CAT gene (GLOB−CAT) was transfected with the RAR expression vector.

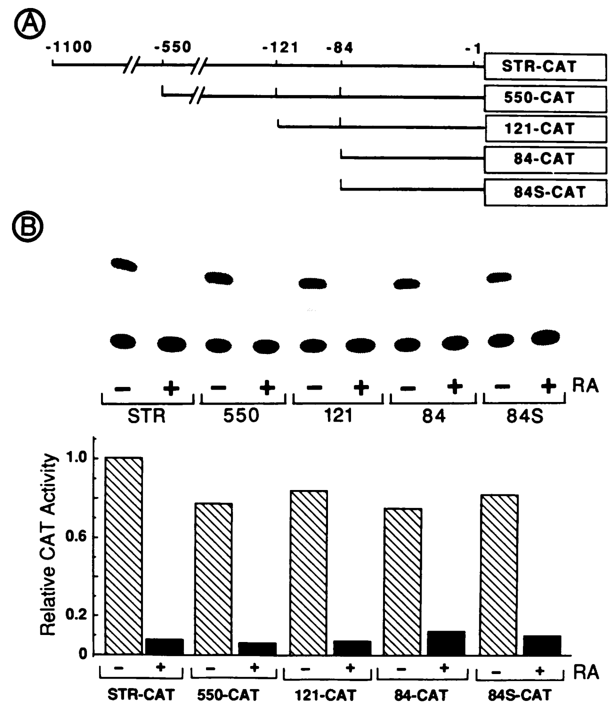

Fig. 3. Localization of RA responsive DNA sequences to within 84 bp of the transcription start site of the stromelysin promoter. (**A**) Diagramatic representation of stromelysin promoter−CAT constructs containing 1100 bp (STR−CAT), 550 bp (550-CAT), 121 bp (121-CAT), 84 bp (84-CAT) of stromelysin gene 5′-flanking DNA sequences, or a synthetic replica (84S-CAT) of 84-CAT, fused to the CAT gene. (**B**) These above plasmids were co-transfected with hRAR-γ0 (1 μg) into PyT21 cells in the presence (+) or absence (−) of RA, and the resulting CAT activities obtained are shown. Averaged values (±15%) of CAT activities from five such transfections are presented in the histogram.

activity when STR−CAT, 550-CAT, 121-CAT, 84-CAT, or a synthetic promoter CAT fusion plasmid (84S-CAT, see below) were transfected into PyT21 cells together with a plasmid expressing RAR-γ. The level of CAT activity in the absence of RA, and the degree of RA dependent repression, was not affected by the removal of 5′-flanking DNA sequences down to 84 bp. This indicates that, under the present conditions of PyT21 cell culture, any DNA sequences necessary for stromelysin gene expression and for RA dependent repression of this expression, are located between nucleotide positions −84 and +8.

Effect of RA concentration

The effect of increasing RA concentrations between 1×10^{-11} M and 1×10^{-5} M on the level of CAT activity obtained from 84S-CAT co-transfected into PyT21 cells with a plasmid expressing either RAR-α, RAR-β, or RAR-γ is shown in Figure 4. A decrease in CAT activity was observed with all three receptors at 1×10^{-10} M RA, and the degree of repression by each receptor increased in magnitude as the RA concentration increased. The RA concentrations at which half maximal repression was obtained with each RAR were $\sim 5 \times 10^{-8}$ M for RAR-α, 1×10^{-9} M for RAR-β, and 5×10^{-9} M for RAR-γ. These concentrations are within the range of RA concentrations which inhibit the ability of malignant cells to form colonies under anchorage independent growth conditions (Udea *et al.*, 1985), and at which RA appears to act as a morphogen in the developing chick limb bud (Brockes, 1989; Eichele, 1989); they correspond also to the RA concentrations which are required for stimulation of transcription by the cloned RARs (Krust *et al.*, 1989; Zelent *et al.*, 1989).

Mutagenesis of the RA responsive region

From the above results we concluded that the DNA sequences required for activity, and RA dependent repression of the rat stromelysin promoter are located between nucleotides −84 and +8. To characterize the DNA sequences necessary for RA dependent repression further, we constructed a plasmid (S-CAT) which contains the stromelysin 5′-flanking DNA sequences from nucleotides −33 to +6 fused to the CAT gene, and which has a *Bam*HI restriction site created such that the C nucleotide at position −33 is the last nucleotide in the *Bam*HI site (see Figure 5B). Insertion of paired oligonucleotides, comprised of wild-type or mutant stromelysin promoter DNA sequences, into the *Bam*HI site of S-CAT has allowed the construction of several mutants of the stromelysin promoter (Figure 5B). As a result of the *Bam*HI site, these synthetic promoter constructs all contained three nucleotide changes between positions −34 and −37. However, these changes had no effect either on the expression or on RA dependent repression (compare 84S-

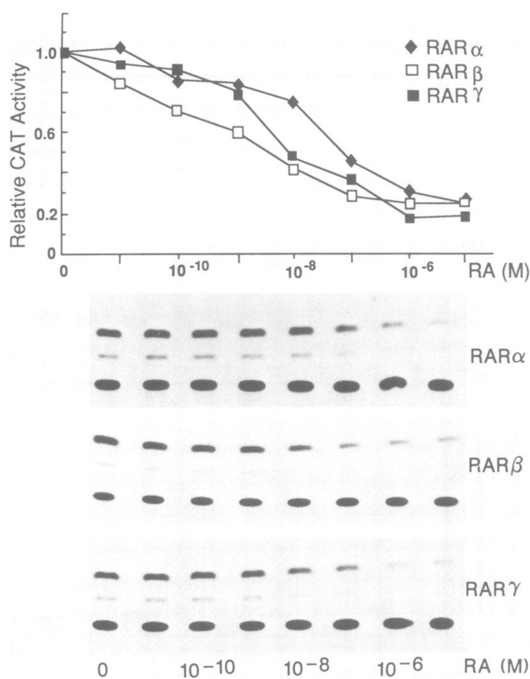

Fig. 4. The effect of RA concentration on repression mediated by the RARs. The effect of RA concentration between 1×10^{-11} and 1×10^{-5} M on CAT activity from 84S-CAT co-transfected with 1 μg of a plasmid expressing either hRAR-α0 (RARα), hRAR-β0 (RARβ) or hRAR-γ0 (RARγ) into PyT21 cells is presented. The graph depicts the average CAT activity observed at each concentration of RA relative to the CAT activity observed with each receptor in the absence of RA.

CAT with 84-CAT, in Figure 3B). Replacement of stromelysin DNA sequences between positions −33 and +6 in 84S-CAT with DNA sequences from the same region of the adenovirus major late promoter (Figure 5B, 84A-CAT) did not affect the extent of RA dependent repression, although the activity of this chimeric promoter was lower than that of the stromelysin one (Figure 5B). Except for the 5′-TATAAAA-3′ DNA sequence, which binds the TATA-box factor, there is no similarity in DNA sequence between the stromelysin and the adenovirus promoters in this region. Therefore the region of the stromelysin promoter responsible for RA dependent repression must be located between nucleotide positions −84 and −38. Examination of the DNA sequence (Figure 5B) of the stromelysin promoter in this region reveals the presence of the DNA sequence 5′-TGCAAAT-3′ (nucleotides −49 to −43), which is nearly identical to the binding site, for the octamer factors which activate transcription from a large number of promoters, 5′-ATGCAAT-3′ (LeBowitz *et al.*, 1989, and refs therein). In addition there are two DNA sequences (underlined in Figure 5B), 5′-TGAGTCA-3′ (nucleotides −71 to −65) and 5′-TGACTCT-3′ (nucleotides −55 to −49) which are identical and closely related, respectively, to the consensus sequence, 5′-TGA$^{G}_{C}$TCA-3′, capable of conferring phorbol ester (TPA) inducibility upon heterologous promoters (Angel *et al.*, 1987), and of binding AP1 (Angel *et al.*, 1987; Lee *et al.*, 1987) which is a heterodimeric complex composed of proteins (Fos and Jun) encoded by the proto-oncogene families, c-*fos* and c-*jun* (Sassone-Corsi *et al.*, 1988a,b; Halazonetis *et al.*, 1988; Curran and Franza, 1988; Cohen *et al.*, 1989; and references therein).

Within this region (from nucleotides −71 to −43) which contains the putative octamer factor and AP1 binding sites, there are two pairs of palindromic DNA sequences. The sequence 5′GCAAA-3′ (Figure 5B, arrow 1) is contained within the putative octamer site and is repeated on the opposite strand between positions −59 and −63 (arrow 3). The palindromic DNA sequence 5′-GAGTCA.N9. TGACTC-3′ (nucleotides −70 to −50, arrows 4 and 2), which overlaps the two putative AP1 binding sites, resembles the DNA sequence of a synthetic RA response element (RARE), 5′-AGGTCA.N6.TGACCT-3′ (Zelent *et al.*, 1989), and also the sequences of a thyroid hormone response element (TRE) from the rat growth hormone gene, 5′-GATCA.N6.TGACC-3′ (Glass *et al.*, 1989) or a synthetic TRE, 5′-AGGTCA.TGACCT-3′ (Glass *et al.*, 1989; Umesono *et al.*, 1988), both of which also function as RAREs. However, there is no apparent similarity between this region of the stromelysin promoter and the sequences of the RAREs found in the human RAR-β gene (de Thé *et al.*, 1990) or the murine laminin B1 gene (Vasios *et al.*, 1989). We have made mutations within each of the arms of the two sets of palindromic sequences to see if we would eliminate the negative response to RA. In all cases where the mutation was introduced within a putative binding site for either AP1 or the octamer factor, the level of CAT activity was reduced relative to the CAT activity obtained with 84S-CAT (Figure 5), suggesting that the activity of the stromelysin promoter in PyT21 cells results from the combined effect of several activating factors. However, none of the mutations affected specifically the RA dependent repression.

We further localized the RA responsive region by deleting the putative octamer site, and thereby moving the region containing the AP1 sites 10 bp closer to the TATA-box. This resulted in an ~50% decrease in CAT activity, but did not prevent the RA dependent repression (Figure 5, mutant m5a). Deleting further the promoter region from −79 to −72 did not affect its activity nor the RA repression (Figure 5, mutant m6a). In this context, where the observed CAT activity was a result of activation through DNA sequences located between −72 and −48, we mutated each arm of the TGACTC palindrome (Figure 5B, arrows 2 and 4). The mutation m7a further reduced the level of CAT activity in the absence of RA, but did not affect the RA-mediated repression. In contrast, the mutation m8a essentially abolished the promoter activity, and thus any possibility of repression by RA.

Taken all together these results indicate that the DNA sequence which is crucial for promoter activity, and contains the AP1 binding site located between −71 and −65, is also essential for mediating the RA dependent repression.

RA-mediated repression of the stromelysin promoter and of an AP1 binding TPA responsive element in HeLa cells

The repression of stromelysin gene expression by RA is not restricted to cells such as PyT21, which express the stromelysin gene at high levels, but is also observed in cells such as the human epitheloid carcinoma cell line HeLa, which are not known to express stromelysin at detectable levels. We found very little CAT activity from STR−CAT or 550-CAT in HeLa cells, but when 5′-flanking DNA sequences were deleted to −121 (as in 121-CAT) or to −84 (as in 84-CAT) the level of CAT activity in HeLa cells

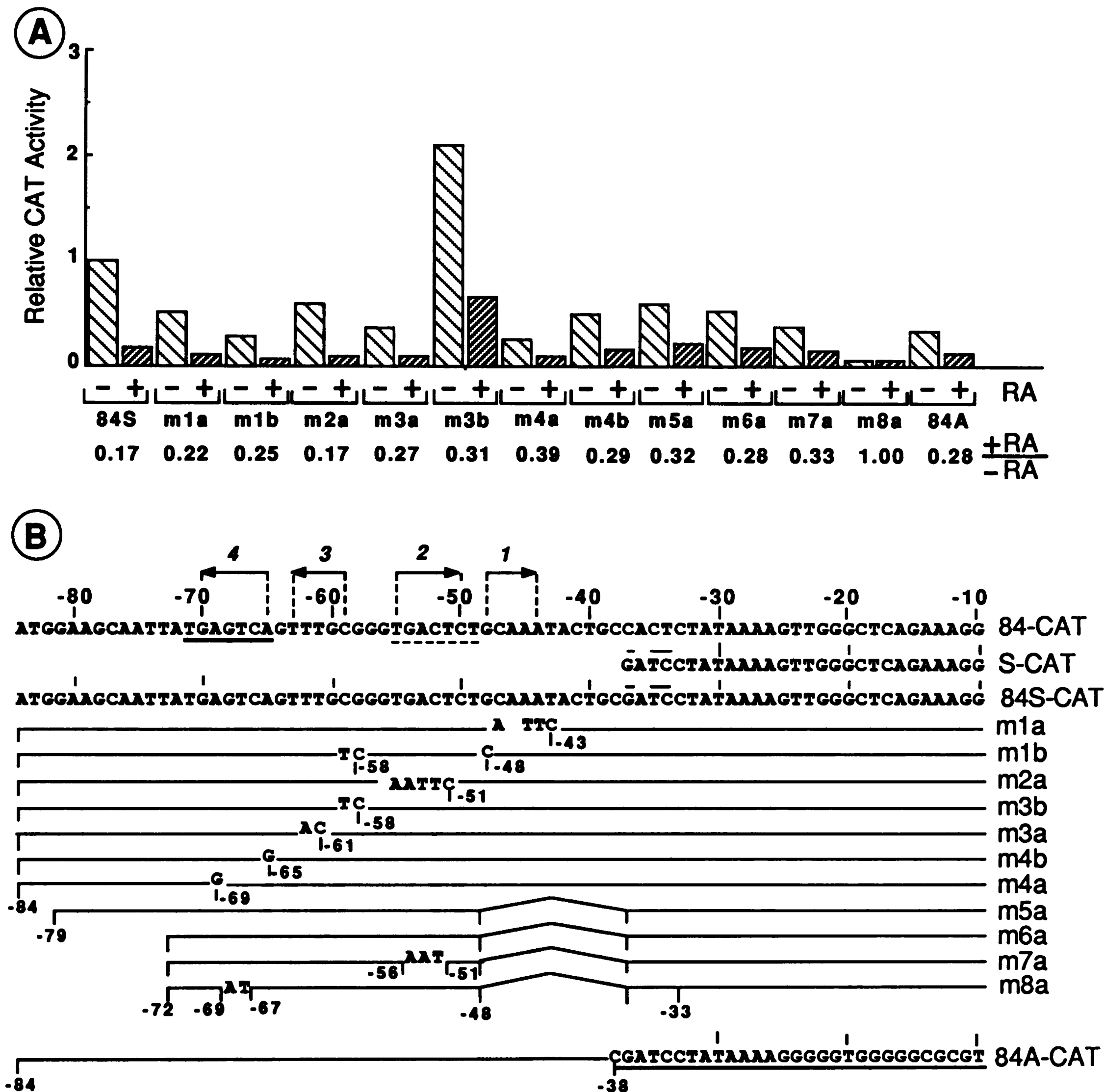

Fig. 5. Mutational analysis of the stromelysin promoter. (**A**) Histogram of averaged values (±15%, at least three independent experiments) for CAT activity obtained from CAT reporter plasmids containing synthetic promoters mutated from the normal rat stromelysin promoter DNA sequence (see panel B), co-transfected into PyT21 cells with hRAR-γ0 (1 μg). The ratio (+/−) of averaged CAT activities obtained from each plasmid in the presence of RA (+) compared with the activity in the absence of RA (−) is given. The average value of CAT activity for each plasmid in the absence of RA relative to the CAT activity from 84S-CAT in the absence of RA are: m1a, 0.51; m1b, 0.28; m2a, 0.59; m3a, 0.37; m3b, 2.11; m4a, 0.26; m4b, 0.41; m5a, 0.49; m6a, 0.43; m7a, 0.24; m8a, 0.03; 84A, 0.21. (**B**) DNA sequence of stromelysin gene between positions −84 and −10 as present in plasmid 84-CAT, compared with the DNA sequence in the synthetic promoter constructs S-CAT and 84S-CAT. The nucleotides in S-CAT and 84S-CAT which differ from those in 84-CAT are overlined. The positions of the palindromic sequences discussed in the text are indicated by arrows. Mutations and their nucleotide coordinates relative to the DNA sequence in 84S-CAT are diagrammed. The AP1 binding site and the AP1 binding site related sequence are underlined in 84-CAT with a solid and a broken line, respectively (see text). The DNA sequence of 84A-CAT which is contained in the adenovirus promoter−CAT fusion plasmid is also presented.

increased several fold (data not shown). This suggests that DNA sequences located between nucleotide positions −550 and −121 of the stromelysin gene are in part responsible for the low levels of stromelysin gene expression in HeLa cells. In Figure 6A, we show that the stromelysin promoter DNA sequences located on 84-CAT (or 84S-CAT, not shown) are sufficient to obtain significant amounts of CAT activity in HeLa cells, and also to direct RA dependent repression upon co-transfection with any one of the RAR expression plasmids. The ~40% decrease in CAT activity which was seen in the absence of RAR expression vector was probably mediated by the HeLa cell endogenous RARs. As expected from the presence in the stromelysin promoter, of a sequence (−71 to −65) identical to an AP1 binding element (see above), transcription from 84S-CAT was stimulated by treatment of the transfected HeLa cells with TPA (Figure 7A). Interestingly, this TPA-stimulated activity was also decreased by RA treatment and the magnitude of the repression was increased upon co-transfection with either one of the three RAR expression plasmids (Figure 7A and B).

To prove definitely that the AP1 binding site was the essential element responsible for the RA dependent repression, HeLa cells were transfected with a reporter gene containing the AP1 binding element located upstream from the HSV thymidine kinase promoter (Figure 6B, AP1−TK−CAT which is identical to the TRE/TK−CAT construct used by Sassone-Corsi *et al.* 1988a). RA dependent

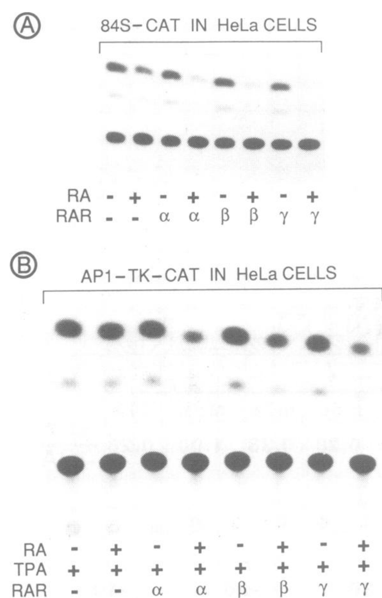

Fig. 6. (**A**) RAR-mediated repression of stromelysin promoter activity in HeLa cells. 84-CAT (see Figure 3A) was transfected along with 1 μg of either hRAR-α0(α), hRAR-β0(β) or hRAR-γ0(γ), or the parental plasmid (−) into HeLa cells in the presence (+) or absence of RA. The percentage decreases in CAT activity of 84-CAT in HeLa cells in the presence of RA relative to CAT activity in the absence of RA were on average (three independent experiments ±15%): 50% (no RAR), 85% (in the presence of hRAR-α), 83% (in the presence of hRAR-β), and 87% (in the presence of hRAR-γ). (**B**) RAR-mediated repression of the activity of a TPA responsive promoter. AP1−TK−CAT (the TRE/TK−CAT construct in Sassone-Corsi *et al.*, 1988b) was transfected into HeLa cells along with one of the hRAR expression vectors (0.5 μg), in the presence (+) or absence (−) of RA. Twenty hours before harvesting, the transfected cells were treated with TPA (100 ng/ml). The decreases (in percentages) in CAT activity of AP1−TK−CAT in the presence of RA relative to CAT activity in the absence of RA were on average (three independent experiments, ±15%): 7% (no RAR), 60% (in the presence of hRAR-α), 52% (in the presence of hRAR-β) and 55% (in the presence of hRAR-γ).

repression was clearly observed upon co-transfection with any one of the RAR expression plasmids. Under similar conditions, the basal activity of thymidine kinase promoter in TK−CAT was not repressed by RA treatment (data not shown).

The RA dependent repression may be the consequence of transcriptional interference/squelching (Meyer *et al.*, 1989, and references therein), thereby RARs unbound to DNA might compete for limiting transcription factors which would also be required for *trans*-activation by AP1. Should this be the case, one might expect to see a decrease in the activity of a RA-activated promoter [(TRE3)3−TK−CAT, see Zelent *et al.*, 1989; de Verneuil and Metzger, 1990] for RAR concentrations in the range of those which result in repression of 84S-CAT or AP1−TK−CAT. As shown in Figure 7C and D, no auto-interference similar to that previously reported for the oestrogen receptor (Bocquel *et al.*, 1989) could be detected when using the same amounts of RAR-γ expression plasmid which resulted in repression of 84S-CAT (Figure 7B and D) or AP1−TK−CAT (Figure 6B and data not shown; note that even higher amounts of RAR-γ expression plasmid did not result in auto-interference—data not shown).

Do RARs bind to the stromelysin promoter or AP1 binding site in vitro?

The RA dependent repression of the stromelysin gene promoter may result from the competitive binding of the retinoic acid receptors to the AP1 binding site. Therefore, we investigated whether human RARs (hRARs) could bind to the relevant stromelysin gene promoter sequence *in vitro* (Figure 8). Extracts of HeLa cells infected with vaccinia virus vectors expressing either hRAR-α, β or γ were incubated with ^{32}P-labelled stromelysin gene promoter oligonucleotide probes and the resulting complexes were analysed using the gel shift/retardation technique (Figure 8). A specific complex (arrow 1 in Figure 8A) was obtained with the wild-type STR1 probe, which disappeared when the −71 to −65 AP1 binding site was mutated (STR2 oligonucleotides) as in the above mutant m8a (Figure 5B). However, this retarded complex migrated distinctly more slowly than the complex (Figure 8A, arrow 2) formed between the same hRARs and an oligonucleotide containing the RA responsive element (RARE) of the hRAR-β promoter (de Thé *et al.*, 1990) (RARE-β in Figure 8; one of the relevant direct repeats is mutated in the control oligonucleotide probe RARE-βm).

The nature of complexes 1 and 2 was further investigated by using antibodies specifically directed against hRAR-γ (Figure 8B; similar results were obtained with hRAR-α or hRAR-β specific antibodies, data not shown). The migration of the specific STR1 complex (arrow 1) was not affected by the addition of hRAR-γ antibodies, in contrast to the RARE-β−hRAR-γ complex (arrow 2) which formed a more slowly migrating complex (arrow 3). Note that the STR3 probe (with the mutations present in m7a, Figure 5B) gave a complex identical to that obtained with the STR1 probe, indicating that the AP1 binding site related sequence present at positions −55 to −49 was not involved in the formation of complex 1. Together with the results obtained with the STR2 probe, this result indicated that complex 1 corresponded most likely to the binding of AP1 to the consensus site located at positions −71 to −65 in the stromelysin gene promoter sequence. This suggestion was fully supported by the competition experiment displayed in Figure 8D, which shows that an oligonucleotide containing the consensus AP1 binding site (AP1 competitor) readily competed out the formation of complex 1. In contrast, under similar conditions, an oligonucleotide containing a mutated AP1 binding site (AP1m), an oligonucleotide containing the AP1 binding site related sequence present in the stromelysin gene promoter (AP1R), as well as oligonucleotides containing the wild-type or mutated RARE-β, did not prevent the formation of complex 1 (Figure 8D). Note that no novel complex appeared in lane 2 of panel D, indicating that the failure of hRAR-γ present in HeLa cell extracts to bind to the STR1 probe (see above) is not simply due to an overlap between the AP1 binding site and a possible RAR binding site.

The above results suggested that none of the three RARs expressed in vaccinia virus recombinant infected HeLa cells could bind to an AP1 binding site to form a stable complex. However, the possibility that the AP1 factors present in the HeLa cell extracts could efficiently compete with the RARs for binding to the AP1 binding site itself was not excluded. To eliminate this possibility, hRAR-α expressed in *Escherichia coli* (RAR-αB) was used (Figure 8C). No specific complex could be detected with the STR1 probe

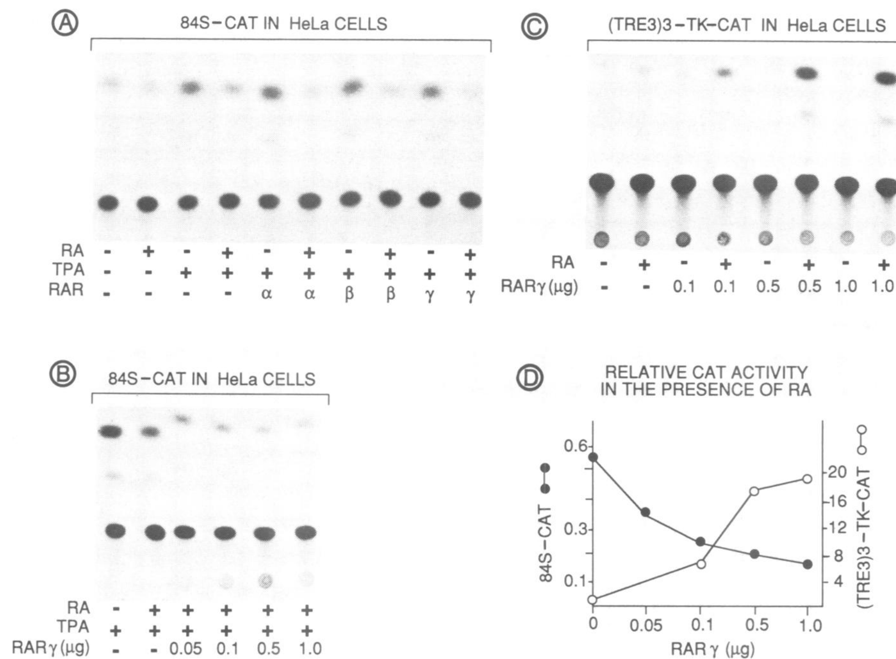

Fig. 7. (**A**) RAR-mediated repression of TPA-stimulated activity of the stromelysin promoter in HeLa cells. 84S-CAT was transfected along with 0.5 μg of either hRAR-α0(α), hRAR-β0(β) or hRAR-γ0(γ) into HeLa cells in the presence (+) or absence (−) of RA and TPA (100 ng/ml, see Figure 6B). The percentage decreases in CAT activity of 84S-CAT in the presence of RA and TPA relative to CAT activity in the absence of RA were on average (three independent experiments ±15%): 51% (no RAR), 81% (in the presence of hRAR-α), 69% (in the presence of RAR-β, and 72% (in the presence of hRAR-γ). (**B**) Effect of increasing RAR-γ concentration on TPA-stimulated activity of 84S-CAT. HeLa cells were transfected with 84S-CAT, along with increasing concentrations of hRAR-γ0 (RAR-γ, as indicated). The corresponding CAT activities expressed relative to the activity of 84S-CAT in the absence of hRAR-γ and RA are plotted in panel (D) (left hand side scale). (**C**) Effect of increasing hRAR-γ amounts on the activity of the RAR reporter gene (TRE3)3−TK−CAT. HeLa cells were transfected with (TRE3)3−TK−CAT, along with increasing concentrations of hRAR-γ0 (RAR-γ, as indicated) in the presence (+) or absence (−) of RA. The corresponding CAT activities expressed relative to the activity of (TRE3)3−TK−CAT in the presence of RA, but in the absence of hRAR-γ are plotted in panel (D) (right hand side scale). (**D**) Graphic representation of the CAT activities of 84S-CAT and (TRE3)3−TK−CAT, shown in panels B and C, respectively.

(compare with STR2 and STR3 probes), whereas RAR-αB bound readily the RARE-β probe (Figure 8C, arrow; no such complex was formed with RARE-βm under similar conditions, and the RARE-β complex could not be competed by a 500-fold excess of AP1 oligonucleotide—data not shown). Note that the same results were obtained when the salt concentrations in the binding buffer varied from 5 to 100 mM KCl and that the RAR-αB−RARE-β complex was shifted in the gel by adding antibodies specific to hRAR-α (data not shown). The binding to hRAR-α, β and γ translated *in vitro* to the STR1 probe was also investigated. No complexes were detected in gel retardation assays, whereas binding to the RARE-β probe was readily seen under identical conditions (data not shown).

From all of the above results, we conclude that AP1, but not the RARs, can bind stably to the AP1 binding element present at positions −71 to −65 of the stromelysin gene promoter. This conclusion was further supported by the observation that a complex was formed between c-*fos* and c-*jun* translated *in vitro* and STR1 and STR3, but not STR2, oligonucleotides (data not shown).

Discussion

We report here a decrease in stromelysin mRNA following RA treatment of PyT21 cells and present nuclear run-on transcription data showing that this decrease was due to a repression of transcription. Moreover, at least part of this repression occurred in the absence of protein synthesis and thus corresponded to a primary transcriptional response. Transcription of stromelysin promoter−CAT fusion plasmids in PyT21 cells was also decreased by addition of RA, and was further repressed by co-transfection of an expression plasmid encoding any one of the three RARs. Thus at least one RAR species appears to be functional in PyT21 cells, albeit probably present in limiting amounts. In this respect we note that RAR-γ is the predominant RAR species in these cells (unpublished result).

The repression in transfected PyT21 cells was mediated through a 25 bp DNA sequence which also contains the essential elements for stromelysin promoter activity in these cells. Most notably, the integrity of a sequence located between −71 and −65, and identical to a consensus AP1 binding site, was essential both for promoter activity and for mediating the RA dependent repression (see m4a, m4b and m8a in Figure 5). In contrast, a related sequence (−55 to −49), which has a T in place of A at position 7 of the consensus AP1 binding site, was not essential for promoter activity (see m2a and m7a in Figure 5), in agreement with a previous report showing that changing the A to a T at this position of the consensus site, greatly reduced AP1 binding (Risse *et al.*, 1989; see also Figure 8).

RA dependent repression of the stromelysin promoter activity was also observed in transiently transfected HeLa cells. As expected from the presence of a consensus AP1 binding site in this promoter, transcription was stimulated in the presence of the tumor promoter TPA. The functionality of this AP1 binding site within the stromelysin promoter

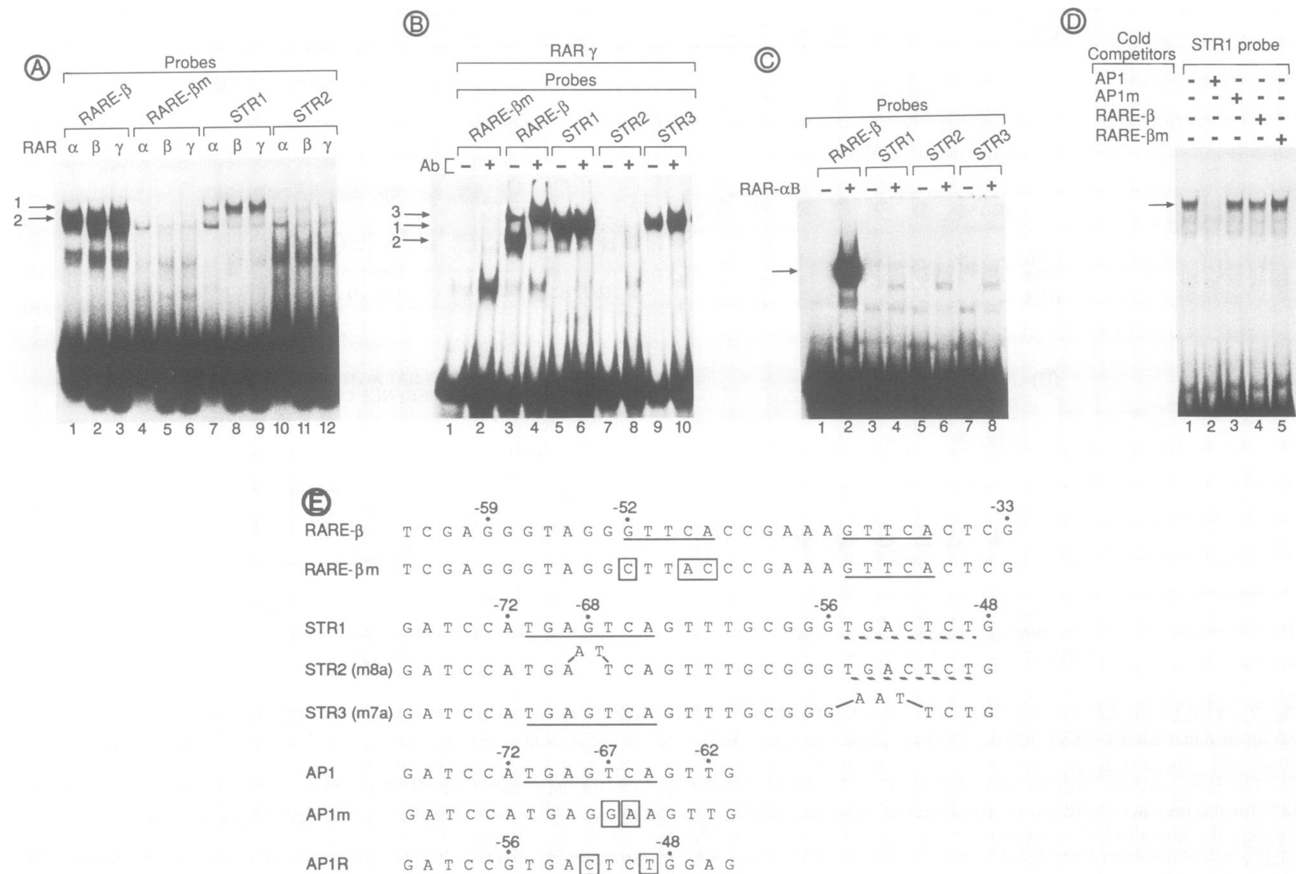

Fig. 8. Characterization of DNA–protein complexes formed *in vitro* with wild-type and mutant stromelysin promoter sequences using gel retardation/shift assays. (**A**) Complexes formed between extracts from HeLa cells infected with recombinant vaccinia viruses expressing the cDNA of either hRAR-α, β or γ and RARE-β, RARE-βm, STR1 and STR2 ^{32}P-labelled oligonucleotide probes as indicated (see Materials and methods). 4 μg of total protein extract (estimated by Bradford Assay) were used for each assay together with 50 000 c.p.m. of probe (10 fmol). The specific complexes formed with the STR1 and RARE-β probes are indicated by arrows 1 and 2, respectively. (**B**) Analysis of the complexes formed with extracts containing hRAR-γ in the presence or absence (as indicated) of a monoclonal antibody (Ab) directed against the A/B region of hRAR-γ (Materials and methods). Gel retardation reactions were carried out with 7 μg of total protein HeLa extracts containing hRAR-γ and the indicated oligonucleotide probes. The complexes formed with STR1 and STR3 probes and the RARE-β probe are indicated as in A by arrows 1 and 2, respectively. The shifted complex formed with the RARE-β probe in the presence of the antibody is indicated by arrow 3. (**C**) Complexes formed at 80 mM KCl with *E.coli* extracts expressing a hRAR-α protein truncated in the A/B domain (RAR-αB). Extracts (10 μg total protein per assay) were prepared from *E.coli* transformed with a vector expressing RAR-αB (+) or with the control parental vector (–), and incubated with various oligonucleotide probes as indicated (see Materials and methods). (**D**) Characterization of the specific complex formed between vaccinia virus extracts expressing hRAR-γ and the STR1 probe by competition with unlabelled oligonucleotides. The gel retardation protocol was essentially as in A, except that the labelled STR1 probe was added either alone (lane 1) or already mixed with a 100-fold excess of an unlabelled oligonucleotide as indicated. The specific complex formed with the STR1 probe (see panel A) is indicated by an arrow. (**E**) Sequences of the oligonucleotide probes used for the gel retardation assay in panels A, B, C and D. The position of the first and the last nucleotides found in the corresponding promoter [hRAR-β gene (de Thé *et al.*, 1990) for RARE-β and RARE-βm; stromelysin gene for STR1, STR2, STR3, AP1, AP1m and APIR] is indicated by the numbers above. The direct repeat in RAR-β probes and the consensus AP1 binding site in the stromelysin probes are underlined with a solid line, whereas the AP1 binding site related sequence located between nucleotides –56 and –48 of the stromelysin promoter is underlined with a broken line. The bases mutated in RARE-βm and in AP1m1 are boxed, as well as the non-consensus bases in the stromelysin AP1 binding site related sequence present in AP1R.

sequence was also supported by results showing that AP1 can bind it *in vitro*. Interestingly, the TPA-stimulated transcription was also repressed in RA-treated HeLa cells. All of these results indicate a co-localization of the DNA sequence required for RA dependent repression with the AP1 binding site which is essential for promoter activity. This conclusion was further supported by experiments showing that transcription from a reporter gene containing the AP1 binding element located upstream from the HSV thymidine kinase promoter was repressed in RA-treated cells upon co-transfection with any one of the RAR expression plasmids. Note that similar results were obtained when the HSV tk promoter was replaced by the rabbit β-globin gene promoter (our unpublished results). Thus the RAR–RA complex appears to repress the activity of the stromelysin gene promoter by preventing AP1 from activating transcription.

Several models have been proposed to explain how transcriptional repression of eukaryotic promoters could occur (Levine and Manley, 1989; Renkawitz, 1990; and refs therein). Co-localization of DNA elements required both for activity and for glucocorticoid dependent repression has been reported for TPA induction of proliferin gene expression (Mordacq and Linzer, 1989), for cAMP induction of the glycoprotein hormone α-subunit gene (Akerblom *et al.*, 1988), and for induction of the bovine prolactin gene by

undefined activating factors (Sakai *et al.*, 1988). Although no DNA sequence resembling a consensus GRE is present in any of those sequences, it was proposed that binding of the glucocorticoid receptor to sites overlapping the binding sites for positive factors was responsible for the repression.

The results of our binding experiments *in vitro* (Figure 8) make it very unlikely that RARs could block the ability of AP1 to activate transcription by binding to a DNA element overlapping the AP1 binding site or to the AP1 binding site itself. Alternatively, RARs may not bind to DNA on its own, but may bind to a component protein of AP1 to form an AP1−RAR complex which could still bind the AP1 binding site, but in which AP1 may not interact 'correctly' with other transcription factors required for stimulation of initiation of transcription. Another possibility would be that the AP1−RAR complex could act negatively on transcription initiation in a silencer-like manner. These two latter possibilities are also unlikely since no such ternary complex could be observed using gel retardation assays (Figure 8). However, we cannot exclude the possible formation of unstable ternary complexes which could not survive the gel electrophoresis separation. RARs could also bind to one or both of the AP1 components and thus prevent their DNA binding. This possibility was investigated by performing immunoprecipitation reactions with anti-RAR antibodies, anti-Fos antibodies and *in vitro* translated labelled RARs, c-*fos* and c-*jun*. No co-precipitation of either c-*fos* or/and c-*jun* with RARs could be observed, although the antibody preparations readily precipitated their cognate protein (data not shown; note that identical negative results were observed in the presence of the stromelysin oligonucleotide STR1). Although the possible existence of weak interactions between RARs and AP1 remains to be explored, these results do not support the idea that AP1−RAR interactions would be the cause of the observed repression. However, the possibility that the antibodies used above can disrupt a possible RAR−AP1 interaction is not excluded. The possibility that the RA dependent repression could be due to the sequestration by RARs of a limiting transcription intermediary factor which would also be required for mediating *trans*-activation by AP1 (the interference/squelching phenomenon; see Ptashne, 1988 and Meyer *et al.*, 1989) is also unlikely in view of the results displayed in Figure 7. Other possible mechanisms which may also account for the present repression remain to be explored. For instance, RA treatment may affect some post-translational modifications of c-*fos* and/or c-*jun* which could be required for their activity; c-*fos* and/or c-*jun* synthesis may also be affected, although the RA dependent repression appears to be, at least in part, a primary transcriptional response (Figure 1).

No matter which mechanism may be used, the colocalization of the sequences required for RA dependent repression with an AP1 binding site is very interesting. Mitogenic signals (eg. growth factors, TPA) arriving at the cell membrane can be transduced to the nucleus where they cause the rapid increase in expression of immediate−early gene products, such as Jun and Fos, which then act to regulate through AP1 binding sites the expression of genes involved in cell division and proliferation (for reviews see Curran and Franza, 1988; Hart *et al.*, 1989; Vogt and Bos, 1989). On the other hand, RA is known to often inhibit cell growth and induce differentiation (see Jetten, 1986 for review). It is particularly noteworthy that in the present case the membrane signalling pathway and the nuclear receptor signalling pathway converge on a common responsive element to exert their opposite effects on transcription of the stromelysin gene.

We have shown here that RA can rapidly repress expression from the stromelysin promoter in cells in culture. It is also known that transcription of the collagenase gene (another member of the metalloproteinase family, see Introduction) is inhibited by RA. Interestingly, the collagenase gene promoter contains an AP1 binding site identical to that present in the stromelysin gene promoter (Angel *et al.*, 1987), suggesting that it may be repressed in a similar manner. Taken together with the ability of RA to activate transcription of genes encoding components of the extracellular matrix (ECM) (Vasios *et al.*, 1989; see also references in Introduction), our present results suggest that RA may play an important dual regulatory role in controlling turnover of the ECM during normal development, differentiation and tissue remodelling, and also uncontrolled degradation of ECM during tumour invasion, metastases and rheumatoid arthritis.

Materials and methods

Determination of RNA levels by Northern analysis and nuclear run-on transcription

Total cytoplasmic RNA was isolated from cells as described previously (Masiakowski *et al.*, 1982). RNA (5 μg) was electrophoretically size-separated on a 1.5% agarose, 6% formaldehyde gel as described elsewhere (Rave *et al.*, 1979) and transferred to nitrocellulose by standard techniques. The filters were hybridized to a nick-translated (5×10^6 c.p.m.) rat stromelysin specific cDNA probe (Breathnach *et al.*, 1987) in a solution containing 50% formamide, 2 × SSC at 37°C for 24 h before washing at equivalent stringency.

Nuclei were isolated as described elsewhere (Brown *et al.*, 1984) and run-on transcription was carried out as previously described (Matrisian *et al.*, 1985a). Filter immobilized cDNAs (2 μg) for rat stromelysin (Breathnach *et al.*, 1987), a rat actin (Matrisian *et al.*, 1985b), rat lactate dehydrogenase (Matrisian *et al.*, 1985b) and plasmid UN121 (Nilsson *et al.*, 1983) were hybridized to 2.5×10^6 c.p.m. of [^{32}P]CTP-labelled RNA for 48 h at 37°C in a solution containing 50% formamide, 1 M NaCl. Filters were washed thoroughly at 37°C in a solution containing 50% formamide, 1 M NaCl, and then in 1 × SSC, 0.1% SDS at 65°C before removal of SDS by washing for 2 × 10 min in 2 × SSC at 25°C. Filters were then treated in 2 × SSC containing 10 μg/ml RNase at 25°C for 30 min, followed by three rapid washes in 2 × SSC at 20°C. The level of transcription was determined by densitometric scanning of autoradiograms with a Hoeffer densitometer. The intensity of hybridization to pUN121 DNA served to determine background.

CAT reporter plasmids

A promoterless CAT plasmid, pCAT, was created by removal of the *Pst*I DNA fragment containing the HSV thymidine kinase (tk) promoter, from pBLCAT8+ (Klein-Hitpass *et al.*, 1988). To construct STR−CAT, the *Bam*HI site at position −1100 bp of the rat stromelysin gene (Matrisian *et al.*, 1986b) was changed to a *Pst*I site by using a specific oligonucleotide linker. A *Pst*I site was created at position +8 bp of the stromelysin gene by oligonucleotide directed site specific mutagenesis using methods described previously (Sanchez-Lopez *et al.*, 1988). The resulting 1110 bp *Pst*I DNA fragment was then inserted into the *Pst*I site of pCAT to create STR−CAT. The plasmid GLOB−CAT was constructed by replacing the tk promoter of pBLCAT8+ with the rabbit β-globin promoter (−109 bp to +10 bp) of pG1 (Wasylyk and Wasylyk, 1986) using a *Pvu*II−*Bgl*II linker.

To construct 550-CAT a *Pst*I site was created by deleting the A at position −550 by oligonucleotide directed mutagenesis, and the resulting 560 bp *Pst*I DNA fragment, containing stromelysin DNA sequences from −550 to +8, was inserted into the *Pst* site of pCAT. Nucleotides −122 to −132 were deleted from 550-CAT, by using oligonucleotide directed site specific mutagenesis in double stranded plasmid DNA (Inouye and Inouye, 1987), to create a *Hin*dIII site. Digestion of this plasmid with *Hin*dIII removed all stromelysin DNA sequences located 5′ nucleotide −121, and created

the plasmid 121-CAT. The digestion of 550-CAT with both *Sph*I and *Bst*XI, trimming the 3′ overhang with T4 DNA polymerase at 11°C for 20 min in the presence of 100 μM of each dNTP, followed by ligation of the blunt ends, removed all stromelysin DNA sequences located 5′ of nucleotide −84, and created plasmid 84-CAT.

The stromelysin TATA-box−CAT gene fusion plasmid, S-CAT, was constructed by inserting synthetic oligonucleotides, containing DNA sequences from nucleotide position −33 to +6 of the stromelysin gene, into the *Pst*I site of pCAT. A *Bam*HI site was designed in S-CAT such that the sixth nucleotide in the *Bam*HI recognition site is the C at position −33 in the stromelysin gene. Insertion of oligonucleotides, containing DNA sequences from −84 to −38 of the stromelysin gene, into the *Bam*HI site of S-CAT created the synthetic stromelysin promoter−CAT gene fusion plasmid 84S-CAT (see Figure 5 for sequence).

Oligonucleotides containing specific nucleotide changes from the DNA sequence in 84S-CAT were inserted into the *Bam*HI site of S-CAT to create a series of plasmids containing mutations of the stromelysin promoter. The mutations (depicted in Figure 5) are as follows; in m1a nucleotide −47 was changed from a C to an A, and nucleotides −45 to −43 were changed from AAT to TTC; in m1b nucleotide −48 was changed from a G to a C and nucleotides −59 and −58 were changed from CG to TC; in m2a nucleotides −55 to −51 were changed from TGACT to AATTC; in m3a nucleotides −62 and −61 were changed from TT to AC; in m3b nucleotides −59 and −58 were changed from CG to TC; in m4a nucleotide −69 was changed from an A to a G, in m4b nucleotide −65 was changed from an A to a G; in m5a nucleotides −84 to −80 and −47 to −38 are not present; in m6a nucleotides −84 to −73 and −47 to −38 are not present; m7a was as m6a except that nucleotides −55 to −52 were changed from TGAC to AAT; m8a was as m6a except that nucleotide −68 was changed from a G to a T.

The adenovirus TATA-box−CAT plasmid A-CAT, was constructed by inserting synthetic oligonucleotides containing the DNA sequence from nucleotide position −32 to +6 of the adenovirus-2 major late promoter (Lee *et al.*, 1988), into the *Pst*I site of pCAT. A *Bam*HI site was designed in A-CAT such that the sixth nucleotide in the *Bam*HI recognition site is the C at position −32 in the adenovirus major late promoter. Insertion of oligonucleotides, containing DNA sequences from −84 to −38 of the stromelysin gene, into the *Bam*HI site of A-CAT created the synthetic stromelysin−adenovirus−CAT plasmid 84A-CAT (see Figure 6 for sequence). (TRE3)3−TK−CAT was a gift from H.de Verneuil (de Verneuil and Metzger, 1990) and AP1−TK−CAT (TRE/TK−CAT) was a gift from P.Sassone-Corsi (Sassone-Corsi *et al.*, 1988a).

The construction of the retinoic acid receptor (RAR) expression vectors hRAR-α0, hRAR-β0 and hRAR-γ0 have been described previously (Petkovich *et al.*, 1987; Brand *et al.*, 1988; Krust *et al.*, 1989).

Transfection of cells and CAT expression analysis

1×10^6 PyT21 or HeLa cells were transfected, using the calcium phosphate procedure (Wigler *et al.*, 1979), with 15 μg of CAT reporter plasmid, together with RAR expression plasmid or the parental expression vectors pSG5 (Green *et al.*, 1988) as indicated in figure legends, and 3 μg of pCH110 (Pharmacia) (a β-galactosidase expression plasmid used as internal control to normalize for variation in transfection efficiency) in Dulbecco medium containing 5% fetal calf serum and retinoic acid (1×10^{-6} M unless indicated otherwise), or 0.02% ethanol. After 15−20 h the cells were washed before incubation for an additional 24 h in new medium as above with the appropriate additions.

Cellular extracts were prepared and normalized for β-galactosidase activity as described (Petkovich *et al.*, 1987). Generally 0.1 β-galactosidase unit of PyT21 cell extract, 0.1 unit of TPA-treated HeLa extract and 1.0 unit of non-treated HeLa cell extract were used per CAT assay. The CAT activity was quantified by counting the amount of [^{14}C]chloramphenicol converted to the mono-acetylated forms.

Preparation of nuclear extracts from HeLa cells infected with vaccinia viruses expressing hRARs

The RAR cDNA present in hRAR-α0, hRAR-β0 and hRAR-γ0 were cloned into the *Bam*HI site of the vaccinia virus transfer vector pTG186 (a gift from Transgene) and the recombinant viruses were isolated and amplified as previously described (Kieny *et al.*, 1984, 1986). HeLa cells were infected (2 p.f.u./cell, 6×10^5 cells/ml) and grown in suspension for an additional 16 h. Cells were harvested by centrifugation, frozen in liquid nitrogen and stored at −80°C until used. Cell pellets, corresponding to 1 l of suspension culture, were thawed on ice and homogenized in 10 ml buffer C (10 mM Tris−HCl pH 7.5, 1 mM $MgCl_2$, 1.5 mM EDTA, 20 mM KCl, 10% glycerol, and a protease inhibitor cocktail) using a glass−glass Dounce homogenizer. The resulting suspension was centrifuged at 3000 r.p.m. for 5 min (Sorvall, RC-2B). The pellet was resuspended in 10 ml of buffer N (10 mM Tris−HCl pH 7.5, 1 mM EDTA, 1 mM DTT, 600 mM KCl, and a protease inhibitor cocktail) vigorously homogenized with a glass−glass Dounce homogenizer and the resulting suspension was centrifuged (55 000 r.p.m. for 1 h at 2°C, Spinco). The supernatant was filtered through a 5 μm Millipore filter (Millex-SV) and glycerol was added to a final concentration of 20% (v/v). The 'nuclear' extract was aliquoted, frozen in liquid nitrogen and stored at −80°C until used. Nuclear extracts for each hRAR possess nearly identical [^{3}H]retinoic acid binding activity (20−30 pmol/mg of protein).

Expression of hRAR-α in E.coli

A truncated hRAR-α cDNA lacking the region encoding amino acids 1−82 was expressed in *E.coli* using a T7 RNA polymerase dependent expression vector constructed as follows. A *Nde*I−*Kpn*I−*Xho*I−*Bam*HI polylinker was introduced between the *Nde*I and *Bam*HI cloning sites of the bacterial expression vector pEt3a (a gift from W.Studier), generating pEt32. A *Kpn*I site was introduced by site directed mutagenesis in the cDNA sequence of hRAR-α0 between nucleotides 330 and 331, and was used to excise a *Kpn*I−*Bgl*II fragment encoding amino acids 83−462 of hRAR-α, that was subsequently cloned into pEt32. The resulting expression vector, pEt32 hRAR-αB, directs the synthesis of a fusion protein, with two amino acids (gly−thr) fused at the N-terminal end of the amino acid 83−462 sequence of hRAR-α. pEt32 hRAR-αB was transformed into the BL21(DE3) pLysE *E.coli* strain, and expression was induced at an OD_{600} of 0.6 by addition of 0.5 mM IPTG (Studier *et al.*, 1990). Cells were collected by centrifugation 1 h later, and suspended in sonication buffer (10% sucrose, 50 mM Tris−HCl, 400 mM NaCl, 10 μM $ZnSO_4$, 0.05 mM DTT) in 3% of the original volume. The suspension was sonicated five times for 15 s with a 150 W Ultrasonic Disintegrator with an amplitude of 18 microns peak to peak, and the soluble fraction was recovered after centrifugation (10 000 r.p.m. for 10 min, and then 40 000 r.p.m. for 1 h), and stored at −80°C after freezing in liquid nitrogen.

Monoclonal antibodies

The monoclonal antibody hRAR-γ/Ab1.8 was prepared as described elsewhere (Rochette-Egly,C., Lutz,Y., Gaub,M.P., Saunders,M., Scheuer,I. and Chambon,P., in preparation). Briefly, 8 week old Balb/c female mice were injected intraperitoneally with 100 μg of an ovalbumin−MBS coupled peptide corresponding to amino acid 39−50 of hRAR-γ (A/B domain). Positive mice received booster injections of 100 μg of coupled peptide 4 days before removing the spleen, and then 10 μg every day until spleen removal (intravenous and intraperitoneal route). Spleen cells were fused with Sp2/0-Agi4 myeloma cells as described (St Groth and Scheidegger, 1980). Hybridomas were selected by ELISA, then by immunocytofluorescence and by Western blot assays as described in Gaub *et al.* (1989) and finally cloned twice on soft agar. 2×10^6 cells were injected intraperitoneally in pristane-primed mice for ascites fluid production.

Gel retardation/shift assay

Extracts from vaccinia infected HeLa cells were diluted to a final KCl concentration of ~100 mM in gel retardation buffer (10 mM Tris−HCl pH 8, 1 mM DTT, 14% glycerol). After addition of poly(dIdC) (2 μg) samples were incubated on ice for 15 min. 50 000 c.p.m. of ^{32}P-end-labelled probe was then added (~10 fmol) and the samples were incubated at 25°C for 15 min. *E.coli* extracts were diluted in the gel retardation buffer to various final salt concentrations (5−100 mM KCl). After 15 min of preincubation on ice with poly(dIdC) (0.5−2 μg were used with similar results), samples were further incubated for 15 min on ice after addition of the probe. For double band shift assays, each sample was further incubated for 15 min on ice with 5 μg of ascites fluid containing hRAR-γ/Ab1.8 monoclonal antibodies. Protein−DNA complexes were resolved on 5% PAGE equilibrated in 0.5 × TBE, run at room temperature for 1 h at 130 V. The gels were dried, and autoradiographed at room temperature.

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