Synergistic transcriptional activation of the mouse urokinase plasminogen activator (uPA) gene and of its enhancer activator protein 1 (AP1) site by cAMP and retinoic acid

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We have investigated the mechanism whereby *all-trans* retinoic acid (tRA) potentiates the 8-bromo-cAMP (8-BrcAMP)-dependent transcription of the urokinase plasminogen activator (uPA) gene in SC115 mouse mammary carcinoma cells. Photoaffinity labelling experiments showed that tRA did not alter the cellular content of cAMP-dependent protein kinase regulatory subunits I and II. In agreement with this, nuclear run-on analysis in the presence of the translational inhibitor puromycin demonstrated that the effect of 8-BrcAMP and its potentiation by tRA were independent of protein synthesis. A transiently transfected 6.6 kb uPA 5'-flanking region-chloramphenicol acetyltransferase (CAT) fusion gene mimicked the response of the endogenous uPA gene. Thus 1 mM 8-BrcAMP induced a 100–200 % increase in CAT content, 100 nM tRA had no effect and 100 nM tRA + 1 mM 8-BrcAMP induced a 300–500 % increase in cells co-transfected with tRA receptor and/or 9-*cis*-RA receptor. Analysis of 5'-

deleted constructs showed that the tRA effect required at least two *cis* regions: –2657 to –2186, encompassing the 100 bp uPA enhancer, and –709 to –324, which exhibited silencing activity. Neither region contained a tRA-response element-like motif. Because tRA receptor and 9-*cis*-RA receptor interact with activator protein 1 (AP1), we tested whether tRA regulated the uPA enhancer AP1 site in the presence of 8-BrcAMP. We found that a dimer of this site fused to a minimal uPA–CAT fusion gene was responsive to 1 mM 8-BrcAMP (100 % CAT increase), not responsive to 100 nM tRA, and synergistically responsive to 100 nM tRA + 1 mM 8-BrcAMP (240 % CAT increase) in cells co-transfected with Fos and Jun. Synergistic activation of the same construct and of the 6.6 kb uPA–CAT fusion gene was also obtained using tRA and 100 nM PMA. We conclude that multiple *cis* elements, probably including the uPA enhancer AP1 site, mediate the tRA potentiation of uPA transcription.

INTRODUCTION

Interaction between the retinoic acid (RA) and cAMP pathways occurs commonly in fundamental biological events such as regulation of growth and differentiation, as well as in the regulation of a variety of specific target genes. Examples include the differentiation of F9 [1], HL60 [2], U937 [2] and neuroblastoma [3,4] cells, the growth of breast cancer cells [5] and the regulation of tissue plasminogen activator [6,7], alkaline phosphatase [8], phosphoenolpyruvate carboxykinase [9], tissue transglutaminase [10], retinoic acid receptor (RAR) β [11] and cytokeratin 8 [12] gene expression, all of which are processes under synergistic RA and cAMP control. In some cases, such as that of the RAR β [13,14] and phosphoenolpyruvate carboxykinase [15] genes, synergy is accounted for by the presence in the promoter region of both an RA- and a cAMP-response element (RARE and CRE). Another important interactive mechanism is the cAMP-dependent protein kinase (PKA) phosphorylation of residue Ser-369 in the ligand-dependent transactivating domain of RAR α 1, which was recently shown to be required for the RA and cAMP induction of F9 cell differentiation into parietal endoderm [16]. Other potential interactive mechanisms are the RA regulation of PKA regulatory subunit content [17–19], and the RA covalent modification (retinoylation) of PKA regulatory subunits [20].

RA regulation of gene expression is mediated by *all-trans*-RA

(tRA) and 9-*cis*-RA receptors (RARs, α , β , γ and RXRs α , β , γ respectively) which bind as dimers to DNA and activate transcription in a ligand-dependent fashion (reviewed in [21]). RXR–RAR heterodimers bind to RAREs consisting of a PuGG/TTCA direct repeat separated by 2 (DR2) or 5 (DR5) bp (although more spaced repeats may also be functional [22]), and RXR–RXR homodimers bind to the same direct repeat when separated by 1 bp (RXRE). In addition, both RAR and RXR can inhibit gene expression by transrepression of activator protein 1 (AP1) (Fos–Jun), which is effected by receptor interaction with other transcription factors rather than DNA binding (reviewed in [23]). The RAR DNA-binding domain, however, is required for AP1 transrepression as a protein-interactive domain.

Gene regulation by cAMP (reviewed in [24]) is mediated by its dissociation of the PKA catalytic (C) subunit from its inhibitory regulatory (R) subunit, followed by nuclear translocation of the C subunit and phosphorylation of transcription factors (CREB, CREM and a subset of ATF family members) which bind as dimers to the CRE TGACGTCA. In addition, the C subunit of PKA can activate AP1 (either Jun–Jun or Fos–Jun) through a mechanism that is independent of protein synthesis and does not involve direct phosphorylation of AP1 [25].

Urokinase plasminogen activator (uPA) is one of the key proteinases involved in normal and neoplastic tissue remodelling (reviewed in [26]) and its transcriptional regulation has been extensively studied. Nerlov et al. [27] identified in the 5'-flanking

Abbreviations used: uPA, urokinase plasminogen activator; RA, retinoic acid; tRA, *all-trans*-RA; 8-BrcAMP, 8-bromo-cAMP; RAR, tRA receptor; RXR, 9-*cis*-RA receptor; RXRdbd[–], RXR mutant lacking the DNA-binding domain; PKA, cAMP-dependent protein kinase; R subunit, PKA regulatory subunit; C subunit, PKA catalytic subunit; AP1, activator protein 1; CAT, chloramphenicol acetyltransferase; β -gal, β -galactosidase; *tk*, thymidine kinase promoter; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RARE, RA-response elements; CRE, cAMP-response element.

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region of the human uPA gene a 100 bp enhancer that is highly conserved in the mouse and pig uPA genes [28]. The uPA enhancer contains a PEA3/AP1 combined site that co-operates with an AP1 site (AP1-1 in Figure 1) through a 'co-operation-mediation region' containing binding sites for multiple transcription factors, one of which has recently been purified [29]. The combined AP1 site is octameric (TGAGGTCA) whereas the AP1-1 site (TGAATCA) differs from the canonical AP1 sequence only at the central position [30]. The relative contribution of each site to the total activity of the uPA enhancer varies from one cell type to another [27]. This is probably related to their differential binding properties. Thus the PEA3/AP1 site preferentially binds Jun/ATF-2, whereas the AP1-1 site preferentially binds Fos-Jun [31]. Several uPA transcriptional responses have been shown to be mediated by the uPA enhancer, including those to epidermal growth factor [32], phorbol ester [32,33], macrophage colony-stimulating factor [34], cytoskeletal reorganization [35] and polyomavirus middle-T transformation [36]. On the other hand, the cAMP induction of uPA in pig kidney cells has been shown to be mediated by co-operation between two upstream CRE sites and a recognition site for LFB3 that are not conserved in the mouse uPA gene [28,37]. In addition to the above sites, which have been characterized functionally, several putative sites (AP1-2, AP1-3 and CRE, Figure 1) can be found in the uPA promoter.

We have reported that uPA expression was up-regulated by 8-bromo-cAMP (8-BrcAMP) and this effect potentiated by tRA in SC115 mouse mammary carcinoma cells; tRA alone was ineffective [38]. Similar observations have been made in myeloid leukaemic cells, where tRA was shown to potentiate the phorbol ester induction of uPA [39]. In both systems, the changes in uPA expression were due to transcriptional regulation [39,40]. Because the nature of the tRA regulation of uPA, in particular its dependence on 8-BrcAMP stimulation, distinguishes it from classical examples of tRA gene regulation, its underlying mechanism was of interest. The data presented here suggest that tRA regulates uPA transcription via a mechanism that is dependent on multiple *cis* elements other than classical tRA-response elements. In particular, the data support a role for the uPA enhancer AP1-1 element.

MATERIALS AND METHODS

Cells and reagents

The SC115 cell line [38] was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 units/ml) and streptomycin (100 µg/ml), and 10% heat-inactivated fetal bovine serum. For most experiments, cells were seeded in growth medium overnight and then washed twice with serum-free DMEM before treatment with 8-BrcAMP and/or tRA in serum-free BSA (0.5 mg/ml)-supplemented DMEM. In dishes receiving both agents, these were added either together or tRA first and 8-BrcAMP after a variable delay, as indicated. 8-BrcAMP and tRA were obtained from Sigma (St. Louis, MO, U.S.A.) as were all other reagents, unless noted otherwise. RA was prepared as a stock solution (1 mM) in ethanol and handled in reduced light.

Cell fractionation

Control or tRA-treated SC115 cells were treated with trypsin, centrifuged and resuspended for 10 min at 4 °C in hypotonic buffer containing proteinase inhibitors (20 mM Hepes, pH 7.4, 25 mM sucrose, 0.1 mM EGTA, 1 mM EDTA, 100 ng/ml leupeptin, 50 µM pepstatin A, 100 µM antipain, 200 U/ml aprotinin and 2 mM PMSF). The cells were then disrupted with a tight-

fitting Potter–Elvehjem homogenizer until more than 90% of the cells included Trypan Blue; they were then centrifuged at 1000 rev./min for 5 min (pellet = nuclear fraction), and the supernatant re-centrifuged at 130 000 g (28 000 rev./min) for 1 h in an SW41 rotor (final supernatant = cytosol fraction). The high-speed pellet (= postnuclear fraction) was washed once to reduce cytosolic contamination.

Photoaffinity labelling

PKA R subunits in aliquots of each cell fraction were covalently labelled with 8-azido-[³²P]cAMP, resolved in SDS/10% polyacrylamide gels and visualized by autoradiography as described previously [40,41].

Run-on transcription analysis

Nuclear run-on analysis was performed exactly as described [40]. Transcripts were hybridized to pDB16 [42] and pCCD32 [43], which contain 1.9 and 1.7 kb inserts of mouse uPA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA respectively. Hybridization to vector DNA was used to control for signal specificity. After densitometry (LKB Ultrosan XL laser densitometer), results were plotted as the ratio of uPA to GAPDH.

Transient transfection

The calcium phosphate method and purified plasmids (Qiagen plasmid kit, Qiagen, Chatsworth, CA, U.S.A.) were used. Cells were seeded at 5×10^5 /60 mm dish and transfected on the following day with 0.8 µg of uPA promoter–chloramphenicol acetyltransferase (CAT) reporter vector, 3 µg of pCH110 encoding β -galactosidase (β -gal; the internal control) and empty vector DNA for a total of 10 µg of DNA. Where indicated, RAR α , RXR α , Fos and Jun expression vectors, all pSG5-based and kindly donated by Pierre Chambon (I.G.B.M.C., Strasbourg, France) were co-transfected (0.5 µg/dish). In Figure 3, an expression vector encoding an RXR mutant lacking the DNA-binding domain (RXRdbd⁻) [44] was also used. Chloroquine was added to a final concentration of 50 µM immediately after addition of DNA. After about 18 h, the DNA was removed by two washes with serum-free DMEM, and serum-free BSA-supplemented DMEM (with or without 8-BrcAMP and/or RA) was added. After 20–24 h, the cells were washed twice with PBS, scraped off the dish using 2 ml of PBS, pelleted by low-speed centrifugation, and lysed in 100 µl of 0.25 M Tris/HCl, pH 7.8, by repeated freeze–thawing (four cycles). Lysate supernatant [12 600 g (12 500 rev./min) for 5 min] were assayed for β -gal activity as described [45], and aliquots of equal activity assayed for CAT protein using a CAT ELISA kit (Boehringer-Mannheim, Indianapolis, IN, U.S.A.). A CAT standard curve (1.25–40 pg) was included in every assay and the results were expressed as pg of CAT/unit of β -gal (1 β -gal unit was operationally defined as the amount yielding an A_{420} of 0.01 after 1 h incubation at 37 °C). In transient transfection experiments using additional plasmids or cells, the same basic transfection protocol was observed.

Reporter constructs

Figure 1 illustrates the reporter constructs used in this study. puPACAT2, the parent construct, contains 5'-flanking sequences of the mouse uPA gene from the *Xba*I site at –6616 to the *Xba*I site at +398 fused upstream of the CAT reporter gene (numbers relative to the transcription start site). puPACAT2 and several 5'-deleted derivatives (5'-termini of –2657, –2186 and –35)

Figure 1 Sketch of puPACAT2 (top) and deleted derivatives

The relative position of the uPA enhancer and its relevant sites, as well as other putative response elements, is shown (not drawn to scale). DR6 stands for a TGTTCa direct repeat with six intervening base-pairs, which was the closest motif to an RARE that we were able to identify.

were previously described [28] and were gifts from Yoshi Nagamine, as was Δ PEA3/AP1, derived from puPACAT2 by an 18 bp internal deletion of the PEA3/AP1 site of the uPA enhancer region [36]. To generate constructs with 5'-termini of -1781 and -324 , puPACAT2 was double digested with *Sma*I (an upstream polylinker site) and either *Bsu*36I (-1781) or *Bln*I (-324), and blunt-end-ligated after filling in with DNA polymerase I (Klenow fragment). To obtain constructs with 5'-termini of -1099 and -709 , PCR-amplified fragments from -1099 to -324 (sense primer: 5'-attaccgggTGCAACCACAATACCAG-3'; antisense primer: 5'-GCTCGCTGAGCAAACCTTAACGCagctc-3') and -709 to -324 (sense primer: 5'-attaccgggACCTTGCCTTTCACCTT-3') were cloned into pCR2.1 (Invitrogen, Carlsbad, CA, U.S.A.) and subcloned into puPACAT2 digested with *Sma*I and *Bln*I. To synthesize AP1₂puPACAT2Apa, which contains a dimer of the uPA enhancer AP1-1 site fused to puPACAT2Apa ($-35/+398$), sense (5'-gggTGTGAATCAGATGTGAATC-ACgggcc-3') and antisense (5'-cGTGATTACATCGTGATTCACAccc-3') oligonucleotides were annealed and ligated to puPACAT2 digested with *Sma*I and *Apa*I. The identity of all new constructs was confirmed by sequencing.

Statistical analysis

Analysis of variance with Bonferroni *post hoc* tests was performed using the SYSTAT program (SYSTAT Inc., Evanston, IL, U.S.A.)

RESULTS

We showed earlier that uPA transcription in SC115 mouse mammary carcinoma cells is increased 300% by 1 mM 8-BrcAMP, not altered by tRA, and increased 700% by 1 mM 8-BrcAMP in cells pretreated with 100 nM tRA [40]. The present study was undertaken to explore the mechanism(s) underlying this potentiating effect of tRA.

RA does not regulate PKA R-subunit content in SC115 cells

We found that tRA did not modify total PKA activity in SC115 cells [38], suggesting that tRA did not target PKA. Nevertheless, on the basis of existing precedent for tRA regulation of PKA R-subunit content [17–19], we wondered whether there was an effect of tRA on R-subunit content in SC115 cells that escaped detection in a total PKA assay. In principle, if SC115 cells have a large pool of free R subunits, a tRA-induced decrease in R-subunit content could enhance the effect of 8-BrcAMP by decreasing either the 'chelation' of 8-BrcAMP by R subunits or the reassociation of free C subunits with R subunits. Although the high concentration of 8-BrcAMP used suggests either possibility is unlikely, we wished to ascertain whether tRA did or did not regulate R-subunit content in SC115 cells. We monitored the RI- and RII-subunit content in subcellular fractions of control and tRA-treated cells by photoaffinity labelling with 8-azido-[³²P]cAMP. We found that 24 h of treatment with tRA had no effect on RI- or RII-subunit content in any of the subcellular fractions analysed (Figure 2).

Figure 2 RA does not regulate PKA R-subunit content in SC115 cells

SC115 cells were treated for 24 h with or without 100 nM tRA and separated into three subcellular fractions: (A) cytosol, (B) nuclear fraction and (C) postnuclear fraction. After SDS/PAGE, PKA R-subunit content was estimated by photoaffinity labelling with 8-azido-[32 P]cAMP as described [40,41]. RI and RII subunits were identified on the basis of their molecular mass, their co-separation with PKAI and PKAII on ion-exchange chromatography, and immunoprecipitation with specific antibodies [40]. The identity of the third band in (C) is not known. Protein loading was controlled by Coomassie Blue staining of the gel; this revealed that the lower intensity of the bands in the RA lane in (C) was due to underloading. Additional experiments confirmed that tRA did not alter R-subunit content.

Figure 3 Puromycin does not block the effects of 8-BrcAMP and RA on uPA transcription

Four dishes of SC115 cells received medium alone and four received medium with 10 μ g/ml puromycin. In each group, one of the four dishes received no further treatment (control), while the others received 100 nM tRA (added at the same time as puromycin), 1 mM 8-BrcAMP (added after a delay of 8.5 h) or 100 nM tRA + 1 mM 8-BrcAMP (each as above). Nuclei were harvested after 9.5 h incubation (1 h after 8-BrcAMP addition), and the run-on assay was performed as described in the Materials and methods section. The uPA/GAPDH densitometric ratio is shown for the two groups of dishes.

RA and 8-BrcAMP exert a primary effect

Nuclear run-on time course studies showed that the inductive effect of 8-BrcAMP peaked at about 30 min, and that even a 2 h pretreatment with tRA could potentiate the peak response to 8-BrcAMP (not shown). This, together with the absence of a tRA effect on PKA R-subunit content, suggested that 8-BrcAMP and tRA might exert a primary effect on uPA transcription, i.e. an effect independent of new protein synthesis. To test this idea, SC115 cells were pretreated with puromycin and tRA, treated with 8-BrcAMP, and uPA transcription assessed by run-on analysis. The concentration of puromycin used (10 μ g/ml) was toxic to SC115 cells (only 43 % of the cells remained attached) but was required to achieve strong inhibition (74 %) of [35 S]-methionine incorporation into trichloroacetate-precipitable protein in the adherent cell fraction (cycloheximide was even more toxic and for this reason was not used). Comparison of puromycin-treated and untreated cells showed that inhibiting protein synthesis actually superinduced the effects of 8-BrcAMP and tRA (Figure 3). Therefore 8-BrcAMP and tRA can regulate uPA

Figure 4 puPACAT2 is responsive to 8-BrcAMP and RA

(A) SC115 cells were transfected overnight with 0.8 μ g of puPACAT2, 3 μ g of pCH110 (for monitoring transfection efficiency) and empty vector DNA (for a total of 10 μ g of DNA/dish). The cells were then washed, and treated for 24 h with medium alone (control) 100 nM tRA, 1 mM 8-BrcAMP or 100 nM tRA + 1 mM 8-BrcAMP. CAT content was assayed in cell lysates (ELISA) and normalized to β -gal activity. Receptor expression plasmids (1 μ g total) were co-transfected as indicated. The data for the six experimental groups shown were taken from three representative experiments and are given as the mean $\pm$ range from duplicate transfections (absence of an error bar in this and other Figures indicates that the measurements were essentially identical). Statistical analysis showed that CAT activity under fully stimulating conditions (tRA + 8-BrcAMP in the presence of RAR + RXR) was significantly different from CAT activity in control ($P = 0.001$), 8-BrcAMP- ($P = 0.013$) and tRA + 8-BrcAMP (in presence of RXRdbd⁻)-treated ($P = 0.010$) cells. Additional experiments confirmed the dependence of the tRA-potentiating effect on co-transfection of receptors with an intact DNA-binding domain. (B) Same as (A) except that RAR and RXR expression vectors were co-transfected throughout. Data shown are representative of many similar experiments and given as the mean $\pm$ range from duplicate transfections. Statistical analysis indicated that all differences between experimental groups were highly significant ($P < 0.001$).

transcription in the apparent absence of new protein synthesis. Superinduction of mouse uPA transcription has also been demonstrated in macrophages treated with cycloheximide [46].

A transiently transfected uPA promoter-CAT fusion gene mimics the response of the endogenous uPA gene

Next, we wanted to establish a transient transfection assay for the analysis of the *cis* regions accounting for the 8-BrcAMP and tRA regulation of uPA transcription. We transiently transfected SC115 cells with puPACAT2 ([28] and Figure 1), and treated the cells with tRA, 8-BrcAMP or tRA + 8-BrcAMP. As shown in Figure 4(A), there was a detectable level of CAT expression in untreated cells, treatment with tRA did not alter this level, and treatment with 8-BrcAMP increased it by 100–200 %. Co-treatment with tRA + 8-BrcAMP resulted in a slightly higher CAT content than with 8-BrcAMP alone. However, when RAR and RXR were co-transfected, the CAT content after treatment with tRA + 8-BrcAMP was double that obtained with 8-BrcAMP alone (Figure 4A). Co-transfection of RAR or RXR alone also supported this potentiating effect of tRA (Figure 4A). Since tRA binds only to RAR, this suggests that SC115 cells overexpressing RXR can rapidly isomerize tRA to 9-*cis*-RA, the RXR ligand, as described for other cells under similar conditions [47]. In contrast with intact RXR, an RXR mutant lacking the DNA-binding domain (RXRdbd⁻) did not support the tRA-potentiating effect (Figure 4A). Co-transfection of RAR and RXR did not confer sensitivity to tRA when this reagent was added without 8-BrcAMP, nor did it modify CAT induction by 8-BrcAMP, which

Figure 5 Deletion analysis of the uPA 5'-flanking region

(A) SC115 cells were transfected overnight with 0.8 μ g of puPACAT2 or one of several 5'-deleted derivatives (5'-end shown in the abscissa), 0.5 μ g each of RAR α and RXR α expression vectors, 3 μ g of pCH110 and empty vector DNA (for a total of 10 μ g/dish). The cells were then washed and treated with medium alone (control), 1 mM 8-BrcAMP or 100 nM tRA + 1 mM 8-BrcAMP. CAT content was assayed in cell lysates and normalized to β -gal activity. Data shown are the median of two to six experiments for each construct. CAT levels in cells treated with tRA alone were always similar to control (within 20%) and are therefore not shown. (B) The effect of tRA + 8-BrcAMP was replotted as a function of the effect of 8-BrcAMP (taken as 100%). The graph also shows the activity of Δ PEA3/AP1. Individual data points and their median are shown.

was still one half of the tRA + 8-BrcAMP induction (Figure 4B). In summary, the tRA and 8-BrcAMP regulation of puPACAT2 mimics the regulation of endogenous uPA transcription as seen by nuclear run-on: no effect of tRA alone, an inductive effect of 8-BrcAMP, and a greater inductive effect of tRA + 8-BrcAMP (compare Figure 4 with Figure 1B in [40]).

Analysis of 5'-deleted constructs implicates two regions of the uPA promoter in the response to RA

To delimit the *cis* regions mediating the response to tRA and 8-BrcAMP, several 5'-deletions of puPACAT2 (Figure 1) were analysed. Figure 5(A) shows the median CAT content obtained after transient transfection of 5'-deleted constructs spanning the region from -6616 to -324 and treatment with medium alone, 8-BrcAMP or tRA + 8-BrcAMP (two to six experiments per construct). Whenever tested, CAT levels in the presence of tRA alone were always similar to basal levels (within 20%) and are therefore not shown. Deletion of about 4 kb of 5'-flanking sequences (from -6616 to -2657) had no significant effect on the response to 8-BrcAMP and tRA + 8-BrcAMP. However, deletion of the next 471 bp (to -2186), which removes the 100 bp uPA enhancer, substantially decreased the potentiating effect of tRA and slightly reduced the 8-BrcAMP response. Progressive deletion to -1781 and -1099 had no significant additional effect, whereas deletion to -709 resulted in an increase in basal CAT expression. Finally, deletion to -324, which deleted a fragment containing a putative CRE (Figure 1), resulted in a further increase in basal CAT expression and loss of the residual response to 8-BrcAMP and tRA + 8-BrcAMP.

The effect of progressive 5'-deletions on the ability of tRA to potentiate the 8-BrcAMP response is highlighted in Figure 5(B) (individual data points are shown). It can be seen that deletion to -2657 did not alter the tRA effect, whereas deletion to -2186 halved it. Abrogation of the residual tRA effect required deletion to -324. Statistical analysis of the data in Figure 5(B) indicated that the decreased effect of tRA on deletion from -2657 to -2186 was significant ($P = 0.01$).

These results demonstrate that the tRA regulation of uPA transcription is rather complex, requiring at least two *cis* elements, i.e. one within each of the two regions identified as relevant to the tRA effect by deletion analysis (-2657 to -2186 and -709 to -324). Moreover, neither of these regions contained an RARE-like sequence. Progress in the definition of alternative *cis* elements responsible for the tRA effect (such as those within the uPA enhancer and the putative CRE at -665) was hampered by their multiplicity and the relatively weak nature of the tRA response, such that site-directed mutagenesis of an individual site might only lead to a small loss of response, the significance of which would be difficult to interpret. A case in point is the response of Δ PEA3/AP1 (Figure 1). Thus we found that Δ PEA3/AP1 had a decreased transcriptional response to tRA that was not statistically significant (Figure 5B), a result that failed to clarify the role, if any, of the PEA3/AP1 site.

Synergistic transactivation of the uPA enhancer AP1 by 8-BrcAMP and RA

As an alternative strategy, we synthesized AP1₂puPACAT2Apa, in which a dimer of the enhancer AP1-1 site was fused to the minimal uPA promoter (-35/+398) in puPACAT2Apa. We chose to study the enhancer AP1-1 site for several reasons: (i) because of the dominant role of the uPA enhancer in uPA transcriptional regulation by a variety of stimuli [32-36]; (ii) because tRA regulation of AP1 activity is a major RARE-independent pathway of tRA gene regulation [23]; (iii) because PKA can activate AP1 [25]; and (iv) because there is precedent for tRA up-regulation rather than down-regulation of AP1 activity [48-50]. After the transient transfection of SC115 cells with AP1₂puPACAT2Apa, very low levels of CAT expression were detected, even in the presence of tRA + 8-BrcAMP (Figure 6). Not surprisingly, when small amounts of Fos and Jun expression vectors were co-transfected, CAT expression increased dramatically. More importantly, treatment with 8-BrcAMP increased CAT expression beyond the basal level, and treatment with tRA + 8-BrcAMP elicited a further increase (Figure 6); tRA

Figure 6 The uPA enhancer AP1 is synergistically activated by 8-BrcAMP and RA

SC115 cells were transfected overnight with 1 μ g of a minimal uPA promoter–CAT fusion gene without or with a dimer of the uPA enhancer AP1-1 site fused upstream. Other transfected plasmids were as for Figure 5, with the addition of Fos or Jun expression vectors (0.5 μ g each), as indicated. The cells were then treated for 24 h with medium alone (control), 1 mM 8-BrcAMP or 100 nM tRA + 1 mM 8-BrcAMP, as indicated. CAT content was normalized to β -gal activity. Data shown are the mean $\pm$ range from duplicate transfections.

Table 1 Synergistic activation of the uPA enhancer AP1 and puPACAT2 by PMA and RA

SC115 cells were transfected overnight with 1 μ g of the uPA reporter plasmid indicated, 3 μ g of pCH110, 0.5 μ g each of RAR α , RXR α , Fos and Jun expression vectors, and empty vector DNA (10 μ g of DNA/dish). The cells were then washed, and treated for 24 h as indicated (agents were combined at the same concentrations as when used singly). CAT content was normalized to β -gal activity and expressed relative to the mean control level (taken as 1). The absolute activity of puPACAT2 was about 50% higher than that of the dimerized uPA enhancer AP1-1 plasmid. Data shown represent the mean $\pm$ S.D. from triplicate transfections.

Treatment	Normalized CAT activity	
	AP1 ₂ puPACAT2Apa	puPACAT2
Control	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1
1 μ M tRA	1.2 $\pm$ 0.2	1.2 $\pm$ 0.3
100 nM PMA	1.1 $\pm$ 0.2	1.3 $\pm$ 0.1
tRA + PMA	2.3 $\pm$ 0.3	2.1 $\pm$ 0.1
1 mM 8-BrcAMP	2.0 $\pm$ 0.1	2.3 $\pm$ 0.2
tRA + 8-BrcAMP	3.8 $\pm$ 0.7	4.2 $\pm$ 0.2

alone had only a minor effect, as shown by additional experiments (Table 1). puPACAT2Apa (without the AP1-1 dimer) yielded only a negligible level of CAT expression under similar conditions. Co-transfection of Jun alone did not support the activity of AP1₂puPACAT2Apa, which is consistent with the preferential binding of Fos–Jun over Jun–Jun to the enhancer AP1-1 site [31]. The 8-BrcAMP up-regulation of AP1-1 activity and the tRA potentiation of this effect were confirmed by three additional experiments; statistical analysis of the combined data showed that both effects were highly significant ($P \leq 0.001$).

8-BrcAMP activation of AP1 is consistent with the reported ability of PKA to activate AP1 [25]. However, since protein kinase C activation is a better established pathway of AP1 activation, we tested the effect of tRA in combination with PMA, a protein kinase C activator. Surprisingly, 100 nM PMA was a poor activator of AP1₂puPACAT2Apa (Table 1). This was not

due to PMA inactivity *per se*, as shown by its ability to activate an API reporter in MCF-7 cells (results not shown). Nevertheless, the combination of tRA, which by itself also had only a minor effect, and PMA, activated AP1₂puPACAT2Apa synergistically (Table 1). Table 1 also shows another example of tRA and 8-BrcAMP synergy. Interestingly, similar results (preferential activation by 8-BrcAMP over PMA, and tRA synergy with both 8-BrcAMP and PMA) were obtained with the full-length promoter, puPACAT2 (Table 1).

DISCUSSION

We have identified in the mouse uPA gene in SC115 cells a model in which tRA alone is inactive as a transcriptional regulator unless 8-BrcAMP or, to a lesser extent, PMA is present. Although the effect of tRA is relatively weak (2-fold), the fact that it occurs on top of a 8-BrcAMP inductive effect suggests that it may have biological significance as a mechanism for the fine-tuning of gene expression.

Photoaffinity labelling experiments (Figure 2) did not show any effect of tRA on PKA R-subunit content in SC115 cells, ruling out an R-subunit-dependent mechanism of tRA action [17–19].

The investigation of whether the effects of 8-BrcAMP and tRA required the synthesis of new proteins revealed that, under conditions that blocked most new protein synthesis (10 μ g/ml puromycin), the effects of 8-BrcAMP and tRA were not blocked, and were in fact enhanced (Figure 3). Thus both effects appear to be mediated by pre-existing proteins. However, a role for induced RAR species in the tRA regulation of uPA cannot be excluded because the effect of tRA increased with longer incubation times (24 h) and was accompanied by induction of RAR β (results not shown). The superinduction of uPA transcription by puromycin is noteworthy because of the ability of translational inhibitors to activate Jun and ATF2 [51]; although the *cis* regions mediating the puromycin superinduction of uPA have not been mapped, it is intriguing that Jun and ATF2 have been shown to bind to the uPA enhancer PEA3/API site [31].

The requirement for transient co-transfection of RA receptors in the experiment of Figure 4(A) is in apparent contrast with the fact that the tRA effect on endogenous uPA transcription is at least partially independent of new protein synthesis. However, it is well known that the calcium phosphate method delivers multiple plasmid copies per cell, and that the full RA induction of transfected RA target genes requires receptor co-transfection (e.g. [52,53]). Therefore a reasonable interpretation is that endogenous RA receptors are at least partly sufficient to mediate the tRA effect on the endogenous uPA gene, but insufficient to mediate the tRA effect on transfected puPACAT2. However, at this point, we cannot exclude the possibility that sequences further upstream of –6616 (the 5'-end of puPACAT2) contribute to the tRA effect, although we consider it unlikely since RA receptor co-transfection resulted in good mimicry of the tRA regulation of endogenous uPA. RAR, RXR, and RAR plus RXR co-transfection supported the tRA effect, suggesting that this effect can be mediated by homodimers of either receptor or by receptor heterodimers.

The uPA region from –1099 to –324 exerted silencing activity, as its deletion increased the basal activity of the promoter (Figure 5A). Interestingly, this region contains a stretch (–735 to –612) that is 66% identical with the human uPA silencer [54]. In addition, it contains a sequence (GCATTAGTGG) closely matching the consensus sequence for the transforming growth factor β inhibitory element identified in many promoters, including human uPA [55].

Deletion of the fragment (−2657 to −2186) containing the uPA enhancer decreased the 8-BrcAMP induction of uPA only slightly (Figure 5A), suggesting that even though 8-BrcAMP can up-regulate API-1 activity (Figure 6 and Table 1), this effect does not contribute significantly to the overall 8-BrcAMP effect. Deletion of a fragment (−709 to −324) containing a putative CRE (Figure 1) was required to abolish essentially all 8-BrcAMP responsiveness. Interestingly, the same deletion also abolished the residual tRA responsiveness (Figure 5A). Whether or not the putative CRE contributes to the response to both 8-BrcAMP and tRA is a subject for future studies.

The only sequence resembling an RARE that we were able to identify by computer search was a TGTTCA direct repeat with six intervening base-pairs (DR6, Figure 1), located in a region that was not relevant to the tRA effect (Figure 5B). As predicted from RARE mutational analysis [56], gel-shift experiments showed that DR6 did not bind RXR–RAR (R. Mira-y-Lopez and Ian de Belle, unpublished work), and transient transfection experiments showed that a DR6tkgrowth hormone reporter gene was unresponsive to tRA, even in the presence of 8-BrcAMP (not shown). The lack of an RARE in the mouse uPA 5′-flanking region is consistent with the lack of an effect of tRA by itself on endogenous uPA transcription [40] and on uPA–CAT fusion genes (Figure 4). The fact that RXRdbd[−] did not support the tRA regulation of uPA (Figure 4) does not imply that an RARE exists, since the DNA-binding domain is also required, as a protein-interactive domain, for interaction with API [23]. In the absence of an RARE, alternative explanations for the effect of tRA on uPA transcription, such as synergy between RARE and CRE [13–15] or PKA-dependent phosphorylation of RAR influencing RARE activation [16], do not seem tenable.

As alluded to above, progress in the definition of the *cis* elements responsible for the tRA effect was hampered by their multiplicity and the relatively weak nature of this effect. The alternative approach of fusing individual sites to a minimal uPA promoter was focused on the enhancer API-1 site for the reasons already given. The remarkable similarity in response pattern with regard to single agents (tRA, 8-BrcAMP, PMA) and agent combinations (tRA+8-BrcAMP and tRA+PMA) strongly suggests that the API-1 site contributes to the regulation of puPACAT2 (Table 1). Further application of this approach until other candidate sites are identified (such as the CRE at −665), followed by multi site-directed mutagenesis in the context of puPACAT2 should help to elucidate further the basis of the intriguing tRA effect studied here.

Two properties of the uPA API-1 site are remarkable in their own right: its preferential activation by 8-BrcAMP over PMA, and its activation rather than repression by tRA (Table 1). Both properties appear to be species- and/or cell-specific, since we found that, in human MCF-7 cells, PMA was a better activator of API₁puPACAT2 than 8-BrcAMP, and tRA had no effect or was slightly inhibitory (results not shown). Several other studies have also shown that activation of API by RA can occur on a cell-specific basis [48–50]. In particular, Kurie et al. [48] showed that RA enhanced the API-dependent activity of the collagenase promoter in NT2/D1 cells, and Schule et al. [57] showed that RA inhibited the same promoter in HeLa and CV-1 cells. Several factors, including cellular abundance of CREB-binding protein [58] and the steric conformation of the DNA–API–RAR complex [59] may determine the outcome (trans-repression versus transactivation) of this interaction.

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