Identification of a Multihormone Responsive Enhancer Far Upstream from the Human Tissue-type Plasminogen Activator Gene*‌

(Received for publication, May 13, 1996, and in revised form, September 30, 1996)

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A 2.4-kilobase (kb) DNA fragment, located 7.1 kb upstream from the human tissue-type plasminogen activator (t-PA) gene (t-PA2.4), acts as an enhancer which is activated by glucocorticoids, progesterone, androgens, and mineralocorticoids. Transient expression of t-PA-chloramphenicol acetyltransferase reporter constructs in HT1080 human fibrosarcoma cells identified a glucocorticoid responsive unit with four functional binding sites for the glucocorticoid receptor, located between bp −7,501 and −7,974. The region from bp −7,145 to −9,578 (t-PA2.4) was found to confer a cooperative induction by dexamethasone and all-trans-retinoic acid (RA) to its homologous and a heterologous promoter, irrespective of its orientation. The minimal enhancer, defined by progressive deletion analysis, comprised the region from −7.1 to −8.0 kb (t-PA0.9) and encompassed the glucocorticoid responsive unit and the previously identified RA-responsive element located at −7.3 kb (Bulens, F., Ibañez-Tallon, I., Van Acker, P., De Vriese, A., Nelles, L., Belayew, A., and Collen, D. (1995) J. Biol. Chem. 270, 7167–7175). The amplitude of the synergistic response to dexamethasone and RA increased by reducing the distance between the enhancer and the proximal t-PA promoter. The synergistic interaction was also observed between the aldosterone and the RA receptors. It is postulated that the t-PA0.9 enhancer might play a role in the hormonal regulation of the expression of human t-PA in vivo.

Vascular patency is the result of a dynamic equilibrium between blood coagulation and fibrinolysis. Injury of the vessel wall can initiate the blood clotting cascade resulting in the formation of a hemostatic clot. To counterbalance fibrin deposition, blood contains the fibrinolytic system, one main function of which is to dissolve blood clots in the circulation. It is composed of the inactive precursor plasminogen, which can be converted into the proteolytic enzyme plasmin by the plasminogen activators (PAs)1 tissue-type and urokinase-type PA (t-PA and u-PA, respectively) leading to the degradation of fibrin. Fibrinolytic activity can be inhibited both at the level of the PAs and plasmin by PA inhibitors (PAI-1 and -2) and α2-antiplasmin, respectively (for a review, see ref. 1). Phenotypic analysis of mice deficient for either t-PA, u-PA, or both suggested that t-PA and u-PA were complementary, not only in the prevention of uncontrolled fibrin deposition in vivo but also in distinct processes which require local extracellular proteolytic activity, such as wound healing and gonadotropin-induced ovulation, as reviewed elsewhere (2).

Dexamethasone, a synthetic glucocorticoid, and androgens increase t-PA synthesis in vitro (3–5) and in vivo (6, 7). The level of t-PA expression in breast carcinoma cells correlates with the endogenous level of progesterone receptor (8), and progesterone induces t-PA-related antigen secretion in primary human endometrial cells (9). Vitamin A, retinoic acid (RA), and some of its (synthetic) analogues induce t-PA-related antigen secretion by human umbilical vein endothelial cells in vitro (10–12) and in the plasma as well as in specific tissues of vitamin A-deficient rats in vivo (11, 13, 14), suggesting that circulating RA might regulate t-PA expression in the vessel wall. The induction of t-PA expression by glucocorticoids and RA can be reproduced in HT1080 human fibrosarcoma cells, and interestingly, both agents induce t-PA mRNA steady state levels and t-PA-related antigen secretion in a cooperative manner.1 Both steroid hormones and RA are triggers of ligand-dependent activation of their respective receptors, which are members of the nuclear receptor superfamily, a class of transcription factors that specifically bind to cis-elements in the regulatory regions of given genes (15). Most hormone responsive genes have a functional RA or steroid responsive element located in the close vicinity of the transcription start site. In the human t-PA gene, however, an RA-responsive element (RARE) consisting of a direct repeat of the GGGTCA motif was identified at −7.3 kb (t-PA/DR5) and shown to mediate induction of t-PA expression by RA (16). The present study provides evi-

1 The abbreviations use are: PA, plasminogen activator; AR, human androgen receptor; ARF, DNA binding domain of AR fused to protein A; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; GR, human glucocorticoid receptor; GRE, glucocorticoid responsive element; GRF, DNA binding domain of GR fused to protein A; GRU, glucocorticoid responsive unit; MMTV, mouse mammary tumor virus; LTR, long terminal repeat; RA, all-trans-retinoic acid; RAR, retinoic acid receptor; RARE, RA-responsive element; RXR, retinoid X receptor; t-PA, tissue-type PA; TK, thymidine kinase; u-PA, urokinase-type PA; bp, base pair(s); kb, kilobase pair(s).
2 F. Bulens, H. Moreau, L. Nelles, and D. Collen, unpublished results.

This paper is available on line at http://www-jbc.stanford.edu/jbc/
dence that the t-PADRE5 RARE is part of a multihormone responsive enhancer covering the upstream fragment from −7.1 to −8.0 kb (t-PA0.9) which contains an unusually complex GRU composed of four binding sites for the GR. It is suggested that the enhancer mediates the synergistic response of t-PA gene transcription to RA and steroids.

MATERIALS AND METHODS

Reagents—Human HT1080 fibrosarcoma cells were obtained from the American Type Culture Collection (Rockville, MD). The expression vector encoding the human androgen receptor (AR, pRSV-hAR) was a gift from Dr. P. Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France), the expression vector encoding the human estrogen receptor (pRSV-hER) and the human progesterone receptor (pRSV-hPR) from Dr. P. Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France), the expression vector encoding the human GR (pRSV-hGR) and the human mineralocorticoid receptor (pRSV-hMR) from Dr. R. M. Evans (The Salk Institute for Biological Studies, La Jolla, CA), the firefly luciferase expression vector (pSVLluc) from Dr. D. R. Helsinki (Department of Biology, University of California, San Diego, CA). G418, Dulbecco’s modified Eagle’s medium, and all medium supplements were purchased from Life Technologies, Inc. (Ghent, Belgium), tissue culture recipients from Corning Inc. (New York, NY) and Becton Dickinson (Franklin Lakes, NJ), d-aldosterone, RA, chloramphenicol, DNA extraction columns from Qiagen (Chatsworth, CA), the synthetic androgen methyltrienolone (R1881), acetyl-CoA, and [3H]acetyl-CoA from ICN Biomedicals (Costa Mesa, CA), reporter lysis buffer and Lipoluma from Lumac-LSC (Olen, The Netherlands), expression vectors encoding the human estrogen receptor (GREafghi-TK-CAT, GREahi-TK-CAT, and t-PA2.0 TK-CAT), yielding t-PA2.4 TK-CAT and t-PA2.4INV-TK-CAT constructs contain a 7.3 kb (bp from 1197 to 8801) DNA fragment (146 bp) yielding t-PA2.0 TK-CAT by deletion of the t-PA2.0-TK-CAT construct was obtained by progressive exonuclease III deletion mutagenesis from t-PA2.4-TK-CAT and from t-PA2.4 INV-TK-CAT. Internal deletions of the t-PA2.4 fragment (from bp −7,145 to −9,578) were created by recombining deletion fragments from t-PA2.4-TK-CAT and from t-PA2.4 INV-TK-CAT, yielding t-PA2.4GREahi-TK-CAT, t-PA2.4TK-CAT, t-PA2.0GREahi-TK-CAT, and t-PA2.0GREeq-TK-CAT (coordinates of the deleted regions are shown in Fig. 2). The region from bp −7896 to −6041 was removed from t-PA2.0GREahi-TK-CAT by deletion of a Rat′ tk fragment (146 bp) yielding t-PA2.0GREaehi-TK-CAT.

Transfection Analysis—To obtain stable expression of t-PA-CAT reporter fragments in HT1080 cells, the calcium phosphate coprecipitation method (19) was applied to a 10-cm dish using a DNA mixture which contained 20–60 μg of PvuI-linearized CAT reporter plasmids (5 μg/kb of plasmid) and 4 μg of PvuI-linearized pCMVβNeo selection plasmid (20). After 48 h of incubation, G418 (500 μg/ml) was added to the medium and selection was performed for 10 days. In order to avoid positional effects, expression experiments were performed using a large pool of resistant colonies (10−7–10−6). After growing to confluency, cells were harvested and seeded in 6-well dishes in Dulbecco’s modified Eagle’s medium containing 0.25% (w/v) bovine serum albumin and 0.5% charcoal-stripped serum. After 24 h of serum starvation incubation fresh medium was added containing dexamethasone and/or RA or excipient. For 24 h after incubation, cells were analyzed as described below.

To obtain transient expression of the t-PA promoter constructs in HT1080 cells the calcium phosphate coprecipitation method (19) was applied to a 10-well dish using a DNA mixture of 20–60 μg reporter plasmid (5 μg/kb plasmid) with 0.1 μg of pBSVluc or pCMVβGal reporter plasmid (5 μg/kb plasmid). Deletion mutants t-PA632-CAT, t-PA1564-CAT, and t-PA3070-CAT from the indicated nuclear receptor expression plasmid. Cells were stimulated with the indicated hormone immediately (treatment for 36 h) or 16 h after the glycerol shock (treatment for 24 h).

Cell extracts were prepared by three freeze-thaw cycles (Tris-HCl 100 mM, pH 7.8, EDTA 5 mM) or by using reporter lysis buffer. Equal amounts of protein were used for the determination of CAT activity by liquid scintillation method (21), a mixture of [3H]acetyl-CoA (0.1 mM), acetyl-CoA, and chloramphenicol (final concentration 0.1 and 0.9 mM, respectively) was added to equal amounts of cell extracts, overlayed with scintillation solution (Lipoluma) and the rate of [3H]-labeled acetyl-chloramphenicol generation was measured using a liquid scintillation counter. Luciferase or β-galactosidase activity used as an indicator for the transfection efficiency was measured in a luminometer after addition of luciferin or Galacton chemiluminescent substrate, respectively. Data obtained from stable and transient expression experiments were corrected for endogenous CAT activity or apparent luciferase or β-galactosidase activity.

All data shown represent values obtained from at least two independent experiments, each performed in triplicate (n = 6 or 9) and for which at least two different plasmid preparations were used.

Electrophoretic Mobility Shift Assays and DNA ′ Protection Analysis—DNA oligonucleotides or fragments were labeled by filling in with the Klenow fragment of DNA polymerase I in the presence of [α32P]dCTP. Electrophoretic mobility shift assays (EMSAs) were performed according to Fried and Crothers (22) as modified by Baes et al. (23). Purified fusion protein (at 50 ng/μl as determined by polycarylamide gel electrophoresis and Western blot analysis) of protein α and the DNA-binding domain of the glucocorticoid or the androgen receptor (GRF and ARF, respectively) (24) were added to the reaction mixture containing 10,000 cpm of labeled DNA fragment or oligonucleotide followed by incubation at 4 °C for 15 min. EMSA reactions were analyzed by a 4–5% polyacrylamide gel electrophoresis at 4 °C in 0.5% Triton X-100 buffer. Bands were visualized by autoradiography. In vitro DNA footprinting analysis was performed according to Galas and Schmitz (25) as modified by Claessens et al. (26). The DNA fragments were end-labeled as described above and incubated with the ARF in a Triton-X100 buffer (20 mM, pH 7.9) containing 50 mM KCI, 6.25 mM MgCl2, 0.5 mM EDTA, 1 μg poly(dI-dC), 2% polyvinyl alcohol, 1 mM dithiothreitol in a final volume of 50 μl. After incubation for 20 min on ice, 50 μl of a 10 mM MgCl2, 5 mM CaCl2 solution were added containing 0.1 unit of DNase I. The reaction was stopped after 1 min by the addition of 100 μl of 10% SDS, 200 mM NaCl, and 20 mM EDTA. Samples were extracted once with phenol-chloroform, and DNA was precipitated with ethanol and resuspended in gel loading dye (98% formamide, 10 mM EDTA, 0.2% bromophenol blue and 0.2% xylene cyanol). The DNA samples were analyzed together with the A and the A/G Maxam and Gilbert degradation reactions of the same radiolabeled DNA fragment as described previously (26).

RESULTS

Regulation of the t-PA4 Enhancer Activity by Steroid Hormones in HT1080 Cells—Co-expression of t-PA5758-CAT construct with different steroid hormone receptors in HT1080 cells revealed a similar induction by 10−7 M progesterone, 10−7 M d-aldosterone, 10−9 M synthetic androgen R1881, and 10−7 M 17β-estradiol. Inductions varied from 2.5 ± 0.13-fold to 4.1 ± 0.73-fold compared to 3.1 ± 0.12-fold for 10−7 M dexamethasone, the latter in the absence of co-expressed receptor (see Fig. 1). In contrast, the t-PA7144-CAT construct was not induced by dexamethasone, progesterone, aldosterone, and R1881. A reproducible induction of 1.8 ± 0.04-fold was observed with 10−7 M 17β-estradiol when the human estrogen receptor was co-expressed. In order to assay putative enhancer
activity of the t-PA2.4 fragment deleted in the shorter construct, it was fused to the TK promoter yielding t-PA2.4-TK-CAT. This construct was not responsive to 17β-estradiol (1.1 ± 0.13-fold) but dexamethasone, progesterone, aldosterone, and R1881 were equally potent, resulting in inductions varying from 6.1 ± 0.64-fold to 10 ± 1.6-fold.

In conclusion, the t-PA2.4 enhancer was equally responsive to all steroid hormones except estrogens and able to confer this induction to both its natural or a heterologous promoter. The regulatory sequences involved in the response of the human t-PA gene to estrogens seemed to be located downstream from the t-PA2.4 enhancer.

Identification of Glucocorticoid Responsive Elements—Table I and Fig. 2, panels A and C, represent putative glucocorticoid responsive elements (GREs; t-PA/GREa, t-PA/GREd, t-PA/GREf, and t-PA/GREg) and GRE half-sites (t-PA/GREh and t-PA/GREi) identified in the t-PA2.4 fragment by the presence of the imperfect palindromic TGTTCT motif (30).

Wild type and mutant t-PA2.0-TK-CAT constructs were evaluated in HT1080 cells by co-expression with the glucocorticoid receptor (GR) in order to obtain a maximal response (see Fig. 2, panel B). Whereas the t-PA2.0-TK-CAT wild-type construct

was induced 11 ± 2.4-fold, elimination of the region from −7,318 to −7,535 (containing the t-PA/GREa, GREh, and GREi elements, construct t-PA2.0GREAhi) or the region from −7,896 to −8,041 bp (containing the t-PA/GREf and GREg, construct t-PA2.0GREGfg) reduced the stimulatory effect of dexamethasone to 5.3 ± 0.65-fold and 6.7 ± 1.6-fold, respectively. Further elimination of the t-PA/GREa, GREh, and GREi elements in the latter construct by deletion of the region from −7,318 to −7,535 bp (t-PA2.0GREAfg-hi-TK-CAT) reduced the induction to 2.0 ± 0.12-fold compared to a 1.6 ± 0.15-fold induction for the TK promoter alone.

Constructs containing site-specific mutations were evaluated in the absence of co-expressed GR in order to increase the sensitivity of the analysis (see Fig. 2, panel D). Site-specific mutation of either the GREa or the GREd element alone had very little effect on the induction by dexamethasone (6.7 ± 0.65 and 6.5 ± 0.25-fold, respectively, compared to 7.5 ± 0.85-fold induction for the wild type construct t-PA2.0-TK-CAT), but a construct with both mutated GREa and GREd elements was only induced 2.6 ± 0.25-fold (Fig. 2, panels C and D). Simultaneous mutation of the GREh and GREi half sites in the wild-type construct and in the t-PA2.0GREAadMUT construct did not alter their response (6.9 ± 0.25-fold compared to 7.5 ± 0.85-fold, data not shown, and 2.5 ± 0.34-fold compared to 2.6 ± 0.25-fold, respectively). In contrast to the results obtained in the presence of co-expressed GR (see Fig. 2, panel B), elimination of the GREf and GREg elements by deletion of the region from bp −7,896 to −8,041 (construct t-PA2.0ΔGREfΔGREG-TK-CAT) had no significant effect on the response of the t-PA enhancer (6.0 ± 1.5-fold versus 7.5 ± 0.65-fold for the wild-type construct t-PA2.0-TK-CAT, data not shown). Elimination of this region and simultaneous mutation of GREa and GREd (t-PA2.0GREAadMUTfg-TK-CAT) abolished the response to dexamethasone completely (0.9 ± 0.15-fold).

Putative GRE elements were linked in two copies to the TK-CAT fusion gene and assayed in transient transfection. Combination of the t-PA/GREi and the GREg elements (with a 3-bp intervening sequence as in their natural context) conferred a 22 ± 0.72-fold induction compared to a 2.5 ± 0.18- and a 1.2 ± 0.1-fold induction for the t-PA/GREi and the GREg elements alone (data not shown). A TK-CAT construct with two copies of the GREa element, the GREd element or two copies of

| Table I Oligonucleotides |

All putative or functional responsive elements are indicated in capitals. Putative recognition motifs in the t-PA genomic sequence were determined using the PC Gene software (IntelliGenetics). Recognition motifs are identified as (pal.)(pal.) (P.A.L.) with the number of intervening nucleotides indicated. For the GREa, GREd, GREh, and GREi elements the minus strand is shown. Nucleotides which are crucial for binding are underlined (29). Coordinates of the elements present in the t-PA2.4 fragment are numbered relative to the start site of transcription (17). Nucleotides substituted in the mutant elements are double underlined.

| Site | Coordinates | Sequence | Motif |
|------|-------------|----------|-------|
| GRE  | −7,428      | CGTCTCT  | GRE half-site/inv |
| t-PA/GREa | −7,428 | CATATCT | GRE half-site/inv |
| t-PA/GREd | −7,474 | ACTCTCT | GRE half-site/inv |
| t-PA/GREh | −7,474 | AATATCT | GRE half-site/inv |
| t-PA/GREi | −7,501 | TCTGTGCT | PAL3/inv |
| t-PA/GREA | −7,501 | GGTGTCGCT | PAL3/inv |
| t-PA/GREd | −7,703 | CACCTCTAGC | PAL3/inv |
| t-PA/GREh | −7,703 | CACCTCTAGC | PAL3/inv |
| t-PA/GREi | −7,942 | GAGGCTCTTC | PAL3 |
| t-PA/GREg | −7,942 | GAGGCTCTTC | PAL3 |
| t-PA/GREG | −7,960 | TGTCGACC | PAL3 |
| t-PA/GREd | −7,960 | TGTCGACC | PAL3 |
| Control oligonucleotides | | | |
| MMTV/GREa | | | |
| RAR/DR5 | | | |
both the GREa and GREd elements did not show any induction (1.16 ± 0.02-fold and 1.06 ± 0.05-fold, respectively; data not show). A 1.1-fold induction was observed for the positive control, the distal GRE located in the long terminal repeat of the mouse mammary tumor virus (MMTV-LTR).

In conclusion, the regulation of the t-PA2.4 enhancer is mediated by a complex hormone responsive unit composed of several GREs, GREa (located at bp 27,501), GREd (bp 27,703), GREg, and GREf (located at bp 27,942 and 27,960, respectively).

**Binding of GR and AR to Dexamethasone-responsive Elements**—Binding of the GR to the t-PA/GREs was evaluated in vitro by using recombinant proteins consisting of the DNA binding domain of the GR or the AR linked to protein A (GRF and ARF, respectively) since the GR and AR were equipotent trans-activators of the t-PA2.4 enhancer (see above). The similarity of the GREs to the consensus sequence is indicated in Table I. In EMSA, GRF specifically bound to a radiolabeled oligonucleotide harboring t-PA/GREa (compare lanes j–l with lane i in Fig. 3A), t-PA/GREf (compare lanes n–p with lane m) and t-PA/GREg (compare lanes r–t with lane q). An oligonucleotide combining both t-PA/GREf and t-PA/GREg with a spacing of 3 bp (as in their natural environment), showed strong binding (compare lanes s–x with lane u) with the appearance of a slower migrating band probably representing tetrameric binding of GRF (see lane x). The consensus GRE located in the MMTV-LTR was used as a positive control (MMTV/GREa, lanes a–d). No binding was observed for a radiolabeled aspecific oligonucleotide (RARb/DR5, lanes e–h) and with the mutated GREa, GREd, and GREf elements (data not shown). Whereas the interaction of GRF with GREa, GREf, and GREg was of the dimeric type, the interaction with GREd in vitro was primarily monomeric; compared to the retarded complex formed with the labeled MMTV/GREa oligonucleotide only a minor retarded complex migrated with similar mobility, whereas the major retarded complex of the labeled t-PA/GREd oligonucleotide migrated at a lower apparent molecular weight (data not shown). The GREh and GREi half-sites were not analyzed by EMSA.

Unlabeled oligonucleotides harboring wild-type t-PA/GREa, GREd, GREf, GREg and GREf competed for binding of GRF to a labeled MMTV/GREa oligonucleotide (see Fig. 3B). Such competition was also observed for an unlabeled oligonucleotide harboring the MMTV/GREa (used as a positive control) but not for the respective mutant GRE oligonucleotides (GREaMUT, GREdMUT, and GREfMUT) and not for an aspecific oligonucleotide (NF1).

Interaction of the ARF with the fragment from bp 7,145 to 7,896 was evaluated in the DNase I protection analysis. Only one region showed an altered DNase I sensitivity (both protection and appearance of hypersensitive sites), which mapped to the GREd element (compare lane a to b in Fig. 3C). This
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Identification of an Enhancer (t-PA2.4) Responsive to Dexamethasone and RA, 7 kb Upstream from the Human t-PA Gene—Addition of dexamethasone (10^{-7} M) or RA (10^{-6} M) to cells stably expressing t-PA9578-CAT led to a 3.7 ± 0.11-fold or 6.2 ± 0.21-fold induction, respectively, whereas the hormone combination had a 21 ± 0.23-fold effect (Fig. 4, panels A and B). The t-PA7144-CAT construct showed no response to dexamethasone nor RA. The t-PA2.4-TK-CAT construct was 5.1 ± 0.14-fold and 5.5 ± 0.50-fold induced by dexamethasone and RA alone, respectively, whereas the combination had a 18 ± 1.5-fold effect. Similar effects were observed for the t-PA2.4INV-TK-CAT construct which contained the enhancer in the reverse orientation. The TK promoter alone (construct TK-CAT) showed no or only a minor induction.

Both dexamethasone and RA induced t-PA2.4-TK-CAT expression in a dose-dependent fashion (see Fig. 4, panels C and D, respectively). Values ranged from 11 ± 0.75-fold (10^{-4} M dexamethasone) to 1.8 ± 0.08-fold (10^{-5} M dexamethasone) and from 8.9 ± 0.29-fold (10^{-6} M RA) to 1.9 ± 0.05-fold (10^{-5} M RA). RA showed a maximal level of induction at 10^{-6} M which was not further increased at higher concentrations. However, for dexamethasone a 2-fold higher induction was seen at 10^{-4} M compared to the level observed for 10^{-6} M probably representing aspecific effects at this high concentration.

Synergistic response of t-PA2.4-TK-CAT to RA and dexamethasone was confirmed in the following way; low doses were identified from the dexamethasone and RA dose-response curves for stably integrated t-PA2.4-CAT that were equipotent for transcriptional induction (1.9 ± 0.08- and 2.0 ± 0.11-fold with 10^{-8} M dexamethasone and 10^{-7} M RA, respectively). When a combined treatment with half of these doses was used, a significantly higher 3.7 ± 0.1-fold induction was observed (see Fig. 4, panel E).

Transient expression of these reporter constructs in HT1080 cells revealed a similar effect of dexamethasone on t-PA9578-CAT and t-PA2.4-TK-CAT as compared to the results obtained by stable integration of the same constructs (respectively 3.1 ± 0.12-fold and 8.8 ± 0.65-fold as compared to 3.7 ± 0.11-fold and 5.1 ± 0.14-fold). However, a substantially lower induction of
tained from separate experiments. B, a competition EMSA was performed using 32P end-labeled oligonucleotides representing MMTV/GREa (lanes a–d), RARβ/DR5 (lanes e–h) and the t-PA/GRE elements identified at −7,501 (lanes i–l), −7,960 (lanes m–p) and −7,942 (lanes q–t) upstream from the human t-PA gene (respectively, t-PA/GREa, t-PA/GREF, and t-PA/ GREg) or the combined t-PA/GREg element (lanes u–x). Oligonucleotides were incubated in the absence or presence of increasing amounts of a fusion protein of protein A and the DNA binding domain of the GRF (50 ng/μl) as indicated. Differences in relative intensity and migration distance of the retarded complex between the oligonucleotides are due to different exposure times and because data were obtained from separate experiments. C, a 32P end-labeled DNA probe covering genomic sequences from bp −7,535 to −7,850 upstream from the t-PA gene was incubated in the absence or the presence of a fusion protein of protein A and the DNA binding domain of the ARF (50 ng/μl, lanes a and b, respectively). Lane c represents the A/G reaction of the Maxam and Gilbert sequencing method of the same fragment. Reaction mixtures were subjected to denaturing polyacrylamide gel electrophoresis and analyzed by autoradiography. The sequence of the protected region is shown.

Figure 3. In vitro binding of glucocorticoid and androgen receptors to the t-PA/GRE elements. A, EMSA was performed using 32P end-labeled oligonucleotides representing MMTV/GREa (lanes a–d), RARβ/DR5 (lanes e–h) and the t-PA/GRE elements identified at −7,501 (lanes i–l), −7,960 (lanes m–p) and −7,942 (lanes q–t) upstream from the human t-PA gene (respectively, t-PA/GREa, t-PA/GREF, and t-PA/ GREg) or the combined t-PA/GREg element (lanes u–x). Oligonucleotides were incubated in the absence or presence of increasing amounts of a fusion protein of protein A and the DNA binding domain of the GRF (50 ng/μl) as indicated. Differences in relative intensity and migration distance of the retarded complex between the oligonucleotides are due to different exposure times and because data were obtained from separate experiments. B, a competition EMSA was performed using 32P end-labeled oligonucleotides representing MMTV/GREs and different amounts (10–250 ng) of competing unlabeled oligonucleotides representing t-PA/GREa, t-PA/GREd, t-PA/GREF, and t-PA/ GREs, and t-PA/GREg were added. The respective mutant oligonucleotides were evaluated at a dose of 250 ng: GREaMUT, GREd- MUT, GRErefMUT, and GREgMUT. An unlabeled oligonucleotide representing aspecific effects at this high concentration.
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![Diagram](http://www.jbc.org/)

**FIG. 4.** Dexamethasone- and RA-mediated induction of t-PA-CAT constructs stably integrated in HT1080 human fibrosarcoma cells. A, schematic representation of the genomic sequences upstream from the transcription initiation site of the human t-PA gene and the t-PA-CAT-reporter constructs obtained thereof (numbering according to Henderson and Sleigh (17)). DNA sequences are represented by the bold lines. CRE-like and AP-2 binding sites involved in basal promoter activity (31) are indicated by an arrow and BamHI restriction sites are indicated by a filled triangle. The CAT reporter gene (■), the TK-promoter (□), and the first exon (■) of the human t-PA gene are shown. B, induction of CAT activity by dexamethasone (DEX) and RA (10⁻⁶ M and 10⁻⁸ M respectively) versus control (Co) of human t-PA promoter/CAT constructs (a-f in panel A). C, dose-response effect of dexamethasone on stably expressed t-PA2.4-TK-CAT construct (e in panel A). D, dose-response effect of RA on stably expressed t-PA2.4-TK-CAT construct (e in panel A). E, induction of CAT activity by dexamethasone and RA versus control (Co) of the TK-CAT (d in panel A) and t-PA2.4-TK-CAT constructs (e in panel A). Cells were stimulated with 10⁻⁸ M dexamethasone, 10⁻⁶ M RA or with 5 x 10⁻⁸ M dexamethasone + 5 x 10⁻¹⁰ M RA. The data represent the generation of CAT activity over time as measured by the liquid scintillation method (21) and are shown as mean ± S.E. (n = 6) of data obtained after 24 h of stimulation.

t-PA9578-CAT and t-PA2.4-TK-CAT reporter activity by RA is seen in transient expression experiments (respectively 1.8 ± 0.25-fold and 2.2 ± 0.15-fold as compared to 6.2 ± 0.21-fold and 5.5 ± 0.50-fold). In HT1080 cells the synergistic effect of dexamethasone and RA on both t-PA9578-CAT and t-PA2.4-TK-CAT was only observed for stably integrated constructs. An increased response to RA of the t-PA2.4-TK-CAT but not of the t-PA9578-CAT construct is obtained by co-expression of the RA nuclear receptors RARβ and RXRα as shown previously (16). However, even under these conditions the synergistic activation of t-PA2.4-TK-CAT and t-PA9578-CAT by dexamethasone and RA could not be reproduced in HT1080 cells (data not shown).

To investigate whether either dexamethasone had an influence on the RA signal transduction pathway or vice versa, control reporter constructs containing two copies of a consensus glucocorticoid responsive element (distal glucocorticoid responsive element of the MMTV-LTR) (27) or two copies of a RA consensus responsive element (DR5 element identified in the murine RARβ2 promoter) (28) linked to the TK promoter (MMTV/GREa- and RARβ/DR5-TK-CAT, respectively) were evaluated in transient expression (data not shown). RA alone had no effect on the induction of MMTV/GREa-TK-CAT promoter activity (1.0 ± 0.07-fold compared to the basal level) and did not alter the induction by dexamethasone (37 ± 5.2-fold in the presence of both dexamethasone and RA compared to 35 ± 3.3-fold in the presence of dexamethasone alone). Similarly, dexamethasone alone did not have any effect on RARβ/DR5-TK-CAT promoter activity (1.1 ± 0.04-fold compared to the basal level) and did not alter the induction by RA (42 ± 6.1-fold in the presence of both dexamethasone and RA compared to 45 ± 4.0-fold in the presence of RA alone).

In summary, stable expression of various t-PA reporter constructs identified the region from −7.1 to −9.6 kb (t-PA2.4) as a dexamethasone- and RA-inducible enhancer since it functioned irrespective of orientation and distance from the promoter and activated a heterologous promoter. Control experiments did not reveal an effect of dexamethasone or RA on the trans-activation potential of RA receptors or the GR, respectively.

**Effect of the Distance from the Transcription Start Site and of the Intervening Sequence on the t-PA2.4 Enhancer Activity—**The t-PA2.4 upstream fragment was cloned at various distances (632, 1564, and 3070 bp) from the transcription start site of the human t-PA gene using its homologous upstream genomic sequence as “spacer” (16). Transient expression of the enhancer-less constructs t-PA632-CAT, t-PA1564-CAT, t-PA3070-CAT, and t-PA7144-CAT in HT1080 cells revealed no effect of dexamethasone (10⁻⁷ M, 36 h; values ranged from 0.81 ± 0.14- to 0.98 ± 0.05-fold control, data not shown) but the presence of the t-PA2.4 enhancer 5’ from these promoter fragments rendered them inducible by dexamethasone (from 3.3 ± 0.16- to 4.8 ± 0.15-fold, data not shown). Similar data have been reported previously for the response of these constructs to RA revealing no or a minor increase in the response with decreasing distance between the enhancer and the promoter (16).

Stable expression in HT1080 cells of promoter deletion constructs containing the t-PA2.4 fragment showed that the induction by dexamethasone (10⁻⁸ M) and RA (10⁻⁷ M) alone is only influenced to a minor extent by the distance or the intervening sequence; whereas the synergistic effect of dexamethasone (5 x 10⁻⁹ M) together with RA (5 x 10⁻⁸ M) increased with decreasing distance between the enhancer and the t-PA proximal promoter elements (from 3.3 ± 0.06-fold for t-PA9578-CAT to 7.3 ± 0.09-fold for t-PA632T2.4-CAT, see Fig. 5).

In aggregate, these data indicate that the t-PA2.4 enhancer did not require the presence of the intervening sequence to confer dexamethasone and RA responsiveness to its natural promoter. The response to dexamethasone and RA alone was not or weakly affected by decreasing the distance between the enhancer and the proximal promoter elements. However, the synergistic effect of both hormones together increased considerably with decreasing distance.

**Synergistic Regulation of the t-PA2.4 Enhancer by d-Aldosterone and RA—**In order to determine whether the synergy would also occur with other members of the steroid hormone family, the mineralocorticoid receptor was transiently expressed in HT1080 cells which contained the t-PA2.4-TK-CAT construct stably integrated in the genome. Whereas treatment with d-aldosterone and RA (both 10⁻⁷ M) induced CAT-activity 3.0 ± 0.18- and 4.9 ± 0.11-fold, respectively, combined treatment with d-aldosterone and RA at half these concentration...
FIG. 5. Effect of the distance and the intervening sequence on the response of the t-PA2.4 enhancer to dexamethasone and/or RA in HT1080 fibrosarcoma cells. A, schematic representation of reporter constructs containing the t-PA2.4 fragment linked to various deletion mutants of the t-PA7144-CAT construct. DNA sequences are represented by the bold lines. B, induction of stably integrated t-PA-CAT constructs containing the t-PA2.4 enhancer by 10^{-8} M dexamethasone (DEX) ( ), 10^{-7} M RA ( ), or 5 \times 10^{-8} M dexamethasone + 5 \times 10^{-8} M RA ( ) for 24 h. The data represent mean ± S.E. (n = 6).

FIG. 6. Delineation of the minimal t-PA enhancer by stable expression of t-PA2.4 deletion reporter constructs in HT1080 cells. A, schematic representation of t-PA2.4 deletion reporter constructs. DNA sequences are represented by the bold lines. B, induction of t-PA-TK-CAT constructs by dexamethasone (DEX) (10^{-7} M), RA (10^{-8} M), or 5 \times 10^{-8} M dexamethasone + 5 \times 10^{-8} M RA as compared to control (stimulation was performed for 24 h). The data represent mean ± S.E. (n = 6).

Delineation of the Minimal Enhancer—In order to delineate the minimal t-PA enhancer, progressive 5' deletions of the t-PA2.4 fragment linked to TK-CAT were evaluated by stable expression in HT1080 cells (see Fig. 6). Stimulation with equi-potent concentrations of dexamethasone and RA (10^{-7} M and 10^{-8} M RA, respectively) led to a 4.5 ± 0.35-fold and a 4.8 ± 0.2-fold induction of the t-PA2.4-TK-CAT construct, whereas the combination of 5 \times 10^{-8} M dexamethasone and 5 \times 10^{-8} M RA had a 12 ± 2-fold effect. The TK promoter alone showed no response. Deletion of the region spanning bp -9,213 to -9,578 (t-PA2.0-TK-CAT), bp -8,559 to -9,578 (t-PA1.4-TK-CAT) or bp -7,535 to -9,578 (t-PA0.7-TK-CAT) did only minimally influence the activation by dexamethasone and RA and did not eliminate the cooperative effect of dexamethasone and RA (see Fig. 6). Deletion of the region from bp -7,897 to -9,578 (t-PA0.7-TK-CAT) reduced the response to dexamethasone or RA as well as the cooperative effect of both agents as compared to the t-PA0.9-TK-CAT construct (from 3.0 ± 0.18- to 2.0 ± 0.69-fold for dexamethasone, from 3.6 ± 0.08- to 2.6 ± 0.58-fold for RA and from 10 ± 2.2- to 5.0 ± 1.0-fold for the combined treatment). Deletion of the regions spanning bp -7,535 to -9,578 or bp -7,324 to -9,578 in (t-PA0.4-TK-CAT and t-PA0.2-TK-CAT, respectively) eliminated or strongly reduced the induction by dexamethasone (0.86 ± 0.04- and 2.1 ± 0.56-fold, respectively) and was not further increased by the combination of dexamethasone and RA (2.0 ± 0.2- and 2.4 ± 0.23-fold).

Similar data were obtained by transient co-expression of the t-PA reporter constructs with the GR, RARβ, and RXRα nuclear receptors in the SK-N-SH neuroblastoma cell line (data not shown). In conclusion, these results show that the enhancer can be reduced to a minimal size of 863 bp spanning the region from bp -7,145 to -8,008.

DISCUSSION

The present data show that the region from bp -7,145 to -9,578 upstream from the human t-PA gene (t-PA2.4) acts as an enhancer which mediates the effect of glucocorticoids, aldosterone, mineralocorticoids and androgens but not estrogens.
Complete elimination of dexamethasone-mediated induction of the t-PA2.4 enhancer activity required the site-specific mutation of GREa (bp −7,501) and GREd (bp −7,703) in combination with deletion of the GREg (bp −7,942) and GREF (bp −7,960) elements. Therefore, the human t-PA gene is a direct target for glucocorticoid action through a unusually complex glucocorticoid responsive unit (GRU) composed of multiple binding sites for the GR, which is located between bp −7,501 and −7,974. All four sites contained the crucial nucleotides of the 3′ half site of the consensus GRE motif shown to direct initial binding of one GR of the dimeric complex (29, 30), and they interacted specifically with a truncated recombinant form of the GR or AR in vitro. In contrast to the other t-PA/GREs in EMSA the interaction of GR with t-PA/GREd was primarily of the monomeric type but dimeric binding might occur in vivo due to the binding of GR to the GREa and GREfg elements and of potential co-activator factors in the GRU. The observation that t-PA/GREa interacted with GR or AR in the electrophoretic mobility shift assay but not in the DNase I protection analysis might be due to the different amounts of purified receptor used in these in vitro assays. Deletion of a fragment containing only GREs but not GREd, GREF, and GREG did also lead to a reduced level of induction. This result is not necessarily conflicting the site-specific mutagenesis data since deletion of a DNA fragment rather than single mutations might eliminate binding sites for transcriptional co-activators which are required for the dexamethasone response of the enhancer.

The GREf and GREg elements activate transcription in response to dexamethasone in a cooperative manner when linked in two copies to the heterologous TK promoter. In contrast, a similar construct with the GREa, GREd, or the combination of GREa and GREd was not responsive to dexamethasone. The reason why GREa and GREd are active within the t-PA enhancer might be the regular spacing of about 200 bp (202 and 239 bp, respectively) observed between the GREa, GREd, and the combination of GREF and GREG. Nucleosome phasing (32) might therefore bring the GREs close enough to each other to allow biological activity for the GREa and GREd by providing multiple contacts with the RNA polymerase II initiation complex. Similar protein-protein interactions mediated by the progesterone receptor bound to distal hormone responsive elements have been reported for the uteroglobin gene (33). Interaction between distal regulatory loci and the basic transcription complex requires looping which depends on protein/protein interactions between transcription factors bound, respectively, to the enhancer and to the proximal promoter (34). The fact that the intervening sequence between the t-PA enhancer and promoter can be deleted without reducing their response to dexamethasone and RA is suggestive for such a mechanism. The Sp1 transcription factor has been shown to mediate looping of DNA (35, 36). Putative Sp1 binding sites are present in the t-PA2.4 enhancer as well as in the promoter. Whether interactions between distally and proximally bound Sp1 are involved in the regulation of the t-PA gene expression by hormones is under investigation.

A steroid hormone responsive unit of similar complexity, as described here, has only been identified in the long terminal repeat of the mouse mammary tumor virus (27). The presence of multiple steroid receptor binding sites increases the amplitude and reduces the dose dependence of the response through cooperative binding (37, 38) and a reduced dissociation rate of the DNA-receptor complex (38).

Treatment of HT1080 fibrosarcoma cells with both dexamethasone and RA led to a cooperative induction of t-PA-related antigen secretion by increasing the level of mRNA steady state levels through a mechanism which requires protein synthesis. This effect is mediated by the t-PA2.4 enhancer as shown by stable expression experiments performed with t-PA promoter constructs. The response of the enhancer to dexamethasone and RA alone increased to some extent when the distance between the enhancer and the proximal promoter elements was decreased, whereas the synergistic effect of both hormones together increased more strongly. Combination of half of multiple equipotent doses of dexamethasone and RA induced significantly higher t-PA2.4 activity than was observed for the corresponding doses alone. According to the definition of Berenbaum (39) and as more recently described by Collen (40), this is indicative for a synergistic rather than an additive interaction between dexamethasone and RA.

The effect of RA in HT1080 cells was found to be more pronounced in stably than in transiently transfected HT1080 cells (16). Moreover, the synergistic interaction on the t-PA2.4 enhancer between dexamethasone and RA in HT1080 cells was only observed for stably integrated CAT-reporter constructs. These phenomena could be due to the more physiological environment of the stably integrated reporter constructs (41). Alternatively, the lower copy number of the reporter construct in stably transfected cell lines may prevent depletion of trans-activating factors. Indeed, stronger induction by RA and/or the synergistic activation by dexamethasone and RA of transiently expressed t-PA-TK-CAT constructs were restored by co-expression of the RA receptors and/or the GR.

RA is able to enhance the translocation of the GR to the nucleus (42), whereas dexamethasone increases the expression of RXR genes in certain cell lines in vitro (43). Neither of these phenomena seem to play a role in the synergistic induction of t-PA expression by dexamethasone and RA; RA was not able to enhance the induction of a consensus glucocorticoid responsive element by dexamethasone when linked to TK-CAT and, similarly, dexamethasone did not influence the RA-mediated induction of a consensus RARE. Therefore, it is concluded that the synergistic response is due to an interaction between the steroid- and RA-signal transduction pathways at the level of the enhancer.

The size of the enhancer could be restricted to the region spanning bp −7,145 to −8,007 (863 bp, t-PA0.9) without loss of response to both dexamethasone and RA. This enhancer contains both the complex GRU and the previously identified RARE located at bp −7,319 and which are responsible for the effect of dexamethasone and RA, respectively (see above) (16). Binding of the GR homodimer as well as the RAR/RXR heterodimer is the initial event in the formation of a functional trans-activation complex in vitro (44, 45). Binding of the GR homodimer has been shown to disrupt the nucleosome structure of the MMTV-LTR (46). Therefore, it is conceivable that the binding of the GR to the t-PA/GREs might facilitate binding of the RAR/RXR receptors to the t-PA/DR5 element and/or vice versa. Alternatively, simultaneous binding of GR and RAR/RXR receptors might facilitate the binding of co-regulators involved in the hormonal response of the enhancer by opening the chromatin structure more efficiently than either the GR or the RAR/RXR would do separately. More experiments are required to confirm such a hypothesis, which would explain the synergistic interaction between both pathways on the t-PA0.9 enhancer.

The present data indicate that in the appropriate cellular context the glucocorticoid and the RA signal transduction pathways can up-regulate t-PA gene expression in a cooperative manner resulting in either an enhanced response or a higher sensitivity of the t-PA gene toward these hormones in vivo. The amplitude of the synergistic response increased considerably with decreasing distance between the enhancer and the promoter. Therefore, it is suggested that the remote localization of
the enhancer permits the dexamethasone and RA pathways to interact synergistically on the level of t-PA expression without exceeding physiological limits of trans-activation. An increased effect was also observed with the aldosterone and the RA receptors suggesting that the synergism could also occur with other steroid receptors (except the estrogen receptor).

Evaluation of 3 kb of upstream human t-PA gene sequence led to an expression pattern in transgenic mice of a linked reporter gene which did not fully resemble the pattern of endogenous t-PA-related antigen and mRNA expression (47). The presence of a far upstream enhancer in the human t-PA gene is inducible by steroid hormones (except estrogen) and by RA is therefore proposed to be important for the expression of the t-PA gene in vivo.

Acknowledgments—We thank Dr. P. Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France) for the hER and hPR expression plasmids, Dr. R. Evans (The Salk Institute for Biological Studies, La Jolla, CA) for the hGR and hMR expression plasmids, Dr. A. O. Brinkmann (Erasmus University, Rotterdam, The Netherlands) for the hAR expression vector and Dr. D. R. Helinski (Department of Biology, University of California, San Diego, CA) for the RSVlac expression vector. We thank Brigitte Verheyden from the Center for Molecular and Vascular Biology for secretarial assistance.

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J. Biol. Chem. 1997, 272:663-671.
doi: 10.1074/jbc.272.1.663

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