Ligand-dependent enhancement of human antithrombin gene expression by retinoid X receptor α and thyroid hormone receptor β

René W. L. M. NIESSEN*†||, Farhad REZAEI*, Pieter H. REITSMA‡, Marjolein PETERS†, Jan J. M. DE VIJLDER† and Augeste STURK§

*Center for Haemostasis, Thrombosis, Atherosclerosis and Inflammation Research, †Emma Children's Hospital AMC, Academic Medical Centre, PO Box 22700, 1100 DE Amsterdam, ‡Haemostasis and Thrombosis Research Center, and §Department of Clinical Chemistry, University Hospital Leiden, PO Box 9600, 2300 RC Leiden, The Netherlands

We studied potential modulators of antithrombin gene expression. A putative hormone response element (HRE) was identified by sequence similarity analysis of the antithrombin promoter, situated between nucleotides –92 and –54 relative to the transcription start site. This HRE contains three hexanucleotide motifs with an AGGTCA consensus, which are potential targets of members of the steroid/thyroid superfamily of nuclear receptors. Stimulation of the hepatoma cell line HepG2 with the receptor ligands L-3,5,3'-tri-iodothyronine, all-*trans* retinoic acid, or their combination, increased production of antithrombin into the culture medium by 1.3-, 1.6-, and 2.0-fold, respectively. In contrast, the receptor ligand 1,25-dihydroxycholecalciferol [1,25-(OH)₂VitD3] did not influence antithrombin production. Analysis of promoter chloramphenicol acetyltransferase (CAT) constructs, showed that the first 86 bp of the antithrombin promoter region are sufficient for basal transcription. The DNA length polymorphism of 32 bp or 108 bp, located upstream of position –276, did not influence antithrombin promoter activity. The antithrombin promoter activity dropped to background values when deleting the region

–97/–49 of promoter fragment –453/+57. Transactivation of the antithrombin promoter by retinoid X receptor α (RXR α) (5–7-fold) or thyroid hormone receptor β (TR β) (4–5-fold) was only observed when at least –167/+57 bp of the promoter region is present in CAT constructs, and when the appropriate ligand of the nuclear receptor was added. This transactivation was not observed upon deletion of the antithrombin promoter region –97/–49. With three copies of the antithrombin promoter fragment –109/–42 in front of the thymidine kinase minimal promoter, transactivation was only obtained with RXR α , and not with TR β . In conclusion, these results indicate that the ligand-dependent enhancement of antithrombin gene expression is regulated by RXR α as well as by TR β . Transactivation of antithrombin gene expression by RXR α and TR β appears to be dependent upon the presence of promoter region up to nucleotide –167. The HRE segment (–109/–42) only confers RXR α responsiveness to a heterologous promoter. Further study is needed to unravel the exact nature of this HRE and its 5'-flanking sequences.

INTRODUCTION

Antithrombin, previously termed antithrombin III, is a single-chain 58.2 kDa glycoprotein, of which the primary structure has been elucidated by protein and cDNA sequencing [1–4]. It is a member of the serine protease inhibitor (serpin) superfamily [5] that inhibits thrombin and several other activated coagulation factors. Heparin accelerates the inhibition at least 1000-fold [6,7]. Its pivotal role in preventing thrombotic events is evident from the increased risk of thromboembolic complications in patients with an acquired or a congenital deficiency of this inhibitor [8,9].

In healthy human term newborns, the plasma antithrombin level is relatively low (40–60%) compared with adult levels. In preterm infants a correlation between maturity and antithrombin levels is observed [10–12]. Even lower plasma antithrombin levels are found in preterm infants with idiopathic respiratory distress syndrome, increasing the risk of thromboembolic complications [11,13]. Furthermore, an inverse correlation between plasma antithrombin level and mortality has been observed in these patients [14]. Currently, several ongoing clinical studies are

investigating the effect of thyroid hormone treatment on the morbidity and mortality in preterm infants [15,16]. The addition of L-3,5,3'-tri-iodothyronine (T3) to the hepatoma cell line HepG2 causes a significant increase in the production of antithrombin [17]. Thyroid hormone treatment may therefore also have a beneficial effect on the plasma antithrombin concentration, but regulation of the antithrombin gene by this hormone has not yet been studied.

The antithrombin gene is situated on chromosome 1q23-25 and its seven exons encode for the 432-amino-acid mature protein and the signal peptide sequence [18–20]. The two functional domains are encoded by sequences in exons 2 and 3A (heparin-binding domain) and exon 6 (reactive site). A TATA element or GC-rich regions are absent in the antithrombin promoter [20–22]. The first 55 bp of the promoter region contain two enhancer elements showing approximately 75% sequence identity with the enhancer sequence of the joining-constant intron of immunoglobulin κ -chain genes [22]. Furthermore, a polymorphism of variably sized DNA segments is present in the antithrombin promoter region at position –276 relative to the

Abbreviations used: HRE, hormone response element; HNF-4, hepatocyte nuclear factor 4; RXR, retinoid X receptor; TR, thyroid hormone receptor; RAR, retinoic acid receptor; VDR, vitamin D3 receptor; T3, L-3,5,3'-tri-iodothyronine; ATRA, all-*trans* retinoic acid; 1,25-(OH)₂VitD3, 1,25-dihydroxycholecalciferol; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; TRE, thyroid hormone response element; RXRE, retinoid X response element; RARE, retinoic acid response element; TK, thymidine kinase; CAT, chloramphenicol acetyltransferase; LUC, luciferase.

|| To whom correspondence should be addressed.

transcription start site [21]. These segments are 32 bp and 108 bp in length, and non-homologous in sequence. The antithrombin promoter (nucleotides -89 to -68) and the promoter of the human transferrin gene (nucleotides -76 to -51) contain a hepatocyte specific *cis*-element which appears to bind the *trans*-acting hepatocyte nuclear factor 4 (HNF-4; previously called Tf-LF1) [23,24]. The antithrombin promoter region -84 to -73, which can interact with HNF-4, is coded by the sequence 5'-AGCCTTTGACCT-3'. This motif also contains a hexanucleotide AGGTCA consensus in the lower strand, which is able to bind members of the steroid/thyroid superfamily of nuclear receptors, e.g. the thyroid hormone receptor (TR), retinoic acid receptor (RAR), retinoid X receptor (RXR), and vitamin D3 receptor (VDR). These nuclear receptors are able to transactivate gene expression after binding their respective hydrophobic ligands T3, all-*trans* retinoic acid (ATRA), or 1,25-dihydroxycholecalciferol [1,25-(OH)₂VitD3].

The aim of this study is to investigate the effects of T3, ATRA, 1,25-(OH)₂VitD3, or combinations of these modulators, on the production of antithrombin by HepG2 cells. The mechanism of action of these modulators is subsequently analysed by transfection studies using antithrombin promoter chloramphenicol acetyltransferase (CAT) constructs, and expression vectors encoding nuclear receptors belonging to a subfamily of the steroid/thyroid nuclear receptors. The results demonstrate that antithrombin production by HepG2 cells is enhanced upon treatment with T3 and ATRA. These ligands, together with their respective nuclear receptor (TR β and RXR α), increase the promoter activity of the antithrombin gene.

MATERIALS AND METHODS

Materials

DNA-modifying enzymes were purchased from Boehringer Mannheim (Mannheim, Germany), Pharmacia Biotech (Brussels, Belgium), and New England Biolabs (Beverly, MA, U.S.A.). Butyryl Co-A, and CAT were purchased from Sigma (St. Louis, MO, U.S.A.). [³H]Chloramphenicol (specific radioactivity 60 Ci/mmol) was purchased from DuPont NEN (Boston, MA, U.S.A.). HepG2 cells were acquired from the American Type Culture Collection (A.T.C.C. HB8065). Cell culture medium Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1, v/v), fetal-calf serum (FCS), gentamicin, trypsin/EDTA [0.05% (w/v)/0.02% (w/v)] and L-glutamine were obtained from Gibco BRL (Paisley, Scotland, U.K.). Oligonucleotides were synthesized on the Gene Assembler Plus (Pharmacia Biotech, Brussels, Belgium). The ligands T3 and ATRA were purchased from Sigma (St. Louis, MO, U.S.A.), and 1,25-(OH)₂VitD3 was a generous gift from Roche Nederland (Mijdrecht, The Netherlands).

Cell culture and stimulation

HepG2 cells [25] were grown in DMEM/F12 (1:1, v/v) supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine and 50 μ g/ml gentamicin at 37 °C, in 5% CO₂ and 95% humidity. The cells were tested to be free of mycoplasma with a cDNA probe (Boehringer Mannheim, Mannheim, Germany). The HepG2 cells were grown in 6-well cluster culture dishes (Costar, Cambridge, MA, U.S.A.) until confluence. Before the stimulation experiment was started the attached cells were washed once with PBS and pre-incubated for 24 h in DMEM/F12 supplemented with L-glutamine and 10% (v/v) FCS pretreated with dextran-coated charcoal [26]. The cells were then stimulated for 48 h in culture medium containing 100 nM T3, 1 μ M ATRA, 100 nM 1,25-(OH)₂VitD3, or combinations of these modulators.

The same procedure was followed for the controls (non-stimulated cells), where the various solvents were added. After the first 24 h of stimulation the cells were supplemented with fresh medium containing the appropriate modulator(s) or solvent(s) and cultured for another 24 h. At the end of the treatment cell supernatants were collected. The amount of antithrombin secreted into the HepG2 cell culture medium was determined by sandwich-type ELISA as described previously [17]. To correct for different amounts of cells per culture well, the cells were washed twice with PBS and lysed in 0.1 M NaOH to determine the whole cell protein content by the BCA protein assay (Pierce, Rockford, IL, U.S.A.). Cell supernatants and lysates were stored at -20 °C until analysis.

Plasmids used for transient transfection analysis

The previously described promoterless vector pCAT00 [27], containing two synthetic poly(A)-addition signals upstream of the CAT gene, was used to generate reporter constructs containing antithrombin promoter sequences. Fragments contained 57 bp of the non-translated region by use of the unique *Bst*XI site in the first exon of the antithrombin gene. To generate blunt-ended, 3' identical fragments to clone into pCAT00.SmaI we subcloned the promoter fragments into the synthetic multiple cloning site *Sall*/*Sna*BI/*Sma*I/*Bam*HI/*Pst*I/*Bst*XI/*Sna*BI/*Ex*-*Bst*XI which was inserted into pBluescript(SK⁺).*Sall*/*Bst*XI, followed by cleavage with *Sna*BI. Fragments from the antithrombin promoter were generated from the *Pst*I genomic subclone pAT1.2 [22], and the *Bam*HI genomic subclones pFast and pSlow which contain the DNA length polymorphism present in the human antithrombin promoter region [21], producing pCAT361 (*Pst*I/*Bst*XI), pCAT434 (*Bbv*I/*Bst*XI) and pCAT1022 (*Bam*HI/*Bst*XI), and pCAT510 (*Bbv*I/*Bst*XI) and pCAT1098 (*Bam*HI/*Bst*XI), respectively. The numbers indicate the nucleotide length of the antithrombin promoter fragment including the 57 bp non-translated region (Sequence numbering according to Bock and Levitan [21] and Genbank U11270; J. B. Rosenberg, unpublished work). The other antithrombin promoter CAT constructs (pCAT102, pCAT143, pCAT160, pCAT224, and pCAT510 Δ HRE) were generated by PCR. After *Bst*XI digestion these promoter fragments were cloned via pBluescript.*Sma*I/*Bst*XI into pCAT00.SmaI. In pCAT510 Δ HRE the antithrombin promoter region -97/-49 is deleted. To test the transactivating effect of the antithrombin promoter region -109/-42 on a heterologous promoter, one or three copies of this region were inserted into the *Kpn*I site upstream of the thymidine kinase (TK) promoter (-81 to +52) in the plasmid pTK81LUC [28]. The region -109/-42 was generated by PCR using 5'-CGTCGGTACCTGCTTAACCTGGGAAGTGGT-3' and 5'-GAC-TGGTCGAGAGACGGGGCCATGGCTGC-3' as primers and pAT1.2 as template. As a positive control an element that contained a direct repeat of AGGTCA with a spacing of four nucleotides (i.e. DR4; 5'-AAGCTTCAGGTCACAGGAGGTCAGAGCTT-3') was inserted into the *Sma*I site upstream of the TK promoter in the plasmid pTK81LUC.

The sequences of all constructs were verified by the use of the dideoxy chain termination method [29] with the Sequenase version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH, U.S.A.). At least two independent DNA preparations were tested for each construct. In the deletion analysis experiments of the human antithrombin gene, the supercoiled plasmid DNA samples were purified by CsCl gradient centrifugation. In the case of co-transfection experiments using RAR (α , β , γ), RXR α and/or TR β expression vectors, the

plasmid DNA samples were purified using Qiagen columns (Qiagen, Hilden, Germany).

Transfection of HepG2 cells

Twenty-four hours prior to transfection, HepG2 cells were plated at a density of 0.5×10^6 per well in 6-well culture dishes (Costar, Cambridge MA, U.S.A.) in DMEM/F12 (1:1, v/v) supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine and 50 μ g/ml gentamicin. The HepG2 cells were maintained overnight in a humidified 5% CO₂-atmosphere at 37 °C. Transfection of supercoiled plasmid DNA was performed using the calcium phosphate co-precipitation method essentially as described in [30]. Transfections were performed with 2 μ g of the antithrombin promoter deletion CAT constructs and 2 μ g of the β -galactosidase expression vector pCH110 (Pharmacia, Piscataway, NJ, U.S.A.), which was co-transfected to monitor the transfection efficiency. Cells were harvested 48 h after transfection, β -galactosidase activity was determined spectrophotometrically using the β -galactosidase assay kit (Promega, Madison, WI, U.S.A.) and CAT activity was determined using the [³H]chloramphenicol-based phase-extraction assay [31]. The CAT activity was then normalized for the β -galactosidase activity, and called the relative CAT activity.

The plasmids pSG5hRAR α , pSG5hRAR β , and pSG5hRAR γ [32], pRShTR β , pRShRXR α , and pRShRXR β [33] were used as receptor expression plasmids. In the co-transfection experiments with these plasmids, the HepG2 cells were grown in the same medium as described above with the exception that the FCS had been treated with dextran-coated charcoal to remove steroids and other hydrophobic ligands. In these co-transfection experiments 2 μ g of antithrombin promoter CAT construct or TK81LUC construct was used, with 100 ng of receptor expression plasmid, at a total of 2.2 μ g of DNA by inclusion of pBluescript. After removing the DNA co-precipitate, culture medium was administered containing either no ligand, 100 nM T3, 1 μ M ATRA, or 100 nM T3 with 1 μ M ATRA. Cells were harvested 48 h after co-transfection, CAT antigen was determined spectrophotometrically using the CAT ELISA (Boehringer Mannheim, Mannheim, Germany), and luciferase (LUC) activity was determined luminometrically using the Luciferase assay kit (Promega, Madison, WI, U.S.A.). The results from both assays were normalized for the total amount of cell protein, and not for the β -galactosidase activity, as these expression constructs were shown to severely repress expression of β -galactosidase expression vectors.

RESULTS

HepG2 stimulation by T3, ATRA and 1,25-(OH)₂VitD3

HepG2 cells were initially stimulated with 100 nM T3, 1 μ M ATRA, 100 nM 1,25-(OH)₂VitD3, or combinations of these ligands (Table 1). Both T3 (1.3-fold) and ATRA (1.6-fold) significantly increased ($P < 0.001$) the antithrombin production into the culture medium by HepG2 cells, whereas 1,25-(OH)₂VitD3 had no effect (Table 1). The most profound effect on antithrombin production (2.0-fold) was obtained when both T3 and ATRA were added to the HepG2 cells. Combined stimulation with 1,25-(OH)₂VitD3 did not significantly increase antithrombin production above the level obtained when only T3 or ATRA was added.

Transfection of antithrombin promoter deletion constructs

To further our understanding of the effect of T3 and ATRA on the antithrombin production by HepG2 cells, regulatory elements

Table 1 Antithrombin production into the culture medium by HepG2 cells upon stimulation with various hormones

After growing HepG2 cells for 24 h in DMEM/F12 (1:1, v/v) supplemented with 10% heat-inactivated charcoal-treated FCS, 2 mM L-glutamine and 50 μ g/ml gentamicin, these cells were treated for 48 h with 100 nM T3, 1 μ M ATRA, 100 nM, 1,25-OHvitD3 (D3), or combinations of these modulators. Stimulation ratios are provided, which represent the amount of antithrombin secreted into the culture medium by the treated cells divided by the amount of antithrombin secreted by the untreated (control) cells. The mean values of five independent experiments $\pm$ S.E.M. are presented.

Modulator	Antithrombin production (fold stimulation)
T3*	1.32 $\pm$ 0.03
ATRA*	1.63 $\pm$ 0.10
D3	0.99 $\pm$ 0.04
T3*/ATRA*	2.03 $\pm$ 0.07
T3*/D3	1.35 $\pm$ 0.06
ATRA*/D3	1.72 $\pm$ 0.10

* Analysis of variance (generalized linear models) shows statistical significant effects ($P < 0.001$) of T3 and ATRA on antithrombin production, whereas no indications for interactions between T3/ATRA, T3/D3, and ATRA/D3 are found.

within the antithrombin promoter region had to be defined first. By sequence similarity analysis of the antithrombin promoter region, a putative hormone response element (HRE) was identified, situated between -92 bp and -54 bp relative to the transcription start site (Figure 1). This HRE contains three hexanucleotide motifs with an AGGTCA consensus, that is potentially capable of binding a subfamily of nuclear receptors belonging to the steroid/thyroid superfamily of nuclear receptors. Accordingly, various antithrombin promoter CAT constructs were designed, and used in transfection experiments in HepG2 cells (Figure 1).

pCAT143, which contains 86 bp of the direct 5'-flanking sequence of the antithrombin gene (containing two halvesites of the HRE), showed an 11-fold increase in CAT activity, i.e. promoter activity, compared with pCAT102. In contrast, pCAT160 (containing all three halvesites of the HRE) increased the antithrombin promoter activity approx. 3-fold. The antithrombin promoter activity remained high (8–12-fold) compared with pCAT102, when more than 167 bp of promoter sequence (up to 1041 bp) was present in the CAT constructs.

Both alleles of the variably sized non-homologous DNA segment polymorphism, situated at position -276 relative to the transcription start site [21], were tested in transfection experiments. The 108 bp-sized DNA fragment is present in the constructs pCAT1098, pCAT510, and partly in pCAT361, whereas the 32 bp-sized DNA fragment is present in pCAT1022 and in pCAT434. The promoter activity of pCAT510 was 1.7-fold higher than pCAT434. However, this difference in promoter activity between these two alleles was not observed with the two larger constructs, pCAT1098 and pCAT1022.

Deletion of the region -97/-49 bp of construct pCAT510, covering the three potential halvesites for steroid/thyroid nuclear receptor binding, decreased the antithrombin promoter activity almost to background levels (pCAT510 Δ HRE, Figure 1).

Co-transfection experiments with pCAT510

To identify which of the presently known vitamin A nuclear receptors are involved in the ATRA-dependent increase in antithrombin production by HepG2 cells, various isoforms of vitamin A nuclear receptors, i.e. RAR (α , β , γ) and RXR (α , β), were investigated in co-transfection experiments using specific

Figure 1 Composition of the human antithrombin promoter and the antithrombin promoter CAT constructs

In the lower part of the Figure the 5' region of the antithrombin gene (up to -1041 bp) and part of the 5' untranslated region are shown. The start of transcription is marked by +1. The promoter region -92 to -54, which contains a potential hormone receptor response element (HRE), is enlarged. This element contains three hexanucleotide motifs similar to the steroid/thyroid nuclear receptor family response element (consensus AGGTCA). A second box is indicated upstream of position -276, which represents the variably sized non-homologous DNA fragment polymorphism present in the promoter region. The various antithrombin promoter CAT constructs which were designed for the study are presented in the upper part of the Figure. Note that for the two longest CAT constructs the promoter region is intercepted. The unique *Bst*XI endonuclease restriction site (position +57) is used to generate identical 3' ends in these CAT constructs. The numbers on the left of each CAT construct represent the length (bp) of antithrombin promoter sequence present in these constructs. The right side of the Figure shows the results of the transfection experiments of HepG2 cells with these deletion CAT constructs. The results are derived from at least four independent transfections. Relative CAT activity is expressed as the percentage of the CAT activity of the pCAT361 construct (mean + S.E.M.).

expression vectors and pCAT510 (Figure 2). Co-transfection with RXR α increased the activity of pCAT510 in the presence of ATRA or a combination of ATRA and T3. This increase in CAT production was not observed with the other vitamin A nuclear receptors, i.e. RXR α , RAR α , RAR β , and RAR γ (Figure 2). Interestingly, a slight increase in CAT production was also observed when only the ligands were added, which reflects the results found in the HepG2 stimulation experiments (Table 1).

When co-transfecting pCAT361, pCAT510 or pCAT1098 together with the RXR α expression vector and ATRA, similar increases were observed in antithrombin promoter activity (results not shown). In further studies we used pCAT510 and pCAT510ΔHRE, lacking the antithrombin promoter region -97/-49, and the eukaryotic expression vectors containing cDNAs encoding RXR α and TR β , to investigate the ligand-dependent enhancement of the antithrombin promoter (Figure 3).

A T3 response of pCAT510 was observed when co-transfecting TR β (4.3-fold) or RXR α /TR β (13-fold), but was almost absent in co-transfection experiments with RXR α (Figure 3A). ATRA response was seen in co-transfection experiments with RXR α (9.4-fold) or with RXR α /TR β (5.5-fold), but was absent with

TR β . The combined ATRA/T3 response of pCAT510 was clearly present in co-transfection of RXR α (5.3-fold) or RXR α /TR β (5.4-fold), but was virtually absent in the co-transfection experiments with TR β . With pCAT510ΔHRE instead of pCAT510 no increase in the CAT production was seen under any of the conditions (Figure 3B).

Co-transfection experiments with deletion CAT constructs

Co-transfection of the constructs pCAT143, pCAT160, pCAT224, pCAT361 and pCAT510, with either RXR α or TR β into HepG2 cells, demonstrated that only the constructs pCAT143 and pCAT160 were not transactivated by either RXR α or TR β (Figure 4). All other promoter CAT constructs, i.e. pCAT224, pCAT361 and pCAT510, showed transactivation upon co-transfection with either RXR α (5-7-fold) or TR β (4-5-fold). This transactivation was only observed if the appropriate ligand of the nuclear receptor was also present.

HRE and heterologous promoter activity

The HRE segment (region -109/-42) was inserted in one or three copies upstream of a TK minimal promoter linked to the

Figure 2 Co-transfection of HepG2 cells with the antithrombin promoter CAT construct pCAT510 and various vitamin A nuclear receptors

pBluescript (pBlue) is used as carrier DNA. To the various culture media either no ligand, 1 μ M ATRA, or 1 μ M ATRA/100 nM T3 were added. The ratio of CAT expression is provided as fold-induction compared with the sample containing the CAT construct plus pBluescript, without ligand. Mean and upper range of three independent experiments are presented.

Figure 3 Ligand-dependent transactivation of the antithrombin promoter by RXR α and TR β

HepG2 cells were co-transfected with antithrombin promoter CAT constructs and eukaryotic expression vectors for RXR α and TR β . pBluescript (pBlue) was used as carrier DNA to ensure that the same amount of DNA was used in each co-transfection. Either no ligand, 100 nM T3, 1 μ M ATRA, or both were added after co-transfecting pBlue, RXR α , TR β , or RXR α /TR β . The ratio of CAT expression is provided as fold-induction compared with the sample containing the CAT construct plus RXR α , without ligand. Means and upper ranges of three independent experiments are presented. (A) pCAT510 used as CAT construct. (B) pCAT510 Δ HRE used as CAT construct.

luciferase (LUC) gene, generating p1xHRE-TKLUC and p3xHRE-TKLUC. The p3xHRE-TKLUC construct conferred RXR α responsiveness to the TK promoter (Figure 5A). Induction of luciferase activity was only found by co-transfection of RXR α in the presence of ATRA or ATRA/T3 in comparison with TKLUC. No TR β responsiveness was conferred to the TK promoter by this HRE segment (Figure 5A). As a positive control for RXR α and TR β responsiveness, we used a DR4 element, containing a direct repeat of AGGTCA with a spacing of four nucleotides. Three copies of this DR4 element were inserted in front of the TK promoter generating p3xDR4-TKLUC. This construct showed ligand-dependent responsiveness to both RXR α and TR β (Figure 5B). This responsiveness was absent in a construct containing only one DR4 element (results not shown). The RXR α responsiveness with p3xHRE-

Figure 4 Transactivation of antithrombin promoter CAT constructs by RXR α and TR β in HepG2 cells

The numbers on the x-axis correspond to the nucleotide length of the antithrombin promoter fragment present in the various CAT constructs. pBluescript (pBlue) was used as carrier DNA to ensure that the same amount of DNA was used in each co-transfection. Co-transfection experiments were performed using RXR α with either no ligand or ATRA, and TR β with either no ligand or T3. The ratio of CAT expression is provided as fold-induction compared with the samples containing the CAT construct, pBluescript and the corresponding ligand (no ligand, T3 or ATRA). Means and upper ranges of three independent experiments are presented.

Figure 5 Transactivation of the TK promoter by the HRE region of the antithrombin promoter and a positive control element DR4 using RXR α and TR β

TK81LUC constructs containing one or three copies of the HRE region (designated 1xHRE and 3xHRE, respectively) or three copies of the DR4 element (designated 3xDR4) were used to transfect HepG2 cells. pBluescript (pBlue) was used as carrier DNA to ensure that the same amount of DNA was used in each co-transfection. TKLUC = TK81LUC. One of three representative experiments is shown. (A) HRE region of antithrombin promoter. (B) DR4 element versus HRE region of the antithrombin promoter. Note that the y-axis is intercepted.

TKLUC was consistently higher (5–12-fold) than with p3xDR4-TKLUC in the three experiments.

DISCUSSION

We have used the human hepatoma cell line HepG2 as a model to study antithrombin gene expression, and observed that ATRA and T3 significantly increase antithrombin production into the culture medium. The 1.3-fold increase found with 100 nM T3 in our HepG2 model using hormone-depleted FCS is comparable with the results previously described using serum-free conditions [17]. This indicates that pretreatment of FCS with charcoal successfully removes steroids and other hydrophobic substances that may influence antithrombin production.

It is known that ATRA and T3 mediate their biological response by binding to their respective nuclear receptors, i.e. RARs and/or RXRs, and TRs [34–36]. The RXRs respond to high concentrations of ATRA (1 μ M), in contrast to the RARs which already respond to ATRA at nanomolar concentrations [37–39]. Therefore, it was postulated that mammalian cells are able to convert ATRA into 9-*cis* retinoic acid, which is the natural ligand for RXRs [40,41]. Our results show that ATRA is only able to enhance antithrombin promoter activity in co-transfection experiments with RXR α and not with RARs, which indicates that RXR α is the vitamin A nuclear receptor which affects antithrombin gene expression.

It could be wondered whether the use of the high concentration of ATRA, 1.0 μ M, could be inhibitory to, and thus potentially obscure, the role of RAR in the presence in RXR. The selection of this ATRA concentration was based upon preliminary experiments with dose–response experiments on the effect of ATRA on the production of antithrombin by HepG2 cells (R. W. L. M. Niessen, unpublished work), and confirmation of the appropriateness of this dose in the literature. This concentration has for instance been used by Durand et al. [32] and Umesono et al. [41a], who clearly established the involvement of RAR under such conditions, albeit in P19 EC and CV-1 cells, respectively. Also, we did not perform co-transfection experiments with both RAR and RXR, and the concentration of endogenous RXR in the RAR co-transfection experiments is in our opinion too low to warrant such a possibility.

The RARs, RXRs and TRs bind as homo- and/or heterodimers to their respective DNA response element. Synthetic and natural DNA response elements have been characterized for TRs, RARs and RXRs [37,41a–47]. All these response elements consist of the repetition of a consensus hexamer motif AGGTCA in different configurations with respect to both the orientation (direct, inverse, or palindromic repetition) and the spacing of the two motifs. The recognition of the response elements by a given receptor appears to be dependent on the actual core sequence, on nucleotides surrounding the hexamer sequence, on orientation, and on spacing of the repeated core motifs [41a,46,48–51]. Recent studies have indicated that the RXRs are the heterodimerization partners of, among others, RARs or TRs [52–54]. The heterodimerization with RXRs is both necessary and sufficient for stable interaction of the receptors with their response elements. This suggests that the ligand-dependent (ATRA, T3 and ATRA/T3) increase of antithrombin promoter activity observed in pCAT510 is also mediated through endogenous RXRs and TRs.

Within the antithrombin promoter region –92/–54 we have concentrated on a putative HRE with the hexamer motif AGGTCA. This antithrombin HRE does not contain the commonly present two halfsites, but three. This is, for example, also found in the rat growth hormone promoter [42,55] and in the

mouse tissue transglutaminase promoter [56]. Another important difference between this HRE and most synthetic and naturally occurring response elements is the spacing of the halfsites. The halfsite in the middle of the HRE pairs with the most 5'-located halfsite as a palindrome with a spacing of 8 nucleotides, and with the most 3'-located halfsite as an inverted repeat with a spacing of 13 nucleotides. It was recently proposed that spacing between direct repeated halfsite motifs of 3, 4 and 5 bp generates responsiveness to 1,25-(OH)₂VitD₃, T3 and ATRA, respectively [41a,48]. In that situation RXR appears to act as the heterodimerization partner of VDR, TR and RAR, respectively. Subsequently, it was shown that a direct repeated halfsite motif, with a spacing of one nucleotide, is recognized by RXR homodimers [46]. Homodimeric responsiveness using halfsite motifs arranged as inverted palindromes was studied by Carlberg [50]. In this study a spacing of three to four nucleotides was optimal for RXR homodimers, whereas TR, RAR and VDR homodimers acted best when halfsites were separated by 8, 10 and 12 nucleotides, respectively. In studies by Tini et al. [57], two (A/G)GGTCA motifs in an everted arrangement spaced by eight nucleotides were identified within the mouse γ F-crystallin promoter, that act as a retinoic acid response element (RARE). Therefore, growing evidence exists that halfsite motifs with a spacing of more than five nucleotides, as found within the antithrombin HRE, can function as thyroid hormone response elements (TREs), RAREs, or retinoid X response elements (RXREs).

In the analysis of the deletion CAT constructs we showed that the first 86 bp of the antithrombin promoter were already sufficient for basal gene expression. Making the antithrombin promoter longer (up to 1041 bp) does not substantially increase promoter activity. The construct pCAT160, that contains all the three halfsites, has a relatively low activity. This is currently not understood and needs further investigation.

When the variably sized DNA segment polymorphism of the antithrombin promoter was analysed, pCAT510 (108 bp fragment) was a somewhat stronger promoter (1.7-fold) than pCAT434 (32 bp fragment). This finding is consistent with the transfection study of Winter et al. [58], who showed a 1.6-fold higher activity of the 108 bp allele compared with the 32 bp allele in promoter fragments corresponding to positions –520/+51 and –444/+51, respectively. The difference in antithrombin promoter activity is no longer present when pCAT1098 (108 bp fragment) and pCAT1022 (32 bp fragment) are compared. This indicates that the promoter activity of the two polymorphic alleles *in vitro* is dependent upon the length of the promoter sequence surrounding these alleles. This fits well with the recent finding that this polymorphism in the antithrombin promoter does not correlate with plasma antithrombin activity levels [58,59].

The results presented here show that RXR α is the vitamin A nuclear receptor which directs enhancement of antithrombin promoter activity. When RXR α and/or TR β are transfected, further increases in ATRA- and T3-dependent antithrombin promoter activity are observed, which may be a consequence of heterodimeric RXR/TR complexes transactivating gene expression. In recent studies using natural TREs within the rat α -myosin heavy chain gene and within the rat malic enzyme gene, Hermann et al. [54] showed that RXR α and TR β formed complexes which strongly and selectively bound to TREs. In our preliminary experiments to explore the T3 response of the antithrombin promoter we showed that this promoter is transactivated T3-dependently upon co-transfection with TR β as well as with TR α (R. W. L. M. Niessen, unpublished work). Comparison of the antithrombin HRE with a DR4 element, a known

TRE that binds heterodimeric complexes of RXR α and TR β [41a], suggests that the HRE present in the antithrombin promoter region $-109/-42$ acts like a RXRE and not like a TRE. These results seem to be discrepant with the finding that pCAT143 (containing two halfsites of HRE) and pCAT160 (containing three halfsites of HRE) do not respond to TR β , and most importantly, not to RXR α . This may indicate that additional sequences up to nucleotide -167 are necessary for the functionality of the HRE within the antithrombin promoter sequence context. On the other hand, the six most 5' nucleotides present in the HRE segment ($-109/-42$), which are absent in pCAT160 (containing $-103/+57$) may be of crucial importance for an adequate RXR α responsiveness. It has recently been shown by site-directed mutagenesis experiments upon the antithrombin promoter motifs $-112/-107$ and $-84/-75$ that these sequences indeed function as essential transcriptional activation elements [60].

RXR α and HNF-4 often bind to the same response elements and thereby activate the same genes such as apolipoprotein AI, cellular retinol-binding protein type II, phosphoenolpyruvate carboxykinase, and transferrin [61]. This is an explanation for the results of Ochoa et al. [23] where competition of the antithrombin promoter region $-89/-68$ (containing the second halfsite of the HRE) was found with the HNF-4 site of the transferrin gene.

In conclusion, we have firm evidence that both T3 and ATRA significantly increase antithrombin production in our HepG2 cell line model. The action of both T3 and ATRA are directed by their respective nuclear receptors TR β and RXR α . Further study is needed to unravel the exact nature of this HRE and its 5'-flanking sequences.

We are grateful to Dr. Edward V. Prochownik and Dr. Susan Bock who kindly provided us with the plasmids pAT1.2, pFast and pSlow, respectively. We thank Dr. Ronald Evans for kindly providing us with the pRShTR β , pRShRXR α , and pRShRXR β expression vectors, Dr. Pierre Chambon for kindly providing us with the pSG5hRAR α , pSG5hRAR β , and pSG5hRAR γ expression vectors, and Dr. K. Dingelhoff of Roche Nederland for his generous gift of 1,25-dihydroxycholecalciferol.

REFERENCES

- Petersen, T. E., Dudek-Wojciechowska, G., Sottrup-Jensen, L. and Magnusson, S. (1979) in *The Physiological Inhibitors of Blood Coagulation and Fibrinolysis* (Collen, D., Wiman, B. and Verstraete, M., eds.), pp. 43–54, Elsevier Science Publishers, Amsterdam
- Bock, S. C., Wion, K. L., Vehar, G. A. and Lawn, R. M. (1982) *Nucleic Acids Res.* **10**, 8113–8125
- Chandra, T., Stackhouse, R., Kidd, V. J. and Woo, S. L. C. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1845–1848
- Prochownik, E. V., Markham, O. F. and Orkin, S. H. (1983) *J. Biol. Chem.* **258**, 8389–8394
- Huber, R. and Carrell, R. W. (1989) *Biochemistry* **28**, 8951–8966
- Damus, P. S., Hicks, M. and Rosenberg, R. D. (1973) *Nature (London)* **246**, 355–357
- Rosenberg, R. D. and Damus, P. S. (1973) *J. Biol. Chem.* **248**, 6490–6505
- Egeberg, O. (1965) *Thromb. Diath. Haemorrh.* **13**, 516–530
- Thaler, E. and Lechner, K. (1981) in *Clinics in Haematology* (Prentice, C. R. M., ed.), vol. 10, pp. 369–390, WB Saunders, London
- Hathaway, W. E. and Bonnar, J. (1978) in *Perinatal Coagulation – Monographs in Neonatology* (Oliver, T., ed.), pp. 53–80, Grune & Stratton, New York
- Peters, M., Jansen, E., ten Cate, J. W., Kahlé, L. H., Ockelford, P. and Breederveld, C. (1984) *Br. J. Haematol.* **58**, 579–587
- Andrew, M., Paes, B., Milner, R., Johnston, M., Mitchell, L., Tollefsen, D. M., Castle, V. and Powers, P. (1988) *Blood* **72**, 1651–1657
- Andrew, M., Massicotte-Nolan, P., Mitchell, L. and Cassidy, K. (1985) *Pediatr. Res.* **19**, 237–239
- Peters, M., ten Cate, J. W., Breederveld, C., de Leeuw, R., Emeis, J. J. and Koppe, J. G. (1984) *Pediatr. Res.* **18**, 273–276
- van Wassenae, A. G., Kok, J. H., Endert, E., Vulsma, T. and de Vijlder, J. J. M. (1993) *Acta Endocrinol.* **129**, 139–146
- van Wassenae, A., Kok, J. and de Vijlder, J. (1995) *J. Endocrinol. Invest. Suppl.* **17**, 75 (Abstr.)
- Niessen, R. W. L. M., Pfaffendorf, B. A., Sturk, A., Lamping, R. J., Schaap, M. C. L., Hack, C. E. and Peters, M. (1995) *Thromb. Haemostasis* **74**, 686–692
- Bock, S. C., Harris, J. F., Balazs, I. and Trent, J. M. (1985) *Cytogenet. Cell. Genet.* **39**, 67–69
- Bock, S. C., Marrinan, J. A. and Radziejewska, E. (1988) *Biochemistry* **27**, 6171–6178
- Olds, R. J., Lane, D. A., Chowdhury, V., De Stefano, V., Leone, G. and Thein, S. L. (1993) *Biochemistry* **32**, 4216–4224
- Bock, S. C. and Levitan, D. J. (1983) *Nucleic Acids Res.* **11**, 8569–8582
- Prochownik, E. V. (1985) *Nature (London)* **316**, 845–848
- Ochoa, A., Brunel, F., Mendelzon, D., Cohen, G. N. and Zakin, M. M. (1989) *Nucleic Acids Res.* **17**, 119–131
- Schaeffer, E., Guillou, F., Part, D. and Zakin, M. M. (1993) *J. Biol. Chem.* **268**, 23399–23408
- Knowles, B. B., Howe, C. C. and Aden, D. P. (1980) *Science* **209**, 497–499
- Samuels, H. H., Stanley, F. and Casanova, J. (1979) *Endocrinology* **105**, 80–85
- Crossley, M. and Brownlee, G. G. (1990) *Nature (London)* **345**, 444–446
- Nordeen, S. K. (1988) *Biotechnology* **5**, 454–457
- Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd. edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
- Seed, B. and Sheen, J. Y. (1989) *Mol. Cell. Biol.* **9**, 1415–1425
- Durand, B., Saunders, M., Leroy, P., Leid, M. and Chambon, P. (1992) *Cell* **71**, 73–85
- Thompson, C. C. and Evans, R. M. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3494–3498
- Yamamoto, K. R. (1985) *Annu. Rev. Genet.* **19**, 209–252
- Evans, R. M. (1988) *Science* **240**, 889–895
- Zhang, X.-K. and Pfahl, M. (1993) *Trends Endocrinol. Metab.* **4**, 156–162
- Mangelsdorf, D. J., Ong, E. S., Dyck, J. A. and Evans, R. M. (1990) *Nature (London)* **345**, 224–229
- Ishikawa, T., Umesono, K., Mangelsdorf, D. J., Aburtani, H., Stanger, B. Z., Shibasaki, Y., Imaware, M., Evans, R. M. and Takaku, F. (1990) *Mol. Endocrinol.* **4**, 837–844
- Yang, N., Schule, R., Mangelsdorf, D. J. and Evans, R. M. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3559–3563
- Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., Stein, R. B., Eichele, G., Evans, R. M. and Thaller, C. (1992) *Cell* **68**, 397–406
- Levin, A. A., Sturzenbecker, L. J., Kazmer, S., Bosakowski, T., Huseilton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberger, M., Lovey, A. and Grippo, J. F. (1992) *Nature (London)* **355**, 359–361
- Umesono, K., Murakami, K. K., Thompson, C. C. and Evans, R. M. (1991) *Cell* **65**, 1255–1266
- Glass, C. K., Franco, R., Weinberger, C., Albert, V. R., Evans, R. M. and Rosenfeld, M. G. (1987) *Nature (London)* **329**, 738–741
- Vasios, G. W., Gold, J. D., Petkovich, M., Chambon, P. and Gudas, L. J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9099–9103
- Reference deleted.
- Leroy, P., Nakshatri, H. and Chambon, P. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10138–10142
- Mangelsdorf, D. J., Umesono, K., Kliewer, S. A., Borgmeyer, U., Ong, E. S. and Evans, R. M. (1991) *Cell* **66**, 555–561
- Rottman, J. N., Widom, R. L., Nadal-Ginard, B., Madhavi, V. and Karathanasis, S. K. (1991) *Mol. Cell. Biol.* **11**, 3814–3820
- Näär, A. M., Boutin, J.-M., Lipkin, S. M., Yu, V. C., Holloway, J. M., Glass, C. K. and Rosenberg, M. G. (1991) *Cell* **65**, 1267–1279
- Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J.-Y., Staub, A., Garnier, J.-M., Mader, S. and Chambon, P. (1992) *Cell* **68**, 377–395
- Carlberg, C. (1993) *Biochem. Biophys. Res. Commun.* **195**, 1345–1353
- Katz, R. W., Subauste, J. S. and Koenig, R. J. (1995) *J. Biol. Chem.* **270**, 5238–5242
- Yu, V. C., Delsert, C., Andersen, B., Holloway, J. M., Devary, O. V., Näär, A. M., Kim, S. Y., Boutin, J.-M., Glass, C. K. and Rosenfeld, M. G. (1991) *Cell* **67**, 1251–1266
- Bugge, T. H., Pohl, J., Lonnoy, O. and Stunnenberg, H. G. (1992) *EMBO J.* **11**, 1409–1418
- Hermann, T., Hoffmann, B., Zhang, X.-K. and Tran, P. (1992) *Mol. Endocrinol.* **6**, 1153–1162
- Garcia-Villalba, P., Au-Fliegner, M., Samuels, H. H. and Aranda, A. (1993) *Biochem. Biophys. Res. Commun.* **191**, 580–586
- Nagy, L., Saydak, M. M., Shipley, N., Basilion, J. P., Yan, Z.-H., Syka, P., Heyman,

- R. A., Chandraratna, R. A. S., Stein, J. P. and Davies, P. J. A. (1995) *J. Biol. Chem.* **270**, in the press
- 57 Tini, M., Otulakowski, G., Breitman, M. L., Tsui, L.-C. and Giguère, V. (1993) *Genes Dev.* **7**, 295–307
- 58 Winter, P. C., Scopes, D. A., Berg, L.-P., Millar, D. S., Kakkar, V. V., Mayne, E. E., Krawczak, M. and Cooper, D. N. (1995) *Blood Coag. Fibrin.* **6**, 659–664
- 59 Niessen, R. W. L. M., Lamping, R. J., Sturk, A. and Peters, M. (1996) *Thromb. Haemostasis* **75**, 376–377 (Letter)
- 60 Rosenberg, J. B., Bergtrom, G. and Amrani, D. L. (1995) *Thromb. Haemostasis* **73**, 1249 (Abstract)
- 61 Sladek, F. M. (1994) in *Liver Gene Expression* (Tronche, F. and Yaniv, M., eds.), pp. 207–230, R. G. Landes Company, Austin, TX, U.S.A.
-

Received 27 November 1995/12 April 1996; accepted 29 April 1996