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Tissue-specific response of the human platelet-activating factor receptor gene to retinoic acid and thyroid hormone by alternative promoter usage

(inflammation/transcriptional control)

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ABSTRACT **We have studied the effects of retinoic acid (RA) and thyroid hormone (3,3′,5-triiodothyronine; T_3) on platelet-activating factor receptor (PAFR) gene expression in intact rats and the ability of two human PAFR gene promoters (PAFR promoters 1 and 2) to generate two transcripts (PAFR transcripts 1 and 2). Northern blotting showed that RA and T_3 regulated PAFR gene expression only in rat tissues that express PAFR transcript 2. Functional analysis of the human PAFR promoter 2 revealed that responsiveness to RA and T_3 was conferred through a 24-bp element [PAFR–hormone response element (HRE)] located from −67 to −44 bp of the transcription start site, whereas PAFR promoter 1 did not respond to these hormones. The PAFR–HRE is composed of three direct repeated TGACCT-like hexamer motifs with 2- and 4-bp spaces, and the two upstream and two downstream motifs were identified as response elements for RA and T_3. Thus, the PAF–PAFR pathway is regulated by the PAFR level altered by a tissue-specific response to RA and T_3 through the PAFR–HRE of the PAFR promoter 2.**

Platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent lipid mediator involved in pathophysiological processes (1–5).

We reported the expression cloning of the PAF receptor (PAFR) (6). Furthermore, two human PAFR transcripts are found (PAFR transcripts 1 and 2) (7, 8). Analysis of the genomic structure encoding the human PAFR showed that two 5′ noncoding exons (exons 1 and 2) directed by two promoters (PAFR promoters 1 and 2) are alternatively spliced to a common splice acceptor site on a third exon (exon 3) that contains the total open reading frame to yield two species of functional mRNA (PAFR transcripts 1 and 2) (9–11) (see Fig. 2*A*). These transcripts are differentially expressed in human tissues (9). The human PAFR transcript 1 is ubiquitous and most abundant in peripheral leukocytes, while transcript 2 is located in the heart, lung, spleen, and kidney but not in the brain or leukocytes (9). Therefore, human PAFR promoter 2 seems to contribute to tissue-specific expression of the PAFR gene.

Vitamin A and thyroid hormone (3,3′-5-triiodothyronine; T_3) participate in inflammatory, immunological, or allergic processes (12). Vitamin A is required for proper maintenance and function of the immune system (12–15). The host-versus-graft reaction, in which PAF is thought to participate, is immunologically enhanced by hypervitaminosis A (16, 17). On the other hand, bronchial asthma is more severe in patients with hyperthyroidism (18).

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These findings led us to further examine the regulation of PAFR by these hormones. Northern blotting showed that retinoic acid (RA) and T_3 regulate in a tissue-specific manner (in the heart and skin but not in the brain) PAFR gene expression at the transcriptional level in intact rats. The tissue-specific expression of PAFR transcript 2 suggested that the response of PAFR gene expression to RA and T_3 is directed by PAFR promoter 2 but not promoter 1. Indeed, a functional analysis of two human promoters demonstrated that a short 24-bp element composed of three TGACCT-like hexamer motifs spaced by 2 and 4 bp in PAFR promoter 2 acts as a hormone response element for RA and T_3. Thus, both *in vivo* and *in vitro* experiments suggest a role for RA and T_3 in tissue-specific hormonal regulation of PAFR gene expression.

MATERIALS AND METHODS

Animals. Wistar rats (3 weeks old) were rendered retinol deficient by feeding them with a vitamin A-deficient diet for 30 days (19). Another group was rendered thyroid hormone deficient by feeding them with a diet containing 6-propyl-2-thiouracil (PTU; 1.5 mg per g of diet) for 30 days (20). In replenishment studies, retinol-depleted rats were given 100 μg of all-*trans*-RA, and thyroid hormone-deficient rats were administered 100 μg of T_3.

RNA Isolation and Northern Blotting. By using total RNA (40 or 80 μg) (21), Northern blots were prepared using a full-length cDNA of rat PAFR (5) as the template for random priming as described by Mano *et al.* (20). The relative abundance of PAFR mRNAs was calculated by densitometrically scanning Northern blots of PAFR-specific bands and is shown as the means ± SD for at least three samples from each rat after normalizing with the β-actin mRNA levels.

Cell Transfection and Chloramphenicol Acetyltransferase (CAT) Assay. Reporter plasmids harboring the rabbit β-globin promoter (2 μg) or the PAFR promoter (−42/+212) (10 μg) were transfected into COS-1 cells by means of calcium phosphate coprecipitation with 500 ng of each expression vector for mouse all-*trans*-RA receptor α (RARα), mouse 9-*cis*-RA receptor α (RXRα) (22), chicken α thyroid hormone receptor (TRα) (23), and rat vitamin D receptor (VDR) (24). The ligand (all-*trans*-RA, T_3, and vitamin D at 10^{-7} M) was added to the medium for 40–44 hr. After normalizing the β-galac-

Abbreviations: PAF, platelet-activating factor; PAFR, PAF receptor; RA, retinoic acid; T_3, 3,3′,5-triiodothyronine; RAR, all-*trans*-RA receptor; RXR, 9-*cis*-RA receptor; RARE, RA response element; TR, thyroid hormone receptor; TRE, thyroid hormone response element; HRE, hormone response element; PTU, 6-propyl-2-thiouracil; CAT, chloramphenicol acetyltransferase; VDR, vitamin D receptor.

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tosidase activity (40 units), the CAT assay was performed as described (25).

Construction of Plasmids. The plasmid pPAF2-6k/CAT was constructed as follows. About −6000 to +212 bp, relative to the transcription start site of human PAFR transcript 2, of the fragment (λPAF20) cloned into the phage vector EMBL3/SP6/T7 was inserted in front of the coding sequence for CAT in vector pUC19. Plasmids pPAF2-265/CAT and pPAF2-42/CAT were constructed by inserting PCR-amplified fragments (−265 to +212 bp or −42 to +212 bp) of promoter 2 of the human PAFR gene. A series of deletion plasmids was constructed containing various PCR-amplified fragments of promoter 2 from the human PAFR gene upstream of the rabbit β-globin short promoter (−109 to +10 bp) of pGCAT (25) (pPAF2GCAT; pDEL1-3, -6, and -7). Synthetic oligonucleotides were also used for the reporter constructs (pDEL4 and -5; pMUT1-4).

Gel Retardation. DNA binding of partially purified mouse RARα, chicken TRα, and mouse RXRα (26) was assayed by electrophoretic mobility shift. Each receptor (10 ng) was incubated with or without each competitor on ice for 15 min in binding buffer [10 mM Tris·HCl, pH 7.5/1 mM dithiothreitol/1 mM EDTA/100 mM KCl/10% (vol/vol) glycerol] in the presence of poly(dIdC) (Pharmacia; 0.5 μg). The binding reaction was started by adding the ^{32}P 5′-end-labeled synthetic oligonucleotide and incubated at 25°C for 15 min. The samples were analyzed on a 5% polyacrylamide gel as described (25).

RESULTS

RA and Thyroid Hormone Regulate in a Tissue-Specific Way PAFR Gene Expression at the Transcriptional Level in Intact Animals. We examined the *in vivo* effects of RA and T_3 on expression of the PAFR gene in the heart, skin, and brain of rats under various RA and T_3 status (19, 20). The levels of PAFR mRNAs decreased in the heart and skin of vitamin A- and thyroid hormone-deficient rats (Fig. 1). Oral administration of all-*trans*-RA (100 μg per rat) to the retinol-deficient rats and intraperitoneal administration of T_3 (100 μg per rat) to the PTU-treated rats restored within 4 hr the levels of PAFR mRNAs in the heart and skin (Fig. 1). Moreover, positive regulation was confirmed by induction of the PAFR gene 6 hr after an excess of all-*trans*-RA (1 mg per rat) or T_3 (500 μg per rat) was given to normal rats (Fig. 1). In contrast, PAFR gene expression in the brain did not respond to RA and T_3 (Fig. 1).

In sharp contrast to RA and T_3, PAFR gene expression was not induced 6 hr after an excess of vitamin D (200 ng per rat) given to normal rats (data not shown).

As previously reported (19, 20), we could confirm the transcriptional nature of the *in vivo* effect of RA and T_3 by sensitivity to actinomycin D but not cycloheximide (data not shown).

Human PAFR Promoter 2, but Not Promoter 1, Confers RA and T_3 Inducibility. We reported that human PAFR transcript 1 is ubiquitous, whereas human PAFR transcript 2 is located in the heart but not in the brain (9). These findings suggest that PAFR promoter 2 might contribute to tissue-specific regulation of the PAFR gene by RA and T_3. To test this notion, we examined the effects of these hormones on human PAFR transcripts 1 and 2 in the human stomach cell line (JR-St cells) (27) and found that only PAFR transcript 2 responded to both RA and T_3 (H.M. and T.S., unpublished results). Therefore, to explore hormone response elements, we analyzed human PAFR promoters 1 and 2 using a transient expression assay with the CAT reporter gene (CAT assay) (Fig. 2*B*). A series of CAT vectors [only three representative CAT vectors are shown as pPAF2-6k, pPAF2-265, pPAF2-42 (each of which contains a sequence from around −6000, −265, or −42 bp to +212 bp, Fig. 2*B*)] was cotransfected with the expression vectors for mouse RARα and mouse RXRα or chicken TRα and RXRα into COS-1 cells. Then the cells were incubated for 40–44 hr in the absence or presence of RA or T_3. Plasmids pPAF2-6k and pPAF2-265, but not pPAF2-42, conferred RA and T_3 responsiveness (7-fold for RA and 4-fold for T_3). With the results of the other CAT vectors (data not shown), these findings indicated that the response element to RA and T_3 is located in the region from −265 to −43 bp of PAFR promoter 2. To assess the enhancer activity of this response element, the PAFR promoter (−42 to +212 bp) of pPAF2-265 was replaced with the rabbit short β-globin promoter (−109 to +10 bp) to construct pPAF2GCAT (−265/−43) because of the presence of silencer-like elements (data not shown) and the weakness of the basal activity of PAFR promoter 2. Both RA and T_3 caused a significant increase in CAT activity of pPAF2GCAT (−265/−43) (Fig. 3). Neither RA nor T_3 affected the transcriptional activity in a series of the PAFR promoter 1 mutants (9) in this assay (data not shown).

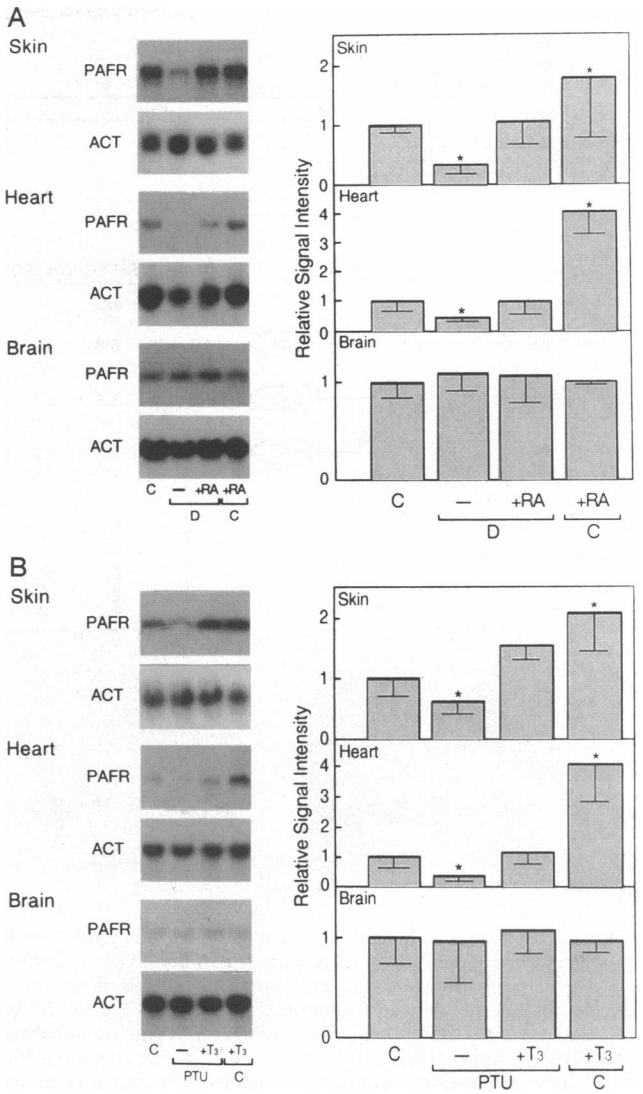

FIG. 1. Positive regulation by RA and thyroid hormone of PAFR gene expression in rat skin and heart but not in brain. (*A*) Tissue-specific response of PAFR gene to RA. (*B*) Tissue-specific response of PAFR gene to thyroid hormone. For replenishment, RA (100 μg per rat) or T_3 (100 μg per rat) was administered to the retinol-deficient rats or the PTU-treated rats (D or PTU). Excess RA (1 mg per rat) (+RA) or T_3 (500 μg per rat) (+T_3) was given to normal rats. Six hours after RA or T_3 administration, the treated (RA or T_3) and control (C) rats were killed and total RNA from the tissues was isolated for analysis. Significant difference ($P < 0.05$; Student's *t* test) from the control (C) is shown (*).

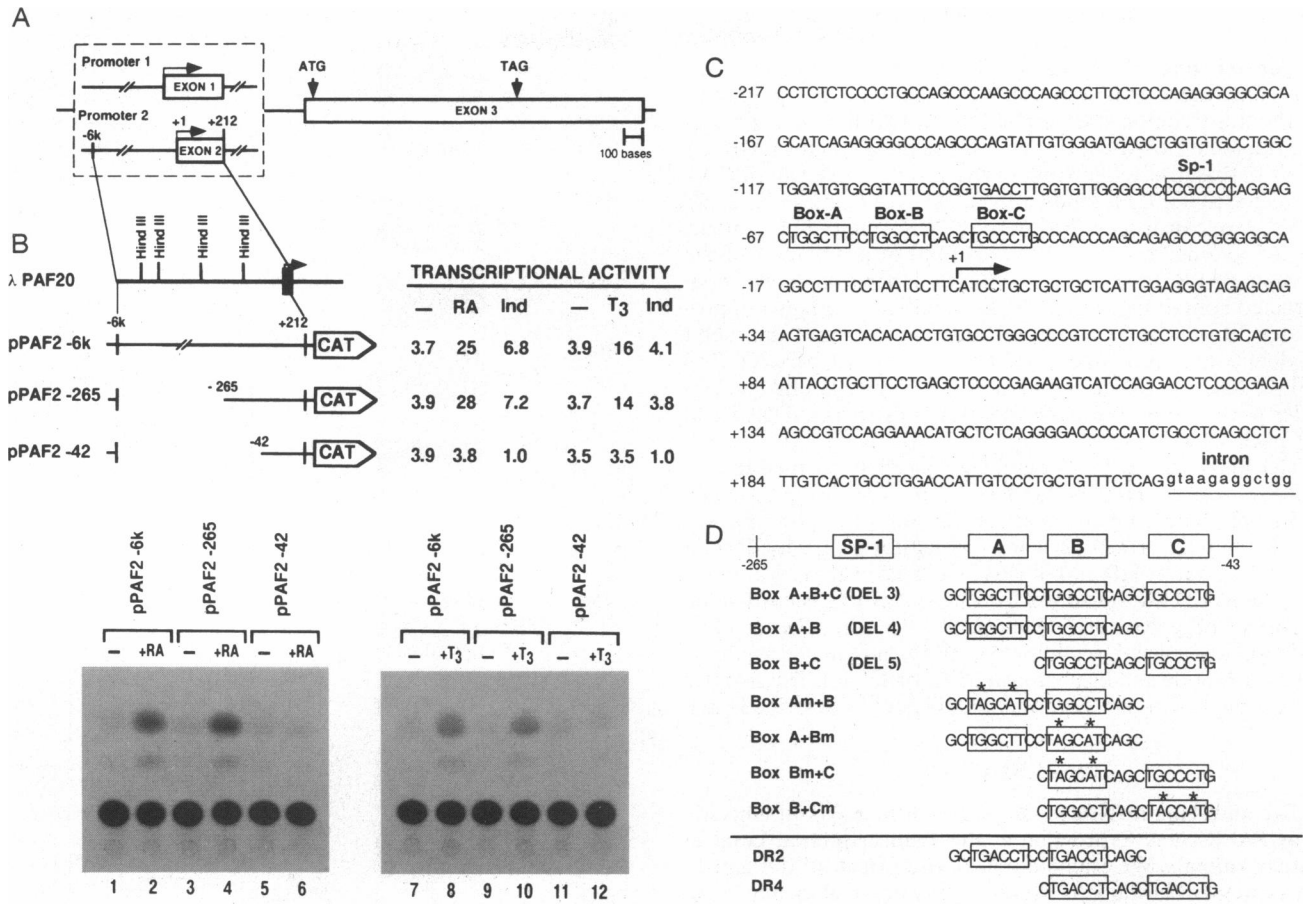

FIG. 2. Structure and function of human PAFR gene promoters. (*A*) Schematic representation of human PAFR gene structure, which generates two human PAFR mRNAs (PAFR transcripts 1 and 2) (9). Locations of the translational start (ATG) and stop (TAG) codons are indicated. The two alternative 5′ exons (exons 1 and 2) with the transcriptional start sites indicated by arrows and the common coding exon (exon 3) are indicated. (*B*) RA and T_3 transactivate human PAFR promoter 2. λPAF20 indicates the insert of λ phage EMBL-3 with the human PAFR gene including PAFR exon 2, which is indicated as a solid box. Restriction enzyme sites for *Hin*dIII are shown. Schematic representation of the CAT reporter plasmids containing three different regions of PAFR promoter 2 is shown (*Upper Left*). Nucleotide numbering is relative to the human PAFR transcription start site designated as +1. Transcriptional activities are mean values from at least two independent experiments. The fold inductions by hormones (Ind) were calculated by dividing CAT activity determined in extracts from hormone-treated cells (lanes +RA or $+T_3$) by that in untreated control cells (lanes −). A representative CAT assay of the reporter plasmids described above is shown (*Lower*). COS-1 cells were cotransfected with the CAT reporter plasmids (10 μg) with the expression vectors for mouse RARα and mouse RXRα (lanes 1–6) or chicken TRα and mouse RXRα (lanes 7–12) and with 3 μg of β-galactosidase expression vector pCH110. The cells were maintained for 48 hr in the absence (lanes −) or presence (lanes +) of 100 nM RA or 10 nM T_3, collected, and used for CAT assay with normalization for β-galactosidase activity. (*C*) DNA sequence of the 5′ flanking region of PAFR transcript 2. The transcription start site is located at +1 bp (arrow). The complete TGACCT motif is represented by underlining. The degenerative TGACCT motifs are indicated as boxes A, B, and C. (*D*) (*Middle*) Boxes A, B, and C indicate wild-type sequences of the TGACCT-like hexamer motifs, whereas boxes Am, Bm, and Cm contain specific nucleotide substitutions for boxes A, B, and C, respectively (*). (*Top*) Schematic illustration of promoter 2 containing SP-1, boxes A, B, and C. (*Bottom*) DR2 and DR4 indicate complete TGACCT motifs spaced with 2 or 4 bp, respectively.

Identification of RA Response Element (RARE) and Thyroid Hormone Response Element (TRE) in Human PAFR Promoter 2. To delineate DNA elements of human PAFR promoter 2 that mediate the response to RA or T_3, we constructed a series of deletion mutants in human PAFR promoter 2 (pDEL1-7) (Figs. 4 and 5). Mutants pDEL1 (−132/−43) and pDEL2 (−91/−43) were still induced 9-fold by RA and 5-fold by T_3 (Figs. 4 and 5). However, the inducibility in

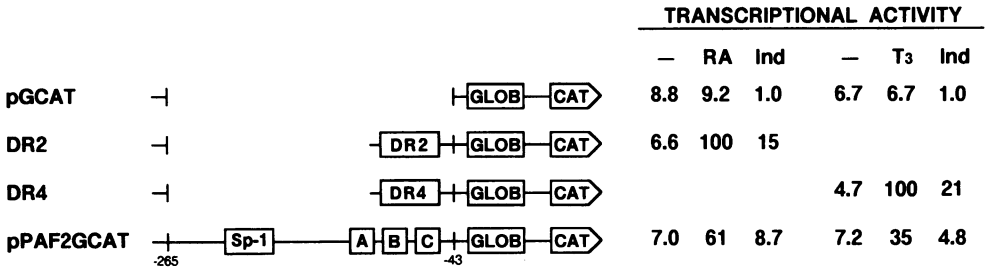

FIG. 3. Activation of human PAFR promoter 2 by RA and T_3. Schematic representation of CAT reporter plasmids containing PAFR promoter 2 region (−265 to −43) in front of rabbit β-globin short promoter (−109 to +10 bp). Positions of boxes A, B, and C and Sp-1 in the promoter are schematically illustrated in Fig. 2*D*. The RA-induced DR2 activities or T_3-induced DR4 activities are taken as 100% in each experiment.

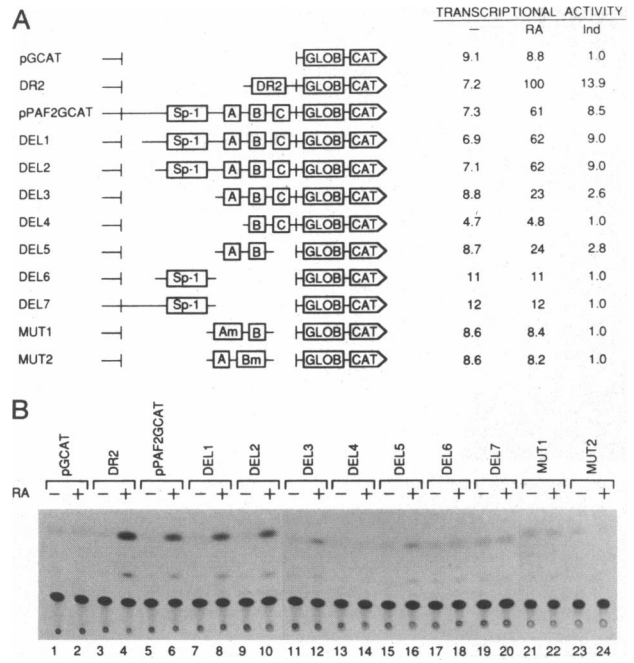

FIG. 4. Characterization of RARE in human PAFR promoter 2. (*A*) Schematic representation of CAT reporter plasmids containing different regions of PAFR promoter 2. RA-induced DR2 activities are taken as 100%. (*B*) Representative results of CAT assay. COS-1 cells were cotransfected with the CAT reporter plasmids (2 μg) together with 0.5 μg of mouse RARα and mouse RXRα expression vectors.

pDEL3 (−68/−43) was reduced to 2.6-fold by RA or 3-fold by T_3, respectively, although neither pDEL6 (−91/−68) nor pDEL7 (−265/−68) was induced. The results suggest involvement of the consensus sequence Sp-1 between −91 and −68 bp in ligand inducibility (Fig. 2*C*). The sequence (−68/−43) inserted into pDEL3 includes three imperfect direct repeats [TGGCTT cc TGGCCT cagc TGCCCT (box A+B+C; Fig. 2*D*)] of the 5′-TGACCT-3′ (5′-AGGTCA-3′) motif, which is the consensus binding half-site motif for RAR, TR, and VDR. Further analyses using pDEL4 (−60/−43; box B+C) and pDEL5 (−68/−50; box A+B) suggested that the sequence responsible for RA is mapped to box A+B (two motifs with a 2-bp spacer) (Fig. 4) and the sequence for T_3 is mapped to box B+C (two motifs with a 4-bp spacer) (Fig. 5). The mutations of 2 bases in each motif (pMUT1-4; see Fig. 2*D*) clearly impaired the responsiveness to RA and T_3 (Figs. 4 and 5). Thus, it is most likely that the two upstream (box A+B) and the two downstream (box B+C) motifs act as response elements for RA and T_3, respectively.

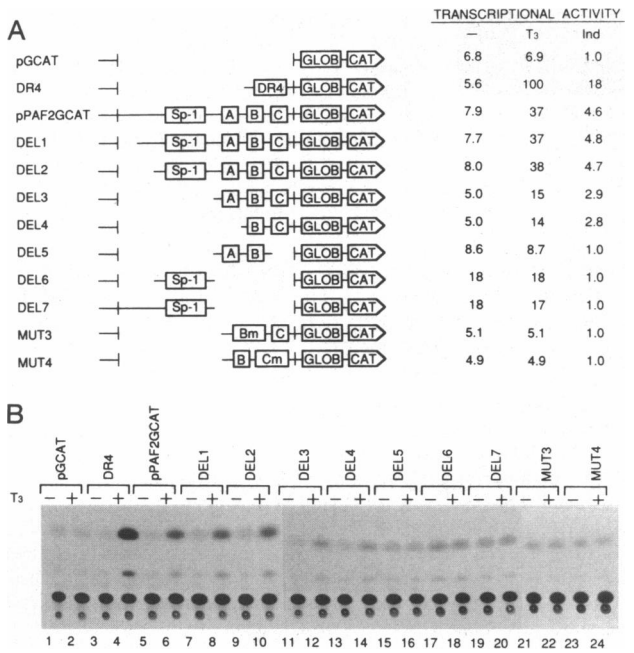

FIG. 5. Characterization of T_3 response element in human PAFR promoter 2. (*A*) Schematic representation of CAT reporter plasmids containing different regions of PAFR promoter 2. T_3-induced DR4 activities are taken as 100%. (*B*) Representative results of CAT assay. COS-1 cells were cotransfected with CAT reporter plasmids (2 μg) together with 0.5 μg of chicken TRα and mouse RXRα expression vectors.

RAR–RXR and TR–RXR Heterodimers Bind Each Regulatory Sequence. We performed an *in vitro* DNA binding assay (gel-shift assay) with partially purified receptors to determine whether the nuclear receptors indeed bind these sequences (Fig. 6). Gel-shift analysis using the labeled synthetic oligonucleotides containing either box A+B or box A+B+C, but not box B+C, revealed a retarded band only in the presence of both RAR and RXR (lanes 1–3 and 13–18 in Fig. 6*A*). These bands were well competed by an excess of a consensus RARE [CRARE (DR2); see Fig. 2*D*] (lanes 19, 20, 23, and 24 in Fig. 6*A*), whereas the stronger retarded band of the labeled CRARE sequence (DR2) in the presence of both RAR and RXR (lane 6) was not fully competed with an excess of the wild-type sequence (lane 21). Mutations of each motif within box A+B abolished binding of the RAR–RXR heterodimer (Fig. 6*A*, box Am+B and box A+Bm, lanes 7–12). Similarly, only the TR and RXR heterodimer bound to box B+C and box A+B+C but not box A+B (Fig. 6*B*, lanes 1–3 and 13–18). Specific binding to TR–RXR was abolished by mutations of each motif within box B+C (Fig. 6*B*, box Bm+C and box B+Cm, lanes 7–12). These results, together with their transcriptional activities, clarified that box A+B is a RARE and box B+C is a TRE in PAFR promoter 2.

DISCUSSION

The results presented here clarified the molecular mechanism of the tissue-specific and positive regulation of PAFR gene expression by RA and T_3 and suggest the significance of RA and T_3 in regulation of the PAF–PAFR pathway through the altered levels of PAFR.

Human PAFR gene expression is directed by two distinct promoters (PAFR promoters 1 and 2) to generate two transcripts (PAFR transcripts 1 and 2). PAFR transcript 1 is ubiquitous, whereas transcript 2 is expressed in a tissue-specific manner (9). The responses of PAFR gene expression to RA and T_3 were tissue specific in rats, and indeed the RA and T_3 response elements [PAFR–hormone response element (HRE)] were located in human PAFR promoter 2. On the other hand, neither RA nor T_3 affected transcriptional activity of PAFR promoter 1. Tissue specificity of PAFR gene regulation by RA and T_3 was thus achieved by using the two promoters properly. As PAF is involved in various pathophysiological processes (1–5), these actions may be controlled by RA and T_3 in a tissue-specific manner through the PAFR–HRE in PAFR promoter 2.

The PAFR–HRE at −67 to −44 bp in PAFR promoter 2 harbors three TGACCT-like hexamer motifs spaced by 2 and 4 bp (5′-TGGCTT cc TGGCCT cagc TGCCCT-3′). The first motif has two mismatches compared with the perfect 5′-TGACCT-3′ (5′-AGGTCA-3′) sequence, and the second and third have one mismatch each.

The 5′-TGACCT-3′ core motif has been described as the consensus half-site for thyroid hormone, RA, and vitamin D_3 receptor binding, while the specificity of DNA binding and transcriptional activation of these receptors are dictated by the

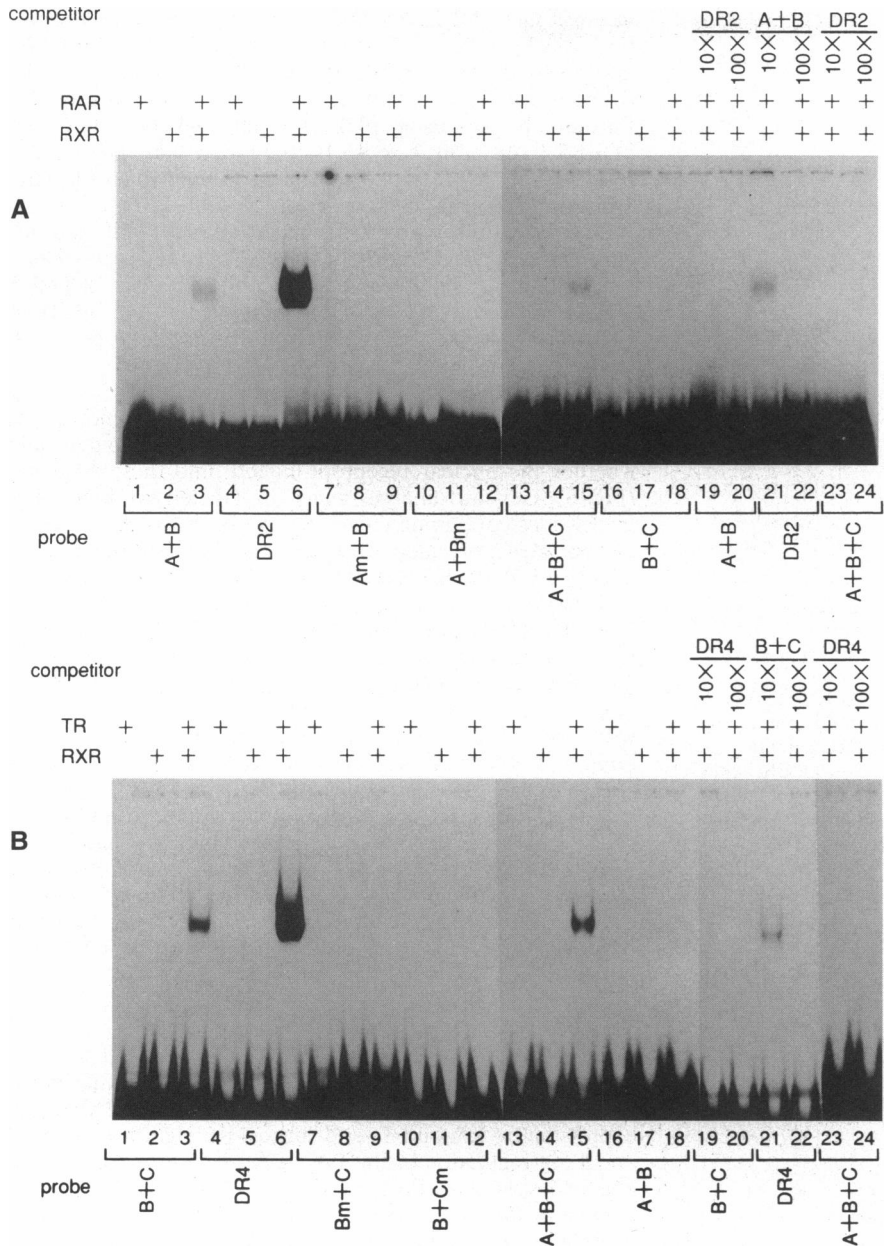

FIG. 6. Specific binding of RAR–RXR and TR–RXR heterodimers to RARE and TRE in human PAFR promoter 2. Gel-retardation shift assay was performed with probes containing DR2 (consensus RARE), DR4 (consensus TRE), PAFR–HRE (box A+B+C), PAFR–RARE (box A+B), PAFR–TRE (box B+C), or mutant sequences (box Am+B, box A+Bm, box Bm+C, box B+Cm) (see sequences in Fig. 2*D*). The 5′ ^{32}P-labeled probes were incubated with purified mouse RAR, mouse RXR, or both (as indicated in *A*) and with partially purified human TR, mouse RXR, or both (as indicated in *B*). Indicated oligonucleotides at either 10- or 100-fold molar excess to the probe were added to the reaction mixture as competitors.

spacing between the two repeated core motifs; HREs with spacers of 3, 4, and 5 bp (DR3, DR4, and DR5) confer a specific response to vitamin D_3, thyroid hormone, and RA, respectively (28–30). Several groups have shown that a direct repeat separated by 2 bp (DR2) serves as a second class of consensus RARE.

Although the RARE and TRE in PAFR promoter 2 included three half-sites, two upstream half-sites with a 2-bp spacer were sufficient for the RARE, and the two downstream sites with a 4-bp spacer for TRE. Namely, the middle half-site was shared by RARE and TRE. The activation by RA and T_3 and the *in vitro* DNA binding of this short element by cognate receptor heterodimers were weaker than that with each complete consensus motif. The reason may be that the half-sites are degenerate from the complete TGACCT motif. However, a consensus sequence for Sp-1 8 bp upstream of this short element cooperatively acted with PAFR–HRE to confer stronger responses than those of PAFR–HRE alone (Figs. 4 and 5). PAFR gene expression in response to RA and T_3 *in vivo* may thus be caused by the cooperative function of RXR–RAR and RXR–TR together with Sp-1. In sharp contrast, the VDR–RXR heterodimer could not bind the PAFR–HRE *in vitro* and vitamin D_3 did not induce the transcriptional activities *in vivo* and *in vitro* (data not shown). This means that the PAFR–HRE is specific for RA and T_3, which might be important for regulation of the PAF–PAFR axis.

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