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Thrombomodulin gene regulation by cAMP and retinoic acid in F9 embryonal carcinoma cells

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ABSTRACT **Thrombomodulin (TM) expression was investigated during differentiation of F9 embryonal carcinoma cells into primitive or parietal endoderm. Exposure of F9 cells to retinoic acid (RA) triggers differentiation into primitive endoderm and induces the appearance of barely detectable amounts of TM mRNA, whereas treatment with dibutyryl cAMP plus theophylline (CT) augments the levels of TM mRNA to a 4-fold greater extent than RA. Exposure of F9 cells to RA plus CT initiates differentiation into parietal endoderm and synergistically increases the levels of TM mRNA by 10- to 12-fold compared with CT. The time-dependent establishment of cooperativity between RA and CT appears to be secondary to RA-induced differentiation to primitive endoderm. The above alterations in TM mRNA levels occur by a transcriptional mechanism as judged by nuclear run-on experiments. Transient gene expression experiments show that the human TM promoter is transactivated by coexpression of the human RA receptor β. Thus, the mechanism of induction of TM expression in F9 cells undergoing differentiation to parietal endoderm appears to be similar, but not identical, to that noted for other late response genes.**

Thrombomodulin (TM) is an endothelial cell receptor that forms a specific complex with thrombin (1). This TM–thrombin complex is able to convert protein C into its activated form, which then cleaves other activated cofactors of the coagulation mechanism, thereby suppressing thrombin generation (2–4). The cloning and sequencing of the bovine and human TM cDNAs revealed that this receptor is structurally similar to coated-pit receptors and is organized into domains that resemble those of the low density lipoprotein receptor (5, 6). The nucleotide sequence of the human TM gene is noteworthy because of the complete absence of introns (6).

F9 mouse embryonal carcinoma cells provide a suitable *in vitro* model to study the developmental regulation of TM gene expression (7). F9 cells can be either maintained *in vitro* as undifferentiated stem cells or hormonally induced to differentiate into a restricted number of cell types found within the developing mouse embryo: retinoic acid (RA) converts F9 stem cells to a phenotype that is functionally similar to primitive endoderm (8, 9), whereas cultivation of the RA-treated cells in the presence of agents that increase intracellular cAMP causes differentiation to a phenotype resembling extraembryonic parietal endoderm (10). The parietal endoderm of the embryo is in contact with the maternal blood and should possess many biological characteristics typical of vascular endothelial cells. RA/cAMP-dependent differentiation of F9 cells initiates production of tissue-type plasminogen activator (tPA) as well as TM, and these two components also appear on the parietal endoderm of mouse embryos before their synthesis by developing blood vessels (11, 12). Therefore, determining the mechanisms that regulate expression of these two natural anticoagulants in differentiating F9 cells may elucidate the events that lead to synthesis of these same molecules in endothelial cells during vasculogenesis. Here we investigate the molecular mechanism by which RA and agents that increase cAMP are able to induce TM expression in F9 cells. We show that this response is mainly due to transcriptional regulation.

MATERIALS AND METHODS

DNA Expression Constructs. Plasmid pβAclacZ was a gift of U. Lendahl (Karolinska Institute, Stockholm) and was used to normalize for transfection efficiency in transient expression experiments. The construct allows *lacZ* expression under the control of a 4-kilobase-pair (kbp) *Eco*RI–*Sal* I fragment of the human β-actin promoter (13). A human RA receptor β (RARβ) expression vector (pβAcRARβ) was obtained from L. G. Lien (Beth Israel Hospital, Boston). It was constructed by joining a 350-bp fragment (positions −340 to +10) of the rat β-actin promoter to an *Sst* I–*Hin*dIII fragment of a human RARβ cDNA (14). Plasmid pTM-CAT was prepared by inserting a 3-kbp *Xba* I–*Bss*HII fragment of the putative human TM gene promoter into a *Bgl* II site of a promoterless plasmid (CAT-3M) carrying the chloramphenicol acetyltransferase (CAT) gene (15).

Cell Culture and Transient Expression Experiments. F9 cells were grown on gelatin-coated (0.1%) tissue culture dishes in Dulbecco's modified Eagle's medium containing 10% heat-inactivated calf donor serum (Irvine Scientific) in 5% CO_2 at 37°C (16). For analyses of hormone induced TM expression, 10^5 cells were plated into 100-mm tissue culture dishes and grown for various periods of time in the absence of hormones or in the presence of 1 μM all-*trans*-retinoic acid (RA), or 0.5 mM dibutyryl cAMP plus 0.5 mM theophylline (CT), or 1 μM RA plus CT (RACT). In experiments where F9 cells were subjected to two consecutive treatments, the cells were exposed to the first set of agents for the stated periods of time as outlined above, rinsed twice with phosphate-buffered saline cultured for 3 hr in medium without agents, and then maintained for the stated periods of time in media containing the second set of agents. Establishment of a differentiated phenotype was evident from morphological changes in response to hormone exposure. Biochemical differentiation was verified by analyzing cell cultures for expression of tPA mRNA. For transient expression experiments, 1×10^5 or 4×10^5 F9 stem cells (for RACT treatment) were seeded per 100-mm tissue culture dish and grown for 3 days in the presence or absence of hormones; the medium

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Abbreviations: CAT, chloramphenicol acetyltransferase; CT, dibutyryl cAMP/theophylline; RA, retinoic acid; RAR, RA receptor; TM, thrombomodulin; tPA, tissue-type plasminogen activator.

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was changed after 2 days as well as 3 hr prior to transfection. Each tissue culture dish received 1 ml of a DNA/calcium phosphate slurry, prepared as outlined by Rickles *et al.* (17), containing 68 mM NaCl, 2.5 mM KCl, 5.6 mM glucose, 104 mM Hepes, 0.7 mM Na_2HPO_4, 125 mM $CaCl_2$, 20 μg of supercoiled pTM-CAT, 6 μg of supercoiled pβAclacZ, 0.05–4 μg of supercoiled pβAcRARβ, and sufficient sheared herring DNA to give a total of 30 μg of DNA. The precipitates were removed from the cells after 12 hr, cells were washed with Tris-buffered saline, and fresh medium with or without hormones was added. Cell extracts were prepared 24 hr later and enzymatic assays for CAT and β-galactosidase activity were conducted (18, 19).

RNA Blot Hybridization. Total cellular RNA was prepared by an acidic guanidinium thiocyanate–phenol/chloroform procedure (20) and quantitated by measuring absorbance at 260 nm. mRNA was isolated from total cellular RNA by affinity chromatography on oligo(dT)-cellulose (21). Equal amounts of RNA were electrophoretically separated in 1.2% agarose/formaldehyde gels and transferred to GeneScreen hybridization membranes (NEN) as described (22). The cDNA inserts encoding β-actin (23), glyceraldehyde-3-phosphate dehydrogenase (24), or mouse TM (25) were labeled with [α-^{32}P]dCTP by random priming (26). Radiolabeled cDNA was hybridized at 10^6 cpm/ml to filter-bound RNA in 50% formamide/0.2% polyvinylpyrrolidone/0.2% Ficoll/0.2% bovine serum albumin/50 mM Tris·HCl, pH 7.5/0.1% sodium pyrophosphate/1% SDS containing sonicated salmon sperm DNA (100 μg/ml) for 12–16 hr at 42°C. Filters were washed for 10 min at 42°C in 2× standard saline citrate (SSC), 30 min at 65°C in 2× SSC/1% SDS, and 15 min at 42°C in 0.1× SSC/0.1% SDS. After quantitation of specific hybridization signals with a Betagen 603 blot analyzer (27), filters were exposed to Kodak XAR film for various periods of time.

Nuclear Run-on Transcription Assay. F9 cells were treated for 72 hr with RA, CT, or RACT as detailed above, and nuclei were isolated as described (16). Labeling of nascent transcripts and hybridization to filter-immobilized DNA were performed essentially as described (28). To this end, 4×10^7 nuclei were incubated for 30 min at 30°C in a 400-μl reaction mixture containing 20% (vol/vol) glycerol, 25 mM Tris·HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.01 mM EDTA, 150 mM KCl, 2.5 mM dithiothreitol, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 160 μCi of [α-^{32}P]UTP (800 Ci/mmol; 1 Ci = 37 GBq), and 80 units of RNasin (Promega). RNA was isolated by treating nuclei with DNase I and proteinase K, extracting with phenol/chloroform, and repeatedly precipitating with ammonium acetate and ethanol. The incorporation of [α-^{32}P]UTP into RNA was determined by precipitating an aliquot of the labeled RNA with trichloroacetic acid (TCA). Denatured plasmid DNA (>2 μg) was transferred to nitrocellulose filters with a dot-blot manifold and hybridized to radiolabeled RNA for 60 hr at 65°C in 10 mM Tes, pH 7.4/0.2% SDS/10 mM EDTA/0.3 M NaCl/0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin containing yeast tRNA (250 μg/ml). Filters were washed for 1 hr at 65°C in 2× SSC/0.2% SDS, rinsed in 2× SSC, incubated for 30 min at 37°C in 2× SSC with RNase A (10 μg/ml) and RNase T1 (100 units/ml), washed for 10 min at 65°C in 0.2× SSC/0.2% SDS, and then dried. The amount of RNA hybridized to the filter-bound DNA probes was determined using a Betagen 603 blot analyzer, and then the filters were exposed to Kodak XAR films for various periods of time.

Polymerase Chain Reaction. Competitive PCR was employed to specifically quantitate low levels of TM RNA (29). RNA for PCR analysis was prepared by CsCl centrifugation (30) and subsequently enriched for mRNA by oligo(dT) affinity chromatography. Oligo(dT)-primed reverse transcription of mRNA was performed by standard procedures (19). The amount of synthesized cDNA was determined by including a small amount of [α-^{32}P]dCTP in the reaction mixture and precipitating an aliquot with trichloroacetic acid. PCR amplifications were performed with a Gene Amp kit (Perkin–Elmer/Cetus). The mouse TM-specific primers d(ACTGATCGGACGCTGCAGAAGTTCTGA) and d(GGCCCAGTATGTCTCAAGATAGCAATG) were prepared with a 380B DNA synthesizer (Applied Biosystems). The competitor cDNA template was generated by deleting an *Nco* I–*Bst*EII restriction fragment from a mouse TM cDNA clone (25), which removes 234 bp from the region amplified by the specific primers. Reaction mixtures contained 30 ng of first-strand cDNA with 50 pmol of mouse TM-specific primers and various amounts of competitor template and were subjected to 30 cycles of denaturation (45 sec, 95°C), annealing (30 sec, 62°C), and extension (2 min, 72°C), followed by a final extension at 72°C for 10 min. PCR products were radioactively labeled by including [α-^{32}P]dCTP for one reaction cycle and were electrophoretically separated in 1.2% agarose gels. The TM-specific primers generated a 917-bp PCR product (positions 1866 to 2783) from the TM cDNA and a 683-bp product from the competitor plasmid. The nucleic acids were precipitated in the gel matrix with 7% trichloroacetic acid, the gel was dried, and the radioactive bands were quantitated with a Betagen 603 blot analyzer.

RESULTS

TM mRNA Expression in F9 Cells Is Regulated by RA and cAMP. Expression of biologically active TM receptor, as measured by an enzymatic assay and RNA slot blot analysis, is induced by dibutyryl cAMP in F9 cells treated with RA (7). The mechanism of action of this effect was examined at the RNA level by exposing F9 cells either to 1 μM RA, to CT, or to RACT. After various times of exposure to these agents, the cells were harvested and then examined for TM mRNA by Northern blot analyses of total cellular RNA (Fig. 1*A*). Sample loadings were equivalent as judged by the intensity of rRNA bands after ethidium bromide staining or by hybrid-

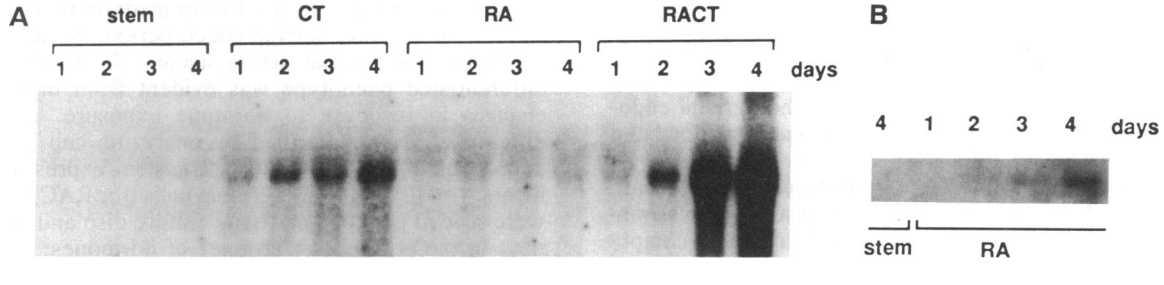

FIG. 1. Northern blot hybridization analysis of hormone-dependent TM mRNA expression. F9 stem cells were cultured for 1–4 days either without hormones (stem) or with RA, CT, or RACT. The RNA was isolated at the indicated times, and 20 μg of total RNA (*A*) or 5 μg of poly(A)$^+$ RNA (*B*) was electrophoresed in formaldehyde/agarose gels, transferred to nylon membranes, and hybridized to radiolabeled TM cDNA fragments.

ization to a β-actin or glyceraldehyde-3-phosphate dehydrogenase cDNA probe (data not shown). In the absence of any treatment, TM mRNA was not detected in F9 cells that had been cultured for up to 96 hr. After 96 hr of RA exposure, TM mRNA in F9 cells was barely detectable (Fig. 1*A*). To enhance sensitivity, Northern blot analyses were carried out with poly(A)$^+$ RNA; these analyses showed that low levels of TM mRNA were present after 48 hr of RA treatment and increased 2- to 3-fold over the next 48 hr (Fig. 1*B*). With CT addition, TM mRNA was observed within 24 hr, and message levels increased 4-fold as compared with RA treated F9 cells at 96 hr. This effect was due to elevated levels of cAMP, since neither 0.5 mM sodium butyrate nor 0.5 mM theophylline affected TM expression (data not shown). After 24 hr of RACT treatment, TM mRNA levels were equivalent to those observed after addition of CT for 24 hr. Exposure of F9 cells to RACT for 96 hr produced TM mRNA levels that were 10- to 12-fold higher than in CT-treated cells (Fig. 1*A*). The data summarized above were confirmed by additional independent experiments. These observations indicate that RA modestly enhances TM expression while inducing stem cells to differentiate into primitive endoderm; CT significantly augments receptor expression in stem cells, and CT functions synergistically with RA after a 24-hr delay to greatly stimulate expression of TM during hormone-induced differentiation into parietal endoderm.

cAMP-Induced TM Expression Is Enhanced in Primary Endoderm. Synergism between RA and CT with regard to TM expression in F9 cells was observed only after 24 hr of exposure to both agents. Therefore, it was important to determine whether the appearance of this synergistic effect was due to the gradual development of an RA-dependent mechanism or was secondary to a delayed cAMP response. To address this issue, F9 stem cells were cultured for 72 hr in the presence of RA to induce differentiation into primitive endoderm, washed thoroughly with phosphate-buffered saline, cultured for 3 hr in RA-free medium, and then exposed to CT. The levels of TM mRNA were measured by competitive PCR as outlined in *Materials and Methods*. This approach involves coamplification of the target TM cDNA with various concentrations of competitor DNA, which requires the same primers but generates a PCR product of different size. In a typical experiment conducted in the absence of competitor DNA, F9 cells exhibited a very low level of TM mRNA prior to exposure to RA, had significantly higher amounts of this message after 72 hr of treatment with RA, and generated steadily increasing levels of TM mRNA over the next 24 hr after CT addition (Fig. 2 *Left*). The complete lack of the PCR product with non-reverse-transcribed RNA obtained from F9 cells exposed for 72 hr to RACT (data not shown) provided evidence that the amplified DNA fragments were derived from mRNA and not from contaminating genomic DNA. When PCR analyses of the same samples were carried out with added known amounts of competitor DNA, F9 cells exposed to RA for 72 hr and CT for an additional 3 hr exhibited TM mRNA levels that were equivalent to 0.5 pg of competitor DNA (Fig. 2*A*, *Center*, 3-hr lane; note equal intensity of target and competitor bands); F9 cells treated with RA for 72 hr and CT for an additional 24 hr possessed TM message levels exceeding the equivalent of 5 pg of competitor DNA (Fig. 2*A*, *Right*, 24-hr lane). The above samples were also examined with various concentrations of added competitor DNA to permit determination of TM mRNA levels at other time points. The compiled data are summarized in Fig. 2*B*. The amounts of TM message increased at a constant rate with a lag time of ≤3 hr, leading to a 10-fold augmentation in the level of receptor message after 24 hr of exposure to CT. The amounts of TM mRNA over this time interval were about twice those expected from an additive effect of RA and CT. Thus, the cooperativity between RA and CT was observed in primitive endoderm almost immediately after exposure to CT and did not require the continued presence of exogenous RA. Furthermore, treatment of F9 cells with CT and then RA did not increase TM mRNA levels beyond those seen with CT alone (data not shown). These observations support the notion that establishment of cooperativity between RA and CT is mediated by RA-dependent cell differentiation.

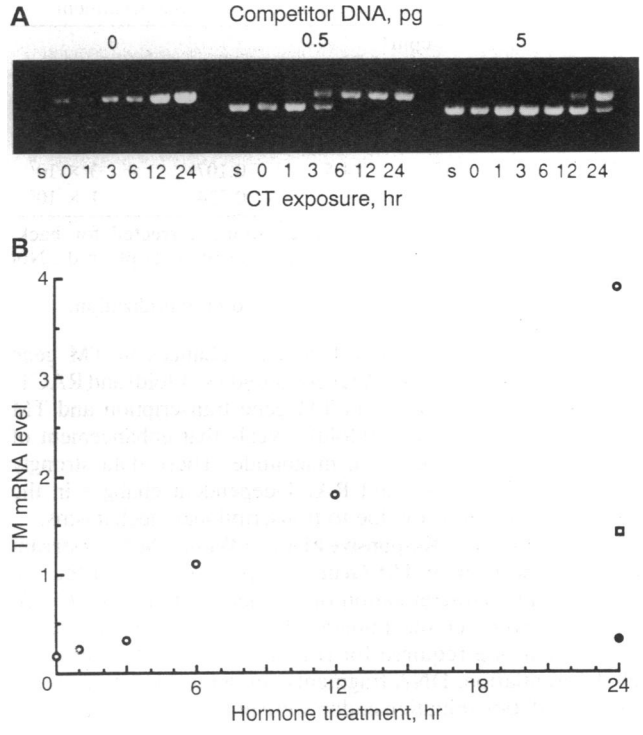

FIG. 2. cAMP-dependent TM mRNA induction in primary endoderm like F9 cells. F9 stem cells were treated first for 72 hr with RA and subsequently with CT for the times shown. Competitive PCR analysis was used to quantitate TM mRNA. (*A*) One-tenth of each reaction mixture was subjected to agarose gel electrophoresis and stained with ethidium bromide. The upper band is a 917-bp product derived from endogenous TM mRNA, and the lower band, 683 bp, is derived from mutated competitor DNA. Lanes s, stem cells. (*B*) Radioactivity associated with specific DNA bands was quantitated as described in *Materials and Methods*. TM mRNA levels are plotted in arbitrary units reflecting the ratio of PCR products derived from endogeneous TM mRNA vs. competitor DNA as a function of time after addition of CT to cells pretreated for 72 hr with RA (○). mRNA levels are also shown for cells treated for 24 hr with CT without exposure to RA (□) and for cells treated for 96 hr with RA (●). For values determined by competitive PCR analysis, each point represents the mean of two determinations at different competitor concentrations. Values for 24-hr CT, 72-hr RA, and 96-hr RA were derived from Northern blot experiments by determining the ratio of TM vs. β-actin hybridization signals. Values were converted into arbitrary units by relating them to the estimates for 72-hr RA cells, which were determined by both PCR and Northern blot analyses.

Transcriptional Activation of TM Expression by RA and CT. We carried out nuclear run-on experiments to determine whether RA- and CT-dependent changes in TM mRNA expression are produced by alterations in transcriptional activity. Cells were exposed to RA, CT, or RACT for 72 hr, nuclei were isolated from the various cell populations, and nascent TM and β-actin transcripts were quantitated (Table 1). β-Actin transcription is not regulated by RA and/or CT in F9 cells (31). Nuclear RNA from untreated F9 stem cells yielded no detectable signal, whereas nuclear RNA from CT- or RA-treated F9 cells exhibited significant hybridization (Fig. 3), which was considerably greater with CT than RA. Nuclear RNA from RACT-treated F9 cells gave a much higher signal than that from CT- or RA-treated cells.

Table 1. TM transcription in response to hormone treatment

Hormone(s)	cpm*			Input cpm†
	β-actin	TM	TM/β-actin	
None	142	n.d.	—	4×10^7
RA	1446	25	0.017	2×10^8
CT	135	14.5	0.107	3×10^7
RACT	636	467	0.734	1×10^8

*Mean values from duplicate hybridizations corrected for background hybridization to vector sequences (pBluescript). n.d., Not detected.
†Trichloroacetic acid-precipitable cpm used in hybridization.

Comparison of CT- vs. RA-dependent changes in TM gene transcription and TM mRNA levels (6-fold vs. 4-fold) and RACT- vs. CT-dependent changes in TM gene transcription and TM mRNA levels (7-fold vs. 10-fold) reveals that enhancement of both parameters is similar in magnitude. These data strongly suggest that RA-, CT-, and RACT-dependent changes in the levels of TM mRNA are due to transcriptional mechanisms.

Evidence for a RA-Responsive Element Within the 5′ Upstream Region of the Human TM Gene. We previously reported the isolation and characterization of the human TM gene (6). To investigate whether the human TM gene contains cis-acting domains that are required for regulated expression during F9 cell differentiation, DNA fragments containing 3 kbp upstream of the translation initiation codon were linked to a CAT reporter gene and analyzed by transient expression assays. In F9 stem cells, pTMCAT-derived CAT activity was about twice the background level after normalization for transfection efficiency with pβAclacZ, and exposure to RA and/or CT failed to augment the above signal (data not shown). We then ascertained whether, in analogy to the laminin B1 gene (32), simultaneous expression of the human RARβ could transactivate the human TM promoter. The data are summarized in Fig. 4. Cotransfection of the two constructs into parietal endoderm-like cells in the presence of exogenous RA resulted in a RARβ dose-dependent augmentation in CAT expression. The maximal induction, 14-fold, was obtained at a 1:9 molar ratio of RARβ expression vector to pTMCAT construct. No additional increase was observed when transfected cells were maintained in RACT as compared with RA alone. Cotransfection of the RARβ expression vector with pTMCAT into stem cells did not produce an increase in CAT activity (data not shown). We suspect that the need for coexpressing RARβ and TM reporter constructs to observe hormone-dependent augmentation of gene expression in parietal endoderm, which already possesses RARs, is most likely due to the relatively large amounts of plasmid DNA used in the transient expression assays. The suppression of TM gene transcription toward baseline values at high levels of RARβ expression suggests the titration of additional transacting factor(s) that may be limiting with regard to gene transcription. The absence of an augmented response to RACT as compared to RA implies that the as yet undefined cAMP-responsive element does not reside within the TM promoter region assayed or that the intracellular factor(s) which constitutes the cAMP pathway is present in insufficient quantities for activation of large amounts of transfected DNA.

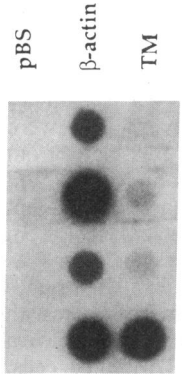

FIG. 3. Nuclear run-on transcription analysis of hormone-dependent TM gene expression. Cells were cultured for 72 hr without hormones (stem cells) or with RA, CT, or RACT. Nuclear run-on transcription assays were performed with isolated nuclei. Labeled nuclear RNA hybridized to filter-immobilized TM cDNA, β-actin cDNA, or vector sequences (pBluescript, pBS) was quantitated as outlined in *Materials and Methods*.

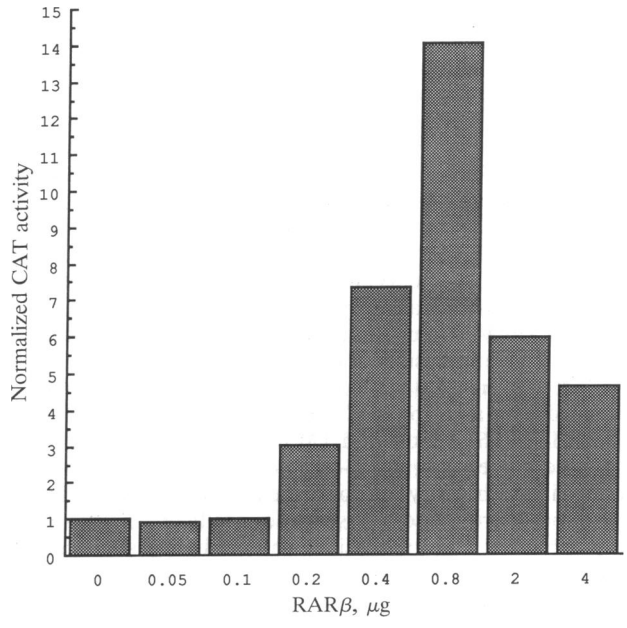

FIG. 4. Transactivation of the human TM promoter by RARβ in transient gene expression experiments. Twenty micrograms of pTMCAT plasmid was cotransfected with the indicated amounts of pβAcRARβ and 6 μg of pβAclacZ into F9 cells exposed to RACT for 3 days. Cell extracts were prepared 36 hr after transfection and analyzed for CAT and β-galactosidase activity. Normalized CAT activity represents the CAT β-galactosidase activity ratio in a given sample. Normalized CAT activity in the absence of cotransfected human RARβ expression plasmid was arbitrarily set at 1. Values represent the mean of duplicate experiments.

DISCUSSION

The RACT-dependent differentiation of F9 stem cells into parietal endoderm leads to the appearance of early and late gene products. Early gene products such as ERA1/Hox-1.6 are synthesized within the first 24 hr of exposure to hormones and may be required for subsequent induction of late gene products. Late gene products, which are expressed 48–72 hr after addition of hormones, include tPA, laminin B1, collagen IV, and platelet-derived growth factor receptors (16, 33–35). In each case, gene transcription is significantly increased by RA and synergistically augmented by addition of CT. Transient expression studies have documented the importance of the 5′ upstream region of the tPA gene with regard to RACT-dependent transcriptional activation (17) and have suggested that Sp1-like proteins may play a critical role in this response (36). Transient expression studies have also demonstrated functionally critical interactions between RARs and specific sites in the 5′ upstream region of the laminin B1 gene (32, 37). Thus, RA-dependent transcriptional activation of late genes may, in part, be due to the direct binding of RARs to the regulatory domains of the above genes rather than the indirect action of early gene products on late genes. These observations suggest a complex two-stage mechanism in which RA and CT interact either directly or indirectly to activate transcription of a battery of genes during hormone-induced differentiation of F9 stem cells into parietal endoderm.

The present results show that RA induces TM mRNA expression by F9 cells within 48–72 hr. CT exerts a much greater effect than RA and activates TM expression within <24 hr. Our results also reveal that addition of both RA and CT, as compared with

RA or CT alone, leads to dramatically augmented TM message levels. The synergistic action of these two hormones requires a time-dependent change in F9 cells induced by RA. These data are somewhat at variance with those of other investigators who employed receptor activity assays to show that RACT, but not RA alone, induced TM expression (7). We suspect that the relative insensitivity of the biologic activity measurements may have been responsible for the lack of detection of RA-induced TM expression. However, we cannot exclude the possibility that CT-dependent TM expression is controlled by a translational mechanism.

Our nuclear run-on data demonstrate that RA- and/or CT-dependent changes in TM mRNA levels are due, in large measure, to alterations in the transcriptional activity of the TM gene. We also provide evidence that the putative promoter region of the human TM gene is involved in regulating transcription during RA-dependent F9 cell differentiation. Our transient expression studies reveal that the TM promoter can be transactivated by the human RARβ, suggesting the presence of a RA-responsive element. These findings are similar to those obtained with the upstream region of the laminin B1 gene, where a specific nucleotide sequence was eventually shown to physically interact with RARs and confer RA-dependent regulation on a heterologous promoter (32, 37). The completion of these types of experiments with the TM promoter construct is necessary to demonstrate that RA directly initiates gene transcription rather than acting in a more indirect fashion via the function of early response gene products.

The results allow us to conclude that hormone-dependent expression of TM during F9 cell differentiation takes place via a mechanism that is similar, but not identical, to that of tPA and laminin B1. On the one hand, transcription of the TM gene is induced by RA and synergistically enhanced by CT. The cooperative interaction of RA and CT requires differentiation of F9 stem cells into primary endoderm. These characteristics of TM gene expression are virtually identical to those observed with other late response genes. On the other hand, exposure of F9 stem cells to CT alone induces transcription of the TM gene, which has not been previously observed with other late response genes. Thus, the differentiation of F9 cells leads to establishment of a RACT-dependent synergistic mechanism of TM gene transcription, rather than the *de novo* appearance of the cAMP-responsive pathway. In this regard, it is of interest that upregulation of TM expression by cAMP has been reported for a variety of cell types, including hematopoietic cell lines (38), human endothelial cells (39), rat and bovine smooth muscle cells (40), CHO cells, and fibroblasts (41). We speculate that the RACT-dependent synergism in augmenting TM gene transcription results from RA-dependent alterations in cAMP-dependent signaling pathways, changes in the effective levels of cAMP-dependent transcription factors, or the occurrence of cooperative interactions between RARs and cAMP-dependent transcription factors. Recent investigations have provided evidence for amplification of cAMP signaling via enhanced expression of the stimulatory subunit ($G_s\alpha$) of adenylate cyclase as well as changes in specific receptor levels (42, 43).

Further studies will be required to identify the precise transacting factors and DNA binding sequences that permit RA- and CT-dependent activation of TM gene transcription. The elucidation of the above mechanism should improve our understanding of how a battery of genes is coordinately induced during F9 cell differentiation to extraembryonic parietal endoderm. The knowledge obtained should also pinpoint critical molecular interactions that initiate development of the natural anticoagulant mechanisms of the blood vessel wall.

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