THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 35, pp. 27279–27288, August 27, 2010
Printed in the U.S.A.

# Retinoic Acid Induces Expression of the Thyroid Hormone Transporter, Monocarboxylate Transporter 8 (Mct8)*[S]

Received for publication, March 13, 2010, and in revised form, June 14, 2010 Published, JBC Papers in Press, June 23, 2010, DOI 10.1074/jbc.M110.123158

**Takahiko Kogai[‡1], Yan-Yun Liu[‡], Laura L. Richter[‡], Kaizeen Mody[‡], Hiroyuki Kagechika[§], and Gregory A. Brent[‡2]**

*From the [‡]Molecular Endocrinology Laboratory, Veterans Affairs Greater Los Angeles Healthcare System, and the Departments of Medicine and Physiology, David Geffen School of Medicine at UCLA, Los Angeles, California 90073 and the [§]Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Tokyo 101-0062, Japan*

**Retinoic acid (RA) and thyroid hormone are critical for differentiation and organogenesis in the embryo. Mct8 (monocarboxylate transporter 8), expressed predominantly in the brain and placenta, mediates thyroid hormone uptake from the circulation and is required for normal neural development. RA induces differentiation of F9 mouse teratocarcinoma cells toward neurons as well as extraembryonal endoderm. We hypothesized that Mct8 is functionally expressed in F9 cells and induced by RA. All-*trans*-RA (tRA) and other RA receptor (RAR) agonists dramatically (>300-fold) induced *Mct8*. tRA treatment significantly increased uptake of triiodothyronine and thyroxine (4.1- and 4.3-fold, respectively), which was abolished by a selective Mct8 inhibitor, bromosulfophthalein. Sequence inspection of the *Mct8* promoter region and 5′-rapid amplification of cDNA ends PCR analysis in F9 cells identified 11 transcription start sites and a proximal Sp1 site but no TATA box. tRA significantly enhanced *Mct8* promoter activity through a consensus RA-responsive element located 6.6 kilobases upstream of the coding region. A chromatin immunoprecipitation assay demonstrated binding of RAR and retinoid X receptor to the RA response element. The promotion of thyroid hormone uptake through the transcriptional up-regulation of *Mct8* by RAR is likely to be important for extraembryonic endoderm development and neural differentiation. This finding demonstrates cross-talk between RA signaling and thyroid hormone signaling in early development at the level of the thyroid hormone transporter.**

Retinoic acid (RA) and thyroid hormones are essential for vertebrate development (1, 2). The actions of these hormones are mediated by specific nuclear hormone receptors, RA receptor (RAR)[3] and thyroid hormone receptor (TR), respectively. RAR signaling plays a critical role in embryonic patterning and in organogenesis (1). TR modulates RA-stimulated neural differentiation as well as expression of some RA-responsive genes in embryonic stem cells (3, 4). The timing of ligand availability and receptor expression is important for normal neural differentiation (4).

RAR and TR form heterodimers with retinoid X (9-*cis*-RA) receptor (RXR) for regulation of most target genes. The consensus sequence of an RA response element (RARE) contains a direct repeat of the consensus half-sites, 5′-PuG(G/T)(T/A)CA-3′, with spacing of 1, 2, or 5 bases (DR-1, DR-2, or DR-5), whereas that of a retinoid X (or 9-*cis*-RA) response element contains the same half-sites separated by 1 base (DR-1) (5). Although the difference of half-site spacing provides selectivity for a specific receptor, there are interactions among the various nuclear receptor signaling pathways. Nuclear receptors also share common co-activator(s) and co-repressor(s) for transcriptional regulation (2). A shared requirement for RXR, interaction at related *cis*-elements, and competition for co-factors are some of the mechanisms underlying cross-talk among nuclear receptor signaling pathways in development (6) and in metabolic regulation (2).

Nuclear receptor ligands are generally lipophilic and have been thought to reach their receptor by passive diffusion through the plasma membrane. Recent studies, however, have demonstrated that uptake of some of these ligands is mediated by selective transporters. Several members of the solute carrier (Slc) family (7–9), including Mct8 (monocarboxylate transporter-8, or Slc16a2) (10), and the solute carrier organic anion transporter family (Slco, or organic anion-transporting polypeptides, Oatp) (9, 11) are known as thyroid hormone transporters. *Mct8* loss of function mutations in humans are associated with profound neurological deficits (7), a common manifestation of thyroid hormone insufficiency in embryos and fetuses. *Mct8* is expressed in many tissues, including brain, placenta, liver, and kidney (7, 10, 12), which are all important thyroid hormone targets.

F9 teratocarcinoma cells have been widely used as an *in vitro* model of embryonic stem cell differentiation. A combination treatment of F9 cells with all-*trans*-RA (tRA), an agonist of RAR, and cAMP induces extraembryonic endoderm (13, 14) or neuron-like cells (15), depending on the composition of culture

* This work was supported, in whole or in part, by Veterans Affairs Merit Review Funds. This work was also supported by National Institutes of Health Grant RO1 CA089364 (to G. A. B.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

[1] To whom correspondence may be addressed: Molecular Endocrinology Laboratory, Bldg. 114, Rm. 230, Veterans Affairs Greater Los Angeles Healthcare System, 11301 Wilshire Blvd., Los Angeles, CA 90073. Fax: 310-268-4035; E-mail: tkogai@ucla.edu.

[2] To whom correspondence may be addressed: Molecular Endocrinology Laboratory, Bldg. 114, Rm. 230, Veterans Affairs Greater Los Angeles Healthcare System, 11301 Wilshire Blvd., Los Angeles, CA 90073. Fax: 310-268-4982; E-mail: gbrent@ucla.edu.

[3] The abbreviations used are: RAR, retinoic acid receptor; BCH, 2-amino-2-norbornane carboxylic acid; BSP, bromosulfophthalein; DBTSS, database of transcriptional start sites; DR, direct repeat; Lat, L-type amino acid transporter; Luc, luciferase; RACE, rapid amplification of cDNA ends; RARE, retinoic acid response element; RXR, retinoid X receptor; $T_3$, triiodothyronine; $T_4$, thyroxine; TR, thyroid hormone receptor; tRA, all-*trans*-retinoic acid; TSS, transcription start site.

media. The extraembryonic endoderm supports the developing embryo and facilitates exchange of small molecules between the maternal circulation and the embryo, functioning as an "early placenta" (16). Because mature placenta expresses abundant Mct8 and transports thyroid hormone (12), it may be expressed in F9 cells differentiated into extraembryonic endoderm. Thyroid hormone is critical for brain development, and thus neural differentiation in F9 cells should be accompanied by induction of thyroid hormone transporter(s). To test these hypotheses, we investigated effects of the differentiation inducer, tRA, on expression of *Mct8* and other thyroid hormone transporter genes as well as thyroid hormone uptake in F9 cells.

## EXPERIMENTAL PROCEDURES

*Cell Culture*—F9 cells, purchased from ATCC (Manassas, VA), were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen) in gelatin-coated flasks or Petri dishes as recommended. Cells were subcultivated at a ratio of 1:10 to 1:20 and used at passages 2–6, unless otherwise noted. Neuron-like cells were induced as described previously (15) with minor modifications. Briefly, $\sim 5 \times 10^5$ cells seeded in gelatin-coated Petri dishes were maintained in DMEM/F-12 medium (Invitrogen) with 2% FBS, 1 $\mu$M tRA, and 1 mM 8-bromo-cAMP for up to 7 days. The cells were fed every 2 days with fresh medium. The cells were split again at a subcultivation ratio of 1:10 2 days after the first seeding. JEG3 cells, MCF7 cells, and SH-SY5Y cells were purchased from ATCC and maintained as recommended.

*Chemicals*—Synthetic retinoids were synthesized as described (17, 18), dissolved in dimethyl sulfoxide to $10^{-2}$ M, and stored at −20 °C. Restriction enzymes and DNA modification enzymes were purchased from New England Biolabs (Ipswich, MA). Other chemicals were purchased from Sigma unless otherwise noted.

*Reverse Transcription (RT)-PCR*—Two-step quantitative RT-PCR was carried out by using the DNA Engene Opticon System (MJ Research, Waltham, MA) as described (19) with minor modifications. Briefly, total RNA from culture cells was prepared with the RNeasy minikit (Qiagen, Valencia, CA) with on-column DNase digestion. Three $\mu$g of total RNA was reverse-transcribed by using 50 units of Superscript III reverse transcriptase (Invitrogen) in a 20-$\mu$l reaction with oligo-$(dT)_{12-18}$ primer or random hexamer. Quantitative PCR of mouse thyroid hormone transporter genes, glyceraldehyde-3-phosphate dehydrogenase gene (*Gapdh*), 18 S ribosomal RNA, and human *MCT8* mRNA was performed with custom DNA primers synthesized by Invitrogen (supplemental Table 1). Quantitative PCR of markers of extraembryonic endoderm and neural differentiation was carried out with the QuantiTect primer assay (Qiagen). Standard curves representing 6-point serial dilution of the corresponding control group were analyzed in each assay and used for calculation of relative expression values. RT-PCR of human *GAPDH* was performed as previously described (19). The sample quantifications were normalized by the internal control *Gapdh* or 18 S RNA. Conventional two-step RT-PCR of the 5′-untranslated region of *Mct8* was performed with custom primers (supplemental Table 1) by using the Expand High Fidelity PCR system (Roche Applied Science). The cycle number was 35.

*Thyroid Hormone Uptake Assay*—Uptake of triiodothyronine ($T_3$) and thyroxine ($T_4$) was measured as described previously (20) with minor modifications. Cells, grown in 12-well plates, as well as empty wells for measurement of nonspecific binding of radiolabeled thyroid hormone to the surface of the side wall of the well were rinsed with 1 ml of Dulbecco's PBS, preincubated with 300 $\mu$l of Hanks' balanced salt solution with 0.1% bovine serum albumin (BSA) for 15 min at 37 °C, and the medium was replaced with 300 $\mu$l of preheated thyroid hormone uptake assay buffer. $T_3$ uptake assay buffer contains Hanks' balanced salt solution, 0.1% BSA, 0.25 $\mu$Ci/ml $^{125}$I-labeled $T_3$ (MP Biomedicals, Solon, OH), and 1.0 nM $T_3$. $T_4$ uptake assay buffer contains Hanks' balanced salt solution, 0.1% BSA, 0.3 $\mu$Ci/ml $^{125}$I-labeled $T_4$ (MP Biomedicals), and 1.0 $\mu$M $T_4$. Cells were incubated for 4–30 min at 37 °C in a humidified incubator, rinsed twice with 1 ml of ice-cold Dulbecco's PBS, and lysed with 200 $\mu$M passive lysis buffer (Promega). Radioactivity of the whole lysate as well as lysis buffer from the duplicate empty wells was measured in a $\gamma$-counter. The background count from the side wall was subtracted from the count of cell lysate. The count was then normalized to the cellular protein content measured in the same cells by using a Bio-Rad protein assay.

*Genomic DNA Sequence Inspection*—To determine putative RARE, consensus half-sites, (A/G)G(G/T)(A/T)CA as well as other reported half-sites (5) were searched on both strands of the mouse *Mct8* locus (NT_000086) by using MacMolly Tetra Lite (Mologen, Berlin, Germany) as described (21). CpG islands around the transcription start site (TSS) of *Mct8* were predicted by the CpG island searcher (hosted on the worldwide web by the laboratory of Dr. P. A. Jones at University of Southern California (USC)) with the following parameters: observed/expected ratio, >0.65; %GC, >50; length, >200 (22). Basic transcription factor binding sites (23, 24) were searched around the TSS of *Mct8* by using MacMolly Tetra Lite.

*5′-Rapid Amplification of cDNA Ends (RACE)*—Total RNA from F9 cells treated with 1 $\mu$M tRA for 72 h was isolated with the RNeasy Plus kit (Qiagen). The 5′-end of the *Mct8* cDNA was identified by the oligo-capping and RNA ligase-mediated RACE method with the GeneRacer kit (Invitrogen) according to the manufacturer's instructions. Briefly, 5 $\mu$g of total RNA was dephosphorylated, decapped, and ligated to GeneRacer RNA oligonucleotides. Reverse transcription was carried out with random primer by using SuperScript III reverse transcriptase (Invitrogen). The 5′-ends of *Mct8* were amplified from the cDNA pool as a template with the GeneRacer 5′-primer and +496 primer (supplemental Table 1). Nested PCR was then performed with the GeneRacer 5′-nested primer and the +496 primer. PCR products were cloned into pCR4-TOPO (Invitrogen) and analyzed by automated DNA sequencer (Laguna Scientific Laboratory, Laguna Niguel, CA).

*Plasmid Construction*—To generate constructs for screening of functional RARE on mouse *Mct8*, annealed synthetic oligonucleotides, containing the putative RAREs as well as an exogenous BamHI site for confirmation of cloning (supplemental Table 1), were inserted to the SmaI site of the pGL3 promoter vector (Promega, Madison, WI). A DNA fragment of the *Mct8* 5′-flanking region (−976 to −54; +1 is A in the translation start site) was obtained by genomic PCR from F9 cells with a forward

primer (5′-CAGATCTTTCGTGCCTCCCTCCTTTC-3′) and a reverse primer (5′-CAAGCTTGTTTCTGCTGCTACTGC-TCCT-3′). To generate pGL3 −976/−54, the PCR products were ligated into the polylinker site of the pGL Basic vector (Promega) with BglII and HindIII. pGL3 −836/−54 and pGL3 −659/−54 were constructed from the pGL3 −976/−54 by deletion of the sequences between ApaI and BglII sites and AgeI and BglII sites, respectively, both of which were treated with T4 DNA polymerase. pGL3 DR5A −976/−54 and pGL3 DR5A −836/−54 were constructed by blunt-end ligation of annealed oligonucleotides of the DR5A (supplemental Table 1) into the SmaI site of the pGL3 −976/−54 and the pGL3 −836/−54, respectively. Mutation of the DR5A element in pGL3 DR5A −836/−54 was generated by using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) with custom primers from Invitrogen (supplemental Table 1). Sequences of the constructed vectors were analyzed by an automated DNA sequencer (Laguna Scientific Laboratory).

*Transient Expression Analysis*—Cells (500–600 cells/well) were seeded in 12-well dishes 24 h before transfection. Unless otherwise noted, 0.2 μg of a Luciferase (Luc) reporter construct and 10 ng of a *Renilla* Luc reporter vector, pRL-CMV (Promega), were transfected to the cells by using Effectene (Qiagen, Valencia, CA) as recommended. The transfection medium was changed to growth media with or without RA at 24 h, and the Luc assay was performed at 48 h with a dual Luc reporter assay system (Promega). Results of the Luc reporter assay were normalized to *Renilla* Luc expression.

*Chromatin Immunoprecipitation (ChIP) Assay*—F9 cells, grown in 100-mm Petri dishes, were treated with tRA (1 μM) for up to 90 min and fixed with 1% formaldehyde. The ChIP assay was carried out with the ChIP-IT express enzymatic kit (Active Motif, Carlsbad, CA) as recommended. Partially digested chromatin was immunoprecipitated with 3 μg of anti-RARα (C-20), anti-RARβ (C-19), or anti-RXRα (D-20) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4 °C. Eluted DNA, as well as the aliquot of sheared chromatin prior to immunoprecipitation (input), was amplified by using the Expand high fidelity PCR system (Roche Applied Science) with primers specific to an RA-responsive region of mouse *Mct8* or the mouse *Lat1* (L-type amino acid transporter 1) promoter (supplemental Table 1). PCR cycle numbers for *Mct8* and *Lat1* were 40 and 37, respectively. The amplicons were analyzed by electrophoresis in a 2% agarose gel.

## RESULTS

*Induction of Mct8 mRNA by RA in F9 Cells*—tRA treatment markedly increased *Mct8* mRNA expression in F9 cells grown in media with 10% serum. A time course study (Fig. 1*A*) showed a significant induction of *Mct8* mRNA by tRA (1 μM) at 12 h (~9.5-fold), reaching a maximum at 96 h (~1120-fold). The effect was dose-dependent with a 50% effective tRA concentration ($EC_{50}$) of $\sim 1.47 \times 10^{-7}$ M and maximum induction at an RA concentration of $10^{-6}$ M (Fig. 1*B*). In the presence of 10% charcoal-stripped FBS, the $EC_{50}$ for tRA was $\sim 1.35 \times 10^{-7}$ M.

tRA is a potent agonist of RAR, although some tRA is converted intracellularly to 9-*cis*-RA, an agonist of RXR. Undifferentiated F9 cells express both RAR and RXR. To determine

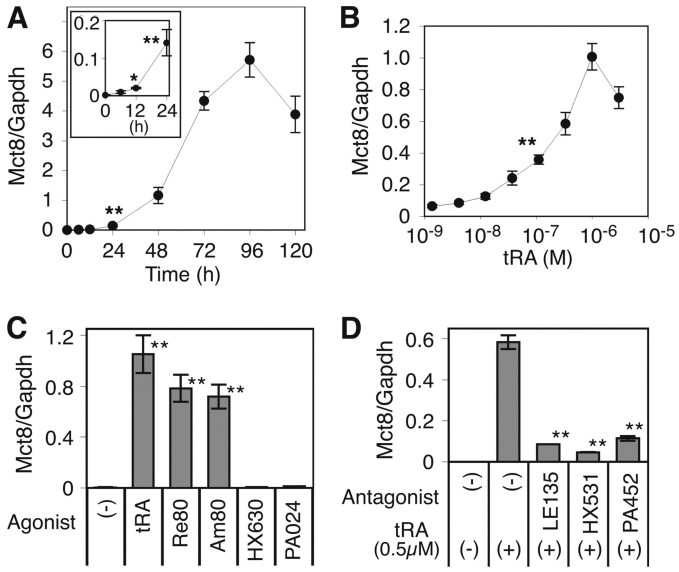


FIGURE 1. **Induction of *Mct8* mRNA expression by retinoids in F9 cells.** Results of quantitative RT-PCR for *Mct8* and *Gapdh* mRNA are shown. *A*, time course of the induction of *Mct8* mRNA by tRA. The *inset graph* shows induction within the first 24 h. Cells were treated with tRA (1 μM) for the indicated times. *B*, dose dependence of the induction of *Mct8* mRNA by tRA. Cells were treated with the indicated concentration of tRA for 48 h. *C*, cells were treated with the indicated retinoid receptor agonist (1 μM) for 48 h (Re80 and Am80-RAR agonists, HX630 and PA024-RXR agonists). *D*, cells were treated with or without tRA (0.5 μM) and the indicated retinoid receptor antagonist (10 μM) for 48 h (LE135-RAR antagonist, HX531 and PA452-RXR antagonists). Treatment with tRA (1 μM) for 48 h was used to generate standard curves to quantify *Mct8* and *Gapdh*. F9 cells were maintained in DMEM with 10% FBS. Values are expressed as means ± S.D. (*error bars*) ($n = 3$). *, $p < 0.05$; **, $p < 0.01$, when compared with untreated cells (*A–C*) or cells with only tRA (*D*).

which receptor is required for *Mct8* induction, we utilized synthetic retinoid receptor agonists (17, 18). Treatment with the pan-RAR agonist, Re80 (1 μM), or with an RARα/β-specific agonist, Am80 (1 μM), mimicked the effects of tRA on *Mct8* mRNA expression. No significant induction was observed with the pan-RXR agonists, HX630 and PA024 (Fig. 1*C*). The induction of *Mct8* by tRA was significantly inhibited by an RAR antagonist, LE135, as well as RXR antagonists HX531 and PA452 (Fig. 1*D*). These data indicate that stimulation of RAR is required for the induction of *Mct8* in F9 cells. Expression of RXR, but not stimulation by ligand, is also probably required for the up-regulation of *Mct8*.

Both RAR and RXR are expressed in many types of cancer cells, including MCF-7 breast cancer cells (25) and SH-SY5Y neuroblastoma cells. Our quantitative RT-PCR study indicated abundant *Mct8* expression in untreated SH-SY5Y cells (~36% of the level in tRA-treated F9 cells) (Table 1). tRA, however, did not significantly increase the *MCT8* mRNA expression in SH-SY5Y or MCF7 cells (Table 1). JEG3 cells express abundant RXR but not RAR, and treatment with tRA did not induce *MCT8* (Table 1).

*Uptake of Thyroid Hormones in tRA-treated F9 Cells*—Transfection of *Mct8* into mammalian cells induces uptake of both $T_3$ and $T_4$ (20). To investigate if the induction of endogenous *Mct8* by tRA increases functional Mct8 expression, we measured the accumulation of $T_3$ as well as $T_4$ in F9 cells treated with or without tRA (1 μM) for 6 days. Cells were incubated with $^{125}$I-labeled $T_3$ or $T_4$ for 4–30 min in the presence of

**TABLE 1**

**Expression of *MCT8* mRNA in various cancer cell lines**

Cells were treated with or without tRA (1 $\mu$M) for 48 h, and quantitative RT-PCR of *Mct8* and *Gapdh* was performed. For comparison of relative *Mct8* expression among the cell lines, *Mct8* expression was quantified with standard curves of *Gapdh* in each cell line and condition, and the average of F9 cells treated without tRA was set as 1. Values are means ± S.D. ($n = 3$).

| | −tRA | +tRA | +tRA/−tRA |
|---|---|---|---|
| F9 | 1.00 ± 0.08 | 77.4 ± 4.46[a] | 76.9 ± 8.32 |
| JEG3 | $2.03 \times 10^{-4} \pm 3.27 \times 10^{-5}$ | $1.41 \times 10^{-4} \pm 8.50 \times 10^{-5}$ | 0.74 ± 0.05 |
| MCF7 | 0.30 ± 0.01 | 0.51 ± 0.04 | 1.71 ± 0.19 |
| SH-SY5Y | 27.8 ± 3.34 | 20.5 ± 3.54 | 0.73 ± 0.04 |

[a] $p < 0.01$, when compared with untreated cells.

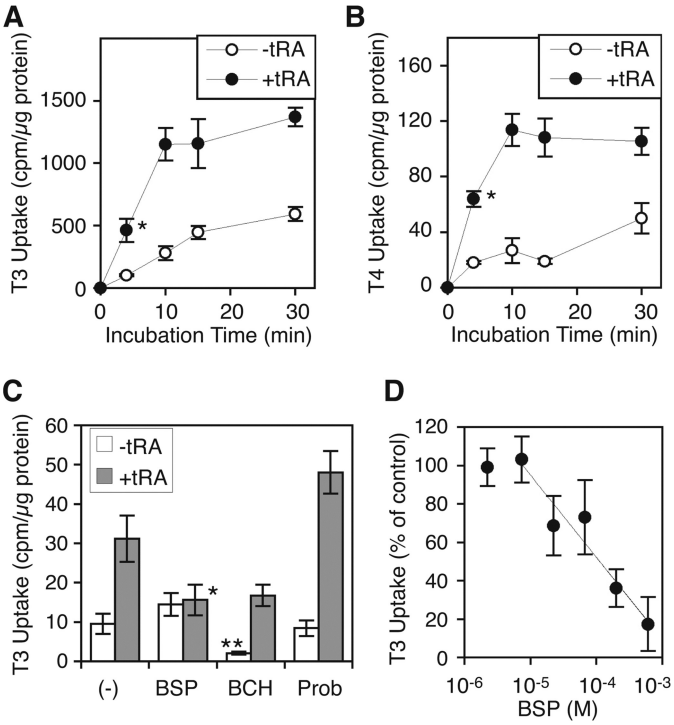


FIGURE 2. **Uptake of thyroid hormones by F9 cells.** Cells were treated with or without tRA (1 $\mu$M) in the presence of 10% FBS for 6 days before the assay. *A* and *B*, time courses of $T_3$ accumulation (*A*) and $T_4$ accumulation (*B*). Cells were incubated with the $^{125}$I-labeled $T_3$ uptake buffer (*A*) or $^{125}$I-labeled $T_4$ uptake buffer (*B*) for the indicated times. Values are expressed as means ± S.D. ($n = 4$). *, $p < 0.01$, when compared with untreated cells. *C*, pharmacological inhibition of $T_3$ uptake. Cells were incubated with the $^{125}$I-labeled $T_3$ uptake buffer for 10 min in the presence of BSP (Mct8 inhibitor), BCH (Lat inhibitor), or probenecid (*Prob*, pan-Oatp inhibitor) (1 mM each). Values are expressed as means ± S.D. ($n = 3$). *, $p < 0.05$, when compared with +tRA/−inhibitor. **, $p < 0.02$, when compared with −tRA/−inhibitor or +tRA/+BCH. *D*, dose dependence of BSP inhibition of tRA-induced $T_3$ uptake. Cells were incubated with $^{125}$I-labeled $T_3$ uptake buffer in the presence of BSP at the indicated concentrations. $T_3$ uptake without BSP was simultaneously measured in tRA-treated and -untreated cells. Uptake, normalized to protein content, without BSP or tRA was subtracted from uptake at each concentration of BSP in tRA-treated cells, and uptake without BSP in tRA-treated cells was set at 100%. Values are expressed as means ± S.D. (*error bars*) ($n = 3$). The *best fit line* ($R^2 = 0.93$) is shown.

0.1% BSA as a carrier. $T_3$ uptake in tRA-treated cells was significantly increased in the first 4 min and partially saturated at 10 min (Fig. 2*A*). $T_3$ uptake in tRA-treated cells at 10 min was significantly higher (~4.1-fold) than that in untreated cells (Fig. 2*A*). $T_4$ uptake was also significantly increased in the first 4 min and saturated at 10 min (Fig. 2*B*). $T_4$ uptake in tRA-treated cells at 10 min was significantly higher (~4.3-fold) than that in untreated cells (Fig. 2*B*). These results are consistent with previous data of thyroid hormone uptake in mammalian cells transfected with vectors expressing *Mct8* (20).

Although Mct8 is one of the most efficient transporters of both $T_3$ and $T_4$, several other transporters have also been reported to mediate thyroid hormone uptake, including other SLC family members, Mct10 (or SLC16A10) (8), sodium/taurocholate-cotransporting polypeptide-1 (NTCP1, or SLC10A1), L-type amino acid transporter-1 (LAT1, or SLC7A5) and LAT2 (or SLC7A8) (9), and eight of the 15 members of the Oatp/Slco family (9, 11). To determine if the tRA-induced thyroid hormone uptake in F9 cells is mediated by Mct8, we utilized pharmacological inhibitors of thyroid hormone transporters (26). An inhibitor of $T_3$ uptake by Mct8, bromosulfophthalein (BSP), but not a broad spectrum inhibitor of Oatp, probenecid, significantly reduced the tRA-induced $T_3$ uptake (Fig. 2*C*) with an $IC_{50}$ of 112 $\mu$M (Fig. 2*D*). Modest $T_3$ uptake was observed in the cells without tRA treatment, although it was not inhibited by BSP or probenecid (Fig. 2*A*). A Lat inhibitor, 2-amino-2-norbornane carboxylic acid (BCH), significantly reduced the $T_3$ uptake in both tRA-treated and -untreated F9 cells, whereas the induction by tRA was not abolished (Fig. 2*C*). These data suggest that the basal modest $T_3$ uptake is mediated by Lat, whereas tRA-induced $T_3$ uptake is dependent on the BSP-sensitive thyroid hormone transporter, *Mct8*.

*Expression of Other Thyroid Hormone Transporter Genes in F9 Cells*—To determine if the tRA-induced thyroid hormone uptake (Fig. 2) was due to induction of *Mct8*, we measured the expression of mouse orthologs of other reported thyroid hormone transporter genes in response to tRA treatment in F9 cells (9, 11). The expression level of each gene was quantified by RT-PCR with a *Gapdh* standard for comparison of the relative expression among each gene.

tRA treatment for 96 h increased expression of *Mct10* and *Lat2* (Table 2), although the magnitudes of induction (~2.1- and ~4.6-fold, respectively) were much less than that of *Mct8* (~678-fold). Interestingly, abundant expression of *Lat1* (~$7 \times 10^{-2}$-fold compared with *Gapdh*), as well as modest expression of *Oatp4a1* (~$10^{-3}$-fold compared with *Gapdh*), was observed in both tRA-treated and untreated F9 cells, although tRA did not significantly influence the expression of these transporters (Table 2). Expression levels of *Ntcp1* and the other seven *Oatp* genes were relatively small (less than $5 \times 10^{-5}$-fold compared with *Gapdh*) and were not significantly increased by the tRA treatment (Table 2). These data indicate that, among the thyroid hormone transporter genes tested in F9 cells, only *Mct8* was markedly induced by tRA.

*Differentiation Status and Mct8 Gene Expression in F9 Cells*—Treatment of F9 cells with tRA and/or cAMP under normal (10%) serum conditions induces extraembryonic endoderm,

**TABLE 2**

**Expression of thyroid hormone transporter genes in F9 cells**

Cells were treated with or without tRA (1 $\mu$M) for 96 h, and quantitative RT-PCR of the indicated genes was performed. For comparison among the genes tested, expression of each gene was quantified with standard curves of *Gapdh* from cDNA mixture of +tRA and −tRA. Values are means ± S.D. ($n = 3$). NA, not applicable.

| | −tRA | +tRA | +tRA/−tRA |
|---|---|---|---|
| *Mct8* (*Slc16a2*) | $1.92 \times 10^{-5} \pm 1.29 \times 10^{-6}$ | $1.29 \times 10^{-2} \pm 1.25 \times 10^{-3}$[a] | 678.1 ± 78.6 |
| *Mct10* (*Slc16a10*) | $1.51 \times 10^{-4} \pm 3.73 \times 10^{-5}$ | $3.11 \times 10^{-4} \pm 2.78 \times 10^{-5}$[a] | 2.05 ± 0.53 |
| *Ntcp1* (*Slc10a1*) | $<10^{-6}$ | $<10^{-6}$ | NA |
| *Lat1* (*Slc7a5*) | $7.26 \times 10^{-2} \pm 2.11 \times 10^{-3}$ | $6.68 \times 10^{-2} \pm 2.08 \times 10^{-3}$ | 0.96 ± 0.18 |
| *Lat2* (*Slc7a8*) | $8.83 \times 10^{-5} \pm 5.51 \times 10^{-6}$ | $4.08 \times 10^{-4} \pm 5.49 \times 10^{-6}$[a] | 4.63 ± 0.21 |
| *Oatp (Slco) 1a1* | $4.66 \times 10^{-5} \pm 5.23 \times 10^{-7}$ | $3.37 \times 10^{-5} \pm 3.21 \times 10^{-6}$ | 0.72 ± 0.05 |
| *Oatp (Slco) 1a4* | $<10^{-6}$ | $<10^{-6}$ | NA |
| *Oatp (Slco) 1a5* | $<10^{-6}$ | $<10^{-6}$ | NA |
| *Oatp (Slco) 1b2* | $1.10 \times 10^{-5} \pm 6.45 \times 10^{-7}$ | $5.20 \times 10^{-6} \pm 4.26 \times 10^{-7}$ | 0.47 ± 0.05 |
| *Oatp (Slco) 1c1* | $<10^{-6}$ | $<10^{-6}$ | NA |
| *Oatp (Slco) 4a1* | $1.45 \times 10^{-3} \pm 9.48 \times 10^{-5}$ | $2.35 \times 10^{-4} \pm 2.15 \times 10^{-5}$ | 0.16 ± 0.002 |
| *Oatp (Slco) 6b1* | $<10^{-6}$ | $<10^{-6}$ | NA |
| *Oatp (Slco) 6c1* | $<10^{-6}$ | $<10^{-6}$ | NA |
| *Gapdh* | 1.29 ± 0.02 | 0.93 ± 0.14 | 0.72 ± 0.07 |

[a] $p < 0.01$, when compared with −tRA.

primitive, parietal, and visceral (13, 14). Combination treatment with tRA and cAMP, under low serum conditions, induces morphologically neuron-like cells with neuron-specific acetylcholinesterase activity (15). tRA treatment induced primitive/visceral endoderm differentiation markers, *Col4a1*, *Lama1*, and *Afp*, in F9 cells, whereas the combination with tRA and 8-bromo-cAMP induced parietal endoderm differentiation markers, *Col4a1*, *Lama1*, and *Thbd* (Fig. 3, *A–C*), consistent with previous reports (13, 14). The expression of *Mct8* mRNA was markedly increased with any extraembryonic endoderm differentiation induced, although the addition of 8-bromo-cAMP modestly reduced the magnitude of induction (Fig. 3*D*). The combination treatment of tRA and 8-bromo-cAMP in low serum (2%) conditions significantly induced *Mct8* (~354-fold; Fig. 3*E*) as well as a neural differentiation marker, *Snca* (Fig. 3*F*), and a neuroectodermal marker, *Otx1* (Fig. 3*G*), but not other neural markers, *Nes* or *NeuroD1* (Fig. 3*H*).

*Transcriptional Regulation of Mct8 Expression in F9 Cells*—To determine if the effects of tRA on *Mct8* expression are at the transcriptional level, we utilized a transcription inhibitor, actinomycin D. Treatment with actinomycin D for 14 h significantly reduced the tRA-induced *Mct8* expression (Fig. 4*A*). A time course study demonstrated that degradation of *Mct8* mRNA in tRA-treated F9 cells was slightly slower than that in untreated cells, although the difference was not significant ($p = 0.062$; Fig. 4*B*). The half-life of *Mct8* mRNA in tRA-treated F9 cells and untreated cells was 15.0 ± 2.53 and 10.3 ± 1.15 h, respectively. Treatment with a protein synthesis inhibitor, cycloheximide, did not significantly decrease the *Mct8* mRNA expression in F9 cells treated with tRA (1 $\mu$M) for 24 h (data not shown). These data indicate that tRA stimulates *Mct8* expression predominantly at the transcriptional level, without a requirement for newly synthesized protein induced by tRA.

*TSSs of the Mct8 Gene in F9 Cells*—To elucidate the mechanism of transcriptional regulation by RA, the proximal promoter region of *Mct8* was characterized. We determined the TSS of *Mct8* in F9 cells by oligo-capping and RNA ligase-mediated 5′-RACE, in which cDNA from full-length 5′-capped mRNA, but not incomplete mRNA, is selectively amplified (27). The identified 5′-end of cDNA, therefore, represents the TSS. PCR amplification of the RACE-ready cDNA from tRA-treated F9 cells with a 5′ adaptor primer and a gene-specific primer (+496, relative to the translation start site of *Mct8*) produced DNA fragments of around 600 bp (Fig. 5*A*). Sequence analysis of 25 clones, following ligation of the PCR products into a pCR4 vector, identified 11 TSSs between −155 and −41 (Fig. 5*B*). A highly utilized TSS has a consensus dinucleotide sequence, pyrimidine-purine, especially CG, TG, or CA (23). Eight of 11 determined TSSs, including the two most used TSSs at −65 and −92, conserve the dinucleotide consensus (Fig. 5*B*). Although the TSS of the reference sequence of *Mct8* mRNA in the National Center for Biotechnology Information data base (MN_009197) is at −186, no clone contained the sequence around −186.

Two isoforms of human MCT8 have been reported: an isoform with high homology to mouse Mct8 and a longer isoform with an additional 74-amino acid-long human specific sequence at the N-terminal portion (7). A comprehensive study of TSS in the human genome (28, 29) has shown at least two putative alternative promoters corresponding to the *Mct8* mRNA variants. The 5′-flanking region of mouse *Mct8* from −343 to +1, containing every TSS detected, had a strong homology (83.2%) with one of the human alternative promoters, AP2, controlling the shorter variant of human *MCT8* (Fig. 5*E*).

Sequence inspection of the core promoter elements indicated that the 5′-flanking region of *Mct8* contained a canonical Sp1 site, between −120 and −110 (GRGGCRGGGW), but no TATA box (Table 3). An epigenetically regulatable CpG island was found between −404 and +124, included in all TSSs determined in our experiments (Fig. 5*E*). These results are compatible with a recent consensus that most of the TATA-less (GC-rich) core promoters initiate transcription at multiple sites and are located in a CpG island (23).

A majority (88%) of determined TSSs in the *Mct8* promoter were mapped to a region downstream of the proximal Sp1 site (Fig. 5*B*), suggesting a role of Sp1 in the *Mct8* promoter activation. Full induction of several genes by RA requires Sp1 (30–32). To assess whether the up-regulation of *Mct8* by RA requires the Sp1 site, we performed RT-PCR of the 5′-untranslated region of *Mct8* in F9 cells treated with or without tRA (Fig. 5*C*). The expression of transcripts containing the Sp1 site was increased by tRA treatment (Fig. 5*D*), indicating that transcription from TSSs upstream of the Sp1 site is also up-regulated by RA. The Sp1 site, therefore, is not required for tRA induction.

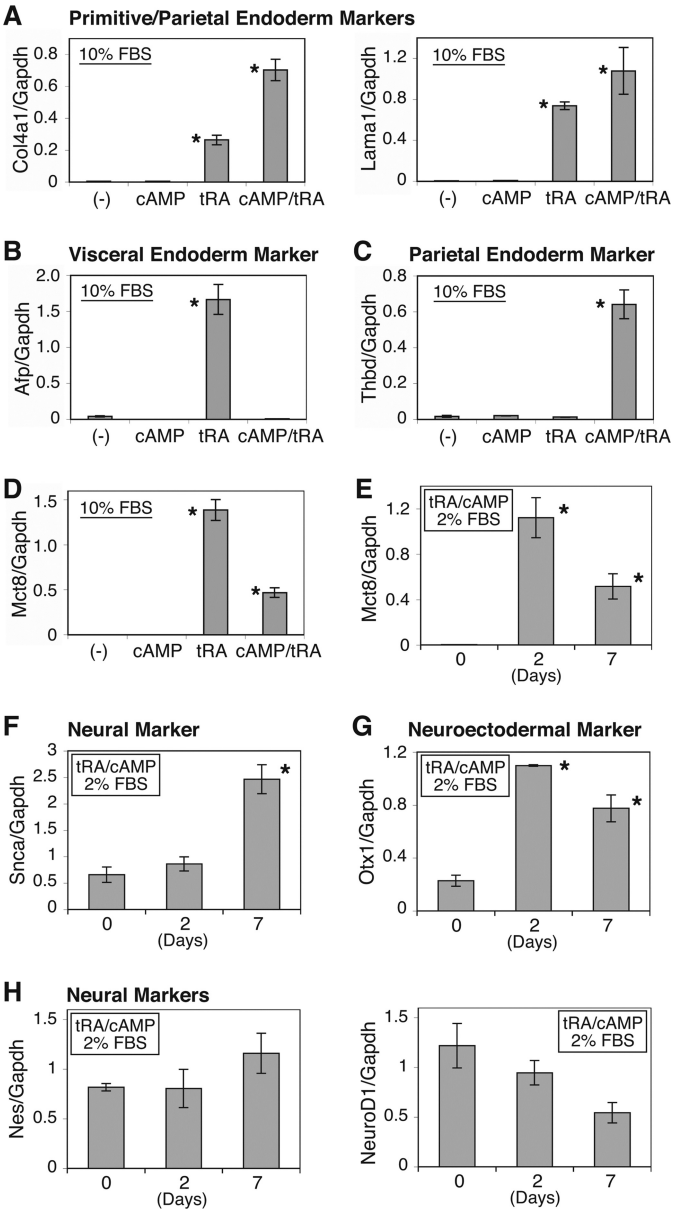


FIGURE 3. **Expression of differentiation markers and *Mct8* mRNA in F9 cells.** *A–D*, induction of markers of endoderm differentiation in F9 cells. Cells were grown in DMEM supplemented with 10% FBS and treated with or without tRA (1 $\mu$M) and/or 8-bromo-cAMP (1 mM) for 96 h, and quantitative RT-PCR of the indicated genes was performed. Quantification of those genes as well as the internal control *Gapdh* was obtained from standard curves of serial dilution series of cDNA mixture of tRA-treated cells and tRA/cAMP-treated cells. The sample quantification was then normalized to *Gapdh*. *E–H*, induction of neural differentiation markers in F9 cells. Cells were treated with or without tRA (1 $\mu$M) and 8-bromo-cAMP (1 mM) in DMEM/F-12 (50:50) with 2% FBS for 2 days or 7 days, and quantitative RT-PCR of the indicated gene was performed. Quantification was obtained from standard curves of serial dilution series of cDNA at day 2 and normalized to *Gapdh*. Values are expressed as means ± S.D. ($n = 3$). *, $p < 0.01$, when compared with untreated cells (day 0). *Col4a1*, collagen type IV $\alpha$-1; *Afp*, $\alpha$-fetoprotein, *Lama1*, laminin $\alpha$1; *Thbd*, thromobomodulin; *Mct8*, monocarboxylate transporter 8; *Snca*, synuclein $\alpha$; *Otx1*, orthodenticle homeobox 1; *Nes*, nestin; *NeuroD1*, neurogenic differentiation 1.

*Functionality of the Mct8 Promoter in F9 Cells*—The 5′-flanking region of mouse *Mct8* was evaluated for promoter activity in firefly Luc reporter vectors containing progressive deletion mutants of the 5′-flanking region from −976 to −226. Significant promoter activity (7.9–13.1-fold compared with the

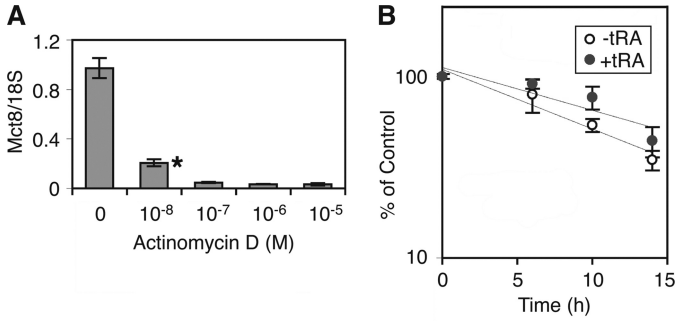


FIGURE 4. **Effects of a transcription inhibitor, actinomycin D, on *Mct8* mRNA expression in F9 cells.** *A*, cells were treated with tRA (1 $\mu$M) and the indicated concentration of actinomycin D for 21 h, and quantitative RT-PCR of *Mct8* was performed. Results were normalized to 18 S ribosomal RNA. Values are expressed as means ± S.D. ($n = 3$). *, $p < 0.01$, when compared with control (0 M). *B*, effects of tRA on degradation of *Mct8* mRNA in F9 cells. To induce abundant *Mct8*, cells were pretreated with tRA (1 $\mu$M) for 24 h. Cells were rinsed twice with Dulbecco's PBS, cultured in growth media for 48 h, treated with actinomycin D (10 $\mu$M) for 1 h, and then treated with or without tRA (1 $\mu$M) in the presence of actinomycin D for the indicated time. Results of quantitative RT-PCR of *Mct8* were normalized to 18 S ribosomal RNA. Data at 0 h were set at 100%. Values are expressed as means ± S.D. ($n = 3$).

background) was observed in the constructs containing the fragment between −659 and −54 (pGL3 −976/−54, pGL3 −836/−54, and pGL3 −659/−54; Fig. 6), whereas the deletion of the sequence between −659 and −227 completely abolished the promoter activity (pGL3 −226/−54; Fig. 6). Deletion of the 3′ portion of the 5′-flanking region (from −146 to −54), containing the Sp1 site, significantly decreased the promoter activity, although a modest activity (~2.8-fold) was still observed (pGL3 −836/−147; Fig. 6). Further deletion of the 3′ side from −226 to −147 abolished the promoter activity (pGL3 −836/−227; Fig. 6). These results demonstrate that the sequence from −659 to −54, including the proximal Sp1 site, is necessary for full promoter activity. This is consistent with the results of TSS mapping, showing that the majority of TSSs are located downstream of the Sp1 site (Fig. 5*B*).

tRA induced *Mct8* expression (Figs. 4 and 5*D*), whereas tRA did not significantly increase proximal promoter activity (Fig. 6). RAREs have been reported both within the proximal promoter (5, 33, 34) and outside of the proximal promoter (35, 36).

*RARE in the Locus of the Mouse Mct8 Gene*—To determine an RA-responsive enhancer in the *Mct8* locus, we inspected the mouse genomic sequence from −10,724 to +134,953, including exon and introns, and more than 10,000 bp of flanking regions (Fig. 7*A*) for consensus sequences of RARE, DR-1, DR-2, and DR-5 (5). Based on the half-site sequences and configuration (21), we identified three candidate elements: two DR-5 elements, DR5A at −6602 (in the 5′-flanking region) and DR5B at +56,356 (in the first intron), and one DR-1 element at +19,291 (in the first intron) (Fig. 7*A*).

The three putative RAREs were inserted into a Luc reporter vector with the heterologous SV40 promoter driving the reporter gene expression (Fig. 7*B*) and evaluated in F9 cells for tRA induction. Our transient transfection study indicated a significant tRA response to the DR5A at −6602 (~4.2-fold) but not the DR5B or DR1 (Fig. 7*B*).

To determine if the identified RARE enhances the mouse *Mct8* promoter, we fused the DR5A element upstream of the

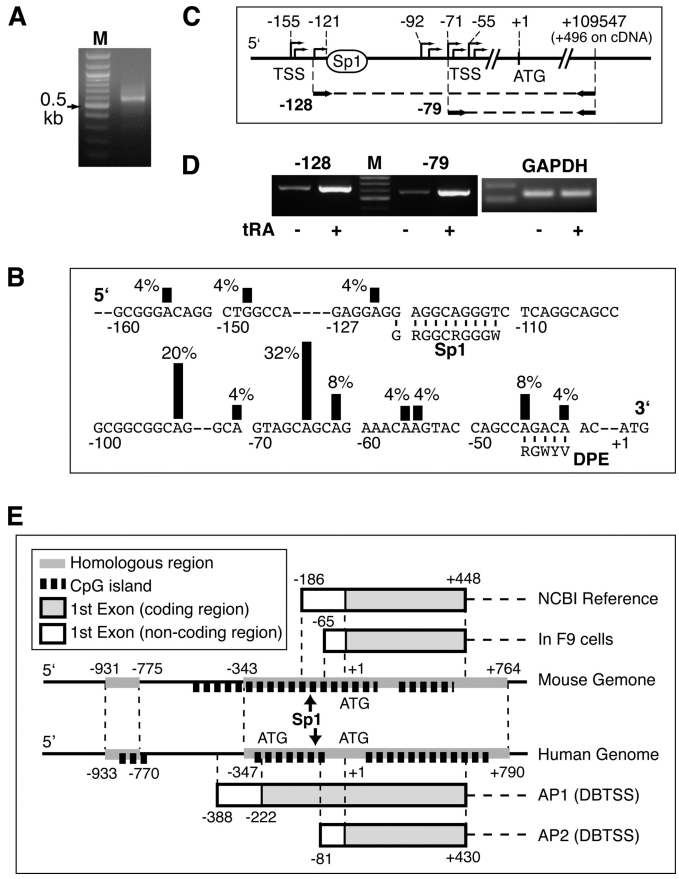


FIGURE 5. **TSS of *Mct8* in F9 cells.** *A*, results of 5′-RACE from tRA-treated F9 cells. Shown is agarose gel electrophoresis of the second round PCR with GeneRacer 5′ nested primer and 3′ +496 primer. Marker (*M*) was a 100-bp ladder. *B*, distribution of the TSS of *Mct8* in F9 cells. The sequence of the 5′-flanking region of *Mct8* is shown. The positions of the identified TSSs are marked with *vertical lines*, the *height* of which indicates the frequency of RACE clones found to initiate at each site. Putative core promoter elements are shown. *DPE*, downstream core promoter element. *C*, diagram of the promoter region and amplification of upstream transcripts, including the Sp1 site (−128) and downstream transcripts without the Sp1 site (−79). *D*, determination of the influence of tRA treatment on transcripts. RT-PCR of the 5′ region of *Mct8* in F9 cells treated with or without tRA (1 $\mu$M) for 48 h is shown. The internal control, *Gapdh*, is shown on the *right*. *E*, comparison of TSS positions in the mouse and human *MCT8* genes. Alternative first exons, CpG islands, canonical Sp1 sites (*arrow*), and homologous regions between the two species are shown. TSSs of mouse *Mct8* as well as that of the short variation of human *MCT8* flanked by AP2 (alternative promoter 2) (*DBTSS*) are located in CpG islands in high homologous regions (79%) between the two species. The long variation of human *MCT8*, flanked by AP1 (DBTSS), is transcribed from a human-specific region outside of the CpG island.

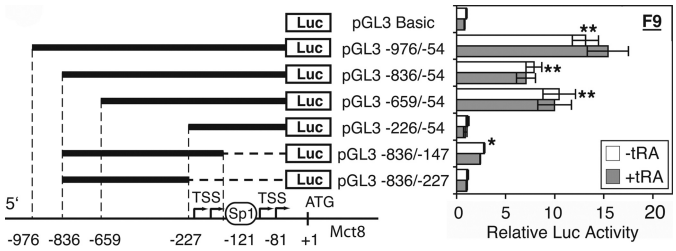


FIGURE 6. **Activity of the *Mct8* proximal promoter in F9 cells.** Cells were transfected with the indicated Luc reporter vector and pRL-CMV, treated with or without tRA (1 $\mu$M) for 24 h, and harvested for the luciferase assay. Data of pGL3 Basic in untreated F9 cells were set at 1. Values are means ± S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$, when compared with pGL3 Basic without tRA.

Mct8 5′-flanking sequence (−976 to −54 or −836 to −54) in Luc reporter vectors and transfected it into F9 cells. tRA treatment (1 $\mu$M) significantly increased the *Mct8* promoter activity (6.2–7.3-fold) with DR5A (pGL3 DR5A −976/−54 and pGL3 DR5A −836/−54; Fig. 7*C*) but not without DR5A (pGL3 −976/−54 and pGL3 −836/−54; Fig. 7*C*). Mutations of the DR5A element completely abolished induction by tRA (Fig. 7*D*), indicating the critical role for the tRA response. The function of RARE was observed even in the Sp1 element-deleted construct (pGL3 DR5A −836/−147; Fig. 7*C*), consistent with the tRA induction of *Mct8* transcripts from the TSSs upstream of the Sp1 site (Fig. 5*D*).

tRA treatment did not increase endogenous *Mct8* mRNA expression in JEG3 cells (Table 1). Significant activity of the *Mct8* proximal promoter was observed in JEG3 cells; however, tRA did not enhance the DR5A RARE (0.8 ± 0.1-fold), even with exogenous RARα expression. This suggests that RA induction of *Mct8* is cell type-specific and may require specific factors to be present or be susceptible to inhibitors.

To determine if retinoid receptors bound directly to the DR5A element, we performed a ChIP assay in F9 cells treated with tRA at various time points (0–90 min). The binding of RARβ to the DR5A region was induced by tRA in 30 min, whereas both RARα and RXRα bound consistently, even in the absence of tRA treatment (Fig. 7*E*). In contrast, no significant binding of RARα, RARβ, or RXRα was observed in the promoter region of *Lat1* (Fig. 7*E*), with no putative RARE or induction of endogenous mRNA by tRA (Table 2). These data demonstrate direct binding of RAR and RXR to the DR5A region in F9 cells.

**TABLE 3**
**Inspection for core promoter elements in mouse *Mct8* and its human ortholog**

| Core promoter elements[a] | Consensus sequence | Mouse *Mct8* | Human *MCT8* |
|---|---|---|---|
| D-BRE | RTDKKKK | NP[b] | NP |
| U-BRE | SSRCGCC | NP | NP |
| Dinucleotide(−1/+1) | YR (Py-Pu) | 8 of 11 TSS | At −81[c] |
| DPE[d] | RGWYVT | Between −45 and −41 | Between −15 and −11[c] |
| Inr | YYANWYY (YYRRWYY) | NP | NP |
| MTE | CSARCSSAAC | NP | NP |
| Sp1 | GRGGCRGGGW | Between −121 and −112 | Between −116 and −107[c] |
| TATA box | TATAWAAR | NP | NP |
| XCPE1 | DSGYGGRASM | NP | NP |

[a] U-BRE, upstream-TFIIB (transcription factor for RNA polymerase IIB) recognition element; D-BRE, downstream-BRE; DPE, downstream core promoter element; Inr, initiator; MTE, motif ten element; XCPE1, X core promoter element-1.
[b] NP, sequence not present.
[c] A in the translation start site of the short variation of *MCT8* mRNA is +1 (see Fig. 5*E*).
[d] A role in transcription and the consensus sequence of DPE are still controversial (23).

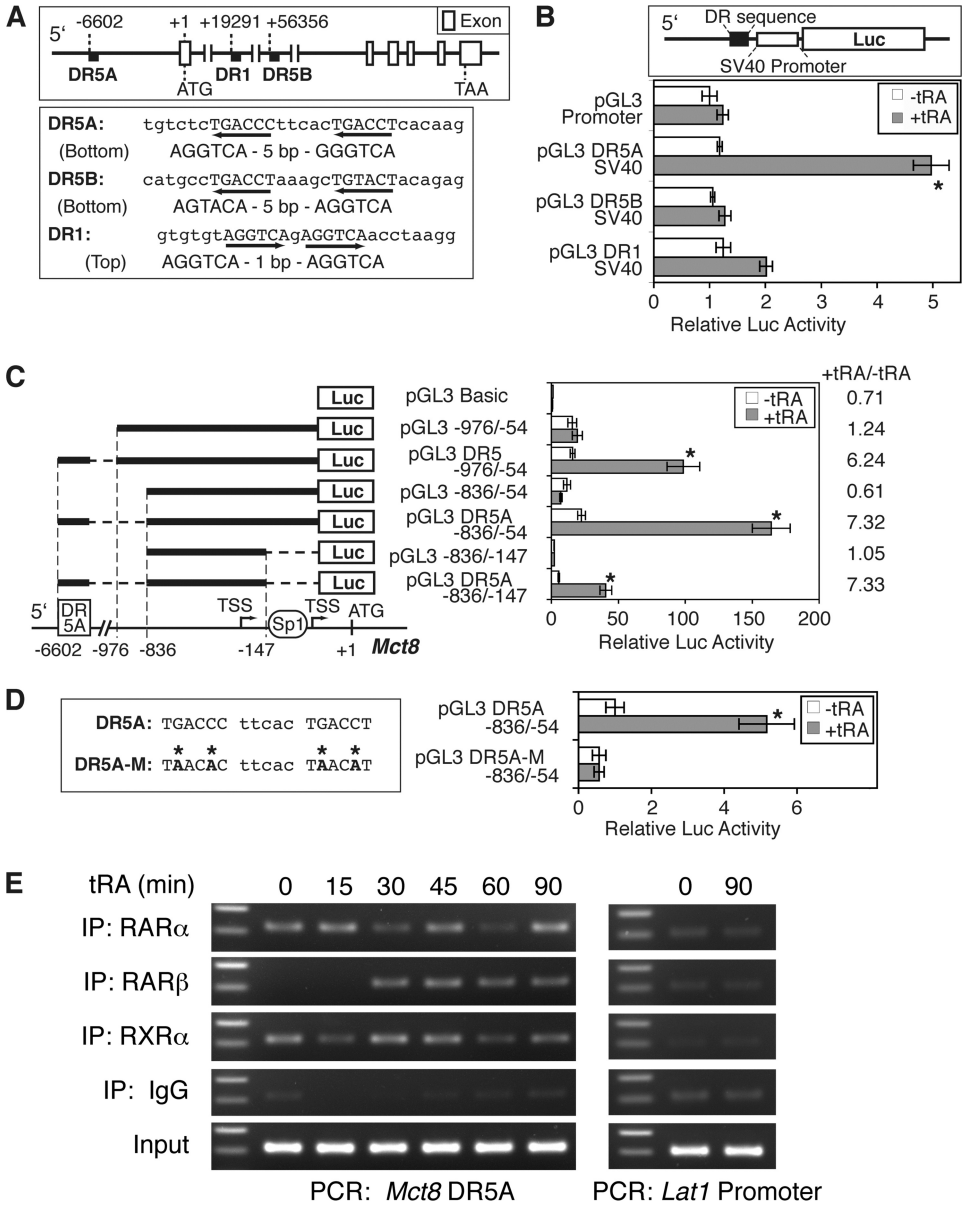


FIGURE 7. **Characterization of mouse *Mct8* RARE in F9 cells.** *A*, location and sequence of putative RAREs in the mouse *Mct8* locus. *B*, functional analysis of putative RAREs in F9 cells. Three putative RAREs were fused to a heterologous SV40 promoter in Luc reporter vector constructs (*upper panel*) and transfected into F9 cells with pRL-CMV. Cells were treated with or without tRA (1 $\mu$M) for 24 h, and the luciferase assay was performed. Normalized data of the pGL promoter (−*tRA*) were set at 1. *C*, enhancer activity of the DR5A RARE with the *Mct8* proximal promoter. Cells were transfected with the indicated Luc reporter constructs as well as pRL-CMV, treated with or without tRA (1 $\mu$M) for 24 h, and harvested for the luciferase assay. Normalized data of pGL3 Basic (−*tRA*) were set at 1. *D*, mutation analysis of the DR5A RARE. In the *left panel*, mutated bases in the DR5A RARE are indicated by an *asterisk*. Cells were transfected with the mutated vector (pGL3 DR5A-M −836/−54) or the original vector with wild DR5A (pGL3 DR5A −836/−54) and treated with or without tRA (1 $\mu$M) for 24 h. Normalized data of pGL3 DR5A −836/−54 (−*tRA*) were set at 1. Values are means ± S.D. ($n = 3$). *, $p < 0.01$, when compared with −tRA in each vector. *E*, binding of endogenous retinoid receptors to the region of DR5A RARE. F9 cells were treated with tRA (1 $\mu$M) for the indicated time, and a ChIP assay was performed with the indicated antibodies or preimmune IgG. The DR5A region, as well as the tRA-unresponsive *Lat1* promoter, was amplified by PCR from the immunoprecipitated (*IP*) chromatin and analyzed by agarose gel electrophoresis. Expected amplicon sizes were 217 and 203 bp, respectively. The DNA size marker indicates 300 and 200 bp.

## DISCUSSION

We have shown that $T_3$ and $T_4$ uptake is significantly increased in F9 teratocarcinoma cells differentiated by tRA. A significant reduction of the tRA-induced $T_3$ uptake by a selective Mct8 inhibitor suggests a dependence of the thyroid hormone uptake on Mct8. Transcription of *Mct8* is markedly induced, at least partially by RAR stimulation of a DR5 RARE identified 6.6 kilobases upstream of the coding region. The induction was dependent on RA treatment, but not limited by differentiation status, in F9 cells.

Five members of the Slc family, including Mct8, and eight members of the Oatp/Slco family have been reported as thyroid hormone transporters (11). We analyzed the expression levels of the known thyroid hormone transporter genes in F9 cells and found that only *Mct8* was significantly induced by tRA. Modest inductions of *Mct10* and *Lat2*, however, were also observed in the tRA-treated cells. Our $T_3$ uptake study demonstrated that a Lat inhibitor, BCH, did not abolish the tRA-induced $T_3$ uptake, indicating a lesser contribution of the modest *Lat2* induction to the increase of uptake. Mct8 transports both $T_3$ and $T_4$, whereas Mct10 transports predominantly $T_3$ in mammalian cells (8). Because the tRA treatment induced uptake of both $T_3$ and $T_4$, the robust induction of *Mct8*, rather than the modest induction of *Mct10*, probably plays a predominant role in the induction of thyroid hormone uptake in tRA-treated F9 cells.

Modest thyroid hormone uptake in undifferentiated F9 cells was sensitive to the Lat inhibitor BCH but not a Mct8 inhibitor, BSP, or a broad spectrum Oatp inhibitor, probenecid, suggesting a central role of a Lat in the thyroid hormone uptake before the tRA treatment. Our RT-PCR in the undifferentiated F9 cells indicated abundant expression of *Lat1* and much less *Lat2* (~1,000-fold less than *Lat1*). Those results indicate that Lat1 probably mediates the modest thyroid hormone uptake in undifferentiated F9 cells. Although Lat1 was originally reported as an amino acid transporter, the apparent $K_m$ for $T_3$ is the lowest of any reported Lat1 substrate (7, 37). LAT1 is also important for cell growth and survival (38). Overexpression of LAT1 has been reported in some cancer tissues as well as many cell lines, including PA1 teratocarcinoma cells (39).

Some nuclear hormone receptors, such as those for glucocorticoid and androgen, reside predominantly in the cyto-

plasm as unliganded receptor bound to heat shock protein. The addition of ligand disrupts binding to heat shock protein and rapidly induces translocation of these receptors from the cytoplasm into the nuclei to induce target genes (40). Although ligand-induced translocation of RAR has been reported (41, 42), unliganded RAR is predominantly localized in the nucleus and binds to RAREs as a heterodimer with RXR, in association with co-repressors (34). Binding of ligand changes the conformation of the RAR/RXR heterodimer, resulting in release of co-repressors and subsequent recruitment of co-activators (34). tRA strongly induces *RARβ* through an RARE in its promoter in association with RARα (33), whereas RAR binding to the RARE is not dependent on tRA (43, 44). Our ChIP assay in F9 cells showed binding of both RARα and RXRα to the RARE in *Mct8*, in the presence or absence of tRA. Induction of RARβ binding to the DR5A element by tRA, shown in our ChIP assay, may be correlated with the induction of *RARβ* expression by tRA. Our study with a protein synthesis inhibitor, cycloheximide, however, showed that *de novo* protein synthesis is not required for tRA induction of *Mct8*.

Recent genome-wide studies of mammalian RNA polymerase II core promoters have identified two classes of promoters, sharp type promoters and broad type promoters. About 20% of mammalian promoters are the sharp type promoter, containing a TATA box and a single TSS, whereas the others are broad type promoters, containing CpG island(s) with multiple TSSs but without a TATA box (23). Our analysis of the *Mct8* promoter demonstrated that the promoter was TATA-less, containing a CpG island with more than 10 TSS, consistent with the broad type promoter.

Several consensus sequences have been identified in the eukaryotic core promoter region, although no universal element has yet been discovered (23). We found a canonical Sp1 site in the mouse *Mct8* promoter, located 56 bases upstream of the most frequently used TSS at −65. The position of the putative Sp1 site is in the consensus region for its efficient functioning, 40–80 bases upstream of the TSS (45). Indeed, our analysis of the *Mct8* promoter demonstrated the requirement of Sp1 site for full promoter activity, although the Sp1 site is not necessary for function of the *Mct8* upstream RARE, DR5A.

Results of a genome-wide study of TSS in several species, based on the oligo-capping method with RNA from multiple organs (28, 29), are available on-line as the data base of transcriptional start sites (DBTSS; available on the World Wide Web). Our *in silico* search for TSS of mouse *Mct8* with the DBTSS identified 12 TSSs between −211 and −81. The results, however, did not match any TSSs identified in our study. The discrepancy may be due to the difference in RNA origin; RNA used in the DBTSS is derived from brain, kidney, spleen, and joint but not testis, the origin of F9 cells. The results of a DBTSS search have shown distinct TSS of *Mct8* among those tissues.

The structure of the mouse *Mct8* proximal promoter was similar to that of the short variant of human *Mct8*. The functional RARE in the mouse is located around 6.6 kilobases upstream of the coding region. A homologous sequence to the mouse RARE was not found in the *MCT8* locus in human; however, there is a similar RARE sequence in an intron of human *MCT8*. The difference in gene structure may reflect the differential regulation of *Mct8* in various species and tissues. There is likely to be cell type specificity because we found RA induction of *Mct8* only in F9 cells.

RA stimulates differentiation of F9 cells into extraembryonic tissues, parietal and visceral endoderm (13). These endoderm-derived tissues support the embryo and transport maternal nutrients as an "early placenta" between 5 and 10 days of gestation (16). Maternal retinol is transported to embryo/fetus through the visceral endoderm (46). TRα is expressed in embryo during the embryogenesis, even in the fertilized egg in *Xenopus laevis* (47), whereas overexposure of thyroid hormone to the embryo causes malformation (48). Visceral endoderm, therefore, might adjust the influx of thyroid hormone into embryonic layers and proamniotic cavity via Mct8, although the detailed pattern of *in vivo* expression of *Mct8* in development has not been described.

F9 cells have been reported to differentiate into neuron-like cells with acetylcholinesterase activity (15). The marked *Mct8* induction was observed in the neuron-like F9 cells. SH-SY5Y neuroblastoma cells expressed abundant *Mct8* even without RA treatment. The simultaneous expression of *Mct8* and neural differentiation markers is consistent with enhancement of neural differentiation by overexpression of *Mct8* in embryonic stem cells (49).

The activity of many nuclear receptors is regulated by the intracellular concentration of its ligand(s), which is modulated by the ligand-selective transporter(s) expressed in the cell membrane. Regulation of such transporters by nuclear receptor signaling has been studied in cholesterol metabolism and inflammation pathways (50). We have demonstrated that RA signaling enhances the TR signaling pathway by up-regulating a thyroid hormone transporter, Mct8. The concept of developmental competence for the thyroid hormone signal in *Xenopus* metamorphosis, linked to TR and RXR expression, is well described (6, 51). This cooperative cross-talk of RA induction of thyroid hormone transport probably plays an important role in signaling pathways of extraembryonic endoderm and neural development.

*Acknowledgments—We thank Drs. Vasanthi Narayan and Sun Wook Kim for assistance with the experiments and Dr. Jerome Hershman for helpful discussions.*

## REFERENCES

1. Mark, M., Ghyselinck, N. B., and Chambon, P. (2009) *Nucl. Recept. Signal.* **7,** e002
2. Yen, P. M. (2001) *Physiol. Rev.* **81,** 1097–1142
3. Lee, L. R., Mortensen, R. M., Larson, C. A., and Brent, G. A. (1994) *Mol. Endocrinol.* **8,** 746–756
4. Liu, Y. Y., Tachiki, K. H., and Brent, G. A. (2002) *Endocrinology* **143,** 2664–2672
5. Giguère, V. (1994) *Endocr. Rev.* **15,** 61–79
6. Furlow, J. D., and Neff, E. S. (2006) *Trends Endocrinol. Metab.* **17,** 40–47
7. Jansen, J., Friesema, E. C., Milici, C., and Visser, T. J. (2005) *Thyroid* **15,** 757–768
8. Friesema, E. C., Jansen, J., Jachtenberg, J. W., Visser, W. E., Kester, M. H., and Visser, T. J. (2008) *Mol. Endocrinol.* **22,** 1357–1369
9. van der Deure, W. M., Peeters, R. P., and Visser, T. J. (2007) *Best Pract. Res. Clin. Endocrinol. Metab.* **21,** 339–350
10. Friesema, E. C., Ganguly, S., Abdalla, A., Manning Fox, J. E., Halestrap,

A. P., and Visser, T. J. (2003) *J. Biol. Chem.* **278,** 40128–40135
11. Hagenbuch, B. (2007) *Best Pract. Res. Clin. Endocrinol. Metab.* **21,** 209–221
12. Chan, S. Y., Franklyn, J. A., Pemberton, H. N., Bulmer, J. N., Visser, T. J., McCabe, C. J., and Kilby, M. D. (2006) *J. Endocrinol.* **189,** 465–471
13. Soprano, D. R., Teets, B. W., and Soprano, K. J. (2007) *Vitam. Horm.* **75,** 69–95
14. Komiya, S., Shimizu, M., Ikenouchi, J., Yonemura, S., Matsui, T., Fukunaga, Y., Liu, H., Endo, F., Tsukita, S., and Nagafuchi, A. (2005) *Genes Cells* **10,** 1065–1080
15. Kuff, E. L., and Fewell, J. W. (1980) *Dev. Biol.* **77,** 103–115
16. Cross, J. C., Werb, Z., and Fisher, S. J. (1994) *Science* **266,** 1508–1518
17. Kagechika, H., Kawachi, E., Hashimoto, Y., Himi, T., and Shudo, K. (1988) *J. Med. Chem.* **31,** 2182–2192
18. Kagechika, H., Kawachi, E., Hashimoto, Y., and Shudo, K. (1989) *J. Med. Chem.* **32,** 834–840
19. Kogai, T., Kanamoto, Y., Li, A. I., Che, L. H., Ohashi, E., Taki, K., Chandraratna, R. A., Saito, T., and Brent, G. A. (2005) *Endocrinology* **146,** 3059–3069
20. Friesema, E. C., Kuiper, G. G., Jansen, J., Visser, T. J., and Kester, M. H. (2006) *Mol. Endocrinol.* **20,** 2761–2772
21. Kogai, T., Ohashi, E., Jacobs, M. S., Sajid-Crockett, S., Fisher, M. L., Kanamoto, Y., and Brent, G. A. (2008) *J. Clin. Endocrinol. Metab.* **93,** 1884–1892
22. Gardiner-Garden, M., and Frommer, M. (1987) *J. Mol. Biol.* **196,** 261–282
23. Sandelin, A., Carninci, P., Lenhard, B., Ponjavic, J., Hayashizaki, Y., and Hume, D. A. (2007) *Nat. Rev. Genet* **8,** 424–436
24. Frith, M. C., Valen, E., Krogh, A., Hayashizaki, Y., Carninci, P., and Sandelin, A. (2008) *Genome Res.* **18,** 1–12
25. Kogai, T., Kanamoto, Y., Che, L. H., Taki, K., Moatamed, F., Schultz, J. J., and Brent, G. A. (2004) *Cancer Res.* **64,** 415–422
26. Wirth, E. K., Roth, S., Blechschmidt, C., Hölter, S. M., Becker, L., Racz, I., Zimmer, A., Klopstock, T., Gailus-Durner, V., Fuchs, H., Wurst, W., Naumann, T., Bräuer, A., de Angelis, M. H., Köhrle, J., Grüters, A., and Schweizer, U. (2009) *J. Neurosci.* **29,** 9439–9449
27. Maruyama, K., and Sugano, S. (1994) *Gene* **138,** 171–174
28. Suzuki, Y., Yamashita, R., Nakai, K., and Sugano, S. (2002) *Nucleic Acids Res.* **30,** 328–331
29. Wakaguri, H., Yamashita, R., Suzuki, Y., Sugano, S., and Nakai, K. (2008) *Nucleic Acids Res.* **36,** D97–D101
30. Lehmann, J. M., Zhang, X. K., and Pfahl, M. (1992) *Mol. Cell. Biol.* **12,** 2976–2985
31. Merchiers, P., Bulens, F., De Vriese, A., Collen, D., and Belayew, A. (1999) *FEBS Lett.* **456,** 149–154
32. Horie, S., Ishii, H., Matsumoto, F., Kusano, M., Kizaki, K., Matsuda, J., and Kazama, M. (2001) *J. Biol. Chem.* **276,** 2440–2450
33. de Thé, H., Vivanco-Ruiz, M. M., Tiollais, P., Stunnenberg, H., and Dejean, A. (1990) *Nature* **343,** 177–180
34. Bastien, J., and Rochette-Egly, C. (2004) *Gene* **328,** 1–16
35. Bulens, F., Merchiers, P., Ibañez-Tallon, I., De Vriese, A., Nelles, L., Claessens, F., Belayew, A., and Collen, D. (1997) *J. Biol. Chem.* **272,** 663–671
36. Liu, Y., Chen, H., and Chiu, J. F. (1994) *Mol. Cell. Endocrinol.* **103,** 149–156
37. Friesema, E. C., Docter, R., Moerings, E. P., Verrey, F., Krenning, E. P., Hennemann, G., and Visser, T. J. (2001) *Endocrinology* **142,** 4339–4348
38. Fuchs, B. C., and Bode, B. P. (2005) *Semin. Cancer Biol.* **15,** 254–266
39. Yanagida, O., Kanai, Y., Chairoungdua, A., Kim, D. K., Segawa, H., Nii, T., Cha, S. H., Matsuo, H., Fukushima, J., Fukasawa, Y., Tani, Y., Taketani, Y., Uchino, H., Kim, J. Y., Inatomi, J., Okayasu, I., Miyamoto, K., Takeda, E., Goya, T., and Endou, H. (2001) *Biochim. Biophys. Acta* **1514,** 291–302
40. Hager, G. L., Lim, C. S., Elbi, C., and Baumann, C. T. (2000) *J. Steroid Biochem. Mol. Biol.* **74,** 249–254
41. Han, Y. H., Zhou, H., Kim, J. H., Yan, T. D., Lee, K. H., Wu, H., Lin, F., Lu, N., Liu, J., Zeng, J. Z., and Zhang, X. K. (2009) *J. Biol. Chem.* **284,** 18503–18514
42. Santos, N. C., and Kim, K. H. (2010) *Endocrinology* **151,** 2361–2372
43. Flajollet, S., Lefebvre, B., Rachez, C., and Lefebvre, P. (2006) *J. Biol. Chem.* **281,** 20338–20348
44. Gillespie, R. F., and Gudas, L. J. (2007) *J. Mol. Biol.* **372,** 298–316
45. Smale, S. T., and Kadonaga, J. T. (2003) *Annu. Rev. Biochem.* **72,** 449–479
46. Johansson, S., Gustafson, A. L., Donovan, M., Romert, A., Eriksson, U., and Dencker, L. (1997) *Anat. Embryol.* **195,** 483–490
47. Banker, D. E., Bigler, J., and Eisenman, R. N. (1991) *Mol. Cell. Biol.* **11,** 5079–5089
48. Kraft, J. C., Willhite, C. C., and Juchau, M. R. (1994) *J. Craniofac. Genet. Dev. Biol.* **14,** 75–86
49. Sugiura, M., Nagaoka, M., Yabuuchi, H., and Akaike, T. (2007) *Biochem. Biophys. Res. Commun.* **360,** 741–745
50. Teng, S., and Piquette-Miller, M. (2008) *Mol. Pharm.* **5,** 67–76
51. Brown, D. D., and Cai, L. (2007) *Dev. Biol.* **306,** 20–33