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A Soluble Transcription Factor, Oct-1, Is Also Found in the Insoluble Nuclear Matrix and Possesses Silencing Activity in Its Alanine-Rich Domain

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Expression of the human thyrotropin β (hTSHβ) gene is restricted to thyrotrophs, at least in part, by silencing. Using transient-transfection assays, we have localized a silencer element to a region between −128 and −480 bp upstream of the transcription initiation site. The silencing activity was overcome in a thyrotroph-specific manner by an unknown enhancer located in the sequences at −∼10000 to −1200 bp. The ubiquitous POU homeodomain protein Oct-1 recognized the A/T-rich silencer element at multiple sites in gel mobility shift assays and in vitro footprinting analyses. The silencing activity of Oct-1 was localized in its C-terminal alanine-rich domain, suggesting that Oct-1 plays a role in silencing of the hTSHβ promoter. Further, a significant fraction of Oct-1 was shown to be associated with the nuclear matrix, and the hTSHβ silencer region was tethered to a nuclear matrix of human cells in vivo, suggesting a possible role of the Oct-1–hTSHβ silencer region interaction in chromatin organization.

Thyrotropin (TSH) is an anterior pituitary hormone that stimulates thyroid hormone production. The expression of the β subunit of this gene is restricted to differentiated thyrotrophs. Although the mechanism for the cell-specific expression of this gene remains largely unknown, the pituitary-specific POU homeoprotein Pit-1 has been shown to be important for TSHβ gene expression in vivo. Certain mutations in the Pit-1 gene in patients with combined pituitary deficiency led to a complete loss of expression of TSH, growth hormone, and prolactin (40, 42, 62). Previous transient-transfection assays with the human and mouse TSHβ promoters also indicated that the proximal Pit-1-binding sites located at −128 to −80 bp (−128/−80) of the human gene (50) and the equivalent sequences in the mouse gene (17, 31) are important for Pit-1-dependent activation. However, Pit-1 alone cannot explain the cell-specific expression of the TSHβ gene because it is also expressed in other pituitary cell types; this suggests an additional regulatory mechanism(s). Previously, it has been noticed that the human TSHβ (hTSHβ) sequences containing the downstream −128 bp and the TATA box support the promoter activity in all cell types tested in both transient-transfection assays (26) and transgenic mice (66). These findings suggest that the hTSHβ gene may be under negative regulation in non-TSH-producing cells to control the constitutive promoter-enhancer activity. In this paper we report that expression of the hTSHβ gene is restricted to thyrotrophs, at least in part, by a silencer element. We found that the hTSHβ silencer element is attached to the nuclear matrix in vivo and that the silencing is mediated by the ubiquitous POU homeoprotein Oct-1.

Oct-1 activates a number of mRNA genes and the small nuclear RNA genes (60) and plays a role in DNA replication in mammalian cells (25, 38, 41, 58). Oct-1 is well known to recognize a broad spectrum of degenerate A/T-rich binding sites through specific interactions with flanking sequences (3). The DNA-binding POU domain of Oct-1 is a bipartite unit composed of two independent DNA-binding modules, the POU-specific domain and the POU homeodomain, tethered by a flexible linker (27). This type of structural arrangement in the POU domain allows the two DNA-binding modules to bind on opposite sides of the double helix and leave the surfaces of the POU-specific domain and the POU homeodomain accessible for interacting with other factors. By recognizing a broad spectrum of DNA sequences and interacting with various nuclear factors, Oct-1 seems to play multiple roles in mammalian cells.

It has been shown that eukaryotic interphase chromosomes are organized into looped domains anchored to the nuclear matrix (13, 22) through DNA regions attached to the matrix; these regions are called MARs or SARs (for matrix or scaffold attachment regions; in this paper, they are called MARs). Only a few nuclear matrix proteins that bind to MARs have been described for mammalian cells: histone H1 (21, 24), topoisomerase II (1), ARBP (61), HMG-I/Y (71), B-type lamin (32), SATB1 (9), and heterogeneous nuclear ribonuclear protein U (12). The MAR-binding proteins generally recognize the minor groove or the intrinsic bend of the A/T-rich DNA and are proposed to modulate higher-order chromatin structure (72). The nuclear matrix also contains factors for DNA and RNA metabolism. These factors have been shown to be localized in discrete domains according to their function, such as DNA replication, transcription, or mRNA splicing (49). The nuclear matrix is thought to support these functions by localizing genes and targeting appropriate factors.

In this paper, we describe the Oct-1-mediated silencing for the tissue-specific expression of the hTSHβ gene. The hTSHβ silencer element, located in the −480/−128 region, is highly A/T rich and consists of multiple degenerate Oct-1-binding sites. The Oct-1-mediated silencing was active in all cell types but was overcome in a thyrotroph-specific manner by an upstream enhancer in the −∼10000 (−∼10K)/−1200 region. Oct-1 was found to possess an intrinsic silencing activity in its C-terminal alanine-rich domain, suggesting that Oct-1 is a pro-

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moter-specific transcription factor with dual regulatory functions (activator and silencer). Further, we showed that the hTSHβ silencer is attached to the nuclear matrix in vivo in human cells and that Oct-1 is a component of the nuclear matrix, indicating that Oct-1 is a MAR-binding nuclear matrix protein. Our findings suggest an interplay between MAR-nuclear matrix interaction and the hTSHβ silencing.

MATERIALS AND METHODS

Plasmid construction. Constructions for the hTSHβ −1200/+8, −128/+8, and −28/+8 constructs were described previously (50). Genomic clones covering the hTSHβ gene were obtained by screening the human genomic P1 library (Genome System, St. Louis, Mo.). The DNA isolated from the P1 clone was digested with several restriction enzymes that do not cut the vector sequences. After Southern blot analysis with the hTSHβ −1200/−800 probe, the fragment containing the −∼10K downstream sequences was subcloned into pKS+ (Stratagene, La Jolla, Calif.). The −∼10K/−128 subfragment was fused upstream to the −128/+8 chloramphenicol acetyltransferase (CAT) construct. For the −∼10K/+8Δ −1200/−128 construct, the *Bam*HI fragment containing hTSHβ −1200/+8 and the CAT gene was removed from the −∼10K/+8 CAT plasmid and replaced with another *Bam*HI fragment that contains hTSHβ −128/+8 and CAT. Internal deletion of the −150/−128 element from the −1200/+8 construct was done by site-directed mutagenesis with the primers containing 20 nucleotides complementary to the regions immediately 5′ and 3′ of the deletion. For the constructs in Fig. 1B, *Sst*I-*Kpn*I PCR fragments containing the indicated hTSHβ promoter regions were fused upstream of the −128/+8 construct. The constructs used for Fig. 4 were made in a similar manner by inserting various synthetic double-stranded DNAs. The Mut (Oct-1) construct was generated by PCR amplification. A pCG CMV-Oct-1 expression vector has been described previously (54). For constructing expression vectors for GAL4–Oct-1 fusion proteins, a *Kpn*I site was created upstream of the Oct-1 coding sequence. The DNA-binding domain of GAL4 (positions 1 to 147) was inserted in frame into the pCG CMV-Oct-1 vector as an *Xba*I-*Kpn*I PCR fragment. Truncated Oct-1 fusion proteins were made by inserting *Kpn*I-*Bam*HI fragments containing various subregions of Oct-1 in place of the full-length Oct-1. All constructs made by PCR were verified by DNA sequencing.

Growth of TtT-97 tumors and primary cell dispersion. Growth of TtT-97 tumors and primary cell dispersion were performed as described previously (68). Briefly, male LAF 1 mice were placed on a low-iodine diet prior to radiothyroidectomy by intraperitoneal injection of $Na^{131}I$. A fine mince of frozen TtT-97 tumor tissue was injected subcutaneously into the hind limbs of hypothyroid mice. The resulting TtT-97 tumor expressed both the α and β subunits of TSH. Dispersions were performed in cells derived from early passages when TSHβ production was relatively high. For primary dispersion, TtT-97 tumor tissue (2 to 3 cm in diameter) was surgically removed, washed in sterile ice-cold phosphate-buffered saline, and finely minced. Trypsin (0.25%; Gibco, Grand Island, N.Y.) was added at 2.0 ml/cm (diameter) of tumor and stirred on a stir plate at 37°C for 4 to 6 h until the minced tissue was completely digested. Monodispersed cells were removed from the digest every hour and passed through a cheesecloth filter, where the trypsin digestion was terminated by addition of medium with 10% fetal calf serum and 1 mg of soybean trypsin inhibitor per ml. Fresh trypsin was added to the remaining tissue every hour until the tissue was completely dispersed. Upon completion of digestion, the combined cell suspension was centrifuged and used for electroporation.

Transient-transfection assays. The enzymatically dispersed TtT-97 cells (5×10^6 to 8×10^6) were transfected by electroporation. HeLa, 293 (human embryonal kidney), and GH_3 (rat pituitary) cells were transfected by the calcium phosphate technique with 20 μg of the hTSHβ reporter plasmids. For some experiments, the expression vectors for the GAL4–Oct-1 fusion proteins, carrier DNA, and/or pXGH5 as an internal control was cotransfected. CAT activities given in this paper were normalized to protein concentration as measured by the Bradford method (7a). Although a growth hormone expression vector, pXGH5, was transfected as an internal control for transfection efficiencies, we found that, as observed in other studies (26, 43), the intra-assay variability due to transfection efficiency was not significant within each cell type.

Gel mobility shift assays. Nuclear extracts from HeLa, GH_3, and TtT-97 cells were prepared as described previously (45) with a slight modification with an isotonic Nonidet P-40 lysis procedure. The extracts were preincubated for 15 min at room temperature with 2 μg of poly(dI/dC) and various competitor DNAs before the end-labeled probe was added. After addition of 10,000 cpm of the ^{32}P-labeled probe, the mixture was incubated for 15 min and loaded on a 4 or 8% nondenaturing polyacrylamide gel. For competition experiments, 300 times more double-stranded unlabeled oligonucleotide was added to the reaction mixture just before the addition of the end-labeled probe. Inhibition of DNA-protein complex formation with antibodies was performed by adding the antisera (2 μl) to the nuclear extracts and incubating overnight at 4°C. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Anti-Oct-1 antibody is a rabbit polyclonal antibody raised against a peptide that corresponds to amino acids 723 to 743 at the carboxy terminus of human Oct-1. Anti-Pit-1 antibody was raised against the full-length rat Pit-1 protein.

DNase I protection assay. The hTSHβ promoter fragment (−310/−180) was generated by PCR in which one of the synthetic oligonucleotides was ^{32}P labeled by using T4 kinase. The DNase protection assays were performed in 50 μl with 40,000 cpm of the probe. Approximately 120 μg of HeLa nuclear protein was used per lane. The footprinting reaction (Promega, Madison, Wis.) was carried out as the manufacturer suggested.

Nuclear matrix preparation and identification of the matrix-attached DNA. The procedure used is a modification of the protocol of Mirkovitch et al. (36). Because the nuclei from 293 cells, which were used with HeLa cells in transient-transfection experiments (see Fig. 1), were too fragile to isolate the nuclear matrix, we used HeLa cells and the human hepatoma HepG2 cells for the MAR identification experiments (see Fig. 6C). The nuclei prepared from 10^8 HeLa and HepG2 cells were resuspended in 50 mM KCl–0.5 mM EDTA–0.05 mM spermine–0.25 mM phenylmethylsulfonyl fluoride–5 mM Tris-HCl (pH 7.4) and incubated for 30 min at 37°C prior to lithium 3′,5′-diiodosalicylate (LIS) extraction. One milliliter of freshly made LIS buffer (12 mM LIS, 0.1% digitonin, 0.05 mM spermine, 0.125 mM spermidine, 0.25 mM phenylmethylsulfonyl fluoride, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-KOH, pH 7.4) was added per 10^6 nuclei, and the nuclei were extracted after three washings with LIS at room temperature. The resulting nuclear preparation was washed five times (14,000 rpm, 5 min) in restriction enzyme digestion buffer with 20 μg of aprotinin per ml and 1 mM phenylmethylsulfonyl fluoride to remove LIS. The resulting pellet was digested with *Bam*HI and *Bst*EII (50 U/10^6 nuclei) for 3 h at 37°C. The DNAs in both fractions (pellet [P] and supernatant [S]) were purified by phenol-chloroform extraction and ethanol precipitation. After electrophoresis, Southern blotting was performed with the ^{32}P-labeled hTSHβ −480/−128 and +181/+300 probes.

Western blot (immunoblot) analysis. For the LIS-extracted nuclear matrix preparation, HeLa cell nuclei were stabilized by incubation at 37°C for 30 min followed by digestion with DNase I (100 to 600 μg/ml) for 2 h and extracted three times with LIS at room temperature. In order to reduce the volume of the LIS-extracted supernatant for immunoblot assay (see Fig. 7A, lane 2), the initial extraction was performed with 10^8 cells per ml of LIS extraction buffer. Two subsequent extractions were done with 10^6 cells per ml of LIS buffer. The pellet fraction was washed once in hypotonic buffer (10 mM HEPES-KOH [pH 7.4], 10 mM KCl, 1.5 mM $MgCl_2$) and analyzed directly (see lanes 5 in Fig. 7A and C) or subjected to additional DNase I digestion (lanes 4 in Fig. 7A and C). For the isolation of nuclear lamina used for lane 2 of Fig. 7D, the heat stabilization step was replaced by an incubation on ice (32). The salt-extracted nuclear matrix preparation used for Fig. 7B was obtained as described by He et al. (18). Briefly, HeLa cells were extracted with Triton X-100 (0.5%) in the physiological-ionic-strength buffer (10 mM HEPES [pH 7.4], 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$), digested with DNase I (100 μg/ml) at room temperature for 30 min, and treated with 0.25 M $(NH_4)_2SO_4$. The resulting insoluble pellet was reported to contain a nuclear matrix protein-intermediate filament complex (18). For immunoblotting, proteins in various nuclear subfractions were dissolved in loading buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 60 mM Tris-HCl, 100 mM dithiothreitol, 0.001% bromophenol blue), boiled for 5 min, and resolved on an SDS-polyacrylamide gel (8 or 12% polyacrylamide). The proteins were transferred to a nitrocellulose filter and blotted with the anti-Oct-1- or the anti-TATA box-binding protein (TBP) antibody (Santa Cruz). Peroxidase-linked anti-rabbit immunoglobulin (Amersham) was used as the second antibody, and immunoblotting was performed as described by the manufacturer. When the primary antibodies were omitted, the Oct-1- or TBP-specific band was not observed (data not shown).

RESULTS

Silencer element is present in the −480/−128 region of the hTSHβ promoter. In transient-transfection assays, we measured the CAT activities of reporter genes containing the −∼10K/+8, −1200/+8, and −128/+8 regions of the hTSHβ promoter in nonthyrotropic cells (HeLa, human embryonal kidney 293, and rat pituitary GH_3) and in primary cultures of the TSH-secreting mouse thyrotropic tumor TtT-97. The −∼10K/+8 gene was active only in TtT-97 cells, whereas the −128/+8 construct showed inappropriate expression in all cell types tested (Fig. 1A). Low-level activity was observed with the −1200/+8 construct in all cell types tested, whereas the internal deletion of the −1200/−128 sequences from the −∼10K/+8 construct (−∼10K/+8Δ −1200/−128) resulted in TtT-97-specific activation. These results indicate that the −1200/−128 region as well as the −∼10K/−1200 region contains silencer elements whose activity is overcome by an unknown activator located between −1200 and −∼10K in thyrotrophs only.

In order to identify the borders of the −1200/−128 silencer

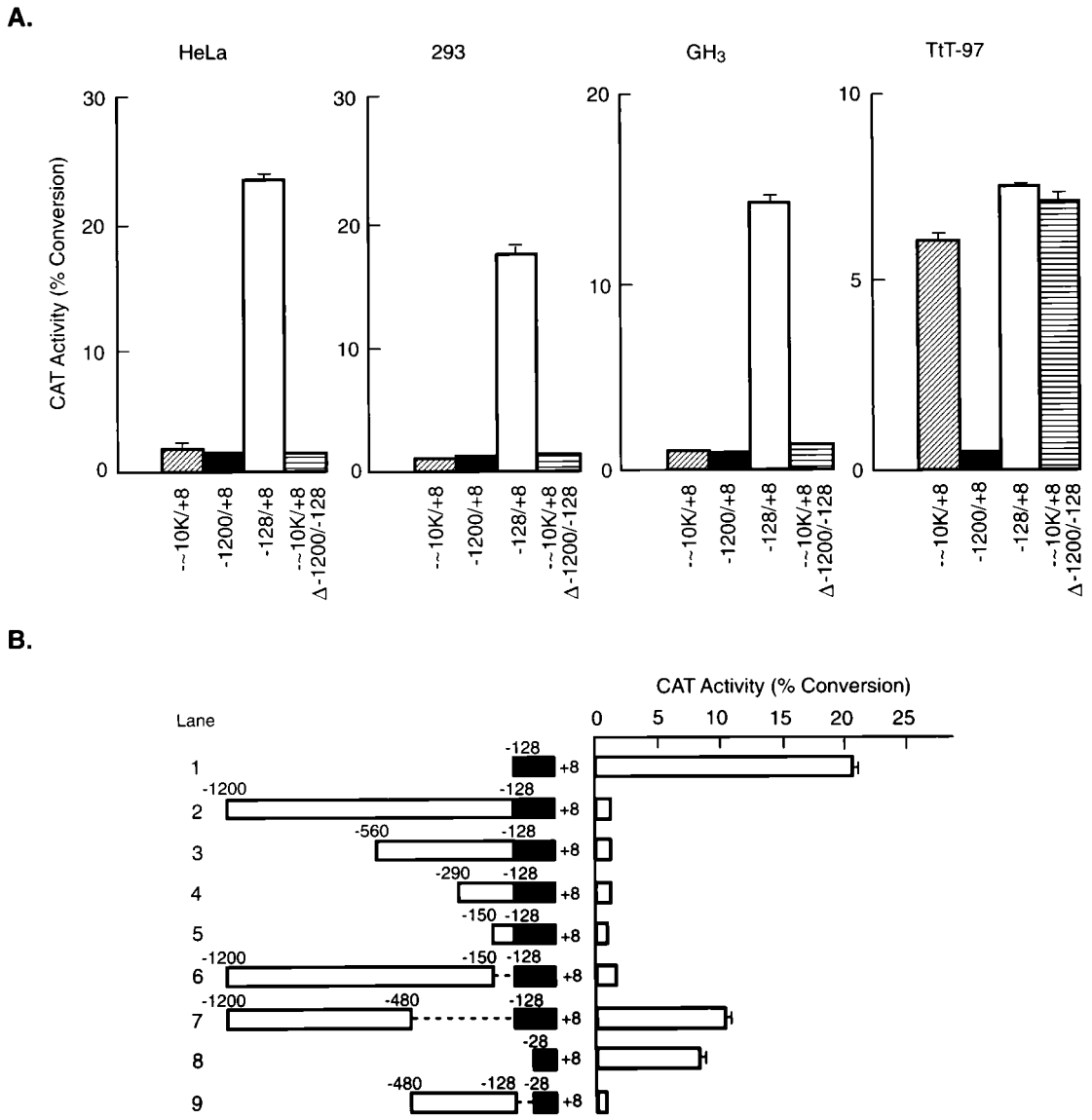

FIG. 1. Localization of a silencer element in the hTSHβ promoter. (A) HeLa, 293, and GH_3 cells were transfected with 20 μg of the reporter genes containing various regions of the hTSHβ promoter. Transfections in these cells were performed by the calcium phosphate precipitation technique. A primary culture of TtT-97 cells was obtained from TSH-secreting thyrotropic tumors. For TtT-97 cells, 20 μg of the indicated plasmids was transfected by electroporation. The cells were harvested 48 h after transfection, and CAT activity was determined. The relatively low-level expression of the hTSHβ constructs in TtT-97 cells might reflect poor transfection efficiency of primary cells. The data for TtT-97 cells were obtained from three independent transfections, while the data with cell lines were from three to six independent transfections; averages plus standard errors are shown. (B) Data obtained with HeLa cells.

element, 5′ or 3′ deletions of the −1200/−128 fragment were cloned 5′ upstream of −128. As shown in Fig. 1B for HeLa cells, a region between −128 and −150 possessed strong silencing activity (lane 5), but deletion of the −150/−128 fragment did not increase expression (lane 6). Among many internal deletions tested (data not shown), deletion of the −480/−128 fragment resulted in the greatest expression (Fig. 1B, lane 7), indicating that this region contains silencer elements. The strong silencing activity of the −480/−128 fragment on the minimal −28/+8 promoter containing only the TATA element (Fig. 1B, lane 9) indicated that the factors that bind this region may interact directly with the basal transcription machinery. Similar results were obtained with 293, GH_3, and TtT-97 cells (data not shown). The silencer activity was evident even when the −480/−128 fragment was placed 200 bp upstream from the proximal −128/+8 promoter, and it functioned in both orientations (data not shown). In addition, overexpression of Pit-1 in HeLa cells did not enhance the activity of the 1200/+8 or −480/+8 construct (data not shown). Although the −1200/−480 region appears to possess an additional negative activity (Fig. 1B, lane 7), we chose to characterize the proximal region (−480/−128) in this study because this region silences more strongly than the −1200/−480 region. In addition, this region is strikingly A/T rich and has other interesting features of the nucleotide sequences (see Fig. 6A).

The −150/−128 silencer region contains the A/T-rich sequences for Oct-1 binding. To identify nuclear proteins that bind the silencer element, we carried out a gel mobility shift analysis, focusing on the short −150/−128 element first. Nuclear extracts prepared from HeLa, GH_3, and TtT-97 cells

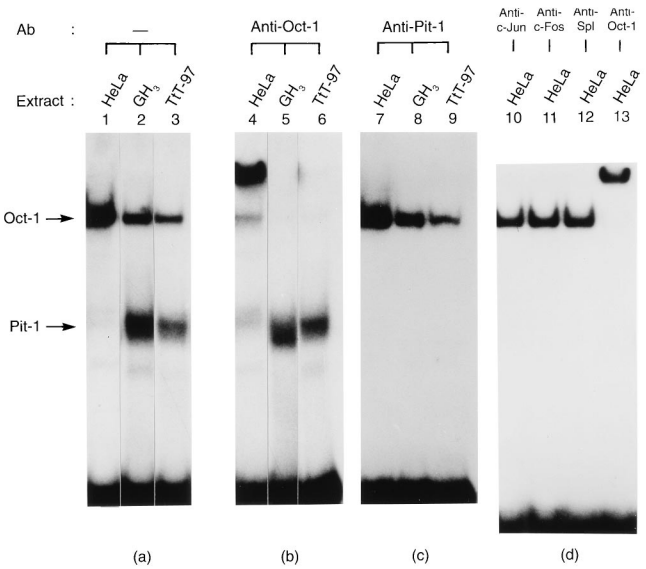

FIG. 2. Identification of a nuclear factor that binds to the hTSHβ −150/−128 silencer fragment. Gel mobility shift assays were carried out with the labeled hTSHβ −150/−120 probe after incubation with nuclear extracts from HeLa, GH_3, and TtT-97 cells. (a) Assays without antibody (Ab). (b to d) The nuclear extracts were preincubated with 2 μl of each antiserum overnight at 4°C before the addition of the labeled hTSHβ probe.

were incubated with the labeled −150/−120 probe. HeLa and 293 (data not shown) cell extracts revealed one nucleoprotein complex (Fig. 2a). In pituitary-derived cells (GH_3 and TtT-97), an additional band migrating at a lower position was observed (lanes 2 and 3). The −150/−120 element contains 21-bp-long A/T-rich sequences (Pal I) (see Fig. 6A). Given the pituitary specificity of the lower band, we thought that it might represent the band formed by Pit-1 that recognizes various A/T-rich elements with high affinity (63). Oct-1 is also well known to recognize degenerate A/T-rich binding sites (3). Because of the highly conserved primary structure of the DNA-binding POU domain of Pit-1 and Oct-1, these two proteins have been reported to possess similar binding specificities (11).

In Fig. 2b, the upper band was supershifted or not formed after incubation with the anti-Oct-1 antibody, which does not cross-react with Pit-1 or other homeodomain proteins. The pituitary-specific lower band was not formed in the presence of the anti-Pit-1 antibody (Fig. 2c, lanes 8 and 9). These bands were not affected by the presence of anti-c-Jun, anti-c-Fos, and anti-Sp1 antibodies (Fig. 2d, lanes 10, 11, and 12). Moreover, formation of both Oct-1- and Pit-1-specific bands was inhibited by excess amounts of unlabeled fragment containing a known Oct-1-binding site in the immunoglobulin heavy-chain (IgH) enhancer (IgH E) (44, 46) but not by the oligonucleotides with multiple Sp1 or AP-1 sites (data not shown). These results indicate that the hTSHβ −150/−120 sequence binds Oct-1 and/or Pit-1, depending on the cell type.

The −480/−128 element consists of multiple A/T-rich Oct-1-binding sites. The −480/−128 silencer region is highly A/T rich (>70%) and appears to contain tandemly repeated sequences with similar A/T-rich motifs (see Fig. 6A). Since Oct-1 or Pit-1 is known to bind sequences such as ATTAATTA/T or ATTTAAAT with high affinity (27, 63), we examined the possibility that the silencer element may contain multiple Oct-1- and Pit-1-binding sites. For this purpose, the previously characterized 30-bp IgH E that binds Oct-1 with high affinity (44) was incubated with extracts from HeLa and GH_3 cells. As reported previously (45), only an Oct-1-specific band was observed in HeLa extracts [Fig. 3A (a), lane 1]. The bands for Oct-1 and Pit-1 were obtained in GH_3 extracts [Fig. 3A (b), lane 1]. The identities of these proteins were confirmed by using specific antisera (data not shown). In gel mobility shift competition analyses with 300-fold molar excesses of various competitors, both bands competed with the unlabeled IgH E probe efficiently (Fig. 3A, lanes 2). The 30-bp oligonucleotides containing nonoverlapping regions of the hTSHβ promoter competed to various degrees. For example, the −210/−180, −240/−210, −270/−240, and −420/−390 fragments (lanes 5, 6, 7, and 12, respectively) showed efficient competition for the Oct-1 band [Fig. 3A (a)], while certain regions, such as −180/−150 and −300/−270 (lanes 4 and 8, respectively), did not compete. Compared with IgH E, which showed a complete inhibition for the Oct-1-specific band formation starting from a 10-fold excess (data not shown), the hTSHβ oligonucleotides were weaker competitors, showing efficient competition at 100- to ~300-fold molar excesses. Degenerate Oct-1-binding sites possess significantly lower affinity than the high-affinity consensus octamer site (60). The differences in Oct-1 binding affinities (10- to ~30-fold) between the IgH E element and various subregions within the hTSHβ silencer are consistent with previous observations that heterogeneous Oct-1-binding sites have a broad spectrum of binding affinities in the range of up to 200-fold (5). Although the competition patterns for Oct-1 and Pit-1 did not always correlate [Fig. 3A (b)] when GH_3 extracts were used, these results indicate that Oct-1 and/or Pit-1, depending on the cell type, interacts with the entire −480/−128 fragment at multiple sites. It is interesting that the −270/−210 sequences that showed a relatively strong binding activity for Oct-1 in the silencer region contain A/T-rich sequences at −279/−248, which is completely devoid of G or C (Pal II) (see Fig. 6A).

To further confirm the Oct-1 interaction with the extremely A/T-rich sequences in the hTSHβ promoter, such as the −279/−248 region, DNase I footprinting analysis was performed with the labeled −310/−180 hTSHβ probe and HeLa nuclear extracts. As shown in Fig. 3B, a broad region was protected (lane 2). The footprint was not formed in the presence of anti-Oct-1 antibody (lane 3) or excess amounts of unlabeled hTSHβ −270/−240 (lane 4), hTSHβ −150/−120 (lane 5), or the 30-bp IgH E oligonucleotides (lane 6). However, competition was not seen with the oligonucleotides bearing tandem Sp1 or AP-1 sites (data not shown). With this additional evidence, we conclude that Oct-1 binds to multiple sites in the hTSHβ silencer region.

Oct-1 confers negative regulation in the context of the hTSHβ promoter. Previously it has been reported that the regulatory function of Oct-1 is determined by the promoter context and not by the specific sequence of an Oct-1-binding site (53). Oct-1 acts as a positive transcriptional regulator in the context of many RNA polymerase II promoters (60). Although it is not known whether Oct-1 itself is responsible, the octamer motif confers negative regulation to human c-*myc* gene (52) and thyroid hormone receptor alpha gene (28) expression. The binding of Oct-1 to the hTSHβ silencer element (Fig. 2 and 3) suggests that Oct-1 might function as the negative regulator of the hTSHβ promoter.

Previously, it has been shown that the −128/+8 promoter contains multiple Pit-1-binding sites and that the transfected −128/+8 promoter is inducible by Pit-1 (26, 50). In Fig. 3A (lanes 17), the gel mobility competition analyses indicate that the previously characterized Pit-1-binding sites located in the −128/−80 region are recognized by Oct-1 as well. Given the high basal activity of the −128/+8 promoter in HeLa or 293 cells, where Oct-1 is abundant (Fig. 1A), these results suggest

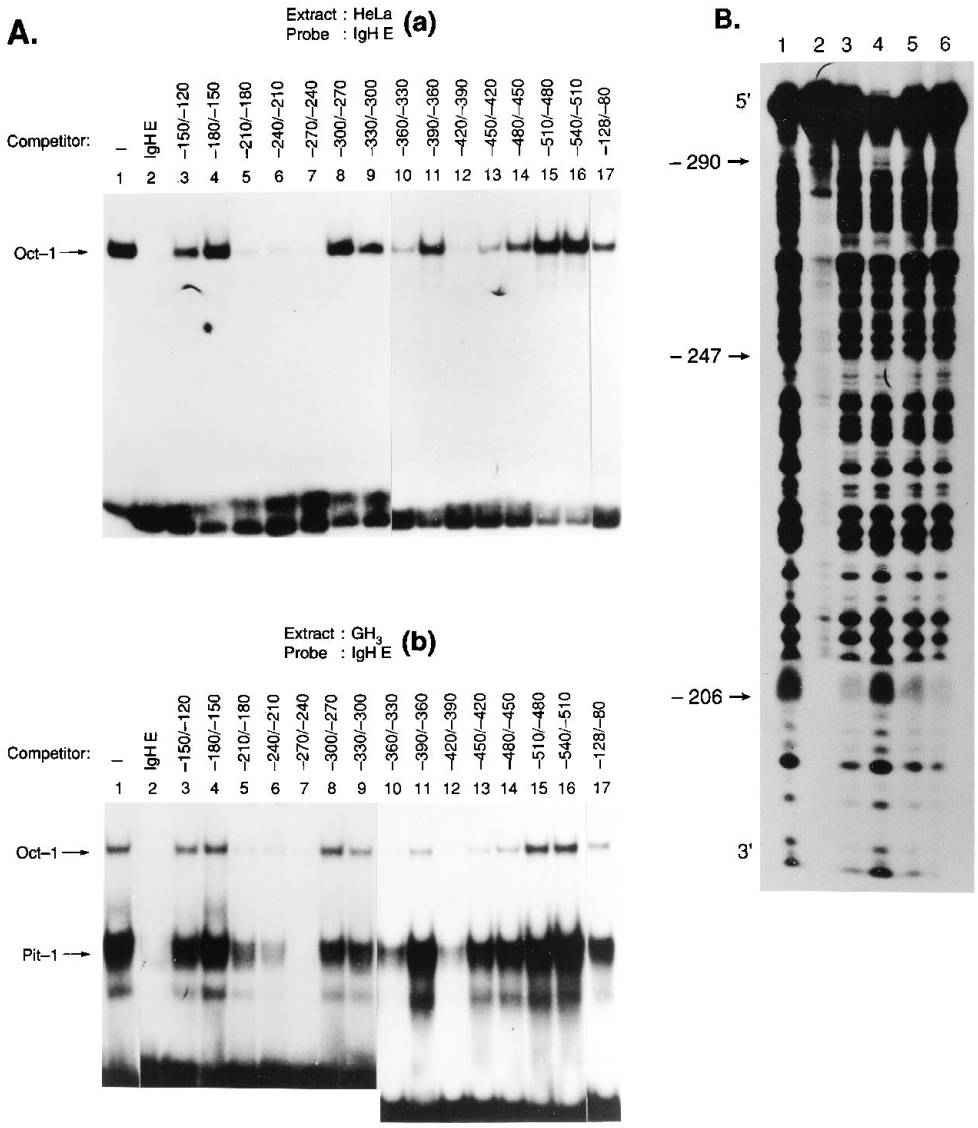

FIG. 3. Multiple Oct-1 binding to the hTSHβ −480/−128 element. (A) Gel mobility shift assays were performed with the labeled 30-bp oligonucleotide derived from the immunoglobulin heavy-chain enhancer containing the known Oct-1-binding site (5′-CCACCTGGGTAATTTGCATTTCTAAAATAA-3′ [octamer site underlined]). In competition experiments, a 300-fold molar excess of each unlabeled competitor was used for the nuclear extracts from HeLa (a) and GH_3 (b) cells. (B) DNase I footprint analysis of the hTSHβ −310/−180 region with HeLa cell extract. The probe was end labeled at position −180. The base pair positions indicated were determined by running a pBR322 *Msp*I-digested radiolabeled standard and Maxam-Gilbert sequencing reaction. Reaction mixtures received no protein (lane 1), HeLa nuclear extract (120 μg) (lane 2), HeLa extract and anti-Oct-1 antibody (lane 3), or HeLa extract and various oligonucleotide competitors (lane 4, hTSHβ −270/−240; lane 5, hTSHβ −150/−120; lane 6, IgH E). The amount of the −150/−120 oligonucleotide competitor was adjusted to show the observed competition in lane 5. The amounts of other competitors used were as same as that of the −150/−120 competitor.

that the Oct-1 binding within the −128/+8 promoter does not result in functional silencing. When the functional Pit-1-binding sites were converted to Oct-1-binding sites by introducing a 6-bp mutation [Mut (Oct-1)], complete silencing was observed in HeLa cells (Fig. 4, bar 2).

The Oct-1-mediated silencing on the hTSHβ promoter was further tested by inserting the 30-bp octamer-containing IgH E fragment upstream of the −128/+8 gene (IgH E construct) (Fig. 4, bar 5). Interestingly, a weaker silencing activity was observed with this fragment than with hTSHβ Pal I (−150/−120) (bar 3) or Pal II (−279/−248) (bar 4). In contrast, the construct containing a 3-bp point mutation within the IgH E fragment that abolishes Oct-1 binding (Mut IgH E) (Fig. 4, bar 6) (46) showed expression comparable to that of the −128/+8 construct, indicating that the silencing is dependent on the binding of Oct-1 to its binding site. Three tandem repeats of octamer sequences (3× Oct-1) (Fig. 4, bar 8) constituted a stronger silencer than one binding site (1× Oct-1) (Fig. 4, bar 7). The control construct bearing three Sp1-binding sites (3× Sp1) (Fig. 4, bar 9) or three AP-1 sites (data not shown) failed to show any silencing effect. As expected, the Oct-1-mediated silencing in the hTSHβ Pal I or Pal II construct was not relieved in GH_3 cells (data not shown). Overall, the results in Fig. 4 demonstrated that Oct-1 is responsible for the silencing of the hTSHβ promoter.

The alanine-rich domain of Oct-1 possesses silencing activity. Oct-1 consists of modular domains (51), as shown in Fig. 5A. The Oct-1-mediated transcription activation of the mRNA

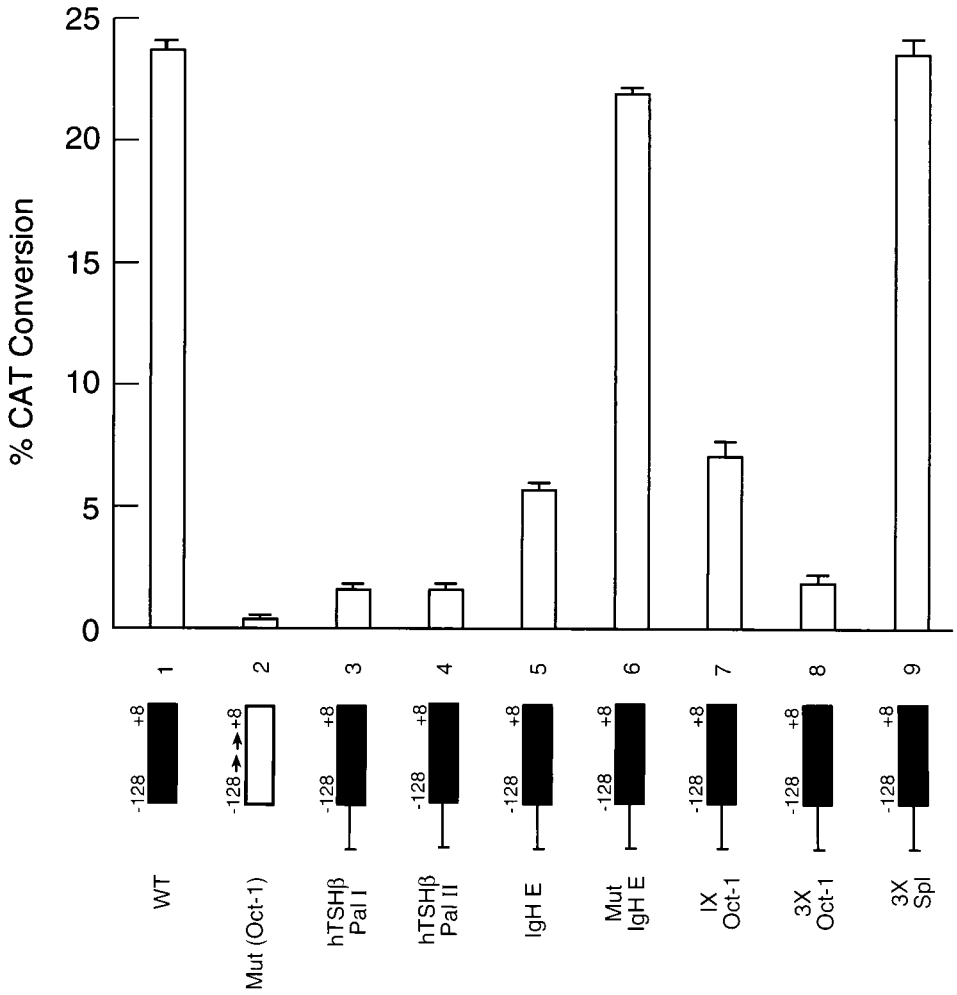

FIG. 4. Effect of Oct-1-binding sites on the CAT expression of the hTSHβ promoter. HeLa cells were transfected with 20 μg of the various reporter constructs as indicated. Bar 1, wild-type (WT) hTSHβ −128/+8 construct; bar 2, Mut (Oct-1) construct in which the Pit-1-binding sites ($^{-120}$ ATGAATTTTCAATAGATGCTT TT^{-108} [Pit-1 sites underlined]) within the −128/+8 promoter were converted to octamer elements ($^{-120}$ ATGCAAATTCAATAGATGCAAAT^{-108} [octamer sequences underlined]). For bars 3 to 9, the indicated synthetic 30-bp oligonucleotides were inserted immediately upstream of the WT hTSHβ −128/+8 promoter. Bar 3, hTSHβ Pal I (−150/−120); bar 4, hTSHβ Pal II (−279/−248); bar 5, IgH E; bar 6, mutated (Mut) IgH E containing a 3-bp point mutation of the octamer site in the IgH E element (5′-CCACCTGGGTAATCTAGATTTCTAAAATAA-3′ [mutations underlined]); bar 7, 1× Oct-1; bar 8, 3× Oct-1; bar 9, 3× Sp1.

promoters requires the N-terminal glutamine-rich domain (54). In order to locate the silencer domain in Oct-1, we tested the regulatory potential of each subdomain. The entire Oct-1 or each subdomain of Oct-1 was expressed as fusion proteins with the DNA-binding domain of GAL4 (1 to 147) (GAL4 DBD) to circumvent the activity of the endogenous Oct-1 (Fig. 5B). The expression vectors were cotransfected into HeLa cells with a CAT reporter vector which contained four synthetic GAL4-binding sites immediately upstream of the hTSHβ −128/+8 promoter element.

As shown in Fig. 5B, cotransfection of GAL4–Oct-1(FL) led to repression, whereas cotransfections with the expression vector for Oct-1 or GAL4 DBD resulted in levels of expression similar to those with GAL4–Oct-1(Glu), GAL4–Oct-1(POU), or GAL4–Oct-1(S/T) (data not shown). Among the subdomains tested, significant silencing activity was exhibited only by GAL4–Oct-1(Ala) fusion. Since GAL4–Oct-1(Ala) lacks the POU domain important for dimerization, it is unlikely that the silencing effect is mediated by the endogenous Oct-1 recruited to the fusion protein. These results suggest that the C-terminal alanine-rich region of Oct-1 possesses a transferable silencing activity when tethered near a promoter.

In order to rule out the possibility that the differences in activities of the fusion proteins could have reflected protein stability, nuclear localization, and/or DNA binding affinity, the levels of these fusion proteins were monitored by gel mobility shift analysis with nuclear extracts prepared from HeLa cells transfected with each expression vector and pXGH5 as an internal control. As shown in Fig. 5C, each fusion protein was competent for DNA binding. The chimeric proteins that lack the silencing activity were expressed as well as or more abundantly than GAL4–Oct-1(Ala) and GAL4–Oct-1(FL).

The hTSHβ silencer is a MAR. The hTSHβ silencer sequence has features often associated with MARs (13). It is A/T rich (>70%) (Fig. 6A) and contains multiple motifs similar to the topoisomerase II consensus site. It also contains motifs that are frequently found at other MARs, such as sequences similar to the A box, T box, and palindromic (or near-palindromic) repeats of an A/T-rich sequence (Pal I and Pal II). Such A/T-rich palindromic sequences are known to form cruciform DNA

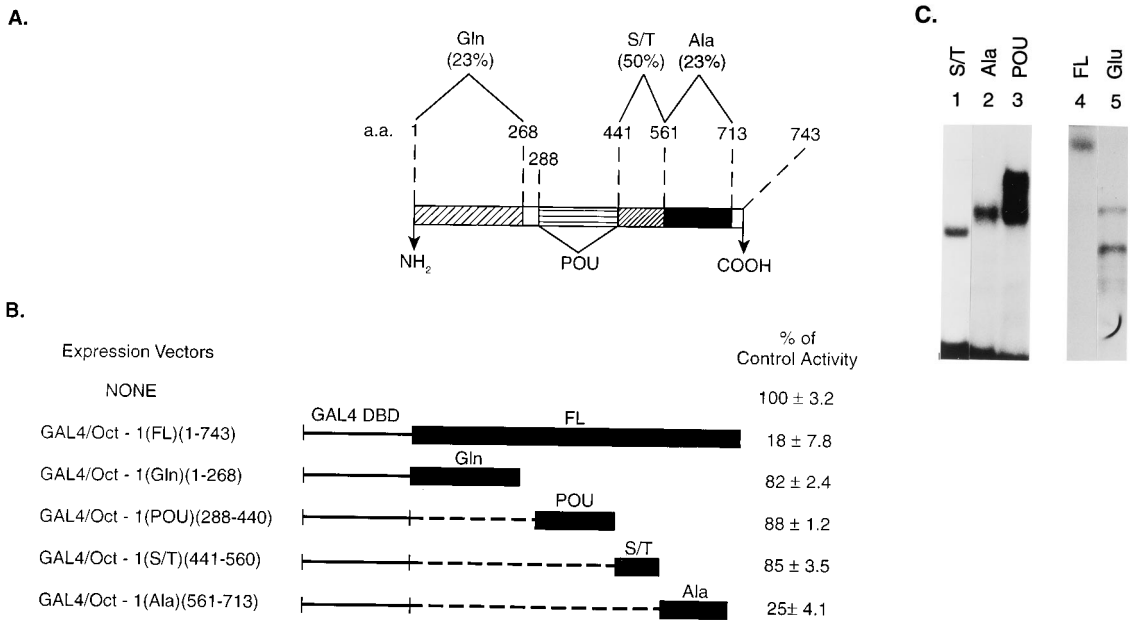

FIG. 5. Identification of a negative regulatory domain from Oct-1. (A) Modular structure of Oct-1. The numbering system for the amino acids (a.a.) is as shown previously (46). (B) Effect of GAL4–Oct-1 fusion proteins on a reporter construct in HeLa cells. The structures of the expression vectors of the GAL4–Oct-1 fusion proteins which contain the full-length human Oct-1 or indicated subdomains fused with the GAL4 DNA-binding domain (amino acids 1 to 147) are shown. A reporter construct (10 μg) (4 × GAL4 hTSHβ −128/+8 CAT) that contains four GAL4-binding sites upstream of the hTSHβ −128/+8 promoter was transfected into HeLa cells with 3 μg of each effector construct, 7 μg of pBluescript as carrier DNA, and 2 μg of pXGH5 as an internal control. The value of the reporter alone (NONE) was arbitrarily set at 100%. The amount of the effector plasmid was determined by dose-response studies to obtain the optimal level of repression with a constant amount of the reporter and various amounts (0, 0.3, 3, 5, or 8 μg) of the GAL4–Oct-1(FL) expression vector (data not shown). CAT activity was normalized according to the amount of total protein, because no significant change in the CAT expression level was found with the internal control value. (C) Gel mobility shift analysis to detect GAL4–Oct-1 fusion proteins in transfected cells. Nuclear extracts were prepared from HeLa cells transfected with the expression vectors encoding the indicated GAL4–Oct-1 fusion proteins. Each transfection contained 20 μg of each expression vector and 2 μg of pXGH5 as an internal control. Two days after transfection, cells were harvested for nuclear extract preparation. The labeled 34-bp oligonucleotide containing two GAL4-binding sites was used as a probe, and the reaction mixture was run on a nondenaturing polyacrylamide gel. In lanes 4 and 5, the gel was electrophoresed until the 34-bp probe migrated into the buffer to show the GAL4–Oct-1(FL) at the position shown here.

under the effect of supercoiling (6), and sequences such as AATATATTT (−246/−238; double underlined in Fig. 6A) have been shown to possess a strong base-unpairing property in the context of other MARs (7). Involvement of these sequences in opening the DNA double helix for transcription and replication has been suggested (20, 39).

In order to determine whether the hTSHβ silencer sequences are anchored to the nuclear matrix of human cells in vivo, a MAR identification experiment was performed with HeLa and HepG2 (human hepatoma) cells, in which the hTSHβ gene is silent. The nuclei were incubated at 37°C for 10 min to preserve the internal structural proteins of the matrix surrounded by the nuclear lamina (32). The isolated nuclei were treated with LIS to extract histones and were digested with *Bam*HI and *Bst*EII. The DNAs solubilized (S fraction) and bound to the nuclear matrix (P fraction) were probed with the labeled hTSHβ −480/−128 or +181/+300 fragment (Fig. 6B). As shown in Fig. 6C, with the −480/−128 probe, the expected 1.2-kb band (5′ *Bam*HI-3′ *Bst*EII) containing the −1200/+8 sequences was detected in the nuclear matrix-associated DNA (P) fraction (lanes 1 and 2) but not in the solubilized (S) fraction (lanes 3 and 4). When the same blot was reprobed with the +181/+300 fragment, the expected 3.2-kb band (5′ *Bst*EII-3′ *Bam*HI) was observed only in the S fraction in both cell lines (lanes 7 and 8) not in the (P) fraction (lanes 5 and 6). These results suggest that the genomic DNA containing the hTSHβ silencer region (−480/−128) is tethered to the nuclear matrix of the non-TSH-expressing human cells. Whether the same region remains attached to the nuclear matrix in human thyrotropic cells is unknown.

Oct-1 is associated with the nuclear matrix. The nuclear matrix binding activity shown in Fig. 6C is thought to reflect sequence-specific interactions between the MAR DNA and anchoring proteins that are insoluble after the LIS extraction procedure. Previously, an octamer-binding activity has been detected in nuclear matrix preparations of several cell lines by gel mobility shift analysis (56). In order to determine whether Oct-1 is associated with the nuclear matrix, nuclear matrix was prepared from heat-stabilized HeLa cell nuclei by DNase I digestion and LIS extraction. When probed with the anti-Oct-1 antibody on a Western blot (Fig. 7A), a large amount of Oct-1 was observed in the LIS supernatant following the first wash (lane 2). When the pellet obtained after additional extractions with LIS was washed with hypotonic solution, no additional Oct-1 was released into the supernatant (lane 3). In the final nuclear matrix preparation (lane 5), a significant amount of Oct-1 remained, indicating that Oct-1 exists in both the soluble and insoluble nuclear fractions. Furthermore, additional DNase digestion did not increase Oct-1 in the supernatant (Fig. 7A, lane 4), ruling out the possibility that Oct-1 is retained in the nuclear matrix (lane 5) by Oct-1 binding to undigested genomic DNA. We confirmed that the band shown in Fig. 7A is indeed Oct-1 by demonstrating that the antibody can be inhibited by excess Oct-1-immunizing peptide (lane 6).

The use of LIS as the extracting agent requires that the nuclei be stabilized by incubation at 37°C (36). When the

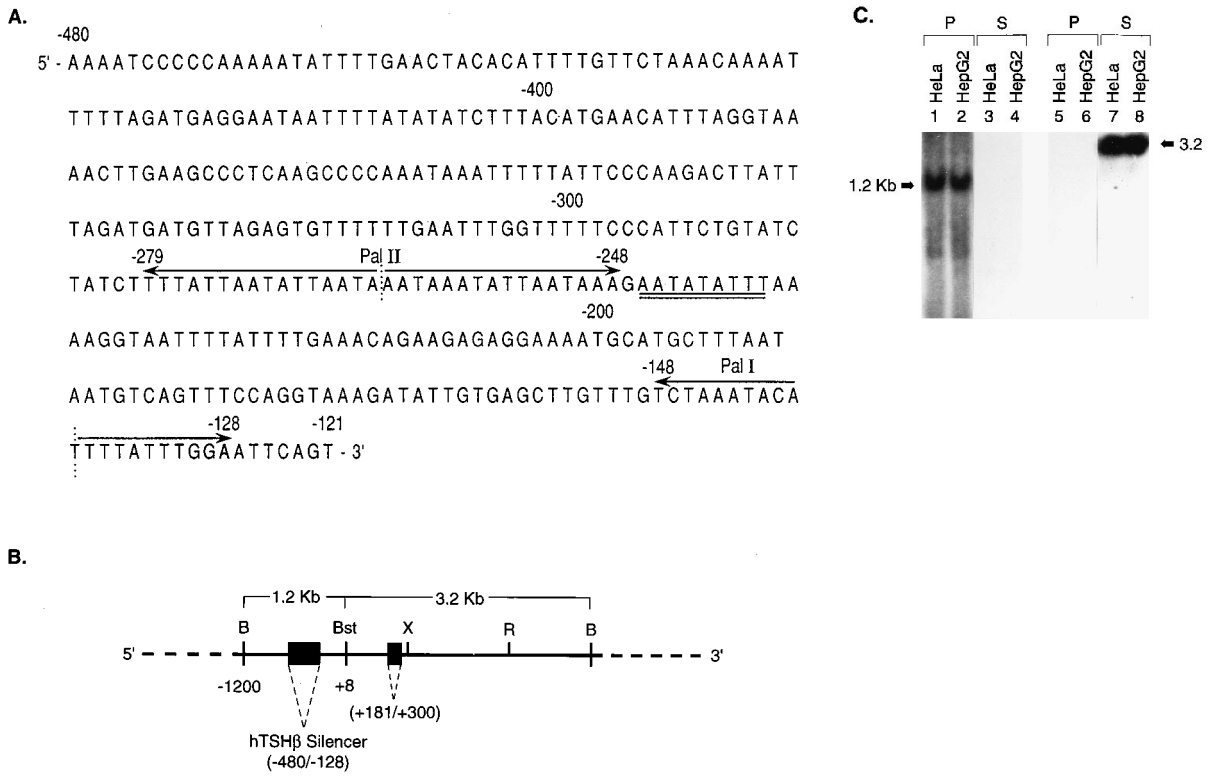

FIG. 6. The hTSHβ silencer element is attached to the nuclear matrix in vivo. (A) DNA sequence of the hTSHβ silencer element. A/T-rich sequences (Pal I and Pal II) are indicated. In the sequences indicated by arrows, the matching bases are separated by dots (Pal I and Pal II contain 80 and 88% matching sequences, respectively). Although these sequences are not perfect palindromes, for the sake of simplicity, they were called Pal I and Pal II in this study. (B) Restriction map of the genomic fragment surrounding the hTSHβ silencer region (67). B, *Bam*HI; Bst, *Bst*EII; X, *Xba*I; R, *Eco*RI. The distance between two *Bam*HI sites was measured by Southern blot analysis with Human Geno Blot (Clontech, Palo Alto, Calif.) probed with the labeled hTSHβ −480/−128 fragment. The 1.2-kb (5′ *Bam*HI-3′ *Bst*EII) fragment and the 3.2-kb (5′ *Bst*EII-3′ *Bam*HI) fragment detected in panel C and the positions of the probes (−480/−128 and +181/+300) are indicated. (C) Identification of the hTSHβ silencer element as a MAR in vivo. DNAs in LIS-treated nuclei from HeLa and HepG2 cells were digested with *Bam*HI and *Bst*EII and centrifuged to separate nuclear matrix-associated (P) and unattached (S) sequences. DNAs obtained from identical numbers of nuclei were run on an agarose gel and hybridized to a probe for hTSHβ −480/−128 (lanes 1 to 4) or +181/+300 (lanes 5 to 8).

nuclei are not stabilized prior to LIS extraction, most of the nuclear matrix proteins were shown to be solubilized, and a nuclear shell enriched with lamins was obtained (4, 32). The required heat stabilization step in the LIS procedure may cause some nuclear proteins to be insoluble. In order to determine whether the apparent association of Oct-1 with the LIS-extracted nuclear matrix (Fig. 7A) is temperature dependent, we prepared nuclear matrix by the salt extraction method (18), which does not require heat stabilization. As shown in Fig. 7B, lane 1, the Oct-1-specific band was found in the salt-extracted nuclear matrix pellet, indicating that the nuclear matrix–Oct-1 association is not restricted to heat-stabilized nuclei. We found that regardless of the extraction procedures, 15 to 23% of Oct-1 is retained in the nuclear matrix preparations as measured by densitometric analysis, whereas over 70% is present in the soluble fraction (data not shown). Previous studies have shown that further extraction of the salt-extracted nuclear matrix complex with 2 M NaCl removes most of the matrix proteins (18). The association of Oct-1 with the nuclear matrix was stable upon a stepwise NaCl extraction up to 500 mM (Fig. 7B, lanes 2 to 4). Most of the matrix-associated Oct-1 was solubilized at 2 M NaCl (lane 5), but a small amount of Oct-1 remained associated even after 2 M NaCl extraction (lane 6).

We then examined the nuclear fractionation pattern of the basal transcription factor TBP following the LIS extraction procedure and compared it with that of Oct-1 (Fig. 7C). TBP is an another ubiquitously expressed, abundant DNA-binding protein in mammalian nuclei that also recognizes A/T-rich TATA box sequences. The TBP-specific band was detected only in the whole-cell lysate (lane 1) and the soluble LIS supernatant (lane 2). The absence of TBP in the insoluble pellet (lane 5) argues against the possibility that the Oct-1 retention in the LIS-extracted nuclear matrix is simply due to contamination with abundant soluble Oct-1. In addition, when the heat stabilization step was omitted to obtain nuclear lamina (4, 32), Oct-1 was not detected (Fig. 7D, lane 2).

Taken together, the results shown in Fig. 7 indicate that Oct-1 in the interphase HeLa nuclei exists in both the soluble nuclear fraction and the insoluble nuclear matrix pellet but not in the nuclear lamina. The nuclear matrix–Oct-1 association was not specific for HeLa cells, because it was also detected in similar preparations from HepG2 cells (data not shown). Overall, these findings are consistent with the possibility that Oct-1 might mediate the hTSHβ MAR-nuclear matrix interaction.

DISCUSSION

The ubiquitous POU homeoprotein Oct-1 activates a number of widely expressed genes and is suggested to play a role in DNA replication in mammalian cells. In this paper, we report novel properties of Oct-1 in gene regulation. We found that the A/T-rich element in the hTSHβ gene (−480/−128), which consists of multiple degenerate Oct-1-binding sites, silences

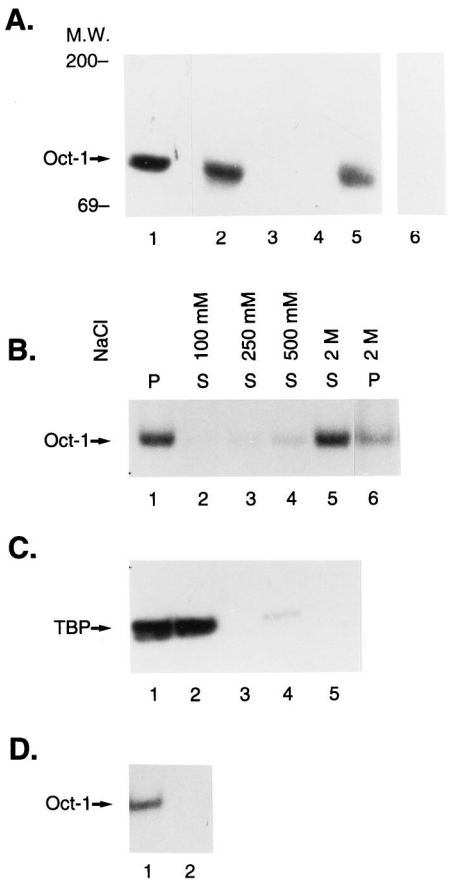

FIG. 7. Oct-1 exists in both the soluble and insoluble nuclear fractions. (A) Immunoblot of HeLa nuclear fractions separated by SDS–8% polyacrylamide gel electrophoresis with anti-Oct-1 antibody (Santa Cruz). HeLa nuclei were subjected to the LIS extraction procedure (32). Protein samples obtained from same number of cells (5×10^6) were loaded in each lane. Lane 1, whole-cell lysate; lane 2, supernatant following the first wash with the LIS extraction buffer; lane 3, supernatant after hypotonic buffer wash. The resulting nuclear pellet was either subjected to further digestion with DNase I (600 μg/ml) (lane 4) or directly analyzed (lane 5). Lane 6, immunoblot of the same sample as in lane 5 in the presence of excess of the immunizing Oct-1 peptide (Santa Cruz). (B) Exposure of the salt-extracted HeLa nuclear matrix preparation (18) to increasing NaCl concentrations. Oct-1 released in the supernatant (S) (lanes 2 to 5) at 100, 200, and 500 mM and 2 M NaCl or remaining in the pellet (P) (lane 1, original pellet; lane 6, after 2 M NaCl wash) was detected by immunoblotting. (C) Immunoblot of samples similar to those used for panel A separated by SDS–12% polyacrylamide gel electrophoresis with anti-TBP antibody (Santa Cruz). Only one band corresponding to the molecular mass of TBP (36 kDa) was observed. Lane 1, whole-cell lysate; lane 2, LIS supernatant; lane 3, hypotonic buffer wash; lane 4, supernatant following redigestion with DNase I; lane 5, insoluble nuclear pellet. (D) Immunoblot of the LIS-extracted insoluble nuclear pellet from heat-stabilized (lane 1) or nonstabilized (lane 2) HeLa cell nuclei.

the tissue-specific hTSHβ gene. By demonstrating that the C-terminal alanine-rich region of Oct-1 fused to the GAL4 DNA-binding domain has the silencing activity, this study suggests, for the first time, that Oct-1 is a promoter-specific factor with dual regulatory functions (activator and silencer). We also found that this A/T-rich hTSHβ silencer element is attached to the nuclear matrix of human cells in vivo. By showing that a significant fraction of Oct-1 is retained in the nuclear matrix preparation, this study suggests that Oct-1 is not only a transcription factor but also a nuclear matrix protein that binds to MARs. Oct-1, therefore, is the first example among the MAR-binding proteins that mediates the promoter-specific activation and silencing. Although the physiological meaning of such colocalization of the silencer element and MARs remains unknown, our findings suggest a possible involvement of the nuclear matrix in hTSHβ gene regulation.

Oct-1-mediated silencing of the hTSHβ gene. The 5′ flanking regions (−520/+8) of the TSHβ promoters in humans, mice, and rats show an overall sequence identity of 70% among all three species, with the mouse and rat sequences having the greatest identity (98%). Interestingly, however, silencing has not been reported to occur in mouse and rat TSHβ gene regulation. It was previously shown that upstream regions of the mouse and rat TSHβ genes that roughly correspond to the hTSHβ silencer element activated heterologous promoters such as those of Rous sarcoma virus or thymidine kinase (TK) in transient-transfection assays with TtT-97 or pituitary cells (34, 48, 69), and they therefore were labeled enhancers. Despite the sequence homology between the mouse and rat genes, the localizations of such enhancers did not coincide. Furthermore, the murine element (−271/−117) that conferred activation to the Rous sarcoma virus promoter failed to show any enhancing activity in the context of the homologous promoter in TtT-97 cells (69). When tested on the homologous promoter, however, the proximal −133/−100 region was shown to be important for basal promoter activity and Pit-1-dependent activation (17, 31). The activity of the rat enhancer elements (34, 48) in the context of the homologous promoter remains unknown.

Cloning of the 80- to 100-kb genomic DNA fragment covering the hTSHβ locus allowed us to examine hTSHβ regulation in the context of a large portion (−~10K/+8) of the regulatory sequences in both the TSH-producing TtT-97 cells and non-TSH-producing cell types. In this study, using transient-transfection assays, we showed that the hTSHβ −480/−128 region that corresponds to the labeled enhancer elements in the mouse and rat genes exerts a silencing activity. This element is composed of multiple degenerate Oct-1-binding sites, and the silencing of the hTSHβ promoter by a chimeric protein containing the Oct-1 alanine-rich domain and the heterologous GAL4 DBD (Fig. 5) suggests that Oct-1 plays a role in silencing of this gene. Although multiple silencer elements appear to be present within the tested −~10K/+8 region, the Oct-1-mediated silencing was overcome in a thyrotroph-specific manner in the presence of a yet-unidentified enhancer located in the −~10K/−1200 region. Since Oct-1 is expressed before the anterior pituitary development (19), the Oct-1-mediated silencing likely plays a role in preventing inappropriate expression of the hTSHβ gene. Our results indicate that the A/T-rich hTSHβ −480/−128 sequences could be a target for many cell- or stage-specific POU proteins such as Pit-1 (Fig. 3) or for homeoproteins that would promote hTSHβ gene expression. In this way, this element may play a pivotal role in both tissue-specific inactivation and activation of this gene in a stage- and cell-specific manner.

In support of this view, previous transgene studies showed that a reporter gene containing hTSHβ −125/+37 was expressed in all tissues (66). However, the reporter gene under the control of the −1192/+37 promoter was expressed in pituitary and muscle. Although the authors did not discuss the silencing effect (66), these transgene data argue that the −1192/−125 region, which includes the hTSHβ silencer described in the present paper, silences the transgene expression in most nonpituitary cells. However, the same region fails to either silence or activate expression in pituitary and muscle. In another transgene study (33), mice that express the simian virus 40 large T antigen under the control of the −1109 hTSHβ promoter developed undifferentiated pituitary tumors that do not express TSHβ. Although the silencing effect mediated by

the −1109/−125 region could not be observed in that study (33) without using the constitutive −125 promoter, both studies argue that the overlapping −1109 promoter region is sufficient for expression in undifferentiated pituitary. Using transient-transfection assays, however, we and others (10, 42) were unable to detect Pit-1-mediated activation of the −1200/+8(+37) hTSHβ promoter in either nonpituitary cell types or fully differentiated GH_3 cells, which express growth hormone. It is possible that such pituitary-specific expression was not detected in transient-transfection assays because it requires a chromatin context, a nuclear matrix interaction, or factors present only in the undifferentiated pituitary. Nevertheless, both the positive and negative regulation by the −1109 sequences are not sufficient for the thyrotroph-specific expression, which is in agreement with the results shown in Fig. 1A. Although it remains to be seen whether the same regulation pattern can be observed in the human thyrotrophs, an unknown enhancer located in the −~10K/−1200 sequences activates the hTSHβ gene in a thyrotroph-specific manner by overcoming the Oct-1-mediated silencing.

Alanine-rich domain of Oct-1 possesses a silencing activity. It has been shown that the promoter-specific activation by Oct-1 is conferred by promoter-selective activation domains. The N terminus of Oct-1, like the activation domains in Sp1 (8) and Oct-2 (54), is glutamine rich and is thought to activate certain mRNA promoters (54). When Oct-1 stimulates the TATA-less U2 small nuclear RNA promoter, however, the region C terminal to the POU domain plays a major role as an activation domain (55).

In this paper, we suggest that Oct-1 plays a role in silencing the hTSHβ gene through the C-terminal alanine-rich domain. Therefore, Oct-1 may be a promoter-specific transcription factor with dual functions for the regulation of mRNA promoters. Other transcription factors, such as the yeast factor RAP-1 and the thyroid hormone receptor, have been reported to function as both a silencer and an activator. Similar to Oct-1, RAP-1 is a promoter context-dependent regulator that contains independent activation, silencing, and DNA-binding domains (47). In the absence of thyroid hormone, the thyroid hormone receptor silences the target genes, and in the presence of hormone, it is thought to adopt a conformational change to activate the promoters (2). In addition to Oct-1, the repressor domains of all three known *Drosophila* homeoprotein repressors, Krüppel, Even, and En, are alanine rich (15, 16, 30). The repressor domain of Dr1, which binds TBP and prevents the interaction of TFIIA and TFIIB with TFIID, is also alanine rich (70).

Promoter specificity of Oct-1. Because a loose covalent linker connects two DNA-binding modules in the POU domain, Oct-1 shows flexibility in binding site recognition. Depending on the structure of its binding sites, the POU domain adopts different conformations, and as a result, different cofactors are recruited to Oct-1 (64). These cofactors may selectively modulate transcription domains (activation and silencing), which might be one of the mechanisms of Oct-1 promoter specificity. In addition, it has been recently shown that the core promoters recruit different sets of TBP-associated factors, depending on the structure of the promoter (57). The transcriptional machineries on the respective core promoters can thus respond differently to the upstream transcription factors. The overall promoter specificity of Oct-1 may then be determined by parameters such as the binding-site-induced conformation of Oct-1 and its interaction with other factors recruited onto the promoter, including those in the core promoter-specific initiation complexes.

The influence of the Oct-1-binding site and promoter on the function of Oct-1 may be seen through the following examples. Compared with the strong silencing activity shown by the native hTSHβ silencer Pal I and Pal II elements (Fig. 4), the IgH E fragment, which contains a higher-affinity Oct-1 binding site (Fig. 4), conferred a weaker silencing activity on the hTSHβ promoter. On the other hand, the hTSHβ Pal I and Pal II subfragments did not silence the enhancerless herpes simplex virus TK promoter (−105/+51) (data not shown). Unlike the A/T-rich hTSHβ promoter, the TK promoter (−105/+51) is G/C rich and recruits Sp1 and CTF/NF-1 (23, 35) to the upstream sites. Although it remains to be studied, the composition of the initiation complex on the TK promoter might also be different from that on the hTSHβ promoter. Oct-1 bound to the hTSHβ silencer may distinguish these two promoters by differential protein-protein interactions.

We found that when connected to the TK promoter, various elements containing the Oct-1-binding sites derived from the hTSHβ silencer DNA not only fail to silence the TK promoter but also mediate Pit-1-dependent activation (25a). Such promoter specificity might be one of the reasons why silencing has not been reported for murine and rat TSHβ gene regulation.

Possible structural role of Oct-1. Oct-1 and other POU homeoproteins, like the high-mobility group (HMG) proteins, bind to and bend the minor groove of the A/T-rich DNA (14, 27, 37, 59). Several HMG proteins may even wrap the DNA into a compact structure (65). It is interesting that the N-terminal arm of the POU homeodomain that makes minor groove contact shows sequence similarity to the N-terminal region of the DNA-binding HMG domain (37). Indeed, the POU domain interaction with HMG-I/Y and HMG 2 has been demonstrated for Oct-1 and other POU proteins (29, 73), suggesting the possibility that similar interaction might play a role in the hTSHβ gene regulation by modulating the promoter architecture. Since Oct-1 (or Pit-1 and perhaps other POU proteins as well) binds the A/T-rich hTSHβ −480/−128 element at multiple sites, it is possible that higher-order nucleoprotein complexes may be formed upon subsequent DNA bending by these proteins. The putative higher-order structure might facilitate an efficient interaction between the factors bound to the A/T-rich element and the basal transcription machinery.

The results obtained in this study suggest that hTSHβ gene regulation may be modulated by the nuclear matrix. The nuclear matrix contains factors for DNA and RNA metabolism and is thought to provide an architecture that organizes transcription, splicing, and DNA replication by localizing factors (49). The possible MAR-mediated chromatin loop formation through the A/T-rich hTSHβ −480/−128 element may bring distant regulatory elements together to modulate basal activities. This type of structural arrangement could be flexible, depending on the cell types and the developmental stages to serve the changing needs in the cell.

Pleiotropic effects of Oct-1. Oct-1 plays multiple roles in the cell, as a positive or negative regulator of gene transcription and DNA replication. As has been suggested for other nuclear matrix proteins that bind to MARs, the hTSHβ MAR–silencer-binding activity and the nuclear matrix association of Oct-1 shown in this study suggest that this protein may also play a role in anchoring chromatin loops to the nuclear matrix. Although the identity of the nuclear matrix structural protein with which Oct-1 interacts remains to be studied, the nuclear retention of Oct-1 may be critical for transcription, DNA replication, and possible chromatin loop formation. By binding to the hTSHβ silencer element that interacts with the nuclear matrix, the multifunctional factor Oct-1 may be able to coor-

dinate transcriptional silencing, chromatin assembly, and/or DNA replication at the hTSHβ locus.

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