Silencing of the Gene for the \( \beta \) Subunit of Human Chorionic Gonadotropin by the Embryonic Transcription Factor Oct-3/4*

(Received for publication, February 21, 1996, and in revised form, April 25, 1996)

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The transcription factor Oct-3/4 may be important in maintaining embryonic cells in an undifferentiated state. It is probably down-regulated at about the time that human chorionic gonadotropin (hCG) is first expressed in embryonic trophoderm. Here we report that Oct-3/4 strongly inhibits the hCG \( \beta \) subunit (hCG\( \beta \)) promoter in JAR choriocarcinoma cells. Oct-3/4 reduced chloramphenicol acetyltransferase (CAT) reporter expression from the \(-305\)hCG\( \beta \) promoter by about 90% in transient co-transfection assays, but had no effect on expression from the \(-249\)hCG\( \beta \) promoter. The \(-305/ -249\) hCG\( \beta \) fragment specifically bound synthetic Oct-3/4 protein as measured in electrophoretic mobility shift assays, and the Oct-3/4-binding site was localized around \(-270\) by methylation interference footprinting. Site-directed mutagenesis of this binding site abolished Oct-3/4 repression. When stably transfected into JAR cells, Oct-3/4 reduced the amounts of both endogenous hCG\( \beta \) messenger RNA and hCG protein to less than 10% of controls. We suggest that silencing of Oct-3/4 in trophoderm is a prerequisite for hCG up-regulation in early human embryos at the time of maternal recognition of pregnancy.

Human chorionic gonadotropin (hCG)\(^1\) is crucial for preventing regression of the corpus luteum during early pregnancy. It is first secreted by trophoderm, the precursor cell layer of the placenta, as the blastocyst forms and begins to implant (1-3). The timing and quantity of hCG release are considered to be key factors in determining whether a human pregnancy succeeds or fails (4-6). hCG is a heterodimer containing an \( \alpha \) subunit (hCG\( \alpha \)), common to all the glycoprotein hormones, and a distinct \( \beta \) subunit (hCG\( \beta \)) responsible for the biological specificity of the hormone. Whereas there is only a single gene for the \( \alpha \) subunit, there are six hCG\( \beta \) subunit genes or pseudogenes (7, 8). Of the latter, hCG\( \beta 5 \) is the one expressed predominantly in placenta and choriocarcinoma cells (7, 9).

Most studies on the control of expression of the hCG genes have concentrated on their transactivation. The upstream promoter region of the hCG\( \alpha \) gene includes two tandem repeats of a cyclic AMP response element (CRE), a complex upstream regulatory element (URE), the \( \alpha \)-activator element, the junctional regulatory element, and the CCAAT region (10-16). Although much less studied, the hCG\( \beta 5 \) gene also contains multiple regulatory regions that contribute toward expression in choriocarcinoma cells (17-20). These elements include several within the \(-310\) to \(-200\) region and further ones more than 1 kilobase upstream of the transcription start site. Although expression of hCG has been studied extensively, little is known about what controls its onset at a time when the corpus luteum must be rescued if the pregnancy is to proceed.

The transcription factor Oct-3/4, characterized by its conserved POU DNA-binding domain, is a strong candidate for a regulator of early embryogenesis (21-25). It is expressed in totipotent/pluripotent embryonic cells and germ cells and in undifferentiated embryonic stem cells and embryonal carcinoma cells, but is rapidly down-regulated when these cells differentiate. Fusion of embryonal carcinoma cells and fibroblasts results in loss of Oct-3/4 expression and neuronal differentiation of the hybrid cells, while introduction of Oct-3/4 transactivating function back into such hybrid cells causes partial dedifferentiation (26). The expression pattern of Oct-3/4 and its correlative relationship with cell pluripotency suggest that Oct-3/4 may be important in maintaining cells in an undifferentiated state and that silencing of its expression could contribute to the process of differentiation. No natural target genes for Oct-3/4 have been unequivocally identified, and it remains unclear whether Oct-3/4 is an activator or repressor of gene expression (27-30).

During the study of the transcriptional regulation of a trophoblast interferon gene (IFNT) (31) in choriocarcinoma cells, an hCG\( \alpha \)-CAT construct was included as an internal control in transient transfection experiments and, surprisingly, was found to be completely silenced by Oct-3/4 co-transfection.\(^2\) Both the Oct-3/4 messenger RNA (mRNA) and the protein have been detected in early stage trophoderm but not in trophoblast cells after the blastocyst has hatched from the zona pellucida in mouse (22, 23, 25). The down-regulation of Oct-3/4 in the human embryo, therefore, probably coincides with the first appearance of hCG in trophoderm. Here we demonstrate that Oct-3/4 is an inhibitor of hCG\( \beta \) expression in JAR human choriocarcinoma cells and suggest that the loss of Oct-3/4 expression in developing trophoderm may be a prerequisite for the onset of hCG expression.

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*This work was supported by Grants R37 HD21896 and HD29483 from the National Institutes of Health (to R. M. R.) and a fellowship from the National Institutes of Health (to R. M. R.) and a fellowship (27-30).

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1 The abbreviations used are: hCG, human chorionic gonadotropin; CRE, cAMP response element; URE, upstream regulatory element; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay.
**Experimental Procedures**

Construction of Plasmids—CAT expression plasmids p-305hCGβ-CAT, p-279hCGβ-CAT, and p-249hCGβ-CAT were provided by Dr. Pamela L. Melkonian (19). Oligonucleotide primers μ-305hCGβ and CATr (Table I) were used in conjunction with p-305hCGβ-CAT to produce the mutant μ-305hCGβ-CAT fragment by PCR. The PCR product was digested with XbaI and XhoI. This fragment (μ-305/6 hCGβ) was used to replace its wild type hCG counterpart in p-305hCGβ-CAT to form the pm-305hCGββ-CAT plasmid.

The Oct-3/4 coding region was synthesized from the pCMV-Oct4 expression plasmid (23) by PCR with the primers Oct45 a and Oct4b (Table I). The PCR product was digested with SalI and BglII, blunted, and cloned into the KpnI site of pDNA3 (Invitrogen) via blunt-end ligation. The hCGβ-CAT and pDNA3-Oct4 constructs were confirmed by DNA sequencing.

Plasmids p0GH, pTKGH, and pXGH5 were purchased from Nichols Institute Diagnostics, San Juan Capistrano, CA. Expression plasmid p0GH was provided by Dr. Pamella L. Mellon (19). Oligonucleotide primers Oct45 a and Oct4b (Table I) were used in conjunction with p0GH, pTKGH, and pXGH5 constructs to produce an80-fold lower specific activity (by diluting the original 3000 Ci/mmol, DuPont NEN). Antisense human β-actin-125-human antisense control template (Ambion, Austin, TX). Expression of the Oct-3/4 messenger RNA in these cells was tested by Northern blot analysis (34) with a 32P-labeled mouse Oct-3/4 cDNA probe.

**RESULTS**

Inhibition of hCGβ-CAT Expression by Oct-3/4—To determine whether Oct-3/4 influences hCGβ gene transcription, the promoter of hCGβ5 gene was fused to the CAT reporter, and the resulting plasmid (p-305hCGββ-CAT) was co-transfected into JAR cells with the Oct-3/4 expression plasmid, pCDNA-Oct3-3′, in which the coding region of a murine Oct-3/4 cDNA had been placed under the control of the cytomegalovirus promoter (Fig. 1). CAT expression was markedly reduced compared to values obtained when the control plasmid vector (pCDNA3) was the co-transfection partner. By contrast, when p-305hCGββ-CAT was co-transfected with an Oct-2 expression plasmid, CAT expression was not affected (94% ± 17% of the control value).
Human growth hormone expression driven either by a viral thymidine kinase promoter or by a mouse metallothionein-I promoter (PTKGH and pXGH5, respectively) was not affected by pcDNA3-Oct-3/4 co-transfection (data not shown). In all subsequent experiments, pTKGH was used as the internal control to normalize transfection efficiencies.

As expected (19), 8-Br-cAMP increased CAT expression from the −279 to 249 hCGβ promoter approximately 2-fold. It did not, however, affect repression by Oct-3/4 (12.8 ± 2.4% expression control in presence of 8-Br-cAMP, 11.9 ± 2.0% in its absence). hCGβ promoters with 5′ truncations were employed to define the region that responded to Oct-3/4. Expression of CAT from the shortest construct (−249 to 275 hCGβ-CAT) was unchanged in the presence of pcDNA3-Oct-3/4, while expression from −279 to 305 hCGβ-CAT was reduced to about 40% of control values (Fig. 1). Clearly, sequences within the −305/-294 promoter region were responsive to Oct-3/4 and probably included positions 268-279 clearly bound Oct-3/4 less well than positions 266-269.

Binding of Oct-3/4 to the hCGβ Promoter in Vitro—Oct-3/4 protein (Fig. 2A), produced by coupled in vitro transcription and translation in a reticulocyte lysate, was able to interact with the 32P-labeled −305/-249 hCGβ promoter fragment in an electrophoretic mobility shift assay (Fig. 2B, lane 2). This complex was abolished when excess unlabeled −305/-249 hCGβ fragment was added to the reaction mixture (data not shown) or when an oligonucleotide competitor (OCT) that contained the consensus octamer motif was present (Fig. 2B, lane 3). The formation of the complex was unaffected by an excess of unrelated oligonucleotide (not shown). In another set of electrophoretic mobility shift assays, radioactive Oct-3/4, which had been synthesized in the presence of L-[35S]methionine, was used in combination with various non-radioabeled DNA fragments (Fig. 2C). A single band of 35S-Oct-3/4 was observed (lane 1) when it was incubated with poly(dI-dC) before electrophoresis. No additional labeled bands were observed when either the −249 to 305 hCGβ or −60 to 44 hCGα fragments were included in the reaction mixture (lanes 4 and 5). In contrast, a faster moving 35S-Oct-3/4 complex appeared when either OCT or the −305/-249 hCGβ fragment were used (lanes 2 and 3). These observations confirm that Oct-3/4 could bind the −305/-249 hCGβ promoter region directly. As expected, Oct-3/4 also bound to a 32P-labeled OCT oligonucleotide (Fig. 2D), and this binding was reduced in the presence of unlabeled −305/-249 hCGβ (lane 4), but not by an unrelated oligonucleotide (lane 3). Clearly, an Oct-3/4 binding site was present in the −305/-249 region of the promoter.

Methylation interference analysis was employed to define the binding site for Oct-3/4 on −305/-249 hCGβ more precisely (Fig. 3A). Probe that had been methylated in the antisense strand at −276 or −269 clearly bound Oct-3/4 less well than probe that had not been methylated at those positions. The −275/-268 region (Fig. 3B) was identical at seven nucleotides out of eight of the optimal POU5 motif, to which the POU-specific domain of Oct-1 would bind (39). Methylation of the sense strand of −305/-249 hCGβ did not interfere with Oct-3/4 binding (data not shown).

Mutation of the Oct-3/4 Binding Site Negates Repression of the hCGβ Gene Promoter by Oct-3/4—When the sequence 5′-AATT (−272 to −269) within the Oct-3/4 binding region of the hCGβ promoter was mutated to 5′-ccag, CAT expression from this mutant promoter was not significantly different from that observed from the nonmutated −305 hCGβ promoter in the absence of Oct-3/4 co-transfection (Fig. 4). However, in contrast to the strong Oct-3/4 inhibition of −305 hCGβ (11.9% of the control), CAT expression from the −305 hCGβ promoter was only slightly decreased (73.4% ± 15 of the control and statistically nonsignificant) when pcDNA3-Oct-3/4 was co-transfected. Therefore, the Oct-3/4 binding site identified in vitro was critical for the ability of this transcription factor to repress the activity of the hCGβ promoter in J Ar cells.

Inhibition of Endogenous hCG Production in J Ar Cells by Oct-3/4 Stable Transfection—To study the effect of Oct-3/4 on endogenous hCGβ gene expression, J Ar cells were transfected with pcDNA3-Oct-3/4. Stably transfected cells were selected by antibiotic G418 and clonally propagated. Such cells expressed Oct-3/4 mRNA, whereas control J Ar cells did not (Fig. 5A). They did not differ morphologically from either wild type cells or from cells that had been stably transfected with the pcDNA3 vector lacking the Oct-3/4 gene. No differences were detected in the rates of protein synthesis (as assessed by incorporation of label from [35S]methionine over 24 h of culture), and analysis of the radioabeled proteins in the medium by one-dimensional SDS-PAGE could not distinguish the transfected and control cells (data not shown). It should be emphasized that the subunits of hCG are produced in such small amounts that they could not be readily detectable by this procedure. The conclusion drawn from these experiments was that stable transfection with Oct-3/4 had no major effect on the phenotype of J Ar cells.

RNA was isolated from both stable Oct-3/4 clones and stable control clones and subjected to a ribonuclease protection assay in the presence of an antisense hCGβ RNA probe expected to hybridize to the first 389-bp part of the coding region of all hCGβ transcripts (7, 32, 37). As shown in Fig. 5B, hCGβ mRNA was barely detectable in either of the stable Oct-3/4 clones tested (S1 and S4) but was present in both control clones (C1 and C2). The content of β-actin mRNA was comparable among all clones, whether they expressed Oct-3/4 or not. When quantitated by densitometry and normalized to β-actin mRNA, the hCGβ mRNA content of the clones expressing Oct-3/4 was about 6% of that in the controls (Fig. 5C).

Production of hCG protein, as determined by a radioimmunoassay specific for the hCGβ subunit, was markedly reduced in clones expressing Oct-3/4 (Fig. 6). For clones S1 and S4, the amount of hCGβ was 8.3% and 3.2%, respectively, of that produced by two control lines C1 and C2.
Here we have demonstrated the transcription factor Oct-3/4 to be a potent repressor of hCGβ gene expression in JAr choriocarcinoma cells. Stable expression of Oct-3/4 reduced the amounts of both endogenous hCGβ messenger RNA and hCGβ protein by over 90% in these cells. Oct-3/4 also strongly inhibited reporter expression from the hCGβ5 gene promoter in transient transfection assays. Furthermore, an Oct-3/4 binding site was present in the hCGβ promoter and was necessary for Oct-3/4 inhibition.

Oct-3/4 can specifically bind to the hCGβ5 promoter in vitro. When the Oct-3/4 binding site in the hCGβ promoter was mutated or deleted, Oct-3/4 repression was lost. This Oct-3/4 binding site (−275/−268; AATAATCA) differed markedly from the previously described octamer consensus sequence (ATG-CAAAT) (21, 38). Despite its unconventional sequence, this site is identical at seven out of eight nucleotides to the motif described as optimal for binding of the Oct-1 POU-specific domain (POU1) (39) and is placed just one base pair upstream from a stretch of six A/T nucleotides (Fig. 3B) that might be capable of interacting with the POU homeodomain (POU1D) of Oct-3/4. It has been found that the arrangement of the binding sites for
the POU\textsubscript{5} and POU\textsubscript{H4} critically influences the orientation of the two DNA binding domains to each other and to the promoter (40). The \(-277/-268\) region (TCAATACTCA) of the hCG\textsubscript{5} promoter is also identical at seven out of ten nucleotides to a weak Oct-3/4 binding sequence (TTAAAATTCA) described by Okamoto et al. (21). This sequence and the consensus ATGCAAAT motif have each been found in two enhancer-promoter units of the mouse genome that are active in undifferentiated P19 embryonal carcinoma cells but inactive in differentiated P19 cells (41). As indicated by competition experiments, Oct-3/4 may possess lower affinity for the recognition sequence in the hCG\textsubscript{5} promoter than it does for the consensus motif (Fig. 2), but it is not uncommon for a low affinity binding site to confer regulatory activity as effectively as one of high affinity (42). In addition, the POU transcription factor Pit1 binds well to the octamer consensus motif (ATGCAAAAT), but fails to transactivate promoters containing that motif (42). Such a consensus sequence is not present in any of the known genes activated by Pit1 (43).

It seems unlikely that Oct-3/4 merely competes for binding with some transcriptional activator whose response element overlaps the Oct-3/4 binding site in the hCG\textsubscript{5} gene. If such were the case, expression from \(-305\)hCG\textsubscript{5}-CAT after Oct-3/4 co-transfection would be anticipated to be at least as high as from \(-249\)hCG\textsubscript{5}-CAT, a construct from which the response element for the putative activator had been deleted. Instead, expression from the \(-305\) construct was much lower than from \(-249\)hCG\textsubscript{5}-CAT in the presence of Oct-3/4 but about the same in its absence (Fig. 1). Oct-3/4, therefore, seems to have an intrinsic ability to repress the hCG\textsubscript{5} promoter. It is unclear whether once bound it directly inhibits the general transcriptional machinery or whether it recruits some other inhibitory factor. Repression of herpes simplex immediate-early promoter by neuronal forms of Oct-2 probably occurs through such a secondary recruitment process (44), and there are several other examples where POU domain proteins function cooperatively with other proteins to regulate transcription (45, 46).

All functional hCG\textsubscript{5} genes possess TATA-less promoters (18), and it could be for this reason that the hCG\textsubscript{5} gene tested here was repressed by Oct-3/4 while another octamer-containing promoter, the one for thymidine kinase, which possesses a conventional TATA box (47), was not affected by Oct-3/4 co-transfection. Some special transcription factor required specifically for proper functioning of such TATA-less promoters (48, 49) could be the target of Oct-3/4 inhibition.

Oct-3/4 dramatically reduced overall expression from the endogenous hCG\textsubscript{5} genes of JAr cells. The mRNA for hCG\textsubscript{5} accounts for about 64% of the total hCG\textsubscript{5} mRNA in first-trimester placenta, whereas hCG\textsubscript{3} and hCG\textsubscript{8} each accounts for about 18% (7). Expression levels of the HCG\textsubscript{5} genes in chorionicarcinoma cells are probably similar to those in placenta (7, 9). Therefore, ectopic expression of Oct-3/4 seems likely to inhibit expression not only from the HCG\textsubscript{5} gene but from other HCG\textsubscript{5} genes as well.

The association of Oct-3/4 expression with the totipotent/pluripotent state of cells has been a subject of considerable speculation, but its target genes have not been unequivocally identified, and whether it is an activator or repressor of gene

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**Fig. 3.** Methylation interference footprint of the hCG\textsubscript{5} promoter in presence of Oct-3/4. A, the \(-305/-249\)hCG\textsubscript{5} fragment was radiolabeled at the 5' end of its antisense (bottom) strand and partially methylated at guanines. The free (F) probes and the Oct-3/4-bound (B) probes were cleaved at the methylated sites and resolved on an 8% sequencing gel. The sites where methylation apparently interfered with Oct-3/4 binding are indicated with asterisks. B, the hCG\textsubscript{5} promoter sequence around the methylation interference sites. The boxed region represents a motif with close similarity (7 nucleotides out of 8) for the optimal binding sequence of the POU-specific domain (POU\textsubscript{5}) established for Oct-1 (39).

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**Fig. 4.** The binding site on the hCG\textsubscript{5} promoter is critical for Oct-3/4 to repress the promoter. The \(-305\)hCG\textsubscript{5}-CAT and \(\mu\)-305hCG\textsubscript{5}-CAT constructs are shown on the left panel. The likely POU\textsubscript{5} binding motif is boxed; mutant substitutions in \(\mu\)-305hCG\textsubscript{5}-CAT are denoted by the lowercase letters. CAT expression from the \(\mu\)-305hCG\textsubscript{5}-CAT obtained in the presence of Oct-3/4 co-transfection do not differ significantly \((p = 0.10)\) from that obtained in the absence of Oct-3/4 co-transfection. The results are the means \((\pm S.E.)\) of six independent transfection experiments.
expression remains unclear (27–30). For example, although Oct-3/4 has recently been shown capable of activating transcription from an enhancer of the fibroblast growth factor 4 Oct-3/4 only coincides in the early stages of mouse embryo development prior to gastrulation (22, 23, 50).

Fig. 5. Decrease of endogenous hCGβ mRNA levels in JAr cells following Oct-3/4 stable transfection. A, Northern blot analysis of Oct-3/4 expression (upper panel). Lane 1 (∼6.8 μg) and lane 2 (∼20 ng) contain partially purified poly(A) RNA from the stable clone S4 that had been transfected with pcDNA3-Oct-3/4. Lane 3 (∼2 μg) contains partially purified poly(A) RNA from normal J Ar cells that had not been transfected with any plasmid. The lower panel is a portion of the ethidium bromide-stained gel showing the 28 S rRNA that was present in the preparation. It is only evident in the two heavily loaded lanes. B, ribonuclease protection assays were carried out as described under “Experimental Procedures” to determine relative amounts of hCGβ mRNA in stable J Ar clones. Clones S1 and S4 had been stably transfected with pcDNA3-Oct-3/4, C1 and C2 with pcDNA3. The protected hCGβ fragment and the internal β-actin control are indicated by arrows. The signals were then quantitated by densitometry. The exposure time used to obtain appropriate optical densities in x-ray film was 30 min for β-actin, and the densitometric values were 0.51 (C1), 0.21 (S1), 0.73 (C2), and 0.46 (S4), respectively. The exposure times for hCGβ were 2 h in C1 and C2 and 5 h in S1 and S2. The densitometric values were 0.82 (C1), 0.04 (S1), 0.85 (C2), and 0.08 (S4), respectively. All hCGβ values were then normalized by comparison with β-actin (C).

Fig. 6. Reduced endogenous hCG production in JAr cells stably transfected with pcDNA3-Oct-3/4. Stable clones C1, C2, S1, and S4 are the same as in Fig. 5. Amounts of hCG secreted by the stable clones were measured by a radioimmunoassay that utilized monoclonal antibodies specific to the hCGβ subunit. The results are the means (±S.E.) of four independent experiments. Values marked with different letters are significantly different (p < 0.001).

Acknowledgments—We thank Dr. H. R. Schöler for pCMV-Oct4, Dr. W. Herr for pcOGCt-2, Dr. J. Boime for hCGβ cDNA clone, and Dr. P. L. Melton for p–305hCGβ-CAT, p–279hCGβ-CAT, and p–249hCGβ-CAT. We also thank Gail Foristol for help in preparing the manuscript.

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