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Extinction of Oct-3/4 Gene Expression in Embryonal Carcinoma × Fibroblast Somatic Cell Hybrids Is Accompanied by Changes in the Methylation Status, Chromatin Structure, and Transcriptional Activity of the Oct-3/4 Upstream Region

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In this study we evaluate, for the first time, the molecular mechanism that underlies the extinction of a tissue-specific transcription factor, Oct-3/4, in somatic cell hybrids and compared it with its down-regulation in retinoic acid (RA)-treated embryonal carcinoma (EC) cells. The Oct-3/4 gene, which belongs to the POU family of transcription factors and is abundantly expressed in EC (OTF9-63) cells, provides an excellent model system with which to study the extinction phenomenon. Unlike other genes whose expression has been repressed in hybrid cells but not during in vivo differentiation, Oct-3/4 expression is dramatically repressed in OTF9-63 × fibroblast hybrids and also during embryogenesis. The ectopic expression of Oct-3/4 in hybrid cells under a constitutive promoter is sufficient for transcriptional activation of an octamer-dependent promoter. These results argue against the possibility that fibroblasts contain a direct repressor which binds directly to the octamer sequence and prevents Oct-3/4 protein from binding. The extinction of Oct-3/4 binding activity in the hybrid cells occurs at the level of mRNA transcription, similarly to the repression of Oct-3/4 transcription during in vivo differentiation. This shutdown of Oct-3/4 transcription in hybrid cells and in RA-treated EC cells is accompanied by de novo methylation of its 1.3-kb upstream region. In contrast to EC cells, in which this region is sensitive to *Msp*I digestion, in hybrid cells and in RA-treated EC cells, the Oct-3/4 upstream region is resistant to *Msp*I digestion, which suggests a change in its chromatin structure. Furthermore, extinction is not restricted to the endogenous Oct-3/4 gene but is also exerted upon a transiently transfected reporter gene driven by the Oct-3/4 upstream region. Thus, changes in the cellular activity of *trans*-acting factors acting on the upstream region also contribute to the inability of the hybrid and RA-treated EC cells to generate Oct-3/4 transcripts. In conclusion, this study draws a connection between the shutdown of Oct-3/4 expression in RA-differentiated EC cells and its extinction in hybrid cells. In both systems, repression of Oct-3/4 expression is achieved through changes in the methylation status, chromatin structure, and transcriptional activity of the Oct-3/4 upstream regulatory region.

The expression of tissue-specific genes is controlled by positive and negative mechanisms. In most instances, somatic cell hybrid cells which are generated by fusing differentiated cell types with fibroblasts extinguish the differentiation-specific traits of the nonfibroblast parental cell (4, 13, 51, 53, 55). The molecular mechanisms which underlie the extinction phenomenon are not completely understood. Several genetic loci responsible for the specific extinction of liver genes have been identified (11, 29, 30, 44).

The POU-specific family of transcription factors contains several proteins sharing two regions of homology: a highly conserved POU-specific domain and a more divergent homeodomain. Members of this family specifically recognize an octameric motif in the DNA. Recently, a new member of this POU family was isolated and termed either Oct-3 (41, 46) or Oct-4 (47–50), here termed Oct-3/4. It has been shown that the Oct-3/4 gene is expressed in primordial germ cells, in oocytes, and in the totipotent and pluripotent stem cells of the pregastrulatory embryo. It is down-regulated during differentiation to endoderm and mesoderm (46, 48). It has been shown that Oct-3/4 is also expressed in embryonal carcinoma (EC) cells, and its expression is down-regulated in EC cells which are induced to differentiate in vitro by using retinoic acid (RA) (41, 46, 48).

The Oct-3/4 gene provides an excellent model system with which to study the extinction phenomenon in somatic cell hybrids, since this is the first pluripotent transcription factor identified whose expression is down-regulated during differentiation in vivo (46, 47). We cloned the Oct-3/4 cDNA from OTF9-63 teratocarcinoma cells. We show that expression of the Oct-3/4 gene product is extinguished in hybrid cells at the transcriptional or RNA processing level and that an exogenously introduced Oct-3/4 cDNA can activate an octamer-dependent promoter in these cells. To understand the molecular mechanisms that underlie Oct-3/4 suppression, we cloned the genomic Oct-3/4 gene. Here we report that the shutdown of Oct-3/4 transcription is accompanied by de novo methylation of the Oct-3/4 upstream region, changes in chromatin structure, and transcriptional activity of this regulatory region. These changes also accompany the repression of Oct-3/4 expression in RA-differentiated EC cells.

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MATERIALS AND METHODS

Cells. Hybrid cells were derived from fusion of OTF9-63 teratocarcinoma cells (OTF9-63 is a hypoxanthine phosphoribosyltransferase [HPRT]-deficient and ouabain-resistant F9 cell line [45]) and thymidine kinase (TK)-deficient L cells (LTK^- cells) which harbor the G418 resistance marker. These hybrids were kindly provided by Dale Frank. Following fusion, hybrid cells were selected and grown in medium containing HAT (10^{-2} M hypoxanthine, 4×10^{-3} M aminopterin, 1.6×10^{-2} M thymidine) and G418. Approximately 500 colonies were pooled and then expanded. Special care was taken to grow the pool of hybrids for the shortest time needed in order to prevent selection for fast growers. For the cytogenetic analysis, cells were arrested by incubation for 1 h in the presence of colcemide (0.05 μg/ml), and metaphase-arrested cells were prepared as described previously (14). RNA, DNA, and nuclear extracts from the hybrids were concomitantly prepared from the same pool of cells. Parental cells were maintained in Dulbecco's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Construction of plasmids. Plasmid p8-5 contains a 1.3-kb *Eco*RI insert encompassing a long open reading frame encoding 352 amino acids. This insert was subcloned from the λZD35 cDNA library (39) that was constructed from poly(A)$^+$ mRNA isolated from OTF9-63 cells. Recombinant phages ($\sim 5 \times 10^5$) were screened with an *Eag*I-*Pst*I Oct-2 fragment encompassing the Oct-2 POU-specific domain, under low-stringency hybridization conditions. Two Oct-3/4 expression vectors were constructed. (i) The 1.3-kb Oct-3/4 cDNA insert was cloned in pcEXV-1, which is a derivative of the pcD-X vector (42). The cDNA was cloned in sense and antisense orientations and was expressed from the simian virus 40 (SV40) promoter/enhancer. (ii) The Oct-3/4 cDNA was cloned in frame in the EVRF vector and was expressed from the cytomegalovirus (CMV) promoter/enhancer region (34). To construct poct-3/4CAT, a 2.0-kb *Hin*dIII fragment, isolated from Oct-3/4 genomic DNA, containing the Oct-3/4 promoter/enhancer region was inserted into the *Hin*dIII site 5′ to the chloramphenicol acetyltransferase (CAT) transcription unit in p106 (19). pCMVCAT contains the CMV regulatory region inserted in front of the CAT transcription unit.

DNA transfections. Hybrid cells were transfected by the calcium phosphate precipitation method (58) with 15 μg of β-globin reporter plasmid, 2 μg of reference plasmid, and 3 μg of either EVRF Oct-3/4 transactivator or control plasmid. The reporter plasmids and the reference plasmid were kindly provided by Patrick Matthias (38). The reporter plasmids were based on the OVEC-1 vector (57) and contained either a wild-type octamer motif derived from the histone H2B promoter or the same octamer motif with three point mutations (described in reference 38), which abolish factor binding and activity. In both reporter plasmids, these wild-type and mutated octamer sequences were located upstream of the β-globin TATA box, and the SV40 enhancer was located 3′ to the β-globin gene. The reference plasmid contains the β-globin TATA box, but has a deletion of 28 bp around the transcription initiation site (between −10 and +19), and is driven by an SV40 enhancer upstream of the TATA box (57). Cytoplasmic RNA was analyzed by RNase protection using SP6 polymerase-generated RNA as a probe. The probe used was generated from the *Sal*I-*Taq*I fragment spanning positions −37 to +179 of the OVEC-1 vector. This probe gives rise to protected fragment 179 nucleotides long with the reporter plasmids and 160 nucleotides long with the reference gene (38). COS cells were transfected by the DEAE-dextran method (52) with the pcEXV-1 expression vector containing the Oct-3/4 cDNA.

Parental hybrid and RA-treated EC cells were transfected with poct-3/4CAT or pCMVCAT by the calcium phosphate precipitation method (58). To correct for differences in transfection efficiency, we cotransfected a plasmid expressing β-galactosidase (pCMV-βgal). At 44 h after transfection, the cells were processed for measuring β-galactosidase (20) and CAT (21) activities. The protein concentrations in the various cell extracts were determined (7). The β-galactosidase activity was measured in 100 μg of protein extract, and the CAT activity was measured in 200 μg of protein of cell extracts, using chloramphenicol (CAM; 53 mCi/mmol; Amersham International, Amersham, United Kingdom) as the substrate, in the presence of acetyl coenzyme A at 37°C for 16 h. CAM was separated from the acetylated forms by silica thin-layer chromatography. The radioactive spots were cut out from the gel and counted in scintillation fluid. Activities were expressed as percent conversion of CAM to the acetylated forms.

In vitro transcription and translation of Oct-3/4 cDNA. mRNA synthesized in vitro was made from the Oct-3/4 cDNA in the Bluescript KS plasmid (Stratagene). The plasmid was linearized, the sense mRNA was made by using T7 polymerase, and the antisense mRNA was made by using T3 polymerase (Stratagene). mRNA (1 μg) was added to a rabbit reticulocyte lysate (Promega). Of a 50-μl total reaction, 5 μl was used for analysis in mobility shift DNA binding assays.

Plasmids and DNA fragments. The β-actin plasmid, which contains a 250-bp cDNA *Pst*I insert of the rat β-actin sequences, was provided by Uri Nudel (40). The octamer oligonucleotide (5′-GATCCATGCAAAT-3′; provided by Ranjan Sen) was cloned into the *Bam*HI site of the pUC18 polylinker. The oligonucleotide was prepared by digesting the plasmid at *Eco*RI and *Hin*dIII flanking sites within the polylinker. Fragments were isolated from a 10% native polyacrylamide gel and quantified by spotting onto ethidium bromide-containing plates (33). As probes, these fragments were end labeled by filling in the ends with the Klenow fragment of DNA polymerase I (33).

Nuclear extracts and DNA binding assays. Nuclear extracts were prepared as described previously (15). The DNA binding reaction mixtures (20 μl) contained 10 mM Tris (pH 7.5), 20 mM KCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 4% glycerol, 2 μg of poly(dI-dC), 12,000 cpm (0.2 ng) of end-labeled DNA fragment, and 20 μg of nuclear extract protein. After incubation for 20 min at room temperature, samples were analyzed by the electrophoresis mobility shift assay (17, 18). The native 4% polyacrylamide gels (acrylamide/bisacrylamide ratio of 30:1) containing one-quarter Tris-borate-EDTA buffer were electrophoresed for 2 h at 200 V. Gels were dried and autoradiographed with an intensifying screen at −70°C.

RNA analyses. Total RNA was prepared and subjected (20 μg) to electrophoresis through a formaldehyde-containing 1% agarose gel and transferred to Nytran filters. The 1.3-kb Oct-3/4 cDNA fragment was labeled with [α-^{32}P]dXTP by using a random priming kit (Amersham) and used as a probe. Hybridization was done under standard conditions (33). The filters were washed at 65°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate (SDS) and autoradiographed with an intensifying screen at −70°C. The filters were stripped by washing in

boiling water and rehybridized with the ^{32}P-labeled β-actin probe to control the amount and integrity of mRNA loaded.

DNA analyses. Genomic DNA was digested with restriction enzymes, electrophoresed on 1% agarose gels, and transferred to Nytran nylon membranes (Schleicher & Schuell). The probe was a random primer-labeled Oct-3/4 1.3-kb fragment. Filters were washed with 0.1 × SSC–0.1% SDS at 65°C.

Isolation and *Msp*I digestion of nuclei. Nuclei were prepared (9) and suspended in a solution of 10 mM Tris (pH 7.4), 10 mM NaCl, and 3 mM $MgCl_2$ in a glass tube on ice. Nuclear concentrations were adjusted to a DNA concentration of 0.5 mg/ml as assayed by dilution into 1% SDS. The nuclei were warmed to 37°C while shaking gently for 1 min and were digested with different concentrations of *Msp*I ranging from 0 to 800 U/ml at 37°C for 20 min. Digestion was terminated by adding an equal volume of a solution of 20 mM Tris (pH 7.4), 200 mM NaCl, 2 mM EDTA, 1% SDS, and 200 μg of proteinase K per ml. After overnight incubation at 37°C (or 1 h at 55°C), 1 volume of a 10 mM Tris (pH 7.4)–1 mM EDTA (TE) solution was added; the DNA was extracted once with phenol and three times with chloroform and precipitated with sodium acetate and ethanol. DNA precipitates were spooled out and dissolved in TE.

RESULTS

OTF9-63 × fibroblast hybrid cells. It has been shown that Oct-3/4 expression is down-regulated in the 8-day mouse embryo and in EC cells which have been induced to differentiate in vitro by using RA (41, 46, 48). To understand the negative regulatory controls operating in eukaryotic cells, we analyzed the expression of the Oct-3/4 gene in OTF9-63 × fibroblast somatic cell hybrids. The latter were generated by fusion of HPRT-deficient F9 cells (OTF9-63 cells) with LTK$^-$ cells which harbor the G418 resistance marker. These hybrid cells grew in selective medium (HAT plus G418). To minimize the influence of unknown parameters in independent, cloned hybrids, we analyzed a pool of about 500 clones. The expanded culture was grown for the shortest time possible (7 to 10 days) in order to prevent selection of fast growers. These cells differed in morphology from the parental cells. In contrast to the fibroblast-like morphology of the parental LTK$^-$ cells, the hybrid cells had an epithelium-like morphology and were larger than either of the parental cells.

Mouse-mouse hybrid cells have been shown to lose a few chromosomes in the first few generations following fusion. After this, the chromosomal makeup of the hybrids becomes stable (16). Previously it had been shown that fusion between EC cells and fibroblast cells yields hybrids which contain only slightly fewer chromosomes than the number expected from the sum of the chromosomes present in the parental cells (24, 35). The hybrid cells examined in the present study contain 10 to 15% fewer chromosomes than the total number of their parental cells (on average, an LTK$^-$ cell contains 46 chromosomes and an OTF9-63 cell contains 38 chromosomes; data not shown).

To determine whether the Oct-3/4 gene is present in the hybrids, we carried out a Southern blot analysis of the parental and hybrid cell DNA. Analysis with nine different enzymes was performed to detect restriction fragment length polymorphism (RFLP) differences between the parental cells DNA. Digestion with two enzymes (*Bam*HI and *Pst*I) followed by probing of the blot with Oct-3/4 cDNA demonstrated hybridization bands which differ between OTF9-63 and LTK$^-$ cell DNAs. Figure 1 shows that the hybrid DNA contains the sum of the polymorphic Oct-3/4 hybridization bands which are found in both parental DNAs (compare lane 2 with lanes 1 and 3 and lane 5 with lanes 4 and 6). Thus, any apparent effects on Oct-3/4 expression are not due to overall loss of the genes in the hybrid pool.

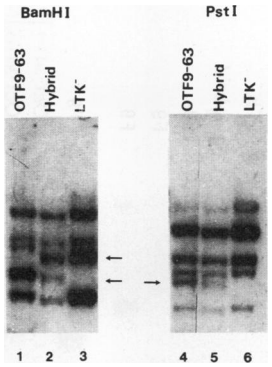

FIG. 1. Southern blot analysis of the Oct-3/4 gene. Genomic DNA from OTF9-63, OTF9-63 × fibroblast, and LTK$^-$ cells was digested with *Bam*HI (lanes 1 to 3) and *Pst*I (lanes 4 to 6), electrophoresed on 1% agarose gels, and transferred to Nytran nylon membranes. Filters were hybridized with Oct-3/4 cDNA (an *Eco*RI fragment of 1.3 kb). The Oct-3/4 bands in OTF9-63 × fibroblast hybrid DNA are less intense than the corresponding DNA bands originating from the parental cell for the following reasons: (i) as judged from ethidium bromide staining of the gel, less DNA was loaded in the hybrid lanes than in the parental cell lanes (data not shown) and (ii) the molar concentration of each of the parental cell Oct-3/4 genes per a constant amount of hybrid DNA is approximately half (assuming 10 to 15% chromosomal loss). Arrows denote the positions of DNA bands which show RFLP differences between the parental cell lines.

Oct-3/4 DNA binding activity is extinguished in OTF9-63 × fibroblast hybrid cells. To determine whether the Oct-3/4 gene is expressed in our hybrid cells, we examined the presence of Oct-3/4 binding activity in the nuclear extracts of the parental and hybrid cells, using the electrophoretic DNA binding assay (17, 18). We used an oligonucleotide fragment which contains the octamer sequence located in the immunoglobulin κ promoter as a target for binding of nuclear extracts. The more slowly migrating protein-DNA complex (Fig. 2, lanes 1 to 3, 5, 6, and 9 to 11) is attributed to the binding of the ubiquitous Oct-1-binding protein (54). Similarly to previously published data (31), OTF9-63 nuclear extracts generated an additional faster retarded band (Fig. 2, lanes 3, 5, 6, and 11) which was specifically competed for by unlabeled octamer oligonucleotide (lane 4) and not by an unrelated cold Tα2 oligonucleotide (originated from the T-cell receptor α enhancer) (lane 5). The in vitro transcription and translation product of our Oct-3/4 cDNA gene produced one shifted band which comigrated with the band in the nuclear extracts from EC cells (Fig. 2; compare lanes 6 and 8). Moreover, nuclear extracts that were prepared from COS cells transfected with an SV40-derived Oct-3/4 expression vector generated a prominent retarded DNA-protein complex which also comigrated with the EC-specific band (Fig. 2; compare lanes 10 and 11). This band was not observed in the nuclear extracts derived from COS cells transfected with the Oct-3/4 antisense expression vector (Fig. 2, lane 9), nor was it observed in the Oct-3/4 antisense in vitro translation products (Fig. 2, lane 7). Thus, the faster-migrating band that is prominent only in the nuclear extract from EC cells represents the Oct-3/4-specific band.

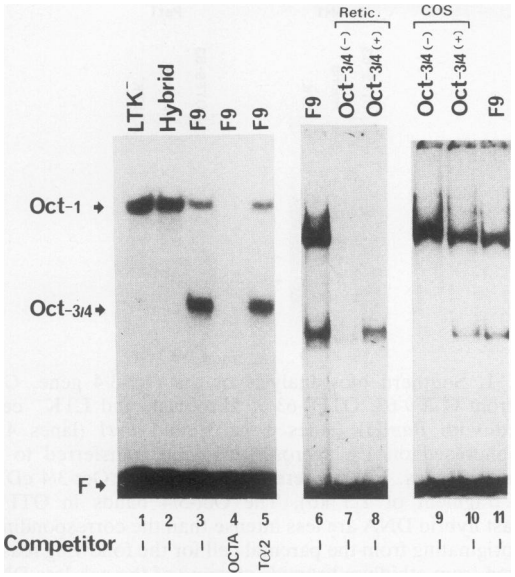

FIG. 2. Repression of Oct-3/4 binding activity in OTF9-63 × fibroblast hybrids. Labeled octamer oligonucleotide (12,000 cpm, 0.2 ng) was incubated with 20 μg of nuclear extracts prepared from parental cells (OTF9-63 [F9] and LTK⁻), OTF9-63 × fibroblast hybrid cells, and COS cells that were transfected with the Oct-3/4 expression vector in which the Oct-3/4 cDNA was transcribed from the SV40 promoter/enhancer in sense (+) and antisense (−) orientations. The KS vector (Stratagene) containing the Oct-3/4 cDNA was linearized, and sense and antisense RNAs were made. Lanes 7 and 8 represent reticulocyte translation products from antisense (−) and sense (+) Oct-3/4 mRNA, respectively. Binding was done in the presence of 2 μg of poly(dI-dC), and analysis was performed by gel mobility shift assay. Binding reactions were done in the absence of oligonucleotide (lanes 1 to 3 and 6 to 11) or in the presence of a 200 molar excess of either a cold octamer oligonucleotide (lane 4) or a cold Tα2 oligonucleotide originated from the T-cell receptor α enhancer (lane 5). The source of each nuclear extract and reticulocyte product is shown above each lane; arrows denote the positions of DNA molecules bound by Oct-1 and Oct-3/4 proteins. The band that is common to lanes 7 and 8 originated from the reticulocyte lysate itself. F, free DNA.

The fibroblast nuclear extract (LTK⁻) lacks the Oct-3/4-specific complex (Fig. 2, lane 1). Interestingly, nuclear extracts generated from OTF9-63 × fibroblast hybrid cells also lack the Oct-3/4 band (Fig. 2, lane 2). The extinction of Oct-3/4 binding activity in the hybrid extracts was not due to proteolytic degradation, because Oct-1 showed similar complex formation in all nuclear extracts that were tested (Fig. 2, lanes 1 to 3, 5, 6, and 9 to 11). Thus, we conclude that the EC × fibroblast hybrids lack any detectable DNA binding activity of the Oct-3/4 transcription factor, despite the presence of the Oct-3/4 genes from the OTF9-63 parental cell line.

Exogenously introduced Oct-3/4 genes activate an octamer-containing promoter in OTF9-63 × fibroblast hybrids. Several explanations could be considered for the absence of Oct-3/4 binding activity in hybrid nuclear extracts. The Oct-3/4 transcription factor may not be able to bind its cognate sequence because of the presence of a fibroblast repressor(s) that competes for binding to the same sequence. Alternatively, Oct-3/4 may not be able to interact with its target sequence because of a different posttranslational modification that prevents binding to its target.

To test these possibilities, transactivation experiments were performed. The Oct-3/4 cDNA was cloned into a mammalian expression vector under the CMV promoter/enhancer (34). The octamer-dependent reporter plasmids (38) and an internal reference plasmid (57) were cotransfected into OTF9-63 × fibroblast hybrid cells with and without the Oct-3/4 expression vector. An RNase protection assay of RNA from the transfected cells yielded two protected fragments, 179 nucleotides long with the reporter plasmid and 160 nucleotides long with the reference gene. This assay showed that the Oct-3/4 protein stimulates the correctly initiated mRNA synthesis from the reporter plasmid that contains a wild-type octamer DNA motif (Fig. 3A; compare lanes 1 and 3). Mutations of the octamer sequence substantially reduced transactivation (Fig. 3A; compare lanes 2 and 4). Thus, exogenously introduced Oct-3/4 cDNA can activate an octamer-dependent promoter in the hybrid cells. We also performed nuclear extract mixing experiments in which we combined nuclear extracts of OTF9-63 and LTK⁻ cells and analyzed their binding patterns on polyacrylamide gels. Binding of the Oct-3/4 product to the octamer motif was not impaired by the presence of LTK⁻-cell nuclear extract (data not shown). Thus, the presence of an interfering fibroblastic repressor, which binds the octamer sequence and prevents Oct-3/4 protein from binding, or incorrect posttranslational modifications are unlikely to explain the absence of Oct-3/4 binding activity in the hybrid nuclear extracts.

Oct-3/4 mRNA is extinguished in EC × fibroblast hybrids. Another possible explanation for the lack of Oct-3/4 binding activity is the shutoff of Oct-3/4 transcription. To determine whether the extinction of Oct-3/4 expression is at the transcriptional level, we analyzed total RNA from parental and hybrid cells for Oct-3/4 expression. No hybridization could be detected with RNA extracted from the parental fibroblast and hybrid cells when samples were probed with radioactive Oct-3/4 cDNA (Fig. 3B, lanes 2 and 3). There were no traces of Oct-3/4 mRNA sequence, neither as mature RNA nor as incomplete transcripts or unprocessed high-molecular-weight precursors, even after a 10-fold increase in exposure of the filter. In contrast, a very prominent wide band representing a 1.5- to 1.6-kb Oct-3/4 transcript was obtained with RNA of EC cells (Fig. 3B, lane 1). Oct-3/4 transcripts are at least 60-fold more abundant in OTF9-63 cells than in hybrids or in LTK⁻ cells (calculated from different exposure times of several autoradiograms). The integrity of the RNAs was monitored with a β-actin probe (Fig. 3C). It is highly unlikely that the lack of Oct-3/4 mRNA in the hybrids is due to chromosomal loss for the following reasons: (i) a relatively small number of chromosomes (<15%) are lost in the hybrid cells, which comprise approximately 500 clones; and (ii) using RFLP analysis, we show that the DNA from the hybrid cells contains the Oct-3/4 alleles originating from both parents. The Oct-3/4 polymorphic DNA bands in the hybrid DNA are three- to fivefold less intense than the corresponding bands originating from the EC parent, partly because a fixed amount of hybrid DNA contains approximately half the amount of DNA originating from each parental cells and partly because less DNA was loaded in the hybrid lanes than in the parental cell lanes (data not shown). However, this difference cannot explain the 60-fold reduction of Oct-3/4 mRNA in the hybrid cells. Thus, our results indicate that the extinction of Oct-3/4 binding activity in the hybrid cells occurs at the level of mRNA transcription, either by regulation of transcription initiation or by differential transcript stability.

The 5′ upstream region of the OTF9-63 Oct-3/4 gene undergoes de novo methylation in the hybrid cells. To under-

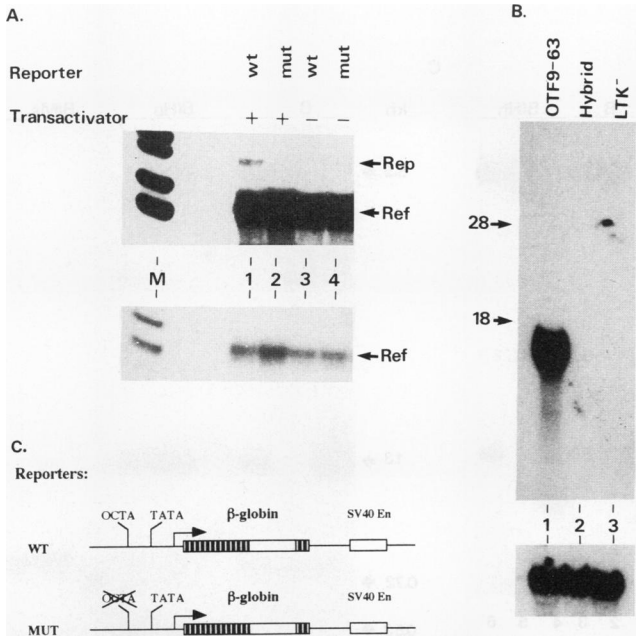

FIG. 3. Evidence that an exogenously introduced Oct-3/4 expression vector can activate an octamer-containing promoter and that endogenous transcription of the Oct-3/4 gene is repressed in OTF9-63 × fibroblast hybrids. (A) RNase protection mapping of 20 μg of cytoplasmic RNA which was isolated from OTF9-63 × fibroblast hybrids that in turn were transiently cotransfected with the following plasmids: 15 μg of reporter plasmid OVEC-wild-type-OCTA (Rep; lanes 1 and 3; octamer sequence is ATTTGCAT), 15 μg of reporter plasmid OVEC-mutant-OCTA (lanes 2 and 4; mutated octamer sequence is ATGTTCAG) (depicted in panel C), 2 μg of a reference plasmid to monitor for transfection efficiency (57) (Ref; lanes 1 to 4), and 3 μg of either Oct-3/4 transactivator plasmid (lanes 1 and 2) or pUC18 plasmid (lanes 3 and 4) DNA. A shorter exposure of the reference plasmid bands is shown in the lower panel. The reference plasmid is expressed in a higher level relative to the reporter plasmid. However, since the levels of transcription of the reference plasmid were similar in the four transfections, it is unlikely that it affects differently the ability of the Oct-3/4 protein to stimulate transcription from the reporter plasmid containing the wild-type (wt) octamer and its inability to induce transcription from the mutated (mut) octamer reporter plasmid. Lane M contains DNA size markers. (B) Expression of Oct-3/4 mRNA in parental and hybrid cell lines. RNAs (20 μg) isolated from parental (OTF9-63 and LTK$^-$) and hybrid cells were electrophoresed on 1% agarose–formaldehyde gels, transferred to Nytran filters, and hybridized with a 1.3-kb *Eco*RI fragment containing the Oct-3/4 cDNA. The source of each RNA sample is shown above each lane. The blot was stripped and rehybridized with a β-actin cDNA probe. The positions of 18S and 28S rRNAs are indicated.

stand the molecular mechanisms that underlie suppression of Oct-3/4 mRNA in hybrid cells, we cloned the genomic Oct-3/4 gene, mapped its exon and intron boundaries, and identified its promoter region (unpublished data). Different mechanisms could have caused the apparent inability of the nonexpressing hybrids to transcribe the Oct-3/4 gene. One of them may involve changes in the methylation pattern of the gene. To address this issue, we sequenced the 1.3-kb upstream region of the Oct-3/4 gene and determined the location of the *Hpa*II and *Hha*I restriction sites. As shown in Fig. 4A, this region contains one *Hha*I site and five *Hpa*II sites. Only one of these sites is colocalized with a potential transcription factor binding site. *Hpa*II site 4 is embedded in the binding site for the epidermal growth factor receptor-specific transcription factor (ETF) (27). We digested DNA isolated from hybrid and parental cell lines with *Bam*HI and then with either *Hha*I, *Hpa*II, or *Msp*I. Digested DNA was analyzed by transfer and hybridization with the *Bam*HI-*Xba*I probe depicted in Fig. 4A. The *Bam*HI digest of OTF9-63 DNA yields a prominent band of 1.3 kb which contains the *Hpa*II and *Hha*I sites mentioned above (Fig. 4B, lane 1). The other bands are due to other Oct-3/4-related genes (our unpublished data). *Bam*HI-digested DNA from LTK$^-$ cells yields a prominent ~5.5-kb fragment (Fig. 4B, lane 2) due to the absence of the 3′ *Bam*HI site present in the OTF9-63 Oct-3/4 first exon (our unpublished data). As expected, DNA from OTF9-63 × fibroblast hybrids contains the sum of the *Bam*HI fragments seen in the two parents (Fig. 4B, lane 3). The *Hha*I site is demethylated in OTF9-63 cells, which express the Oct-3/4 gene (Fig. 4B, lane 4). In contrast, this *Hha*I site is methylated in fibroblast cells, which do not express the Oct-3/4 gene (Fig. 4B, lane 5). However, the *Bam*HI-*Hha*I digest of the hybrid cell DNA shows that the majority of the Oct-3/4 gene originating from OTF9-63 cells underwent de novo methylation at this site, since the DNA was resistant to *Hha*I digestion (Fig. 4B, lane 6).

*Bam*HI-*Hpa*II digestion of OTF9-63 DNA reveals that the *Hpa*II sites located at the 1.3-kb Oct-3/4 5′ upstream region are unmethylated (Fig. 4C, lane 4). The presence of the 1.3-kb fragment in the *Bam*HI-*Hpa*II digest of LTK$^-$ and hybrid cell DNA (Fig. 4C, lanes 5 and 6) reveals that most of the *Hpa*II sites are methylated, indicating that in the hybrid cells, the *Hpa*II sites located at the Oct-3/4 upstream region, like the *Hha*I sites, underwent de novo methylation. Probing with smaller fragments from the 1.3-kb upstream region indicated that the band representing the 0.72-kb fragment (present only in hybrid cells; lane 6) is due to partial demethylation of *Hpa*II site 2 and demethylation of site 5 and that the 0.53-kb fragment (present in LTK$^-$ and hybrid cells; lanes 5 and 6) is due to partial demethylation of *Hpa*II site 2. The *Msp*I digest of DNA from LTK$^-$ and hybrid cells yields the 0.4- and 0.45-kb fragments present in the *Hpa*II digest of OTF9-63 DNA (Fig. 4C, lanes 7 to 9). The de novo methylation of the 1.3-kb Oct-3/4 upstream region in hybrid cells is particularly interesting in light of the fact that this fragment directs the expression of a reporter gene transiently introduced into OTF9-63 cells (see below).

***Msp*I sensitivity of nuclear DNA in the 5′ region of the Oct-3/4 gene.** The results presented above showed that in hybrid cells, the Oct-3/4 upstream region underwent de novo methylation. What is the correlation between this change and the chromatin structure of this region in the hybrid cells? Previous work has shown that methylated CpGs in the intact nucleus are preferentially inaccessible to restriction endonucleases recognizing CpG, perhaps because of the presence of nuclear proteins that bind to methyl-CpG (1, 37). More explicitly, *Msp*I cuts at CCGG in naked DNA whether or not the internal C residue is methylated; however, in an intact nucleus, *Msp*I preferentially cleaves nonmethylated CpGs. We therefore digested isolated nuclei from parental and hybrid cells with increasing concentrations of *Msp*I, purified the DNA, and redigested it with *Bam*HI and *Hin*dIII. The different patterns of *Msp*I sensitivity that were observed are shown in Fig. 4D. OTF9-63 cells, expressing Oct-3/4, were sensitive to *Msp*I digestion. At the lowest *Msp*I concentration (40 U/ml), the DNA was cleaved at all four *Msp*I sites analyzed, as revealed by probing with the most-upstream *Bam*HI-*Msp*I 139-bp fragment (Fig. 4D, lanes 1 to 7). There is an extra band, located between bands 3 and 4, which we

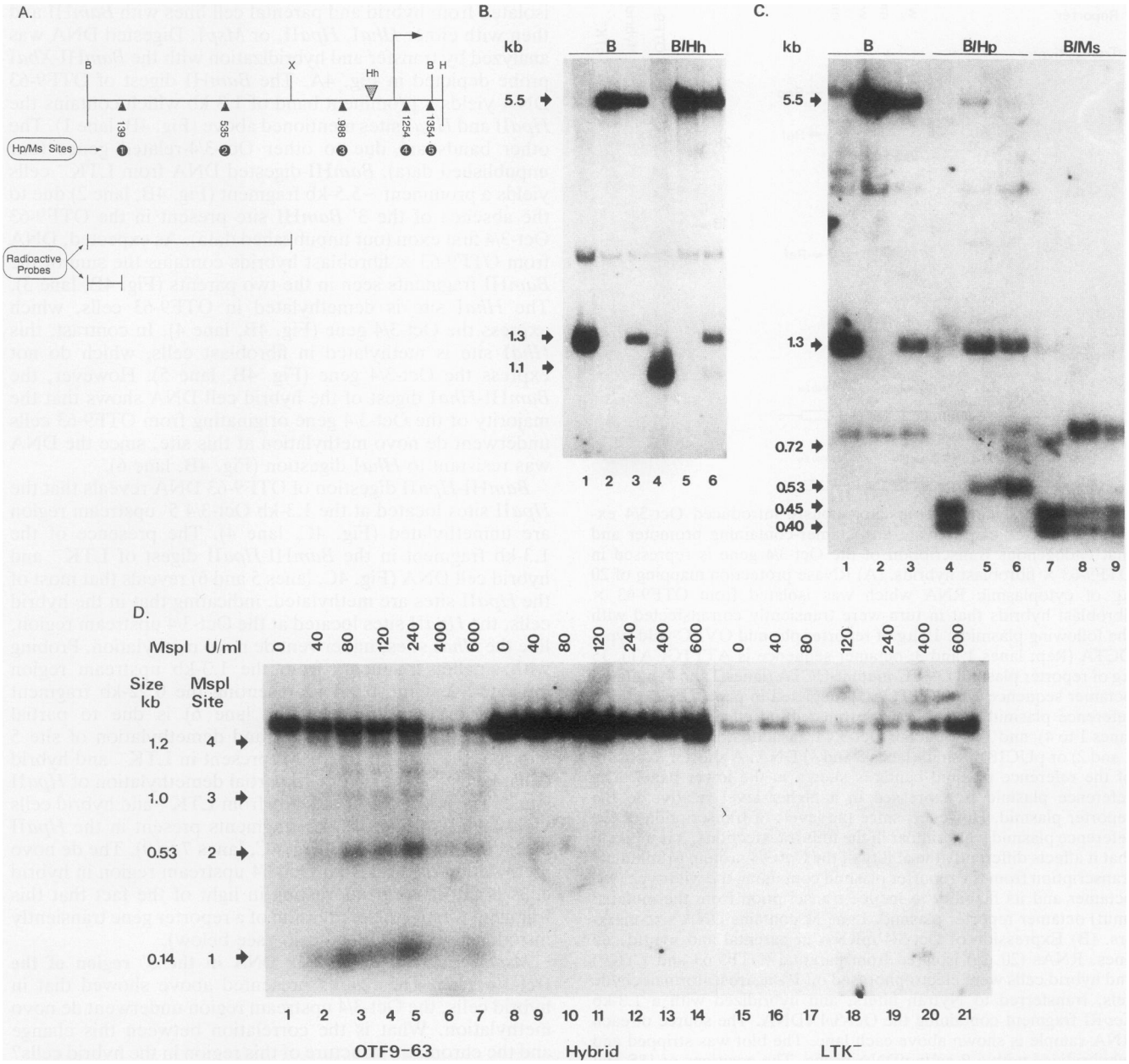

FIG. 4. Evidence that in the hybrid cells, the 5′ upstream region of the OTF9-63 Oct-3/4 gene undergoes de novo methylation and a change in the chromatin sensitivity to *Msp*I digestion. (A) Map of the 5′ region of the Oct-3/4 gene. The initiation site is shown. The *Bam*HI (B), *Xba*I (X), *Hin*dIII (H), *Hha*I (Hh), and *Hpa*II (Hp)/*Msp*I (Ms) sites are indicated. The *Hpa*II/*Msp*I sites are numbered 1 to 5. The 3′ *Bam*HI site is present in OTF9-63 DNA but is absent in L-cell DNA. The radioactive probes used are indicated. (B) OTF9-63 (lanes 1 and 4), LTK$^-$ (lanes 2 and 5), and hybrid (lanes 3 and 6) cell DNA was digested with *Bam*HI alone (B; lanes 1 to 3) or in combination with *Hha*I (B/Hh; lanes 4 to 6). The arrows represent the Oct-3/4 fragments identified on the basis of the sequence of the cloned Oct-3/4 gene. Restriction fragments were electrophoresed on a 1.2% agarose gel, blotted, and probed with the 802-bp *Bam*HI-*Xba*I fragment depicted in panel A. (C) OTF9-63 (lanes 1, 4, and 7), LTK$^-$ (lanes 2, 5, and 8), and hybrid (lanes 3, 6, and 9) cell DNA was digested with *Bam*HI alone (B; lanes 1 to 3) or in combination with either *Hpa*II (B/Hp; lanes 4 to 6) or *Msp*I (B/Ms; lanes 7 to 9). The blot was probed with the 802-bp *Bam*HI-*Xba*I fragment depicted in panel A. Arrows represent the identified cloned Oct-3/4 gene fragments. The additional bands observed between the 0.72- and 1.3-kb fragments are resistant to *Msp*I digestion and are probably due to hybridization of the probe with a different but related Oct-3/4 gene (our unpublished data). (D) Nuclei of parental (lanes 1 to 7 and 15 to 21) and hybrid (lanes 8 to 14) cells were digested with the indicated concentrations of *Msp*I at 37°C prior to the isolation of DNA. Nuclei incubated with no *Msp*I at 0°C gave a pattern identical to the pattern obtained following incubation at 37°C (data not shown). Purified DNA was digested with *Bam*HI and *Hin*dIII and hybridized with the *Bam*HI-*Msp*I 139-bp probe depicted in panel A. Arrows indicate the positions corresponding to each of the CCGG sites shown in panel A, which were calculated relative to molecular size standards. *Msp*I site 5 was not analyzed because of its proximity (34 bp) to the *Hin*dIII site.

could not assign to a known *Msp*I site in this sequenced fragment. This extra band could have arisen from a related Oct-3/4 gene, since probing of a genomic Southern blot with the Oct-3/4 cDNA probe does not yield a pattern of a single-copy gene (our unpublished data). Alternatively, it could correspond to an additional polymorphic *Msp*I site which is absent in our isolate of the genomic Oct-3/4 clone. Interestingly, in LTK$^-$ and hybrid cells, the Oct-3/4 upstream region was completely insensitive to *Msp*I digestion, as shown by the undigested 1.3-kb band (Fig. 4D, lanes 7 to 21). In hybrid cells, the insensitivity of the Oct-3/4 upstream region to digestion with *Msp*I indicates a change in the chromatin structure of this region that is probably related to the change in methylation status of the Oct-3/4 upstream sequences.

The 2.0-kb Oct-3/4 upstream region does not confer activation upon a reporter gene in OTF9-63 × fibroblast hybrid cells. To determine whether extinction of Oct-3/4 mRNA in hybrids correlates not only with changes in the methylation status and chromatin structure of the endogenous gene but also with changes in the cellular activities of *trans*-acting factors regulating Oct-3/4 gene expression, we transiently transfected parental and hybrid cells with a CAT reporter gene driven by the Oct-3/4 2.0-kb upstream region (Fig. 5A). This 2.0-kb fragment has been shown to confer cell-specific expression on a reporter gene in mouse embryos and in EC cells (43). It contains a low-basal-activity promoter element and an enhancer which is active in EC cells and inactive in RA-differentiated EC cells (43). As a positive control for transfection, parental and hybrid cells were also transfected with a construct containing the CAT gene driven by a CMV promoter/enhancer region. We compared the CAT expression driven by the L32 ribosomal promoter, β-actin promoter, SV40 promoter/enhancer, TK promoter, and CMV regulatory regions. The CMV-CAT construct yielded the highest CAT expression in EC cells, and this construct served as our positive control.

As shown in Fig. 5B, the poct-3/4CAT construct is efficiently expressed in EC cells, while in hybrid and LTK$^-$ cells, only very low levels of CAT activity are produced (compare lane 1 with lanes 3 and 5). These experiments indicate that extinction is not restricted to the endogenous Oct-3/4 promoter but is also exerted upon the newly exogenously transfected Oct-3/4 regulatory region. Thus, changes in the cellular activity of *trans*-acting factors, either repression of activators or induction of repressors, acting on the Oct-3/4 upstream region probably contribute to the inability of the hybrid cells to generate Oct-3/4 transcripts.

Oct-3/4 gene expression is repressed in RA-treated EC cells. The Oct-3/4 gene provides a unique model with which to compare the molecular parameters that underlie the extinction of its expression in somatic cell hybrids and during cellular differentiation. Oct-3/4 mRNA is down-regulated in EC cells which are induced to differentiate in vitro by using RA (41, 46, 50) (Fig. 6A). To examine the presence of Oct-3/4 binding activity in nuclear extracts of RA-treated EC cells, we used the electrophoretic DNA binding assay (17, 18), using the octamer oligonucleotide used for the assay shown in Fig. 2. Nuclear extracts generated from EC cells treated for 96 h with RA show a very low level of the Oct-3/4 band (Fig. 6B). EC cells treated for 7 days with RA almost completely lack Oct-3/4 binding activity (data not shown). Thus, in RA-treated cells, the level of Oct-3/4 mRNA and the level of Oct-3/4 protein, which bind to the octamer oligonucleotide, were reduced considerably.

To determine whether the Oct-3/4 upstream region undergoes de novo methylation in RA-treated EC cells as it does in hybrid cells, we digested DNA from untreated and RA-treated EC cells with *Bam*HI alone and in combination with *Msp*I, *Hpa*II, and *Hha*I. Digested DNA was analyzed by Southern blotting using the 562-bp *Xba*I-*Bam*HI fragment as a radioactive probe. The *Bam*HI digest of EC DNA yields a prominent band of 1.3 kb. The *Hha*I site is demethylated in EC cells (Fig. 4B, lane 4; Fig. 6C, lane 4). However, the *Bam*HI-*Hha*I digest of RA-treated cells shows that the

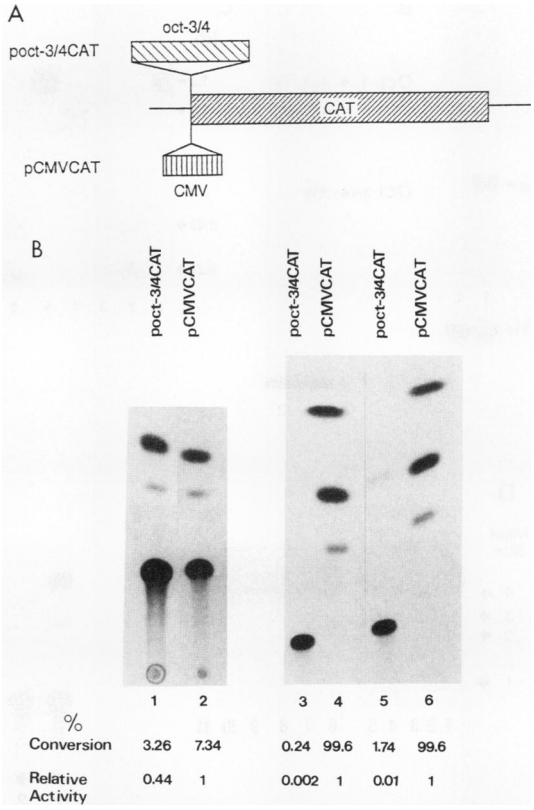

FIG. 5. Down-regulation of expression of the CAT gene linked to the Oct-3/4 2.0-kb upstream region in OTF9-63 × fibroblast hybrids. (A) Constructs used. pCMVCAT carries the CMV promoter and enhancer cloned upstream of the CAT gene, and poct-3/4CAT contain the 2.0-kb Oct-3/4 upstream region inserted in front of the CAT transcription unit. (B) CAT expression. Parental (OTF9-63 [lanes 1 and 2], LTK$^-$ [lanes 5 and 6]), and hybrid cells (lanes 3 and 4) were cotransfected with 15 μg of either poct-3/4CAT or pCMV CAT DNA together with 5 μg of pRSV-βgal DNA. CAT and β-galactosidase activities were assayed. In the CAT assay, the autoradiography analysis was quantified by excising the acetylated and unacetylated forms of the ^{14}C-labeled CAM and determining the amount of radioactivity by liquid scintillation counting. The percent conversion of each separate transfection was normalized to the β-galactosidase activity and is depicted below the autoradiogram. The experiments were repeated three times, and the calculated standard deviation between experiments was less than 10%. The CAT activity driven by the CMV regulatory region expressed in L and hybrid cells is not in the logarithmic range, since we carried out the assay for 16 h in order to detect CAT activity driven by the Oct-3/4 promoter/enhancer region. The percent conversion of pCMVCAT transfected into LTK$^-$ and hybrid cells was 36.6 and 11.3, respectively, when assayed for 2 h. Thus, the relative activity of poct-3/4CAT in the hybrid and LTK$^-$ cells is even lower than the values depicted in the figure, indicating almost undetectable levels of CAT activity in the hybrid cells.

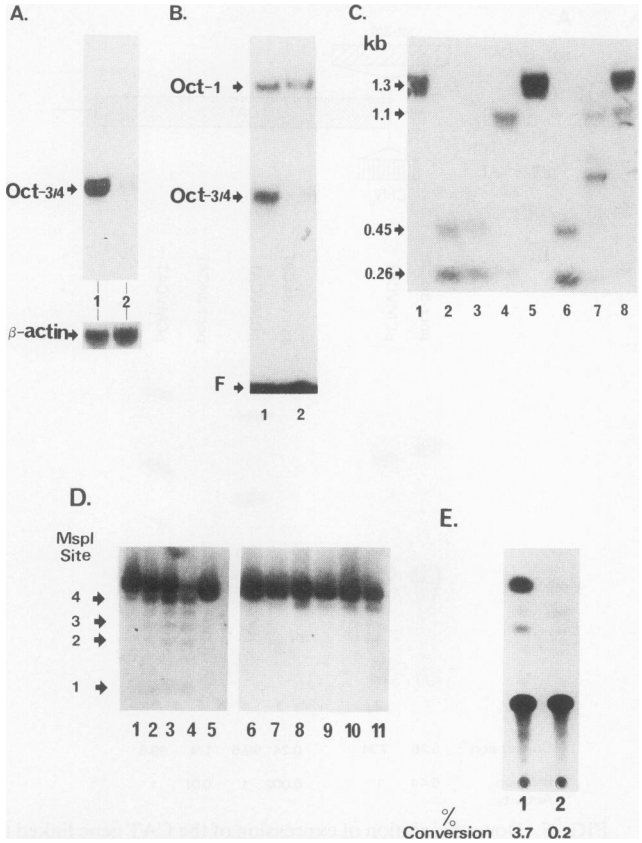

FIG. 6. Repression of Oct-3/4 gene expression in RA-treated EC cells. (A) Oct-3/4 mRNA is repressed in RA-treated cells. RNA (20 μg) isolated from F9 (lane 1) and RA-treated F9 (lane 2) cells was electrophoresed on a 1% agarose–formaldehyde gel, transferred to a Nytran filter, and hybridized with a 1.3-kb *Eco*RI fragment containing the Oct-3/4 cDNA. The blot was stripped and rehybridized with a β-actin cDNA probe (shown in the lower part of the panel). (B) Oct-3/4 binding activity is repressed in RA-treated EC cells. Labeled octamer oligonucleotide was incubated with 20 μg of nuclear extracts prepared from F9 cells (lane 1) and from F9 cells treated with RA for 96 h (lane 2). Binding was done in the presence of 2 μg of poly(dI-dC). Arrows denote the positions of DNA molecules bound by Oct-1 and Oct-3/4 proteins. F, free DNA. (C) The 5′ upstream region of the Oct-3/4 gene undergoes de novo methylation. DNA from F9 cells (lanes 1 to 4) and F9 cells treated for 4 days with RA (lanes 5 to 8) was digested with *Bam*HI alone (lanes 1 and 5) or in combination with *Msp*I (lanes 2 and 6), with *Hpa*II (lanes 3 and 7), and with *Hha*I (lanes 4 and 8). Restriction digests were electrophoresed on 1.2% agarose gel, blotted, and probed with the 562-bp *Xba*I-*Bam*HI fragment. (D) Chromatin sensitivity to *Msp*I digestion in the 5′ upstream region of Oct-3/4 in untreated and in RA-treated EC cells. *Msp*I digestion at 37°C prior to isolation of DNA was performed on nuclei isolated from EC cells (lanes 1 to 5; 40, 120, 400, 800, and 0 U of *Msp*I per ml, respectively) and from EC cells treated with RA for 7 days (lanes 6 to 11; 0, 40, 120, 400, 600, and 800 U of *Msp*I per ml, respectively). Purified DNA was digested with *Bam*HI and hybridized with the *Bam*HI-*Msp*I 139-bp probe depicted in Fig. 4A. Arrows indicate the positions corresponding to each of the CCGG sites shown in Fig. 4A. (E) Expression of the CAT gene linked to the Oct-3/4 2.0-kb upstream region is downregulated in RA-treated EC cells. Untreated (lane 1) and RA-treated (lane 2) EC cells were cotransfected with 10 μg of poct-3/4 CAT DNA together with 10 μg of pUC18 DNA and 5 μg of pCMV-βgal DNA. For transfection into RA-treated EC cells, 1 μM RA was added when the cells were plated. RA was present until the cells were harvested for the CAT assay. The percent conversion of each transfection was normalized to the β-galactosidase activity and is shown below the autoradiogram.

majority of the Oct-3/4 gene underwent de novo methylation at this site (Fig. 6C, lane 8). The *Hpa*II sites located in the 1.3-kb Oct-3/4 upstream region are unmethylated (Fig. 6C, lane 3). After 4 days of RA treatment, partial de novo methylation is observed, emphasized by the disappearance of the 0.26- and 0.45-kb bands and the appearance of higher-molecular-weight bands (Fig. 6C, lane 7). In EC cells treated for 14 days with RA, most of the *Hpa*II sites undergo de novo methylation (data not shown). Thus, in EC × fibroblast somatic cell hybrids and in RA-treated cells, in which Oct-3/4 expression is repressed, the Oct-3/4 regulatory upstream region underwent de novo methylation.

To address the issue of whether in RA-treated EC cells the chromatin structure of the Oct-3/4 upstream region undergoes a change, we digested isolated nuclei from EC cells treated with RA with increasing concentrations of *Msp*I. Purified DNA was redigested with *Bam*HI, and the patterns of *Msp*I sensitivity from untreated and RA-treated EC cells were compared (Fig. 6D). Whereas in EC cells all sites were sensitive to *Msp*I digestion, in RA-treated cells these sites were much less accessible to this digestion. Probing with the *Bam*HI-*Msp*I 139-bp fragment shows that mostly *Msp*I site 4 remains sensitive to digestion. Upon a longer treatment of EC cells with RA, site 4 also becomes insensitive to *Msp*I digestion (data not shown). Thus, in RA-treated cells, as in hybrid cells, the chromatin structure of the Oct-3/4 upstream region undergoes a change.

As shown in Fig. 6E, in RA-treated EC cells, the 2.0-kb Oct-3/4 upstream region does not confer activation upon a CAT reporter gene. In conclusion, as in EC × fibroblast hybrids, in RA-treated cells the Oct-3/4 mRNA and binding activities are repressed and the upstream regulatory region is inactive, undergoes de novo methylation, and undergoes changes in chromatin structure.

DISCUSSION

The formation of hybrids between somatic cells can result in various patterns of gene expression, depending on the nature of both parental cell types. Among the different phenotypes that appear after fusion, extinction of specific cellular functions is most frequently observed (12). Previous studies utilizing several different hybrid cell systems, notably the pituitary cell × fibroblast, B-cell × fibroblast, and hepatoma × fibroblast hybrids, have analyzed the extinction of tissue-specific gene expression of growth hormone, immunoglobulin, α_1-antitrypsin, and albumin, respectively (2, 3, 8, 10, 26, 36, 56, 59). These studies concentrated on assessing the expression of transcription factors known to play a pivotal role in expression of the above-mentioned tissue-specific genes. In most cases, it was found that extinction is accompanied by repression of transactivators (Pit-1/GHF1, Oct-2, LF-B1, and HNF-1) that bind to crucial sequences in the promoter/enhancer regulatory element. Very little is known concerning the molecular mechanisms underlying repression of expression of these transcription factors in hybrid cells.

In this study we evaluate, for the first time, the molecular mechanism that underlies the extinction of a tissue-specific transcription factor, Oct-3/4, in an attempt to understand the essence of the extinction phenomenon. Oct-3/4 belongs to the POU-homeo transcription factor family, which includes Oct-2 and Pit-1/GHF1 as well. The Oct-3/4 gene product is the first transcription factor described that is specific for early stages of mouse development. It is expressed in primordial germ cells, unfertilized oocytes, and mouse blas-

tocysts. After day 8 of embryogenesis, Oct-3/4 expression decreases (46, 48). It has also been shown that Oct-3/4 mRNA is repressed in RA-treated EC cells. Exposure of EC cells to RA leads to acquisition of a fibroblast-like morphology (41). Until now, very little has been elucidated concerning the molecular mechanisms that repress Oct-3/4 gene expression during embryogenesis and in differentiated EC cells.

Our study demonstrates that Oct-3/4 expression is repressed in somatic cell hybrids between OTF9-63 cells and LTK^- cells. The extinction of Oct-3/4 expression occurred in a pool of approximately 500 different clones that lost only about 10 to 15% of their chromosomes. In addition, our RFLP analysis shows that the hybrid cells contain Oct-3/4 alleles from both parents. Thus, we think it highly unlikely that the absence of Oct-3/4 expression in our hybrid cells is due to chromosomal loss. The absence of Oct-3/4 binding activity in the hybrid nuclear extracts is not due to a fibroblast-encoded repressor that binds to the octamer sequence and prevents Oct-3/4 from binding to the same sequence. Our transient transfections using the Oct-3/4 expression vector together with an octamer-dependent reporter plasmid, and the nuclear extract mixing experiments, argue against this possibility. Rather, we have shown that the extinction of Oct-3/4 binding activity is at the level of mRNA transcription, similar to the repression of Oct-3/4 expression in RA-treated EC cells.

We have studied the molecular mechanism that underlies extinction of Oct-3/4 mRNA. Interestingly, the shutdown of Oct-3/4 transcription is accompanied by de novo methylation of 1.3-kb upstream sequences of the Oct-3/4 gene. The primary role of CpG methylation in regulation of gene expression is a matter of debate, but a number of studies have established that methylation of the 5′ end of several genes is incompatible with their transcription. Methylation probably inhibits gene expression by altering DNA-protein interactions through two possible routes. The direct route envisages that DNA methylation interferes with the binding of specific transcription factors such as in the case of the cyclic AMP-responsive element-binding protein (CREB) (23). However, not all transcription factors are methyl sensitive; Sp1 can bind and activate regardless of the methylation pattern of its binding site (22). Interestingly, in the Oct-3/4 upstream regulatory region, *Hpa*II site 4 is colocalized with an ETF binding site (27). It has been shown that ETF specifically stimulates transcription from promoters without a TATA sequence (28). We have not yet evaluated the importance of this binding site to Oct-3/4 transcription or whether ETF can bind a methylated sequence, but it is interesting that Oct-3/4 is also a TATA-less promoter. The indirect route, on the other hand, suggests that methylated DNA is bound by proteins that secondarily prevent specific transcription activators from binding DNA. Recently, it has been shown that methylated DNA is bound by nuclear proteins, such as MeCP1, that secondarily prevent interaction of transcription factors and lead to transcriptional inactivation (6) and by MeCP2 (32). We do not know whether either MeCP1 or MeCP2 is involved in mediating Oct-3/4 repression in the OTF9-63 × fibroblast hybrid cells, but it is interesting that EC cells contain very low levels of MeCP1 and MeCP2 proteins (32, 37).

It has been shown that in the nucleus, methylated DNA has reduced accessibility to nucleases (1, 37). Indeed, in the hybrid cells, the Oct-3/4 upstream region that underwent de novo methylation became resistant to the naked DNA methyl-insensitive restriction endonuclease *Msp*I. This finding implies that methylated sites in the hybrid cells nuclei are made inaccessible, presumably by association with nuclear proteins.

Our transient transfection experiments employing the CAT reporter gene driven by the Oct-3/4 upstream sequences show that extinction is not restricted to the endogenous Oct-3/4 gene but is also exerted upon a newly transfected reporter gene driven by the Oct-3/4 regulatory region. Thus, Oct-3/4 inactivation is accompanied by a change in the cellular activity of *trans*-acting factors (either a loss of activators or induction of repressors) acting on the Oct-3/4 upstream region of the gene.

Since Oct-3/4 expression is down-regulated in RA-treated EC cells, Oct-3/4 provides a unique model system with which to compare the molecular mechanisms that underlie its extinction in somatic cell hybrids versus those that play a role during cellular differentiation. We have compared Oct-3/4 mRNA levels, DNA binding abilities, chromatin and methylation patterns, and activities of the Oct-3/4 upstream regulatory sequences in hybrids versus RA-treated EC cells. In both systems, the suppression of Oct-3/4 expression is achieved through changes in the methylation status, chromatin structure, and *trans*-acting factors that act on the Oct-3/4 upstream regulatory region. This finding may imply that at least similar repression mechanisms operate in RA-differentiated EC cells and in EC × fibroblast somatic cell hybrids. Thus, somatic cell hybrids could probably be used to identify regulatory circuits that are less apparent in biochemical approaches.

The molecular mechanism that underlies Oct-3/4 extinction in hybrid cells is very different from the one previously reported to be involved in extinction of the CREB transcription factor, which regulates tyrosine aminotransferase gene expression, in hybrid cells. Extinction in that case is mediated by a specific genetic locus (TSE1) recently shown to encode the regulatory subunit of protein kinase A (5, 25).

What could be the primary mechanisms that repress Oct-3/4 transcription in hybrid cells? One mechanism postulates that the fibroblast suppresses transactivators which are critical for tissue-specific expression. Most of the studies on hybrids cells reveal a coordinate negative regulation of a transactivator and a target gene. However, this coordination does not prove that extinction is mediated by the loss of one transcription factor only. It has been shown that constitutive expression of LF-B1 was not sufficient to prevent α_1-antitrypsin extinction (8). Furthermore, in the case of Oct-3/4, it is unlikely that transfection of OTF9-63 × fibroblast hybrid cells with a transactivator that may be missing in these cells and that is capable of binding to the Oct-3/4 upstream region will activate the dormant Oct-3/4 gene. This region is probably inaccessible to transcription factors because of its altered methylation and chromatin structure.

Extinction of Oct-3/4 expression could operate through suppression of one, but more likely more than one, transcription factor, which is then followed by a change in the methylation status and chromatin structure of the DNA. Thus, it is reasonable to suggest that in order to prevent extinction of a tissue-specific gene, one would have to supply the nonfibroblast parent cell prior to fusion with fibroblasts with a battery of constitutively expressed transcription factor genes that control the regulatory expression of a particular tissue-specific gene. This approach may rescue the gene from extinction only if extinction operates at the level of transcription and not at subsequent levels of regulation.

It seems that repression of tissue-specific structural genes,

such as those encoding albumin, immunoglobulin, and growth hormone, and suppression of the expression of tissue-specific transcription factors, such as Oct-3/4, occur at the transcription level, possibly utilizing very similar mechanisms. This inference points to the alternative possibility that extinction of a tissue-specific gene may not be the result of suppression of crucial transcription factors but rather may be the consequence of activation of a dominant repressor(s). This dominant negative factor(s) may shut off a battery of tissue-specific genes, including those encoding tissue-specific transcription factors, by acting directly on the genes themselves or by influencing the locus control regions. Use of somatic cell hybrids may help to genetically identify these negative repressors.

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