Protein – DNA interactions in the cAMP responsive promoter region of the murine ornithine decarboxylase gene

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ABSTRACT

To evaluate the function of the murine ornithine decarboxylase (ODC) gene promoter, expression of chimeric ODC-chloramphenicol acetyltransferase (CAT) plasmids (pODCcat) containing 1,658 nt of the ODC promoter sequence and its various 5'-deletions was analyzed. In transient expression assays with NIH/3T3 mouse cells, pODCcat constructs exhibited fairly strong promoter activity yielding CAT values up to 40% of those obtained with the viral promoter RSV. Interestingly, 5'-deletions of the pODCcat constructs increased the promoter activity over that achieved using the entire 1.6-kb 5'-flanking region, with the highest activity being observed with about 750 nt of the ODC promoter. This finding suggests that the distal part of the promoter includes DNA elements which are involved in repressing its function. The promoter region could be deleted down to the proximal 97 nt and still be stimulated by cAMP to the same extent as the 1.6-kb promoter. DNase I footprinting and methylation interference studies showed that a specific protein binds to the region from –59 to –39, which encompasses a DNA motif resembling the consensus cyclic AMP response element (CRE). However, comparative gel retardation and Southwestern blotting experiments with the putative ODC-CRE and the somatostatin promoter CRE indicated that the 70-kDa protein interacting with the CRE-like element of the ODC promoter is different from the well-characterized nuclear CRE-binding protein CREB.

INTRODUCTION

Ornithine decarboxylase (ODC) is the first and key regulatory enzyme in the biosynthesis of polyamines, which appear to be indispensable for mammalian cell growth (1–3). ODC is a ubiquitous housekeeping enzyme which is highly regulated and responds to a large number of stimuli affecting cell growth and differentiation, such as male and female sex steroids and compounds that mediate their actions *via* cyclic AMP (4, 5). The

enzyme activity seems to be regulated at multiple levels, including the rate of transcription, the stability and translational efficiency of mRNA, and the rate of degradation and/or covalent modifications of the enzyme protein (1–10).

To elucidate the nature of *trans*-acting protein factors and *cis*-acting DNA elements involved in the function and regulation of the murine ODC gene, we have previously isolated and sequenced about 1.6 kb of the 5'-flanking region of the expressed mouse gene (11). The mouse and rat ODC promoters are very similar, with about 92% sequence conservation in the first 380 nt of the 5'-flanking DNA that is very GC rich (76%) (11–14). Likewise, the proximal promoter of the human ODC gene exhibits over 70% sequence identity with the murine gene (15). The mouse promoter sequence was found to contain several putative DNA motifs for binding of various transcription factors (11–13); however, no studies on the functional aspects of these elements have been described. In the present communication, we report the localization of a cyclic AMP response element within the proximal promoter region of the murine ODC gene and characterize protein-DNA complexes formed within this region.

MATERIALS AND METHODS

Materials

Restriction endonucleases were purchased from Boehringer Mannheim (Mannheim, Germany) or New England Biolabs (Beverly, MA). T4 polynucleotide kinase and DNase I were from BRL-Gibco (Gaithersburg, MD). Poly(dI-dC).(dI-dC) was from Pharmacia (Piscataway, NJ). 8-Bromo-cyclic AMP (8Br-cAMP) and o-nitrophenyl- β -D-galactopyranoside were from Sigma (St. Louis, MO). pSV0cat, pSV2cat, and pRSVcat were obtained from the American Type Culture Collection (Rockville, MD). pPLcat was prepared by inserting a polylinker containing multiple restriction enzyme sites in front of the CAT gene of pSV0cat. pSV- β -galactosidase (pSV- β -gal) control plasmid was from Promega (Madison, WI). Double-stranded DNA oligonucleotides were obtained by annealing chemically synthesized (Pharmacia Gene Assembler) complementary strands as previously described

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(16). Oligonucleotides containing the consensus sequences for AP-1, AP-2, NF-1/CTF, and Sp-1 binding sites were purchased from Stratagene (La Jolla, CA). The following oligonucleotides were used (only the upper strand is shown):

ODC-s: GCGTATGGGCGGGTGGGTGGGCACGCGCTGC-GCCCCGCCCCACTGACGCG (−218 to −169 of the murine ODC promoter);
 ODC-a: TCCCGGCCGGAACCGATCGCGGCTGGTTT-GAGC (−94 to −62);
 ODC-b: AGCTGGTGGCTCTCCATGACGACGTGC (−64 to −37);
 ODC-c: GTGCTCGGCGTATAAGTAGCGGCGCGTCGC (−41 to −12);
 ODC-d: TGGCTCTCCATGACGAC (−58 to −42);
 ODC-e: TGACGACGTGCTCGGCG (−48 to −32);
 ODC-H: TGGCTCTCCATGGCGAC (−52 to −42);
 SOM-CRE: CCTCCTGGCTGACGTCAGAGAGAGAG (−58 to −32);
 HSP-70: AAGGCGGGTCTCCGTGACGACTTATAA (−52 to −26);
 AP-1: CTAGTGATGAGTCAGCCGGATC;
 AP-2: GATCGAACTGACCGCCCCGCGGCCCGT;
 NF-1: ATTTTGGCTTGAAGCCAATATG; and
 Sp-1: GATCGATCGGGGCGGGGCGATC.

Prestained molecular weight markers were from Bio-Rad (Richmond, CA). [γ - 32 P]ATP (3000 Ci/mmol) and [3 H]acetyl-CoA (0.2 Ci/mmol) were purchased from New England Nuclear (Boston, MA). HeLa cell nuclear extract, recombinant upstream stimulatory factor (USF, ref. 17), and USF antiserum were gifts from Dr. R. Roeder (The Rockefeller University, New York, NY).

Plasmid constructions

The 1,658-nt long mouse ODC promoter sequence plus the first 13 nt of exon I were isolated as a single *Pst* I fragment (11) and inserted into the *Nsi* I site of a promoterless chloramphenicol acetyltransferase reporter vector (pPLcat), which contained a multiple cloning site in front of the CAT gene. 5'-Deletion mutants were generated using the Erase-a-base exonuclease III digestion system (Promega). The length of all deletion mutants was verified by DNA sequencing using double-stranded plasmid templates and the dideoxy chain-termination method (18).

Cell culture and transfections

NIH/3T3 mouse cells were obtained from the American Type Culture Collection. Cells were cultured in a medium containing Dulbecco's modified Eagle's medium supplemented with 10% calf serum. For transfection experiments, cells from a subconfluent dish were seeded at a density of 5×10^5 cells/dish and transfected 24 h later with 10 μ g of CAT plasmid DNA using the calcium phosphate co-precipitation method as described in (19). Seven μ g of pSV- β -gal plasmid was cotransfected with the CAT constructs in each experiment to serve as an internal control for transfection efficiency. To further minimize experimental variation, plasmids were transfected onto duplicate or triplicate dishes for each treatment. After an 18-h incubation with the precipitate, the cells were washed with phosphate-buffered saline and fresh medium containing either 0.5 mM 8Br-cAMP or no additives was added. Cells were harvested and extracts prepared after a 24-h induction. CAT assays were performed as described

by Neuman *et al.* (20) and Eastman (21), and care was taken to keep the enzyme reaction time and sample volume such that CAT activities were within the linear range of the assay. β -Galactosidase activity was determined according to Rosenthal (22). For each sample, CAT activity was divided by β -galactosidase activity to normalize for transfection efficiency.

Preparation of nuclear extracts

Nuclear extracts were prepared from mouse tissues essentially according to Gorski *et al.* (23), except that nuclear pellets were resuspended directly in the nuclear lysis buffer that contained 0.75 mM spermidine and 0.15 mM spermine instead of MgCl_2 . KCl concentration in the dialysis buffer was 90 mM. Proteinase inhibitors (phenylmethylsulfonyl fluoride, 0.25 mM; antipain, 1 μ M; pepstatin A, 1 μ M; and leupeptin, 1 μ M) were added to all buffers before use. Extracts from the cultured cells were prepared according to the method of Shapiro *et al.* (24), frozen in small aliquots in liquid nitrogen and stored at -70°C . Protein concentration was determined using the Bio-Rad protein assay system according to manufacturer's instructions.

Gel retardation assay

The binding reaction mixture (20 μ l) contained typically 10 μ g nuclear protein with 6 μ g poly(dI-dC)(dI-dC) in 20 mM HEPES (pH 7.9), 45 to 135 mM KCl, 1 mM dithiothreitol, 2.5 mM MgCl_2 , 0.4 mM EDTA, and 10% (v/v) glycerol. After a preincubation on ice for 10 min, 0.5 ng of a double-stranded oligonucleotide or 2–3 ng of a restriction fragment labeled with T4 polynucleotide kinase was added and the incubation was continued at room temperature for 20 min. The protein-bound DNA complexes were separated from the free probe immediately after the incubation on a 4% polyacrylamide gel (29:1) run in $0.25 \times \text{TBE}$ (1 $\times \text{TBE}$: 90 mM Tris base, 90 mM boric acid, and 2 mM EDTA). Gels were dried and autoradiographed under Kodak X-Omat AR films.

DNase I protection experiments

The −136 to +13 ODC promoter fragment, which was isolated from pODCcat by digestion with *Ava* I and *Xba* I, was end-labeled with T4 polynucleotide kinase and recut with *Alw*NI to obtain the −136 to +4 fragment for DNase I protection of the upper strand. The end-labeled −308 to +13 fragment generated by digestion with *Sph* I and *Xba* I was recut with *Hinf* I to yield the fragment −118 to +13 for DNase I protection of the lower strand. These labeled fragments were purified by polyacrylamide gel electrophoresis. Preincubations and incubations were carried out as in the gel retardation assay, except that the amount of protein used was 35 μ g in a 20- μ l reaction. Competition reactions included 45 ng of a specific oligonucleotide in the binding reaction. After an incubation at room temperature for 20 min, 40 ng (2 μ l) of freshly diluted DNase I [dilution buffer: 50 mM Tris-Cl (pH 8.0), 100 mM NaCl, 1 mM dithiothreitol, 1 mM CaCl_2 , and 30% glycerol] was added to the binding mixture. The digestions were terminated after 60 s at room temperature by addition of 80 μ l of proteinase K buffer [125 mM Tris-Cl (pH 7.5), 250 mM NaCl, 30 mM EDTA, and 1.25% SDS] plus 10 μ g of glycogen and incubated with 10 μ g of proteinase K for 15 min at 65°C . DNA was recovered after phenol-chloroform extraction by ethanol precipitation and analyzed on 10% sequencing gels containing 10 M urea along with G and G+A lanes (Maxam–Gilbert) as guides for identifying the protected regions.

Methylation interference analysis of G residues was performed as described in (19). The end-labeled -136 to $+4$ promoter fragment was generated as described above for DNase I protection. The labeled fragment was partially methylated with dimethyl sulfate and incubated with nuclear extract in a 5-fold scaled-up gel retardation assay mixture. The bound and free DNA regions were isolated from a preparative band-shift run, cleaved with piperidine, and analyzed on a sequencing gel.

Southwestern blotting

Nuclear proteins (50 μ g protein/lane) were resolved by 10% SDS-polyacrylamide gel electrophoresis along with prestained molecular weight markers and transferred to a nitrocellulose membrane. The transfer buffer contained 25 mM Tris base, 192 mM glycine, 1 mM EDTA, and 0.01% SDS. After the transfer, the filter was renaturated (25) overnight at 4°C in buffer SW [20 mM HEPES (pH 7.9), 100 mM NaCl, 0.1 mM EDTA, 2 mM MgCl_2 , 1 mM dithiothreitol, and 10% glycerol] containing 5% (w/v) Carnation nonfat dry milk with gentle agitation. Before addition of the probe, the filter was briefly washed once with buffer SW containing 0.25% nonfat dry milk and preincubated in the same buffer in the presence of 35 μ g poly(dI-dC)(dI-dC)/ml. After a gentle shaking for 30 min at room temperature, the end-labeled oligomer was added to 0.8×10^6 cpm/ml and the incubation was continued for 60 min. The filter was then washed three times (10 min/wash) in buffer SW containing 150 mM NaCl and 0.25% (w/v) nonfat dry milk.

Southwestern blotting was also performed utilizing the renaturation procedure of Silva *et al.* (26). Briefly, after SDS-polyacrylamide gel electrophoresis, the gel was immersed in renaturation buffer containing 50 mM NaCl, 10 mM Tris-Cl (pH 7.5), 20 mM EDTA, 0.1 mM dithiothreitol, and 4 M urea for two 1-h cycles with gentle agitation. Thereafter, the proteins were transferred to nitrocellulose and the filter was processed as described above.

Fig. 1. Effect of 5'-deletions on the constitutive activity of the murine ODC promoter. NIH/3T3 cells were transiently transfected with the indicated 5'-deletion plasmids of the parent vector pODCcat ($-1,658/+13$) and with the pSV- β -gal vector as an internal control, using the calcium phosphate coprecipitation technique. The cell extracts were prepared 48 h after transfection. All CAT activities were first normalized using the β -gal activity in the same extract, and the relative values for the pODCcat ($-1,658/+13$) were set as 100. The histogram shows the mean values (S.D.) for four separate transfection experiments, each performed with triplicate dishes. Background CAT activity produced by the promoterless pPLcat vector was constantly less than 4% of that of the pODCcat ($-1,658/+13$).

RESULTS

Functional analysis of the ODC promoter

We have previously reported the nucleotide sequence of the mouse ODC promoter up to the position $-1,658$ (11). To test the function of this region as a promoter in a reporter gene construct, nucleotides $-1,658$ to $+13$ relative to the mRNA cap site were inserted 5' of the CAT reporter gene. In transient expression studies with NIH/3T3 mouse cells, this construct exhibited a fairly strong promoter activity and yielded CAT values that were up to 40% of those achieved with a vector containing the Rous sarcoma virus promoter (range: 25–40%). For functional analysis of the promoter, a series of CAT constructs containing progressive deletions of the 5'-flanking region were transfected into NIH/3T3 cells. Fig. 1 shows that the deletion of the region from -1658 to -752 of pODCcat increased the promoter activity 2-fold. This suggests that the distal part of the ODC promoter contains DNA elements involved in repressing its function. The promoter activity was decreased dramatically by a deletion that removed the sequence from -427 to -208 , suggesting that major determinants of the basal ODC promoter function are within this region. Interestingly, a further deletion of the region from -208 to -97 had only a minor effect on the basal CAT activity.

Protein binding to the murine ODC promoter

In these experiments, we screened the promoter region up to position -564 for nuclear protein binding using the gel retardation technique. There are several potential DNA elements for binding of *trans*-activating factors within this region, such as four Sp-1 sites, a putative CAAT element, two CRE-like sequences and a TATA/TFIID factor binding site. We used the overlapping restriction fragments -564 to -268 (ODC564), -308 to $+13$ (ODC308), -267 to $+13$ (ODC267) and -136

Fig. 2. Detection of DNA-protein interaction(s) in the murine ODC promoter by the gel retardation technique. **Panel A:** ^{32}P -labeled DNA fragment from -136 to $+13$ was incubated with duplicate nuclear extracts from mouse kidney (lanes 2 and 3), liver (4 and 5), and spleen (6 and 7) or without protein (lane 1). The bracket depicts the four different protein-DNA complexes and 'F' refers to unbound DNA. **Panel B:** Competition analysis with synthetic double-stranded oligomers using ^{32}P -labeled DNA fragment from -308 to $+13$ as a probe and mouse liver nuclear extract. In the absence of competitor (lane 1); in the presence of a 250-fold molar excess of a 17-mer, 5'-TGCGTCTCCATGACGAC-3', corresponding to the region from -58 to -42 of the ODC promoter (lane 2); in the presence of a 120-fold molar excess of a 22-mer containing the AP-1 consensus sequence 5'-TGAGTCA-3'; in the presence of a 250- and 1000-fold excess of a 17-mer, 5'-TGACGACGTGCTCGGCG-3', corresponding to the region from -48 to -32 of the ODC promoter (lanes 4 and 5, respectively).

Fig. 3. Detection of DNA-protein interactions in the murine ODC proximal promoter by methylation interference and competition analysis with double-stranded oligomers containing single base substitutions. **Panel A:** Methylation interference analysis. The upper strand of the ODC promoter from -136 to $+4$ was end-labeled, partially methylated with dimethyl sulfate, and incubated with mouse kidney nuclear extract. The bound (B) and the free DNA (F) were isolated from a preparative band-shift gel, cleaved with piperidine and analyzed in a sequencing gel. The important contact G residues are indicated by arrowheads. **Panel B:** Competition analysis. A 32 P-labeled 27-mer corresponding to the region from -64 to -37 (ODC-b) was used as a probe. Shown is the position of each single base substitution within core binding motif in oligonucleotides M-1, M-2, and M-3. These oligomers were used as unlabeled competitors. Fold molar excess of each mutated competitor over the unsubstituted ODC-d oligomer (WT, 200-fold excess) is shown above the lanes.

to $+13$ (ODC136) as labeled probes with nuclear extracts from mouse tissues in gel retardation studies, when addressing the question of whether protein binding to these elements can be demonstrated *in vitro*. Complexes formed with ODC564 were very weak and were not further characterized in the present work. Fragments ODC308, ODC267 and ODC136 all formed a similar, strongly retarded major complex with nuclear extracts from mouse liver, spleen and kidney (Fig. 2A). This specific complex, which consisted of a subset of four separate bands (numbered I to IV in Fig. 2), could not be demonstrated with the fragment -308 to -136 (although it formed other, weaker complexes), indicating that the binding site was located within the proximal promoter region. The complex was stable up to 200 mM KCl, and the presence of $MgCl_2$ in the incubation buffer slightly enhanced the DNA-protein complex formation (data not shown).

We were able to demonstrate that the core DNA region involved in the major complex formation is localized to the region -58 to -42 of the promoter, by using a series of synthetic DNA fragments as competitors in gel retardation assays. The formation of the complexes corresponding to the bands I-III were clearly abolished by competition with the oligomer -64 to -37 as well as the oligomer -58 to -42 , but not with the oligomer -94 to -62 or the oligomer -48 to -32 (Fig. 2B, and data not shown). An oligomer containing the AP-1 factor consensus binding sequence ($5'$ -TGAGTCA- $3'$) was unable to compete for the labeled complex formation (Fig. 2B). Moreover, no competition was observed using oligonucleotides containing AP-2 or NF-1/CTF binding sites (data not shown).

Fig. 4. DNase I footprinting analysis of the proximal promoter of the murine ODC gene. **Lower strand:** a promoter fragment from -118 to $+13$ was end-labeled and footprinted with nuclear extract from mouse liver. Lane 1, intact probe; lanes 2 and 6, no added nuclear extract; lanes 3–5, each reaction contained 35 μ g nuclear extract in the absence (lanes 3 and 5) or presence of 45 ng of unlabeled competitor oligomer ODC-d (lane 4). Total DNase I added to each reaction was 40 ng and mixtures were incubated at room temperature for 60 s. The reaction products were analyzed on a 10% sequencing gel containing 10 M urea. The DNase I-protected sequence is indicated by the shaded bar on the left. Maxam–Gilbert A and A+G sequencing reactions are shown. **Upper strand:** a promoter fragment from -136 to $+4$ was end-labeled and used for footprinting with mouse liver nuclear extract. Lanes 1 and 5, without added nuclear extract; lanes 2–4, each reaction contained 35 μ g nuclear extract without (lanes 2 and 4) or with 45 ng of unlabeled competitor oligomer ODC-d (lane 3).

In order to identify precisely the G nucleotide contact sites, labeled DNA was partially methylated with dimethyl sulfate, incubated with mouse nuclear extracts, and free and bound probes resolved and examined. As seen in Fig. 3A, methylation of the three G nucleotides (positions -55 , -47 , and -44) prevented binding. The methylation interference data was confirmed by competition experiments with oligomers containing point mutations of the critical G residues that were identified by the methylation interference analysis. While the wild-type ODC oligomer (ODC-d) abolished efficiently the formation of a labeled complex at a 200 -fold molar excess, oligonucleotides M-1, M-2 and M-3 failed to compete equally well, even when five times higher concentrations of these competitors were used (Fig. 3B). However, oligonucleotide M-3, in which the G at position -44 was mutated to a T, competed clearly better than oligomers M-1 and M-2. The data with the mutated ODC-d sequences were in agreement with those shown in Fig. 2B, in which case the oligomer ODC-e (-48 to -32) failed to influence protein complex formation with the fragment ODC136. These experiments thus indicated that the three G residues (-55 , -47 , and -44) underlined in the sequence $5'$ -GCGTCTCCATGACGAC- $3'$ are all important for the DNA-protein complex formation, with the two more distal Gs being more significant than the the most $3'$ one. The corresponding region of the rat ODC gene (14) is identical with the sequence above, and the human gene differs from the murine sequence only in one nucleotide as it contains a G instead of an A in position -46 (15). This difference between the murine and human sequences did not affect the DNA-protein complex formation, as analyzed

Fig. 5. Reciprocal competition analysis between the ODC-CRE-like element-containing oligomer (ODC) and the somatostatin CRE oligomer (SOM-CRE). The nuclear proteins were incubated with 32 P-labeled 27-nt long oligomers corresponding to the region from -64 to -37 of the ODC promoter or the region from -51 to -29 of the somatostatin promoter. The molar excess of each unlabeled competing oligomer is shown. Nuclear extract from mouse liver (M) and HeLa cells (H) were used.

Fig. 6. Reciprocal competition analysis between the 27-mer oligonucleotide ODC-b (ODC) and a 27-mer oligonucleotide corresponding to the region from -52 to -26 of the human HSP-70 gene promoter (HSP-70). The molar excess of each unlabeled competing oligomer is shown. The nuclear extract used in this experiment was from HeLa cells; similar results were obtained using nuclear extracts from mouse tissues.

by gel retardation assays with an oligonucleotide corresponding to the human sequence (data not shown).

The results from gel retardation and methylation interference studies were further verified using DNase I protection analysis. Nuclear extract from mouse liver demonstrated the presence of a protein that bound to DNA encompassing the region from -59 to -39 (Fig. 4). To further evaluate the competition seen with the oligomer ODC-d in gel retardation assays, we used the same oligomer in the footprinting analysis and verified that it competes also under these experimental conditions (Fig. 4).

The binding element described above contains the sequence 5'-TGACGACG-3' which is similar to the well-characterized palindromic cAMP response element (CRE), 5'-TGACGTCA-3'. Therefore, we synthesized for comparative studies the CRE of the rat somatostatin gene (SOM-CRE) flanked by sequences of its natural environment [nucleotides -58 to -32 of the somatostatin gene (27)]. Both the putative ODC-CRE and SOM-CRE were labeled and used as probes in gel retardation assays. Strong and specific SOM-CRE-binding activity was observed in nuclear extracts from various mouse tissues, such as liver, kidney and spleen, and from cultured mouse (3T3) and human cells (HepG2). This activity was clearly different from that seen with the CRE-like element of the ODC promoter, as illustrated in Fig. 5 using nuclear extracts from mouse liver. Extracts from other mouse tissues and murine and human cell lines yielded identical results (data not shown). Furthermore, reciprocal competition experiments with the putative ODC-CRE and SOM-CRE showed that distinctly different proteins bind to these two elements (Fig. 5). Phosphorylation of nuclear extracts with the catalytic subunit of the cAMP-dependent protein kinase (Sigma) did not change the affinity of the proteins for the CRE-like element of the ODC promoter, or alter the mobility of the DNA-protein complexes in the band-shift gels (data not shown).

The footprinted region of the murine ODC gene (from -59 to -39) was used for a computer-assisted search for homologous

Fig. 7. Characterization of DNA-protein complexes by Southwestern blotting. Nuclear protein extracts (50 μ g protein/lane) were separated on a 10% SDS-PAGE and transferred onto nitrocellulose membranes either directly (panel A) or after a renaturation step in 4 M urea (panels B and C). The filters were probed with 32 P-labeled 27-nt long oligomers corresponding to the region from -64 to -37 of the ODC promoter (panels A and B) or the region from -58 to -32 of the rat somatostatin CRE (panel C). **Panel A:** HepG2 cells (lane 1), mouse liver (lane 2), mouse spleen (lane 3), mouse kidney (lane 4), and NIH/3T3 cells (lane 5). **Panels B and C:** HepG2 cells (lane 1) and mouse spleen (lane 2).

DNA sequences in the most recent GenBank/EMBL data resources. Intriguingly, the human heat-shock protein 70 (HSP-70) gene (28) was found to contain a very similar sequence, 5'-GCGGGTCTCCGTGACGACTTA-3' (-49 to -29), partly overlapping with the TATA box. The DNA-protein complexes formed with the oligomer containing this sequence were efficiently competed for by the corresponding ODC oligomer, suggesting that both ODC and HSP-70 elements bind similar nuclear proteins present in mouse and human cells (Fig. 6).

Southwestern blotting technique was utilized to further characterize the protein(s) binding to the CRE-like motif of the ODC promoter. Renatured proteins bound to nitrocellulose filters were probed with labeled oligomers in the presence of poly(dI-dC)(dI-dC). This technique revealed that a 70-kDa nuclear protein is the major component interacting with the ODC-CRE, whereas

proteins with relative molecular masses of about 45 kDa are the major components binding to the SOM-CRE (Fig. 7). These results provided, therefore, additional support to the idea that the putative ODC-CRE binding protein is different from the well-characterized SOM-CRE binding protein CREB (29).

Cyclic AMP responsiveness of pODCc constructs

To test whether the murine ODC gene promoter contains functional CRE sequences, we studied regulation of pODCc activity, transiently expressed in mouse NIH/3T3 cells, by an elevated intracellular cAMP concentration. The construct containing the entire 5'-flanking region (−1,658 to +13) responded significantly to the treatment with 8Br-cAMP (0.5 mM) with a stimulation factor of 3.6 ± 0.5 ($n=4$), suggesting that the ODC promoter contains one or more cis-acting elements mediating the cAMP responsiveness. Inspection of the promoter sequence revealed that, in addition to the CRE-like element described above (commencing at position −48), another sequence with 5 out of 8 nucleotides matching with the CRE is present at the position −174. Since we were not capable of demonstrating protein-DNA interaction around this latter position in our initial gel retardation experiments (see above), we proceeded to study whether the elements within the proximal 97 nt of the ODC promoter are sufficient to confer cAMP inducibility to CAT expression. Although the basal expression of the most proximal pODCc (−97 to +13) construct was only about 30% of that of the entire 5'-flanking region, its relative cAMP responsiveness was undiminished, being 4.3 ± 0.7 ($n=4$) times higher with 0.5 mM cAMP than without it. Under these conditions, the CAT activities elicited by transient expression of pRSVcat and pPLcat constructs were influenced minimally, or not at all by cAMP [in all experiments ($n=8$) by a factor of 1.3 or less]. These results suggest strongly that the element mediating cAMP induction resides within the proximal promoter region and that the DNA motif at position −58 to −42 is a good candidate for this function.

DISCUSSION

The relative strength of the murine ODC promoter in our transient expression assays with the bacterial CAT gene as a reporter was similar to that described by Brabant *et al.* (13) and Katz and Kahana (12), *i.e.*, the 1.6-kb flanking region was about one-third to one-half as strong as of the RSV promoter. 5'-Deletions increased significantly the constitutive promoter activity in a mouse fibroblast cell line (NIH/3T3), with the maximal CAT expression being achieved with constructs containing about 750 nt of the murine ODC promoter. In this respect, our results are distinctly different from those of Zheng *et al.* (30), who reported maximal promoter activity in a mouse macrophage-like cell line, RAW264, with a CAT construct containing only the most proximal 90 nt of the murine ODC promoter. Whether this disparity results from differences in the experimental conditions or represents tissue- and/or cell-specific regulation of the ODC promoter remains to be investigated. The constitutive activity of the proximal rat ODC promoter in 3T3-like, fibroblast cells (Rat-1) was, however, very similar to that of the murine promoter, in that the maximal activity was about 40% of that of the RSV promoter and that deletions from position −398 to −105 decreased its activity to one-third (31). This behavior is in good agreement with the highly conserved nature of the proximal promoters of the two rodent ODC genes (11–14, 31).

The mouse ODC promoter is extremely GC-rich up to approximately −500 nt from the transcription start site (11–13), and it contains a number of potential Sp-1 binding elements. Deletion of 220 nt of the 5'-sequence (from −427 to −208) reduced the promoter strength markedly, indicating that the GC-rich region plays an important role in determining its basal function. It was, therefore, somewhat surprising that our initial DNA-protein interaction studies revealed only weak protein complex formation with the fragment ODC564 (−564 to −268). The most likely explanation is that complexes formed with the GC-rich region have relatively low binding affinity, and they did not survive under the conditions of our band-shift studies, using relatively long DNA fragments. This notion is supported by the experiments we performed with an oligonucleotide encompassing the region −208 to −168 and containing the two proximal Sp-1 elements of the promoter: it was only this probe that permitted detection of nuclear protein binding to these sites in the mouse ODC promoter. The binding was readily abolished by a 100-fold molar excess of an oligonucleotide containing the Sp-1 consensus sequence 5'-GGGGCGGGG-3', implying that mouse tissues do contain nuclear proteins that interact with at least one of the Sp-1 sites of the proximal ODC promoter (JJ Palvimo and OA Jänne, unpublished data).

There are several reports in the literature to indicate that ODC mRNA accumulation and the rate of ODC gene transcription are stimulated by cAMP or factors utilizing this signalling pathway (1–3, 32). At least in one study using 3T3 cells, forskolin that elevates intracellular cAMP concentration was found to elicit a several fold increase in the transcription rate of the ODC gene, as measured directly by nuclear run-on assays (32). Our studies with the chimeric ODC promoter-CAT constructs revealed that the cis-acting DNA element conferring cAMP responsiveness to the murine ODC promoter is located within its most proximal 90 nucleotides, most likely in the region from −58 to −42. Whilst this region contains an element (5'-TGACGACG-3') which is very similar to the consensus sequence of the classical CRE (5'-TGACGTCA-3'), the protein that forms a complex with the CRE-like element of the ODC promoter was shown to be different from CREB. In view of this, it is not surprising that the two mutations of the ODC promoter sequence (5'-AGACGACG-3' and 5'-TGTTACG-3'), known abolish the interaction of CRE with the CRE-binding protein CREB (33, 34), did not influence the responsiveness of the promoter to the combined exposure of a lipopolysaccharide and 8Br-cAMP of RAW264 cells (30).

Although the proximal promoter of the murine ODC gene contains an CRE-like element, the *trans*-acting factor conferring cAMP responsiveness to this gene is most likely not CREB. The protein binding to the region −58 to −42 has a molecular mass of about 70 kDa as opposed to 45 kDa for CREB (Fig. 7). In addition, somatostatin CRE failed to interfere with the complex formation between the ODC element and the 70-kDa protein. Several other proteins besides CREB have been shown to interact with CRE, including phosphoproteins ranging in molecular mass from 120, 72–65, 43–38, and 37–31 kDa (35, 36), and the protein binding to the *cis*-element of the ODC promoter may be one of these additional nuclear factors. In contrast to the above-mentioned proteins, however, the ODC promoter-binding protein bound poorly to the consensus CRE, suggesting that the sequences adjacent to the CRE-like element are more important determinants for its binding.

There are a number of recent reports to indicate that *cis*-

elements other than the CRE may mediate cAMP responsiveness. For example, several steroid hydroxylase genes contain their own sets of cAMP-responsive sequences, which are unrelated to that of CRE (37–39). Although it has been difficult to find areas of homology in the promoters of the steroid hydroxylase genes, the sequence 5'-CTTGATG-3' is conserved between the cAMP-responsive sequences of the CYP17 and CYP11A genes (38). A similar sequence (5'-CTCCATG-3') is located in the middle of the footprinted region from –58 to –42 of the murine ODC promoter. It remains to be studied whether this similarity has any bearing on the cAMP responsiveness of the above-mentioned genes, including the murine ODC gene.

Regulation of the human heat shock protein 70 gene is similar to that of the ODC gene in many respects, in that both of them respond to a variety of environmental stimuli and growth-promoting factors. In the case of the HSP-70 gene, DNA elements mediating responses to serum and adenovirus E1a protein appear to be different from the heat shock element (40). In view of this, it was of interest to observe a significant sequence identity between the proximal promoters of the human HSP-70 and mouse ODC genes: the sequence covering the mouse promoter region from –55 to –40 (5'-GTCTCCATGACGAGTT-3') has only two mismatches with the sequence from –45 to –30 of the HSP-70 promoter, and the corresponding region (–52 to –37) of the human ODC gene (15) has 12 of 16 nucleotides identical with those of the HSP-70 promoter. Another intriguing feature of these promoter elements was that they bear significant sequence similarity to the DNA motifs interacting with the human upstream stimulatory factor (USF), such as the mE3 enhancer and the ML promoter (17). However, when tested directly in band-shift experiments, recombinant USF protein did not bind to ODC-b oligomer, anti-USF antiserum did not influence the mobility of ODC-b oligomer-protein complexes, and the consensus oligomer for USF binding did not compete for the interaction between the 70-kDa protein and the ODC promoter (our unpublished results). In view of this, it is highly unlikely that the 70-kDa protein that we have described in this work is related to USF.

In conclusion, the present studies have described some functional aspects of the mouse ODC promoter. In particular, we have defined a region in the proximal promoter that contains a CRE-like element and appears to be sufficient to confer cAMP responsiveness to the promoter. However, the protein that interacts with this region is different from CREB and other proteins so far described to form complexes with the consensus CRE. Further studies are needed to characterize the nature of this ubiquitous protein, and to determine directly that it is indeed involved in mediating the response to cAMP of the ODC promoter.

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