Multiple promoter elements govern expression of the human ornithine decarboxylase gene in colon carcinoma cells

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ABSTRACT

Overexpression of the ornithine decarboxylase (ODC) gene may be important to the development and maintenance of colonic neoplasms, as well as tumors in general. In this study, we examined the promoter elements governing constitutive expression of the human ODC gene in HCT 116 human colon carcinoma cells and, for comparison, K562 human erythroleukemia cells. It was determined by functional analysis that the promoter elements responsible reside within the 378 bp immediately upstream from the transcription start site. Within this sequence, there are at least three regions that modulate the efficiency of the ODC promoter cooperatively. Both DNA bandshift and footprint assays demonstrated all three regions to be rich in sites that bind to nuclear proteins isolated from HCT 116 and K562 cells; the protein binding pattern of non-transformed, diploid fibroblasts was found to be much less complex. Several of the protein binding sequences have little or no homology to common regulatory elements. We suggest that the constitutive activity of the ODC gene in HCT 116 colon carcinoma cells, and perhaps transformed cells in general, involves a complex interaction of multiple regulatory sequences and their associated nuclear proteins. Finally, the saturation of the promoter in these transformed cell lines suggests that high levels of protein binding in the ODC promoter may contribute to elevated constitutive expression of this gene.

INTRODUCTION

Ornithine decarboxylase (ODC¹, EC 4.1.1.17), the rate-controlling enzyme in polyamine biosynthesis, is tightly coupled to mammalian cell proliferation and neoplastic transformation (reviewed in refs. 1–4). Numerous studies have demonstrated that ODC activity is substantially increased in tumors (5, 6), transformed cells (7) and, in some cases, in tissues predisposed

to malignant transformation (8–10). ODC mRNA levels are also constitutively elevated in most transformed cells examined to date including; human small cell and non-small cell lung carcinomas (11), hepatocellular carcinomas (12), and *ras*-transformed NIH 3T3 fibroblasts (13). The strong correlation between ODC gene expression and tumorigenesis is clearly evident in the development of human colonic neoplasms. Both ODC activity and mRNA levels are enhanced in human colon adenomas and carcinomas relative to pathologically uninvolved, adjacent mucosa and control mucosa (9, 14–16). These observations suggest that overexpression of the ODC gene is important in the development and maintenance of neoplastic growth in general and colonic tumors in particular.

ODC has been shown to be highly regulated by multiple processes including gene transcription, mRNA translation and enzyme degradation (17–21). We have cloned the human ODC gene (22, 23) to elucidate the molecular mechanisms controlling ODC gene expression in normal and neoplastic cells. Others have also reported the sequence of the human gene (24, 25), as well as that of other mammals: rat (26, 27), and mouse (28, 29). The proximal promoter region of the mammalian ODC genes are highly conserved (22, 23) and contain multiple sequences homologous to known transcription factor binding sites. However, with the exception of a CAAT box (30) and a CRE element in rodent ODC promoters (31,32), the actual function and protein binding capability of these putative regulatory elements has not been investigated.

In this report, we examined the promoter elements controlling constitutive expression of the human ODC gene in HCT 116 human colon carcinoma cells. Chimeric constructs consisting of ODC gene 5'-flanking DNA fused to a chloramphenicol acetyltransferase (CAT) reporter gene were transiently expressed in HCT 116 cells to define functional promoter sequences. In addition, specific protein-binding sites within the constitutive promoter were identified by DNA bandshift and DNase I protection assays. These studies demonstrate that the ODC gene promoter contains a strikingly high number of nuclear protein

binding sites, some of which have little or no homology to common regulatory elements. Constitutive ODC gene expression in colon carcinoma cells involves the cooperative interaction of these multiple regulatory elements and associated nuclear proteins. We also show that ODC promoter function and nuclear protein binding in another transformed cell line, K562 human erythroleukemia, is strikingly similar to that of HCT 116. Interestingly, non-transformed, diploid fibroblast IMR90 cells expressing lower steady-state levels of ODC mRNA display a less complex pattern of protein binding to the ODC promoter.

METHODS

Materials

Reagents were obtained from the following sources: chemicals (reagent grade or better) from Sigma Chemical Co. or J.T.Baker Chemical Co.; cell culture reagents from GIBCO (Grand Island, N.Y.); restriction enzymes and other nucleic acid modifying enzymes from New England Biolabs (Beverly, MA), Boehringer Mannheim (Indianapolis, IN) or Bethesda Research Laboratories (Gaithersburg, MD) unless otherwise specified; [α -³²P]dNTPs, [γ -³²P]ATP, and [α -³⁵S]dATP from Amersham (Arlington Hts., IL) or New England Nuclear (Boston, MA); Sequenase II from U.S. Biochemicals, Inc. (Cleveland, OH); unmodified T7 polymerase from Pharmacia (Piscataway, NJ); dideoxy sequencing reagents from U.S. Biochemicals and Pharmacia in kit form; chemical degradation sequencing reagents from New England Nuclear (Boston, MA) in kit form; DNase I and other reagents for DNase I protection studies from Stratagene (LaJolla, CA) in kit form; and sequencing gel reagents from Bio-Rad (Richmond, CA) and National Diagnostics (Manville, NJ). Standard molecular biology procedures were performed according to published protocols (33, 34).

ODC Sequence

Two human ODC gene clones were independently isolated: λ hODC49 (22) and ODC709-A2 (23). The clones are virtually identical out to -378 bp upstream of the transcription start site on the basis of both restriction site mapping and sequence analysis (see 'Results'). In this study, the two ODC clones were used interchangeably from -378 to +77. Sequences from -1215 to -379 were derived exclusively from clone λ hODC49. Nested deletions of λ hODC49 were obtained as previously described (22) and the sequence was extended in the 5' direction by a combination of the methods of chain-termination (35) and base-specific chemical cleavage (36). Unmodified T7 polymerase and the modified T7 polymerase, Sequenase II, were used in the chain-termination method with single-stranded template and [α -³⁵S]dATP (400 Ci/mmol). Both strands were sequenced completely. The unmodified T7 polymerase was found to be the most successful for reading through the GC-rich regions. Compressions were resolved by the use of 7-deaza dGTP (Pharmacia) in the sequencing reactions together with independent sequencing of opposing strands. Sequence data were collated and aligned with the aid of the DNASTAR sequence analysis program (DNASTAR, Inc., Madison, WI).

Plasmid Constructions

ODC promoter/CAT reporter gene constructs were prepared by ligating fragments from the ODC gene 5' flanking region into plasmid pKK232-8 (Pharmacia) containing a promoterless gene for the bacterial enzyme CAT (Fig.2). Initially, the *Nco*I-*Nae*I

fragment from ODC709-A2 that includes 52 bp of the ODC gene 5'-flanking region, the cap site and the first 77 bp of exon 1 was subcloned into Bluescript SK(+) vector (Stratagene) with *Hind*III restriction sites on either side. The base construct PRODC-8 was made by ligating this *Hind*III fragment into the *Hind*III site of pKK232-8 in the correct orientation to drive CAT expression. Insertion of a subcloned *Pvu*II-*Nco*I fragment into the *Nco*I site of PRODC-8 produced PRODC-2 and extended the length of the 5' flanking region to 378 bp. Deletion subclones PRODC-6, PRODC-7 and PRODC-9 were derived from PRODC-2 by restriction endonuclease digestion at *Aat*II, *Xma*I and *Not*I sites, respectively, and intramolecular ligation of the remaining large fragments. Similarly, PRODC-4, PRODC-5 and PRODC-10 were made by double-digesting PRODC-2 with *Bam*HI (vector site)/*Not*I, *Bam*HI (vector site)/*Mlu*I, and *Not*I/*Mlu*I restriction enzymes, respectively, and ligating the blunted-ends of the large fragments. To form PRODC-3, the *Bst*NI-*Hind*III fragment of PRODC-2 was ligated into the *Sma*I-*Hind*III sites of pKK232-8. PRODC-11 was produced by digesting PRODC-2 with *Cfr*10I and re-ligating the two largest fragments. Lastly, PRODC-1 was constructed by joining the *Bgl*II-*Mlu*I fragment of clone λ hODC49 into *Bam*HI (vector site)-*Mlu*I (insert site) deleted PRODC-2. The structure of the fusion constructs were confirmed by DNA sequence analysis and/or restriction site mapping.

Cell Culture and Transfection

Human colon carcinoma cell line HCT 116 (American Type Culture Collection, Rockville, MD) was maintained in Dulbecco's modified Eagles medium (DMEM) with high glucose and 10% fetal bovine serum (FBS). Human erythroleukemia cell line K562 (American Type Culture Collection) was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2 mM glutamine and 10% FBS. Human diploid IMR90 fibroblasts (Coriell Institute of Medical Research, Camden, NJ) were cultured in Minimal Essential Medium (MEM) supplemented with antibiotics, 2 mM glutamine and 10% FBS. The cells were used between 18 and 30 population doublings, and were harvested 24 h after the addition of fresh serum at 75% confluence. ODC promoter/CAT gene constructs were introduced into HCT 116 and K562 cells by calcium phosphate (37) and DEAE-Dextran (38) transfection protocols, respectively, using CellPhect™ transfection kits (Pharmacia). The DNA used for transfection was purified by two successive cesium chloride density gradient centrifugations. Briefly, HCT 116 cells seeded at a density of 1×10^6 cells per 100-mm² plate were transfected 24 h later with 10 μ g of ODC promoter-CAT gene DNA and 1 μ g of pCH110 DNA (Pharmacia) encoding the enzyme β -galactosidase under transcriptional control of the SV40 early promoter. After 6 h of incubation with calcium phosphate-precipitated DNA, cells were shocked for 30 s with 15% glycerol in Hepes-buffered saline (pH 7.12) at room temperature. The cells were then cultured for 40 h, harvested, resuspended in 60 μ l of 0.25 M Tris-HCl, pH 7.8, and lysed by 5 freeze-thaw cycles in liquid nitrogen. K562 cells at a density of 5×10^6 cells/ml were transfected with 10 μ g/ml of DNA and 100 μ g/ml of DEAE-Dextran at 37°C for 45 min. The cells were shocked with 10% DMSO for 2 min, incubated in culture medium for 40 hr and harvested as described above.

Northern Blot Analysis

Cells were homogenized in guanidine monothiocyanate and total RNA was isolated and fractionated by electrophoresis in a 1.5%

agarose gel containing 6% formaldehyde and 20 mM sodium phosphate, pH 6.8, and transferred to Gene ScreenTM membrane (New England Nuclear, Boston, MA). Human ODC cDNA probe from clone ODC2H/10 (39), generously provided by Dr. O.A.Jänne (University of Helsinki, Finland), was radiolabelled with [α -³²P]dCTP using a random primer labelling kit (Pharmacia, Piscataway, NJ) and hybridized to the filter as published (23). The final washing of the membrane was performed with 0.5×SSC, 1.0% SDS at 55°C. The intensity of the hybridization signal was quantitated using the AMBIS Radioanalytical Imaging System (AMBIS, San Diego, CA). The membrane was deprobed and rehybridized with [³²P]dCTP radiolabelled 18S rDNA to correct for variation in the concentration of RNA loaded onto each lane.

Enzyme Assays

CAT assays were performed as previously described (37) except that cell lysates were incubated at 60°C for 10 min prior to the CAT assay to inactivate any endogenous acetyltransferases. The acetylated and non-acetylated forms of [¹⁴C]-chloramphenicol were fractionated by thin layer chromatography and quantitated directly by scanning the TLC plate with an AMBIS Radioanalytical Imaging System (AMBIS Systems Inc., San Diego CA). The percentage conversion of chloramphenicol to acetylated derivatives was determined by averaging the results of 5 to 17 transfections for HCT 116 cells and 8 transfections of K562 cells using two different DNA preparations for each construct. β -Galactosidase activity was assayed as described (40). The protein concentration of each extract was determined using a Bio-Rad kit (41). CAT activity was normalized with respect to β -galactosidase activity and/or protein concentration.

Preparation of nuclear extracts

Nuclear extracts were made from HCT 116, K562 and IMR90 cells as described (42) except that for HCT 116 extracts 2 μ g/ml each of antipain, leupeptin and pepstatin A (Sigma) were added to buffer A (43). Also, HCT 116 and IMR90 cells were lysed in buffer A supplemented with Nonidet P-40 to a final concentration of 0.3% and 1.3%, respectively. The extracts were finally dialyzed against 50 to 100-fold buffer D: 20 mM HEPES pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 20% glycerol. Nuclear extract protein concentrations were determined using a Bio-Rad assay kit (41).

Bandshift assays

DNA restriction fragments were end-labelled with 100 μ Ci of [α -³²P]dCTP (3000 Ci/mmol) by filling-in recessed 3' ends using DNA polymerase Klenow fragment and isolated by gel electrophoresis. Approximately 20,000 cpm (0.5 ng) of test DNA was incubated with the appropriate amount of nuclear extract for 30 min at room temperature in 25 μ l of buffer composed of 17 mM HEPES (pH 7.9), 32 mM Tris-HCl (pH 7.9), 10% glycerol, 52 mM KCl, 0.8 mM EDTA, 0.8 mM DTT, 20 μ M PMSF, 1 μ g poly(dI-dC)·poly(dI-dC) (Pharmacia) and 15 μ g BSA (BRL). Competition experiments included non-radiolabeled construct PRODC-2 containing ODC promoter DNA sequences or control plasmid vector pKK232-8 DNA. Each binding reaction was fractionated by electrophoresis in a 4% acrylamide gel in 50 mM Tris-glycine (pH 8.5) and 0.2 mM EDTA buffer at 4°C. Afterwards, the gel was dried and exposed to X-Omat film with a Cronex intensifying screen.

Figure 1. Promoter of the human ornithine decarboxylase gene. A) Schematic representation of positions of putative regulatory elements in the human ODC gene promoter region, and sequencing strategy. The 5'-flanking region is shown as a thin line, exon 1 as a bar. Positions of putative regulatory elements are indicated: Z-DNA, potential Z-DNA forming region; GC, GC-box, potential Sp1 binding site; CRE, cAMP responsive element; CCAAT, CCAAT binding protein (CTF) binding site; CORE, enhancer core sequence; TATA, TFIID binding site. An arrow indicates the transcription start site. The sequencing strategy is shown below the schematic. Solid lines indicate chain termination sequencing reactions (34), dashed lines indicate chemical cleavage sequencing reactions (35). All overlaps have been obtained. B) Nucleotide sequence of the 5'-flanking region and 80 bp of the first exon. The putative regulatory elements shown schematically in part A are shaded and labelled, as in A, except that the Z-DNA is labelled zzzz. The two 29 bp repeats are underlined. An arrow indicates the transcription start site.

DNase I Footprint Assays

Footprinting assays were done essentially as published (44) using the HOTFOOT™ DNase I Footprinting kit (Stratagene). Overlapping restriction fragments of λ hODC49 with a single 3' recessed end (Fig.4) were labelled with 40–80 μ Ci of [α - 32 P]dNTPs (6000 Ci/mmol) catalyzed by AMV reverse transcriptase (Boehringer Mannheim) or MMLV reverse transcriptase (Bethesda Research Labs). The desired fragments were purified from the remainder by native gel electrophoresis. 20,000 cpm of gel-purified fragment, approximately 1 ng, was used per reaction. 0–90 μ g of nuclear protein in 25 μ l Buffer D (see above) was added to 25 μ l reaction buffer on ice, which contained 4% polyvinyl alcohol, 1 μ g poly dI-dC and 13 μ l incubation buffer (Stratagene), and incubated on ice for 15 min. The end-labelled fragment was then added in 1 μ l and incubated for an additional 15 min on ice. Samples were incubated at room temperature for 2 min, 50 μ l of DNase I buffer (Stratagene) was added, and then digested with empirically determined amounts of DNase I for 2 min. The digestion was terminated by the addition of 100 μ l stop buffer (Stratagene) and extraction with an equal volume of phenol-chloroform (1:1). Samples were precipitated with 2.5 volumes of ethanol in the presence of 0.3

M NaOAc, washed once with 70% ethanol and resuspended in formamide dye (Stratagene), then run on a 5% or 6% acrylamide-8M urea gel (0.4–1.2 mm) at 50–55°C. Sequencing reactions (35) of the labelled fragments were run as size markers. The gels were dried and exposed to X-Omat film for 4–14 days without screens.

RESULTS AND DISCUSSION

Sequence of the Human ODC Gene 5'-Flanking Region

Multiple overlapping subclones of the human ODC gene 5'-flanking region derived from genomic clone λ hODC49 (22) were sequenced according to the strategy shown in Fig. 1A. The sequence of the 5' flanking region is extended to nucleotide –1473 (Fig. 1B). Comparison of this sequence to that of genomic clone ODC709-A2 (23) reveals a single base substitution at position –117; λ hODC49 contains a cytosine and ODC709-A2 a thymidine. Another human ODC gene sequence (24) has a cytosine at position –117 and is otherwise identical to clones λ hODC49 and ODC709-A2. The difference at –117 in clone ODC709-A2 may represent a polymorphism in the 5'-flanking region or simply a cloning artifact.

Figure 1 shows that the ODC gene 5'-flanking region contains numerous regulatory element sequence motifs: a TATA element at position –31 that varies from the consensus sequence TATAAA (45) by one base; two CCAAT motifs (46), CCGAT at –82 and CCAAT at –1069; a one-base altered enhancer 'core' element (47), GTGGTTTG, at –72; and two variants of

Figure 2. Relative promoter activities of ODC/CAT constructs in HCT 116 cells. The structure of the human ODC promoter and exon 1 sequences fused to the promoterless CAT gene is shown at the top with the restriction enzymes used to make the PRODC constructs. The restriction sites indicated are Bg, *Bgl*II; P, *Pvu*II; B, *Bst*NI; N, *Not*I; M, *Mlu*I, A, *Aat*II; C, *Cfr*10I; X, *Xma*I, Nc, *Nco*I. ODC promoter sequences retained in each construct are represented by solid horizontal bars, internal deleted sequences are indicated by thin horizontal lines and CAT gene coding sequences are depicted by striped bars. Transfection of HCT 116 cells and enzyme assays were performed as described in 'Methods'. Relative CAT activities are expressed as a percentage of construct PRODC-2 ± S.E.

Figure 3. DNA bandshift analysis of the binding of HCT 116 nuclear factors to ODC promoter DNA. DNA bandshift assays were performed as described in 'Methods'. DNA fragments containing ODC promoter regions *Mlu*I/*Nco*I (–206 to –50 bp) and *Pvu*II/*Xma*I (–378 to –137 bp) were analysed (see restriction maps in Fig. 2). The *Pvu*II site in the ODC promoter is within 10 bp of a vector *Xma*I site in a plasmid subclone. This *Xma*I/*Xma*I fragment containing ODC sequences *Pvu*II/*Xma*I was [32 P]-labelled and used as probe in lanes 8 to 12. Approximately 0.5 ng of the [32 P]-labelled DNA fragments were incubated with 0 μ g (lanes 1 and 8), 7.5 μ g (lanes 2–7), or 2.5 μ g (lanes 9–12) of total protein from HCT 116 nuclear extract. The reaction mixes also included PRODC-2 competitor DNA and pKK232–8 non-specific control DNA in the amounts indicated. 100 ng of PRODC-2 DNA represents a 6- and 9- fold molar excess of the *Mlu*I/*Nco*I and *Pvu*II/*Xma*I fragments, respectively. Arrowheads refer to DNA/protein complexes and free DNA probe.

Figure 4. DNase I footprint analysis of the location of nuclear protein binding sites in the ODC promoter region. A) A schematic diagram of the ODC promoter region. B) HCT 116 cells. DNase I protection pattern on four overlapping DNA fragments spanning the area -424 to +180: *XmaI/BstXI* (-137 to +180); *KpnI/NcoI* (-424 to -50); *KpnI/XmaI* (-424 to -137); and *KpnI/MluI* (-424 to -206). The indicated restriction fragments were [32 P]-end-labelled and isolated as described in 'Methods'. The [32 P]-labelled end is indicated by an asterisk. In each panel, the first two lanes, G and C, are guanine and cytosine sequencing ladders generated by the method of base specific chemical cleavage (35). The adjacent lanes, labelled '-', are the DNase I digestion products of end-labelled DNA in the absence of any added nuclear extract. One or two empirically determined concentrations of DNase I were used. The final two lanes, labelled '+', are the DNase I digestion products of end-labelled DNA in the presence of 52 μ g of HCT 116 cell nuclear protein. Two different concentrations of DNase I were used, as for the '-' lanes. The boxes indicate regions protected from DNase I digestion. The areas of protection are numbered starting at the transcription initiation site: roman numerals for those in the 5'-flanking region, and arabic numerals for those in the first exon. C) K562 cells. Identical to 4B, with the exception that 60 μ g of K562 cell nuclear protein was used in the final two lanes of each panel. The *KpnI/MluI* fragment is not shown. D) IMR90 cells. Identical to 4C with the exception that 60 μ g of IMR90 cell nuclear extract was used.

the cAMP response element (CRE) (48), TGACGTCA, at positions -48 and -177. The 5'-flanking region also contains two regions of potential Z-DNA and twelve GC boxes that represent putative binding sites for the general transcription factor Sp1. Four of the GC boxes are located within two 29 bp repeats between -230 and -288 separated by a single adenosine residue. Most of these putative regulatory elements are located between -1 and -350. There are also two GC boxes and a CRE motif within the first 55 nucleotides of exon 1.

Deletion Analysis of the ODC Gene Promoter

To delineate the promoter regions directing ODC gene transcription in a human carcinoma, ODC/reporter gene constructs were transiently transfected into HCT 116 human colon carcinoma cells. DNA restriction fragments from the ODC gene 5'-flanking region were inserted immediately upstream from the bacterial chloramphenicol acetyltransferase (CAT) gene in plasmid pKK232-8 as detailed in 'Methods'. The promoter activity of each DNA fragment was ascertained by measuring the CAT activity in crude lysates of transfected cells. Each ODC/CAT construct was assayed 5 to 17 times with at least two different plasmid DNA preparations. Variations in transfection efficiency were corrected by co-transfecting a β -galactosidase expression vector and using the resultant β -galactosidase enzyme activities to normalize CAT activity. It should be noted that each construct encodes the first 77 nt of the 5' untranslated region (UTR) of ODC mRNA. Larger portions of the 5' UTR of hamster ODC mRNA have been shown to decrease translational efficiency of heterologous mRNAs (49). If the presence of 5' UTR sequences influences translation, we assume that the effects are equivalent for each construct and that differences in CAT activity reflect changes in promoter activity.

Initially, HCT 116 cells were transfected with constructs PRODC-2, PRODC-4, PRODC-6, PRODC-8 and promoterless control pKK232-8 (Fig.2). The CAT activity expressed from PRODC-2 (-378 to +77) was arbitrarily designated as 100%. PRODC-4 (-254 to +77) and PRODC-6 (-178 to +77) produced 33% ($p=0.032$) and 11% ($p=0.004$) CAT activity relative to PRODC-2, respectively. The sequences deleted from PRODC-2 to form PRODC-4 contained a region of Z-DNA and four putative GC boxes; whereas, PRODC-6 lost an additional three GC boxes. PRODC-8 (-52 to +77) and negative control

pKK232-8 yielded insignificant levels of CAT activity. PRODC-8 retains most of one CRE element and the TATA sequence from the 5'-flanking region and two GC boxes and a potential CRE element from ODC exon 1. These sequences alone appear incapable of supporting even minimal promoter activity.

We also transfected K562 human erythroleukemia cells with PRODC-1 (-1215 to +77, see below) and the constructs described above to compare promoter activities in two transformed cell lines of different cell types. The relative activities of the deletion constructs in K562 cells were virtually identical to those exhibited by HCT 116 cells. The difference in promoter activity between PRODC-1 and the PRODC-2 standard was not statistically significant ($p=0.54$), but PRODC-4 and PRODC-6 exhibited CAT activity was 28% ($p=0.015$) and 11% ($p=0.002$) that of PRODC-2, respectively. Again, PRODC-8 expressed no significant CAT activity above background levels ($p=0.7$). Poor transfection efficiency and interfering background levels of β -galactosidase prohibited further investigation of ODC promoter activity in K562 cells.

PRODC-1 (-1215 to +77) and PRODC-3 (-324 to +77) were constructed to estimate the 5' distal border of the ODC promoter. The extension of PRODC-2 from -378 bp to -1215 bp had no significant influence on the level of CAT activity expressed ($p=0.7$). However, deletion of 54 bp from PRODC-2 resulted in a 4-fold reduction in promoter strength. The difference in CAT activity between PRODC-3 and PRODC-4 was not significant ($p=0.08$). These data indicate that the promoter sequences responsible for constitutive expression of the ODC gene are contained within the 378 bp of flanking DNA present in PRODC-2. Additional deletions within PRODC-2 were assayed to refine the mapping of functional promoter elements. The CAT activity from PRODC-5 (-206 to +77) was about

Figure 5. Northern blot analysis of ODC mRNA in IMR90, HCT 116 and K562 cells. Total RNA from log growing cells was isolated, fractionated (10 μ g per lane) by gel electrophoresis and transferred to a nylon membrane as described in 'Methods'. The membrane was initially hybridized to [32 P]-labelled ODC cDNA and subsequently rehybridized with [32 P]-18S rDNA to normalize RNA sample concentrations. The intensity of the hybridization signals were quantitated directly from the membrane using an AMBIS Radioanalytical Imaging SystemTM.

Figure 6. Resolution of nuclear protein binding sites in footprint I. A) The *Xma*I/*Bst*XI fragment (-137 to +180) was [32 P]-end-labelled and isolated as described in 'Methods'. Lanes 1 and 2 are guanine and cytosine sequencing ladders as in Fig. 4. Lanes 3 through 10 are the DNase I digestion products of the end-labelled *Xma*I/*Bst*XI fragment in the presence of increasing concentrations of nuclear protein from K562 cells: 3 and 4, 0 μ g; 5 and 6, 24 μ g; 7 and 8, 100 μ g; 9 and 10, 200 μ g. As in Fig. 4, two different empirically determined concentrations of DNase I were used for each pair of samples. Boxes indicate protected regions resolved by protection from DNase I digestion at different concentrations of nuclear protein. B) Same as 6A with the exception that lanes 5 and 6 were digested with 60 μ g of K562 cell nuclear extract, lanes 7 and 8 were digested with 60 μ g of IMR90 cell nuclear extract. Lanes 3 to 6 are also shown as part of Fig. 4C.

half of PRODC-4 (−254 to +77) ($p=0.01$) and approximately 2-fold greater than PRODC-6 (−178 to +77) ($p=0.002$). PRODC-7 (−137 to +77) represents the minimal amount of 5'-flanking DNA in these constructs that still display promoter activity. This construct retains the TATA box, the proximal CRE, an enhancer core element and the CCAAT box. Constructs PRODC-9, PRODC-10 and PRODC-11 contain internal deletions within PRODC-2. The loss of a 30 bp region containing two GC boxes (corresponding approximately to the first 29 bp repeat) in PRODC-9 resulted in a 34% decrease in CAT activity relative to PRODC-2. There was a slight, but statistically significant ($p=0.03$), additional drop in CAT activity when this deletion was extended to include three more GC boxes in PRODC-10. Lastly, the results from PRODC-11 show that there is a 6-fold reduction in CAT activity relative to PRODC-2 upon deletion of sequences from −164 to −39.

Interaction of HCT 116 Nuclear Proteins with ODC Promoter Elements

Specific binding of HCT 116 nuclear proteins to the ODC gene 5'-flanking region was analyzed by DNA bandshift and DNase I footprinting assays. For bandshift assays, [32 P]-end-labelled DNA fragments from the ODC gene 5'-flanking region were incubated with HCT 116 nuclear extracts and subsequently fractionated by gel electrophoresis as detailed in 'Methods'. Multiple DNA-protein complexes indicative of specific interactions were detected when sequences from −378 to −50 were used as the labelled probes. Figure 3 shows five major bands were detected when DNA fragment *Mlu*I to *Nco*I (−206 to −50) was used as probe, and that four of these bands were sensitive to competition with non-radiolabelled PRODC-2 DNA (−378 to +77). Likewise, probe *Pvu*II to *Xma*I (−378 to −137) produced six bands sensitive to competition with PRODC-2 DNA. These DNA bandshift assays demonstrate that nuclear proteins from HCT 116 extracts are capable of forming multiple nucleoprotein complexes with the portion of the ODC promoter that is sufficient for constitutive activity. These DNA probes produced the same bandshift patterns when complexed with nuclear protein from K562 human erythroleukemia cells (data not shown).

DNase I footprinting was used to define the sequences that together with HCT 116 nuclear proteins form the nucleoprotein complexes detected in the bandshift assays. Four overlapping fragments spanning the region −424 to +180 (Fig. 4A) were [32 P]-end-labelled and incubated with HCT 116 nuclear extracts. They were then digested with DNase I and fractionated on sequencing gels as described in 'Methods' (Fig. 4B). The regions of protection from DNase I cleavage in the presence of protein suggest that there are at least eight protein binding sites within the 5'-flanking region (roman numerals), and two within exon 1 (arabic numerals). Footprint I appears to have at least two components as shown by the additional protection of fragment *Kpn*I/*Nco*I compared to fragment *Xma*I/*Bst*XI.

Three of the same four fragments were incubated with K562 nuclear extracts (Fig. 4C). The K562 cell nuclear extracts produce a footprint pattern almost identical to that of HCT 116, protecting identical sequences within each footprint. The exceptions are the truncation of footprint I at the 3' end and the absence of footprints 1 and 2 within exon 1. Using several different preparations of K562 extract, the 3' end of footprint I (see Fig. 6A) and footprint 2 (data not shown) have been observed. For comparison, the same three fragments were

incubated with nuclear extract prepared from human non-transformed, diploid fibroblasts, IMR90 cells (Fig. 4D). It was found that footprint I is much smaller than when HCT 116 or K562 cell nuclear extracts are used, and that the only other protected sequences were footprints IV, V and VI. The region protected by footprint IV extends in the 3' direction further than it does with the transformed cell extracts, and is therefore labelled IV'. Interestingly, steady-state levels of ODC mRNA are 3 and 3.5 times higher, respectively, in K562 and HCT116 cells than in IMR90 fibroblasts (Fig. 5).

The variable size of footprint I (Fig. 4) suggests that it is composed of several protein-binding sites in addition to that detected by the additional binding to fragment *Kpn*I/*Nco*I

Figure 7. Nucleotide sequences in the human ODC promoter protected by nuclear proteins. The nucleotide sequences of the regions protected from DNase I digestion by the binding of nuclear proteins (Figs. 4 and 6) are listed, together with their sequence location. The shaded sequences correspond to putative regulatory sequences; the abbreviations below the sequences have the same meaning as in Fig. 1B. HCT 116 and K562 nuclear extracts protect all sequences listed with the exception of footprints 1 (K562) and IV'. IMR90 nuclear extracts protect only a subset of these sequences: IA, ID, IV', V and VI.

described above. It was found that footprint I can be further resolved into at least four protein-binding sites on the *XmaI/BstXI* fragment (Fig. 6). Fig. 6A shows a titration of nuclear protein from K562 cells. This demonstrates a resolution of the 3' end of footprint I into three parts, IA, IB and IC. Similar results were obtained with HCT116 nuclear extract (data not shown). Fig. 6B demonstrates that the 5' end of footprint I on the *XmaI/BstXI* fragment, ID, is present only with the 3'-most portion, IA, in IMR90 cell nuclear extracts (lanes 7 and 8). This contrasts with the larger footprint I protected by an equivalent amount of nuclear protein from K562 cells (lanes 5 and 6). The presence of footprint IA in this experiment with the same batch of IMR90 nuclear extract that was used in Fig. 4D (showing no footprint IA binding), together with the problem of detection in the K562 extracts, suggests that the binding of the protein that protects this region is difficult to detect with the DNase I protection assay. A summary of the sequences in Fig. 4 and 6 protected from DNase I digestion by the nuclear extracts is shown in Fig. 7.

Comparison of Nuclear Protein Binding Sites and Deletion Mutant Analysis

The promoter sequences responsible for constitutive expression of the human ODC gene in HCT 116 human colon carcinoma and K562 erythroleukemic cells reside within the 378 bp immediately upstream from the transcription start site as determined by ODC/CAT function analysis (Figure 2). Both DNA bandshift and footprint assays reveal multiple interactions between HCT 116 and K562 nuclear proteins and the DNA sequences within this region (Figures 3, 4, and 6). Twelve distinct regions covering 62% of the base pairs in this proximal 5' flanking DNA are protected from DNase I digestion by nuclear factors from HCT 116 cells. These protected sequences include 1 of 2 putative CRE's, a potential CCAAT protein binding site, an enhancer core element, and 8 GC boxes (Figure 7). There are, however, five footprints (IC, IE, II, VII and VIII) that span DNA sequences with no significant homology to the common cis-acting elements already mentioned. Three of these footprints (IE, II and VIII), as well as IB, ID, III, IV, V and VI, contain GC-rich sequences that resemble the consensus binding site for transcription factor *egr-1* (50, 51). In addition, two regions within exon 1 also interact with nuclear proteins. One of these protected

areas (footprint 1) covers exon sequences +1 to +13 and, as described for other RNA polymerase II transcribed genes, may be involved in initiation site determination. The other exon 1 footprint (footprint 2) includes a putative CRE which, in conjunction with the putative TATA box, was unable to support significant transcriptional activity (PRODC-8; Figure 2).

Three regions within the promoter appear to be particularly important to constitutive expression of the ODC gene in HCT 116 cells: distal (-378 to -325), middle (-284 to -206), and proximal (-164 to -39). The deletion of distal sequences (PRODC-3) resulted in a 4-fold reduction in maximum promoter activity. This deletion removed all of the sequences protected by footprint VIII, one-half of those within footprint VII and a stretch of putative Z-DNA between the two footprints. As discussed above, neither of these footprints have significant homology to any common cis-acting elements. The deletion of proximal sequences (PRODC-11) resulted in a 6-fold reduction in maximum promoter activity, but not its abolition. This sequence includes footprints IA-E and II containing a putative CRE, CCAAT, enhancer core element and GC box. The deletion of one (PRODC-9) or both (PRODC-10) of the 29 bp repeats lead to an approximately 2-fold reduction in promoter activity. These deletions remove footprint V (PRODC-9) or footprints III, IV and V (PRODC-10).

Evidence for Cooperative Interactions of the Three Functional Regions

The data in Figure 2 indicates that these three functional regions interact cooperatively to activate transcription. The maximum activity of the intact promoter is greater than the sum of the individual regions. From the 5'deletion mutants, distal (-378 to -325) and proximal (-164 to -39) regions should reconstitute 96% of the intact promoter activity, i.e. PRODC-2, but the combination of these two regions in PRODC-10 is only 56% as effective as PRODC-2. Since the middle region region has virtually no influence on the proximal region (PRODC-3 to PRODC-5), the distal region must interact with the middle region for maximum promoter activity. Similarly, distal and middle (-378 to -206) regions should produce 81% of full activity, but these sequences in PRODC-11 without the proximal region display only 16% of the activity of PRODC-2. Thus the distal/middle region must interact with the proximal region for maximum constitutive expression. These results strongly suggest that individual sites within these regions interact, either directly or through an intermediary protein, to produce a more active transcription complex.

The binding of Sp1 or Sp1-like molecules could account for the cooperative effects seen in the present study. Sp1 transcription factors, which bind to GC boxes such as those clustered within the middle portion of the human ODC promoter as well as footprint ID in the proximal region, have been shown to activate transcription by interacting with factors that bind to both TATA boxes and initiator regions (52). The plausibility of cooperative interactions involving GC-rich motifs is also supported by the fact that the Sp1 transcription factors are capable of interacting cooperatively with each other (53) as well as with other factors such as OTF-1 (54) and the TATA-binding protein TFIID (52). In fact, we have found that Sp1 binding site sequences successfully compete for some of the complexes formed on footprints ID, III, IV, V and IV (J.R.Cooper and M.A.Flanagan, unpublished data).

Figure 8. Summary of the human ODC gene promoter elements and function. (Top) The schematic shows the ODC gene 5'-flanking region (thin line) and proximal portion of exon 1 (thick line). Circles represent sequences protected from DNase I protection by the binding of nuclear factors from HCT 116 extracts. These 'footprints' are identified by roman or arabic numerals as described in Figures 4 and 6. Where protected sequences coincide with putative regulatory elements, this is indicated directly below the promoter schematic. The abbreviations have the same meaning as in Fig. 1. (Below) Hatched boxes depict the location of the distal, middle, and proximal functional regions, as determined by ODC/CAT promoter analysis, relative to the DNase I footprints. Similarly filled circles map to the same promoter domain.

Comparison of Mammalian ODC Promoters

The human, mouse and rat ODC promoters are 82% homologous from nucleotides -1 to -148, roughly corresponding to the proximal promoter region delineated in this study; within this region is a stretch of 89 bp (-48 to -94) with a single mismatch (22). Although homology is substantially reduced in the upstream sequences, all three mammalian promoters have multiple GC boxes in the corresponding middle regions (23). As explained below, the sequence of the human promoter distal region is not conserved in the cloned rodent ODC genes. This may explain why only 264 bp of 5'-flanking region is required for full constitutive activity of the mouse ODC promoter (55).

A more detailed investigation into the function of a rat ODC promoter in Rat-1 embryo fibroblast cells (30) offers some informative comparisons to the human ODC promoter activity in HCT 116 cells. The activity of the rat promoter decreased gradually to 34% of maximum activity as the GC-box-containing sequences from -398 to -105 were deleted. This resembles the loss of human ODC promoter activity upon deletion of sequences -324 to -137 which contain most of the human GC-box binding sites. In addition, deletion of sequences 3' to -78 completely abolished promoter activity, similar to the data presented here. In contrast, the function of the distal sequences appear to be different in these two studies. Human ODC promoter activity dropped four-fold with the loss of sequences from -378 to -324; there was no corresponding decrease in the rat study. There is little or no sequence homology at this region in the human and rat promoters. The lack of structural and functional homologies in the distal regions of these two ODC promoters may be due to one or more of the following reasons. First, there may be functionally homologous sequences in the rat promoter that are not utilized in rat embryo fibroblast cells. Second, the missing sequences may be located elsewhere within the rat gene or flanking regions. Third, since there appear to be multiple rat ODC genes or pseudogenes, it is possible that another rat ODC gene is responsive to the factor(s) that interact with the 5' end of the human ODC promoter.

Constitutive Expression Mediated by Multiple Regulatory Elements

In this report, we have focused on constitutive control of ODC gene expression. The significance of constitutive expression is underscored by the sustained elevation of ODC activity in virtually all tumors and transformed cells (see Introduction). Furthermore, growth arrest of proliferating cells both *in vitro* and *in vivo* is effected by lowering constitutive ODC activity with enzyme-specific inhibitors (56). These studies raise the question: Why is the ODC promoter comprised of so many and varied regulatory elements? While it is not uncommon to find multiple elements in eukaryotic promoters, few match the concentration of nuclear protein binding sites found in the ODC promoter. It has been proposed that the combinatorial use of low affinity, low specificity components results in transcription complexes that are highly specific and flexible (57). Both specificity and flexibility would be expected to be salient features of mechanisms designed to control expression of such a highly regulated gene in a myriad of cell types. The question of interest then becomes: Is the saturated binding of the promoter in HCT 116 and K562 cells related to the elevated constitutive expression of ODC in neoplastic cells? It has been shown recently that transcription activation parallels promoter element binding and that

promiscuous cooperativity can occur between factors when binding sites are saturated (58). Interestingly, we observe that only a fraction of the ODC promoter elements are occupied by nuclear factors from non-transformed, diploid human fibroblasts (Figure 4D). These results suggest that increased occupancy of ODC promoter binding sites in HCT 116 and K562 cells may contribute to elevated constitutive expression.

CONCLUSIONS

In this study it has been shown that the sequences responsible for constitutive expression of the human ODC gene in HCT 116 human colon carcinoma and K562 human erythroleukemia cells reside within the 378 bp immediately upstream from the transcription start site. Within this sequence, there are at least three regions that modulate the efficiency of the ODC promoter cooperatively. All three regions are rich in sites that bind to nuclear proteins isolated from HCT 116 cells. One of these regions contains five GC boxes, suggesting that Sp1 may bind to these sequences and contribute to the cooperative interaction of this region with the other two. Several of the protected sequences in the regulatory regions have little or no homology to common regulatory elements, suggesting that further characterization of these regions will result in the identification of novel transcription factors. Finally, the saturation of the promoter in these transformed cell lines suggests that high levels of protein binding in the ODC promoter may contribute to elevated constitutive expression of this gene. The similarities of function and nuclear protein binding of the ODC promoter in two malignant cell lines of different tissue origin indicate that these promoter properties are not tissue specific but transformation related. Further comparisons of ODC promoter activity and protein binding in cells of different tissue specificity and neoplastic status will enhance our understanding of the contribution of the individual transcription factors and their cooperative interactions to constitutive expression of the ODC gene.

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