

Identification of α -enolase as a nuclear DNA-binding protein in the zona fasciculata but not the zona reticularis of the human adrenal cortex

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Abstract

In order to establish whether there are differences in DNA-binding proteins between zona fasciculata (ZF) and zona reticularis (ZR) cells of the human adrenal cortex, we prepared nuclear extracts from separated ZF and ZR cells. The formation of DNA-protein complexes was studied using an element in the first intron of the type I and type II 3β -hydroxysteroid dehydrogenase genes (HSD3B1 and HSD3B2). Using the element in the HSD3B2 gene as a probe, a complex (C1) was formed with extracts from ZF cells but was formed only at a low level with ZR cell extracts. Another pair of complexes (C2/C3) was formed with both ZF and ZR cell extracts. The ZF-specific protein forming C1 was enriched by column chromatography on DEAE-Sepharose and carboxymethyl-Sepharose. Oligonucleotide competition analysis on the enriched fraction gave results consistent with those ob-

tained on the unfractionated material. A further enrichment was brought about by passing the protein over an oligonucleotide affinity column based on the HSD3B2 element. The protein bound to the column was identified as α -enolase by mass spectrometry. Although α -enolase is a glycolytic enzyme, it binds to specific DNA sequences and has been found to be present in nuclei of various cell types. We performed immunohistochemistry on sections of adult human adrenal cortex and found α -enolase to be located in nuclei of ZF cells but to be predominantly cytoplasmic in ZR cells. Transfection of an α -enolase expression vector into NCI-H295R human adrenocortical cells increased HSD3B2 promoter activity, suggesting a possible functional role for this protein in regulation of HSD3B2 expression.

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Introduction

In the human adrenal cortex the zona reticularis (ZR) is biochemically and functionally distinct from the zona fasciculata (ZF; Hornsby 1995). The most significant difference is the level of 3β -hydroxysteroid dehydrogenase (3β -HSD) activity. The type II enzyme, the product of the HSD3B2 gene, is expressed in steroidogenic tissues, whereas the type I enzyme, the product of the HSD3B1 gene, is expressed in non-steroidogenic tissues (Lachance *et al.* 1991). HSD3B2 mRNA is present at high levels in ZF cells but is almost absent from ZR cells (Endoh *et al.* 1996). This difference is likely the key factor in causing ZR cells to secrete dehydroepiandrosterone and its sulfate (DHEAS) rather than cortisol (Hornsby 1995). In previous experiments we demonstrated differences in cortisol/DHEAS production ratios and HSD3B2 mRNA in ZR

and ZF cells isolated by microdissection (Endoh *et al.* 1996). Immunocytochemical data also show striking zonal differences in 3β -HSD protein levels (Sasano *et al.* 1990, Parker 1997, Gell *et al.* 1998). There are a variety of other differences between ZF and ZR cells. DHEA sulfotransferase is expressed at a higher level in ZR cells (Kennerson *et al.* 1983, Parker 1997). MHC class II gene expression also differs between the zones (Marx *et al.* 1997). A survey of differences in gene expression between ZF and ZR cells revealed several other differentially expressed genes (Wang *et al.* 2001). The molecular basis for functional differences between ZF and ZR cells remains unknown, but recent data implicate the transcription factor GATA-6 (Jimenez *et al.* 2003). Moreover functional features of ZR cells can be produced by treating an adrenocortical cell line with Src tyrosine kinase inhibitors (Sirianni *et al.* 2003).

In the present experiments we sought to elucidate other differences between ZR and ZF cells, specifically differences in nuclear DNA-binding proteins. In beginning this work we took advantage of experiments previously performed by Guerin *et al.* (1995) on the HSD3B1 gene, showing that an element in the first intron of the gene binds a 37 kDa protein. These authors showed that the first intron is important in transcriptional regulation, and they pointed out that this element differs in sequence between HSD3B1 and HSD3B2, although the two genes are otherwise very similar in sequence. We show that this element in HSD3B2 binds nuclear proteins; some of these are also bound by the element from HSD3B1, but some are bound only by the element from HSD3B2. One protein is present in nuclear extracts from ZF cells but is present only at low levels in ZR cell nuclear extracts. We identified this protein as α -enolase, a multifunctional protein already established as a DNA-binding protein and transcriptional regulator (Feo *et al.* 2000, Subramanian and Miller 2000).

Materials and Methods

Preparation of nuclear proteins from human and bovine adrenal glands

Adrenal glands were obtained from kidney organ donors or from patients undergoing resection of the kidney for renal neoplasms. Using microdissection, the ZR was separated from the ZF on the basis of color (brown for ZR and bright yellow for ZF; Endoh *et al.* 1996). Glands were trimmed free of fat and placed in culture medium. Under the dissecting microscope glands were sliced, the boundary between the ZR and ZF was identified, and fragments of zonal tissue were excised by inspection of color. Tissue fragments were dissociated to cell suspensions using enzymatic and mechanical dispersal (3 h incubation with 1 mg/ml type I collagenase and 0.1 mg/ml DNase, both from Sigma Chemical Co; Hornsby and McAllister 1991). Larger fragments and debris were removed by filtration. Cells were washed by low-speed centrifugation, once in serum-containing medium, and then three times in PBS.

The preparation of nuclear extracts was based on a procedure published previously (Wang and Klein 1996). Cell pellets (about 0.3 ml by volume) were resuspended in 600 μ l 10 mM Tris-HCl, pH 7.6, 1.5 mM MgCl₂, 10 mM KCl and 0.5 mM dithiothreitol (DTT). After 15 min incubation on ice, cells were homogenized by passing them through a 23-gauge needle and the sample was centrifuged for 5 min at 12 000 g. The nuclear pellet was resuspended and salt extracted in 100 μ l 20 mM Tris-HCl, pH 7.6, 25% sucrose, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA and 0.5 mM DTT, and was

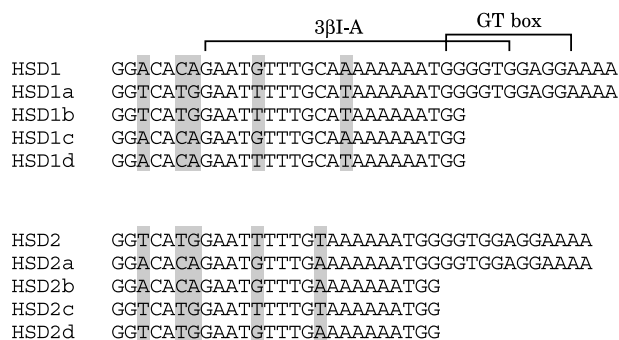


Figure 1 Sequences of oligonucleotides used in these experiments. The region of the first intron of HSD3B1 previously characterized by Guerin *et al.* (1995) is shown, together with the sub-elements within this region termed 3 β I-A and GT box by these authors. The 3 β I-A sequence was shown to bind a 37 kDa protein (Guerin *et al.* 1995). The upper set of oligonucleotides is based on HSD3B1 and the lower set is based on HSD3B2. The oligonucleotides designated 1 and 2 are the unmodified sequences. The other oligonucleotides have been modified from these by truncation at the 3' end and/or by mutation of the indicated nucleotides.

incubated on ice for 30 min. After centrifugation at 15 000 g for 10 min, the supernatant (nuclear protein) was removed for storage at -80°C .

Gel-mobility shift assays

Gel-mobility shift assays were performed as previously described (Wang and Klein 1996). Double-stranded DNA oligonucleotides were prepared by annealing and were then purified by electrophoresis on 15% acrylamide gel. Oligonucleotides were 5' end-labeled with [γ -³²P]ATP and T4 polynucleotide kinase. Usually 2 μ g nuclear protein and 2×10^4 c.p.m. probe (2–5 fmol) were used in each binding reaction. A volume of 10–20 μ l buffer comprising 20 mM Tris-HCl, pH 7.6, 100 mM KCl, 5 mM MgCl₂, 1 mM DTT, 10% glycerol and 2 μ g poly(dI-dC) was used. After 30 min at room temperature complexes were separated by non-denaturing gel electrophoresis on 5% polyacrylamide. Complexes were visualized by autoradiography.

Probe sequences for the HSD3B1 and HSD3B2 genes are shown in Fig. 1. The *c-myc* P2 promoter oligonucleotide (see text) had the sequence 5'-AGGGATCGCGCTGAGTATAAAAGCCGGTTTTTCGGGG-3' (Ray and Miller 1991).

Chromatography on DEAE and carboxymethyl columns

Protein fractionation by DEAE ion-exchange chromatography was based on published procedures for other DNA-binding proteins (Thomas *et al.* 1995, An *et al.* 1996, Vostrov and Quitschke 1997). A 200 μ l DEAE-Sepharose column (Amersham Pharmacia Biotech) was

prepared in a 1 ml syringe and equilibrated with binding buffer containing 40 mM KCl. Nuclear extract was applied to the column. Proteins were eluted in the same buffer. Fractions were assayed by gel-mobility shift assay as described above. Carboxymethyl-Sepharose chromatography was performed in the same way.

Purification of protein on oligonucleotide affinity column

The starting material for protein purification was 1.5 mg nuclear extract protein (IMR32; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The protein was first passed through DEAE- and carboxymethyl-Sepharose columns as described above. The eluate was then further purified on an oligonucleotide affinity column. The column was prepared as follows: a double-stranded oligonucleotide was prepared in which the 5' end of the top strand of the HSD3B2 element (sequence 2 in Fig. 1) was linked to the 3' end of the bottom strand with a short hairpin. The hairpin had an amino-modified base which was used to bind the oligonucleotide to the column. The complete oligonucleotide was synthesized from three constituent oligonucleotides, comprising (part 1, hairpin) 5'-pAATTCCATGACCTTXXTTGGTCAT-3', where X is amino-C₆-deoxyuridine, (part 2, top strand) 5'-pGGAATTTTTGTAAAAAATGGGGTGAAGGAA AA-3' and (part 3, bottom strand) 5'-TTTTCTCC ACCCATTTTTTTTACAA-3'. To construct the hairpin 20 nmol part 1 and part 2 were annealed, and then the product was incubated with part 3 together with 30 000 units T4 DNA ligase in a reaction volume of 30 μ l at 37 °C for 18 h. The ligated double-stranded oligonucleotide was then isolated by non-denaturing PAGE. The appropriate band was visualized by ethidium bromide staining, eluted in Tris buffer and precipitated with LiCl/ethanol.

The purified double-stranded oligonucleotide was conjugated to DSB-X biotin succinimidyl ester (comprising desthiobiotin linked to succinimidyl ester by a seven-atom aminohexanoyl X spacer; Molecular Probes, Eugene, OR, USA). Eight nmoles purified double-stranded oligonucleotide was reacted with 200 μ g DSB-X biotin succinimidyl ester in 0.1 M sodium tetraborate, pH 8.5, at 23 °C for 6 h. The product (conjugate of double-stranded oligonucleotide with DSB-X biotin) was precipitated with LiCl/ethanol. It was redissolved in Tris buffer (20 mM Tris, 100 mM KCl, 5 mM MgCl₂, 1 mM DTT and 100 μ g/ml poly(dI-dC), pH 7.6) and added to a column of streptavidin-agarose beads (Molecular Probes), equivalent to 20 nmol streptavidin (0.3 ml). The column was washed with 20 ml of the same buffer.

The material from the DEAE and carboxymethyl columns (approximately 30 ml) was added to the oligonucleotide affinity column. The column was then washed with 10 ml of the same buffer. The oligonucleotide with attached protein was eluted from the beads by incubation

with shaking with 100 mM biotin in 80 mM Tris and 2 mM sodium bicarbonate, pH 6.5, for 20 min. The protein was captured on a PVDF membrane by passing the eluate through the membrane, and the membrane was then washed with buffer and dried. The dried protein on PVDF was subjected to analysis by mass spectrometry (nano-liquid chromatography electrospray ionization tandem MS). This analysis was performed by Proteome Factory AG (Berlin, Germany). Database matching of identified peptide sequences was performed using the Mascot program (Matrix Science, London, UK).

Immunohistochemistry

Portions of human adrenal glands were fixed in 4% paraformaldehyde and were dehydrated and embedded in paraffin using standard techniques. Sections (4 μ m) were deparaffinized and rehydrated using graded alcohol concentrations. Antigen retrieval was performed by incubation in 100 mM sodium citrate, pH 6.0, and were subjected to three cycles of heating in a microwave oven for 3 min followed by 10 min of cooling. After non-specific binding was blocked with 10% horse serum (10 min), sections were incubated with a rabbit anti-human α -enolase antibody (product 6880-0410; Biogenesis, Kingston, NH, USA) at a 1:100 dilution for 40 min at room temperature. Bound primary antibody was visualized by incubation of sections with a secondary antibody (biotinylated goat anti-rabbit; Vector Laboratories, Burlingame, CA, USA) at a 1:100 dilution for 1 h. The sections were washed in buffer and then a quantum dot conjugate (Qdot 655 streptavidin conjugate; Quantum Dot Corp., Hayward, CA, USA) was added at 10 nM in the manufacturer's buffer for 5 min followed by washing. The sections were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) at 10 ng/ml and photographed using fluorescence microscopy.

Promoter activity assay

The -1019/+202 and -1019/+13 regions of the human HSD3B2 gene were generated by PCR. For the PCR reaction, the 5' and 3' primers contained, respectively, KpnI and XhoI sites at their 5' ends. The PCR products were subcloned into the pGL3-Basic luciferase reporter plasmid (Promega). An expression vector for human α -enolase, in which the full-length α -enolase cDNA is expressed from a cytomegalovirus promoter, was obtained from Invitrogen.

NCI-H295R human adrenocortical cells were cultured in 24-well plates at a density of (1–5) $\times 10^5$ cells/well in medium with 2.5% Nu-Serum (BD Biosciences, Franklin Lakes, NJ, USA) for 24 h before transfection. Cells were transfected with Lipofectamine 2000 (Invitrogen) using the manufacturer's protocol. Luciferase reporter constructs and the α -enolase expression plasmid were co-transfected

with the internal control vector pRL-TK (encoding *Renilla* luciferase; Promega). Total DNA transfected per well (2 μ g) was kept constant by adjusting the amount of empty pGL3-Basic vector. 48 h after transfection, cells were harvested and the cell lysates were assayed for luciferase activities with the dual-luciferase reporter assay system (Promega). Firefly luciferase activities were normalized to *Renilla* luciferase activity.

A one-way ANOVA was used to compare the influence of α -enolase on reporter activity, accepting $P < 0.05$ as significant. Statistical analyses were performed using the StatView 5.0 program (Abacus Concepts, Berkeley, CA, USA).

Results

In these experiments we took advantage of the previous characterization of an element in the first intron in HSD3B1 (Guerin *et al.* 1995). The sequences of this region in HSD3B1 and HSD3B2 are shown in Fig. 1. The sequences differ by the presence of two bases (CA) found in HSD3B1 but not in HSD3B2 and by base differences at five other positions, as indicated in the figure. This element in HSD3B1 was shown to bind a 37 kDa protein in gel-mobility shift assays, producing a complex designated R1 (Guerin *et al.* 1995). The R1 complex was formed by extracts from a variety of established cell lines, both steroidogenic and non-steroidogenic, but human adrenocortical tissue was not investigated. Here, we investigated the formation of the R1 complex using nuclear extracts from human adrenocortical tissue, separated into ZF and ZR cells (Fig. 2). The R1 complex was observed using extracts from both zones, as well as with HeLa cell nuclear extract. Moreover, the R1 complex was not disrupted by the presence of an excess of an oligonucleotide based on HSD3B2, indicating that the R1 protein does not bind to this element in HSD3B2.

Guerin *et al.* (1995) noted several less-prominent DNA-protein complexes when using an element from HSD3B1 as probe, which required higher protein concentrations for visualization (Guerin *et al.* 1995). We observed that the other complexes formed by the element from HSD3B1 were disrupted in the presence of an oligonucleotide based on HSD3B2. Therefore, in the following experiments, we investigated proteins binding to the HSD3B2 sequence. Several complexes were formed by the element from HSD3B2 using extracts from ZF and ZR cells (Fig. 3). In four separated ZF/ZR-cell preparation pairs, ZF cell extracts consistently formed a complex (designated C1) that was present at a much lower level in ZR cell extracts, whereas other larger complexes were formed in all extracts. A complex of similar size to the C1 complex was also formed from bovine adrenal cortex nuclear extract. When the same cell extracts were used with a control oligonucleotide (Sp1), no differences in complex formation was noted between samples (Fig. 3).

HeLa ZF ZF ZR ZR
- +2 - +2 - +2

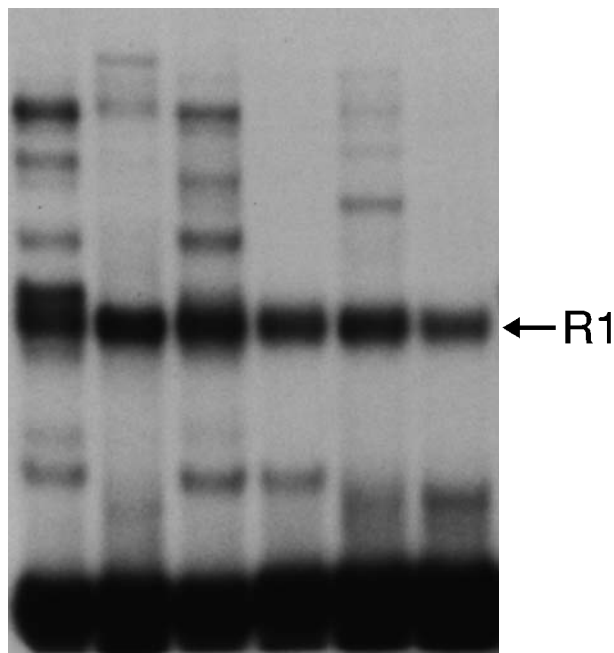


Figure 2 Formation of complex R1 in extracts from separated ZF and ZR cells. A labeled double-stranded oligonucleotide corresponding to an element in HSD3B1 (sequence 1 in Fig. 1) was incubated with nuclear extracts from adrenocortical cells (ZF or ZR) or from HeLa cells. +2 indicates that a 100-fold molar excess of an oligonucleotide based on HSD3B2 (sequence 2 in Fig. 1) was added during the binding reaction.

We performed gel-mobility shift analysis with competitor oligonucleotides to investigate the formation of the C1 complex with elements from HSD3B1 and HSD3B2. These studies were designed to characterize the region of the element required for C1 complex formation and to find out whether a shorter sequence would still efficiently form a C1 complex. This information was subsequently used in the design of an oligonucleotide affinity column used for purification of the protein. Figure 4 shows that, as expected, all complexes formed by the oligonucleotide based on HSD3B2 were disrupted in the presence of an excess of the same sequence. A similar result was obtained with an excess of an oligonucleotide based on HSD3B1 in which bases in the 5' part of the sequence have been changed to those of HSD3B2 (sequence 1a in Fig. 1). Thus the ZF-specific protein forming C1 appears to bind both to HSD3B2 and HSD3B1, presumably to the 3' part of the element where the two sequences are most similar. In the presence of the unmutated HSD3B1 sequence the C1 complex was disrupted, but two rapidly migrating complexes were formed (designated C2/C3), concomitant with the disappearance of two slower-migrating

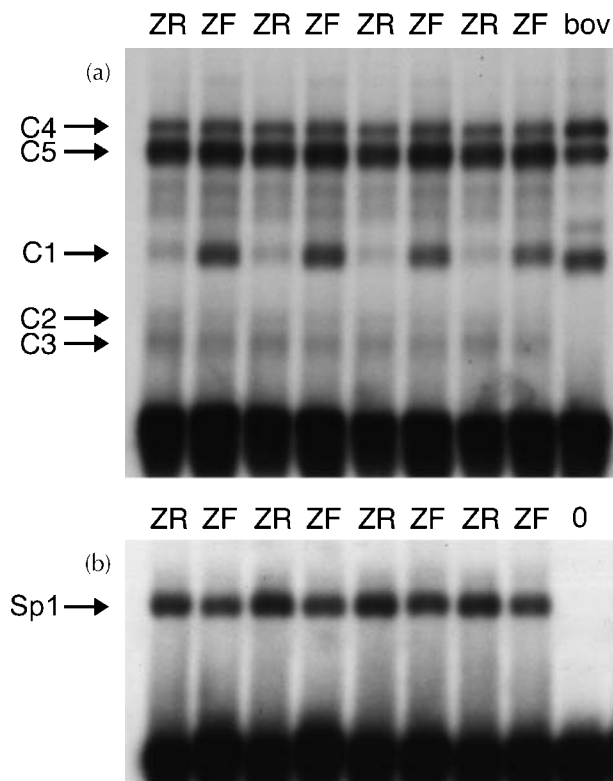


Figure 3 Complexes formed with a probe based on HSD3B2. In (a) nuclear extracts from four pairs of ZF and ZR cells were incubated with labeled oligonucleotide (sequence 2 in Fig. 1). Cells were obtained from four adrenal glands from donors of 36, 44, 53 and 60 years of age. Additionally nuclear extract from bovine adrenal cortex (bov) was used. The ZF-specific complex C1 is indicated. In (b) ZR and ZF cell extracts were incubated with a labeled oligonucleotide of a sequence that binds Sp1. 0 indicates the no-extract control.

complexes (designated C4/C5). Because the C2/C3 pair is not formed in the presence of sequence 1a, but is formed in the presence of the unmutated sequence, the proteins involved appear to bind to the 5' part of the HSD3B2 element. The C4/C5 pair is presumably formed when the C1 protein is also bound by the probe. This conclusion is consistent with results obtained when competition experiments were performed using ZR cell extract (Fig. 4). ZR cell extract formed a less-prominent C4/C5 pair whereas the C2/C3 pair was formed even without the HSD3B1 competitor. This indicates that the proteins forming C2/C3 are found in both ZR and ZF cells.

These conclusions were confirmed by analysis of the effects of competitor oligonucleotides based on the 5' part of the element, extending to the boundary of the 3 β I-A and GT box sub-elements, as previously defined by Guerin *et al.* (1995; Fig. 1). Oligonucleotides based on the 5' part of HSD3B2 (2c and 2d) disrupted the C4/C5 pair of complexes without disrupting C1 (Fig. 4). However sequence 2b, in which five bases have been mutated to those of HSD3B1, did not affect C4/C5. Sequence 2d differs from 2b in that two bases only have been mutated. Taken together these findings indicate that formation of the C4/C5 complexes depends on an unmutated HSD3B2 sequence in bases beyond the 5' end of the 3 β I-A sub-element (Fig. 1). None of the truncated oligonucleotides based on HSD3B1, even when bases were mutated to those of HSD3B2 (1b, 1c, 1d) disrupted the C4/C5 complexes. This indicates that the central pair of bases (CA) present in HSD3B1 does not permit binding of the proteins forming these complexes. The competition analysis also confirmed that the formation of complex C1 requires the 3' end of HSD3B1 or HSD3B2. C1 was disrupted by sequences 1, 1a, 2 and 2a. It was not disrupted by truncated oligonucleotides, except partially

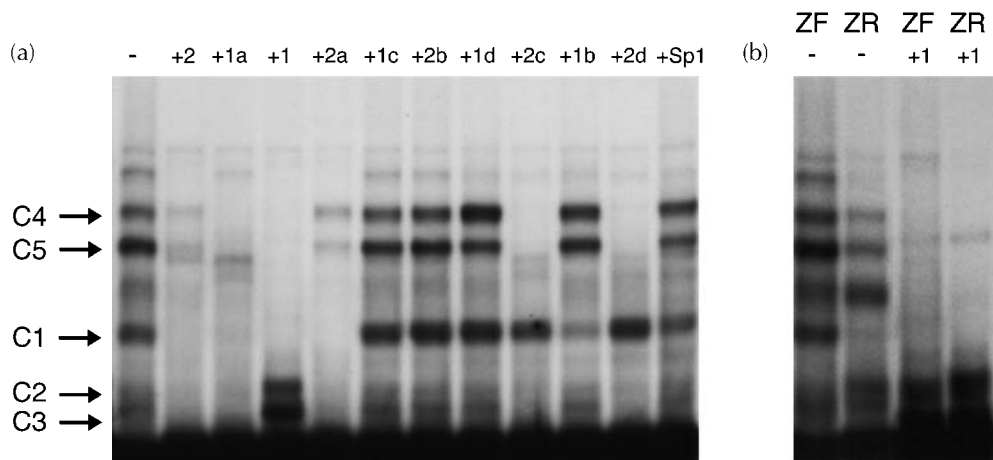


Figure 4 Gel-mobility shift competition analysis on nuclear extract proteins bound to HSD3B2. All reactions comprised nuclear extract proteins (all from human ZF cells except where indicated) and the labeled probe based on HSD3B2 (sequence 2 in Fig. 1). Competitor oligonucleotides were added at 100-fold molar excess in the binding reaction. The oligonucleotides used are indicated above the lanes; the sequences are shown in Fig. 1. Positions of complexes designated C1 to C5 are indicated (see text).

by sequence 1b, which was based on HSD3B1 with five bases mutated to the sequence of HSD3B2. This indicates that although C1 formation requires the 3' end of the sequence, formation of this complex is still affected by the context of the 5' part of the sequence. Consistent with this conclusion, we found that shorter oligonucleotides, in which bases have been removed from the 5' end of the HSD3B2 element, did not strongly bind proteins from adrenocortical cell extracts when used as labeled probes (results not shown).

We concluded that an oligonucleotide affinity column for purification of the ZF-specific nuclear protein forming the C1 complex would need to be formed from the full-length HSD3B2 element. Because such an oligonucleotide would bind more than one protein, some fractionation of the nuclear extract would be required before affinity-column purification. We investigated whether chromatography on DEAE and carboxymethyl ion-exchange columns would be useful for separation of the C1 complex protein. Human adrenocortical nuclear extract was applied to a DEAE ion-exchange column and proteins were eluted with 40 mM KCl (Fig. 5). Fractions were assayed by gel-mobility shift using the probe based on HSD3B2. The protein forming the C1 complex eluted rapidly from the column. Other proteins that bind to HSD3B2, producing slower-migrating complexes from unfractionated extract, eluted later. The early fractions therefore comprise a partially purified preparation of a protein forming the C1 complex. Further elution with 70 and 100 mM KCl buffers did not yield fractions with more C1 complex. Figure 5 also shows that a protein producing a similar gel-shift pattern also eluted rapidly with 40 mM KCl when bovine adrenocortical nuclear extract was fractionated. Moreover the same pattern was observed when we tested nuclear extracts from a variety of human cell lines (results not shown). C1 complex protein also eluted rapidly from a carboxymethyl ion-exchange column (results not shown).

Competition assays performed with partially purified C1 complex protein gave results that were consistent with those obtained with crude extracts. The complex was disrupted by those oligonucleotides that disrupt the C1 complex formed from the unfractionated extract (Fig. 6). It was partially disrupted by 1b and fully disrupted by the unmutated HSD3B2 element. It was not disrupted by unrelated competitor oligonucleotides comprising binding sequences for SF-1 and Sp1 (Fig. 6, and data not shown).

Based on these analyses we sought to purify and identify the protein forming the C1 complex. An oligonucleotide affinity column was constructed using double-stranded DNA based on the sequence of the HSD3B2 element. Covalently attached biotin was used to attach the DNA to streptavidin-modified beads. Because of the limited supply of human adrenocortical tissue, and because we observed that a C1 complex was formed by extracts derived from a variety of human cells, we used a human cell line

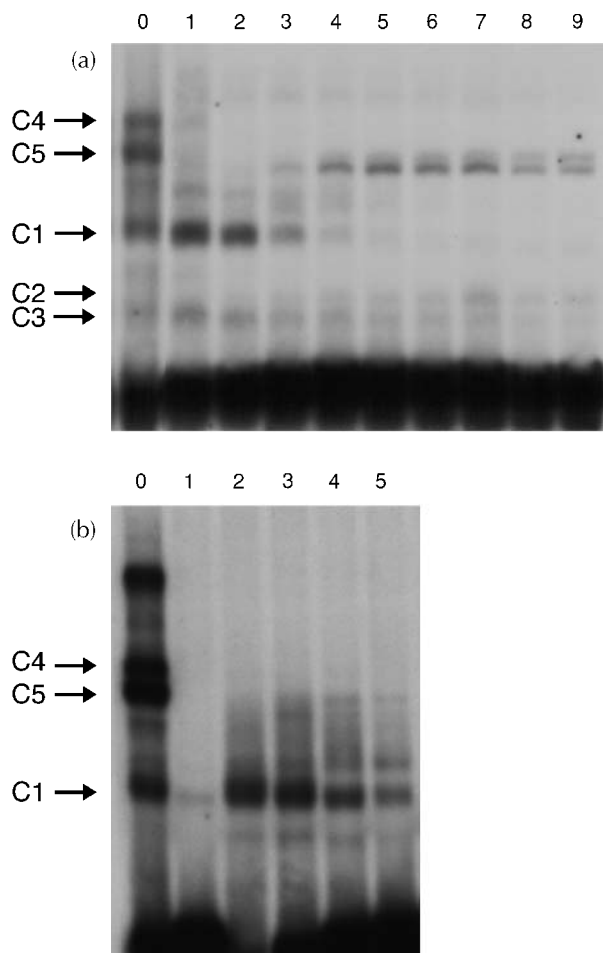


Figure 5 Gel-mobility shift analysis of fractionated proteins from human (a) and bovine (b) adrenal cortex. Unfractionated nuclear extract incubated with labeled HSD3B2 probe was used in lane 0. In lanes 1–9 (human) or 1–5 (bovine) fractions eluted from a DEAE ion-exchange column were incubated with labeled probe (see the Materials and Methods section).

(IMR-32) as a source of nuclear extract proteins. To remove other proteins that bind to the HSD3B2 element the extract was passed through DEAE and carboxymethyl columns and the early-eluting proteins were applied to the oligonucleotide affinity column. The oligonucleotide-protein complex was eluted with biotin and adsorbed on to PVDF membrane. The adsorbed protein was subjected to mass spectrometry analysis. The only significant hit (probability-based MOWSE score of 94) was α -enolase. Scores >56 indicate that the observed match is significant, i.e. that the probability of a random match is <0.05.

α -Enolase is a multifunctional protein that has been shown to bind to DNA. Although the entire protein can bind (Feo *et al.* 2000, Subramanian and Miller 2000), binding also resides in an N-terminal-truncated form of α -enolase that arises by use of an internal translational start site.

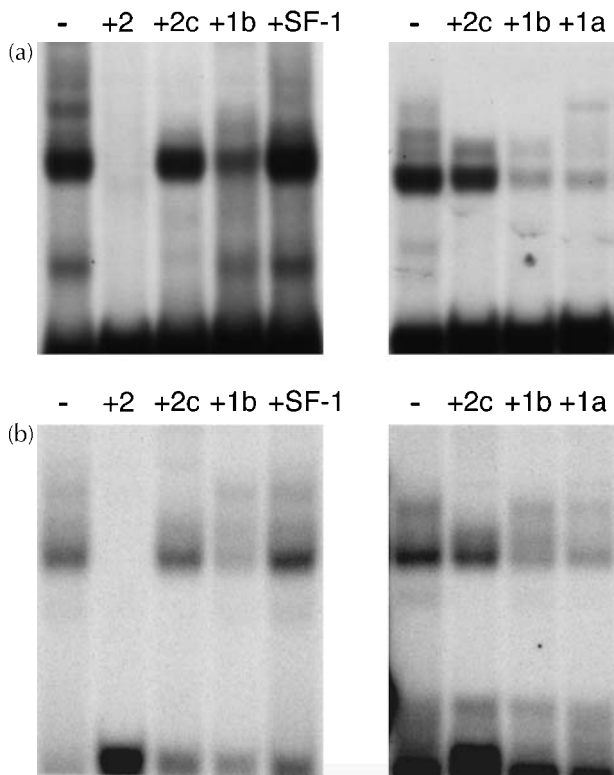


Figure 6 Gel-mobility shift competition analysis of partially purified C1 complex protein from human (a) and bovine (b) nuclear extracts. Two different preparations from each source were used. Each binding reaction contained HSD3B2 probe with 5 μ g protein pooled from fractions 1 and 2 (human) or 2–4 (bovine) (see Fig. 5). Competitor oligonucleotides as indicated above the lanes were added at 100-fold molar excess.

This protein, termed *c-myc* promoter-binding protein (MBP-1), binds to a region around the major (P2) promoter of the *c-myc* gene (Ray and Miller 1991). We tested whether the C1 complex was disrupted by a double-stranded oligonucleotide that was initially characterized for binding of MBP-1 (Ray and Miller 1991) and which was subsequently used in binding the full α -enolase protein (Feo *et al.* 2000, Subramanian and Miller 2000). Figure 7 shows that the MBP-1 oligonucleotide does in fact disrupt the C1 complex. As controls we tested double-stranded oligonucleotides based on the binding sites for eight other DNA-binding proteins; none of these oligonucleotides disrupted the C1 complex at a 100-fold molar ratio, indicating the specificity of disruption by the MBP-1 oligonucleotide (results not shown).

The competitive gel-shift and protein-purification experiments lead to the conclusion that α -enolase is a protein with a nuclear localization in ZF cells but not in ZR cells. In order to study this further, the subcellular localization of α -enolase was examined by immunohistochemistry. In sections of adrenal cortex from several different donors we

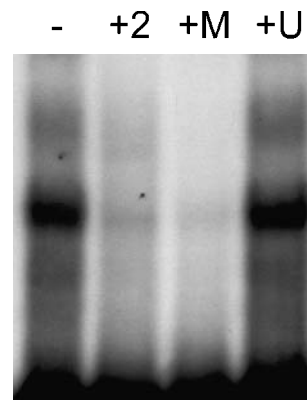


Figure 7 Competition for C1 complex with an oligonucleotide that binds α -enolase and its alternate translational form MBP-1. Bovine adrenal nuclear extract that had been passed over DEAE- and carboxymethyl-Sepharose columns, as described in the text, was incubated with labeled HSD3B2 probe (-). In the other lanes 100-fold molar excess of competitor oligonucleotides were added: the HSD3B2 element (+2), the *c-myc* P2 promoter (+M) and an unrelated sequence (+U).

found that α -enolase staining was nuclear in the ZF and cytoplasmic in the ZR (Fig. 8). In ZF cells staining was almost entirely nuclear with little cytoplasmic staining. In ZR cells staining in many cells was confined to the cytoplasm and the nucleus was negative; in others staining appeared to be both cytoplasmic and nuclear. This could be caused by cellular heterogeneity or alternatively it could result from some overlap of cytoplasm and nucleus in the section.

These experiments establish that α -enolase is a DNA-binding protein with a zone-specific subcellular location in the human adrenal cortex, but leave open the question of whether α -enolase affects the activity of the HSD3B2 promoter. To address this question we assessed the effects of α -enolase on HSD3B2 promoter activity in co-transfection experiments (Fig. 9). These studies used the functional human adrenocortical cell line NCI-H295R (Rainey *et al.* 1994). Increasing amounts of an α -enolase expression plasmid increased HSD3B2 promoter activity, but only when the construct contained the first intron (-1019/+203). The shorter construct (-1019/+13) was unaffected by co-transfected α -enolase. In separate experiments the increase in promoter activity induced by α -enolase was variable but statistically significant. Possible causes of the variability are discussed below.

Discussion

An adrenocortical DNA-binding protein was identified as α -enolase and was shown to have a nuclear localization in human ZF cells, whereas it was predominantly

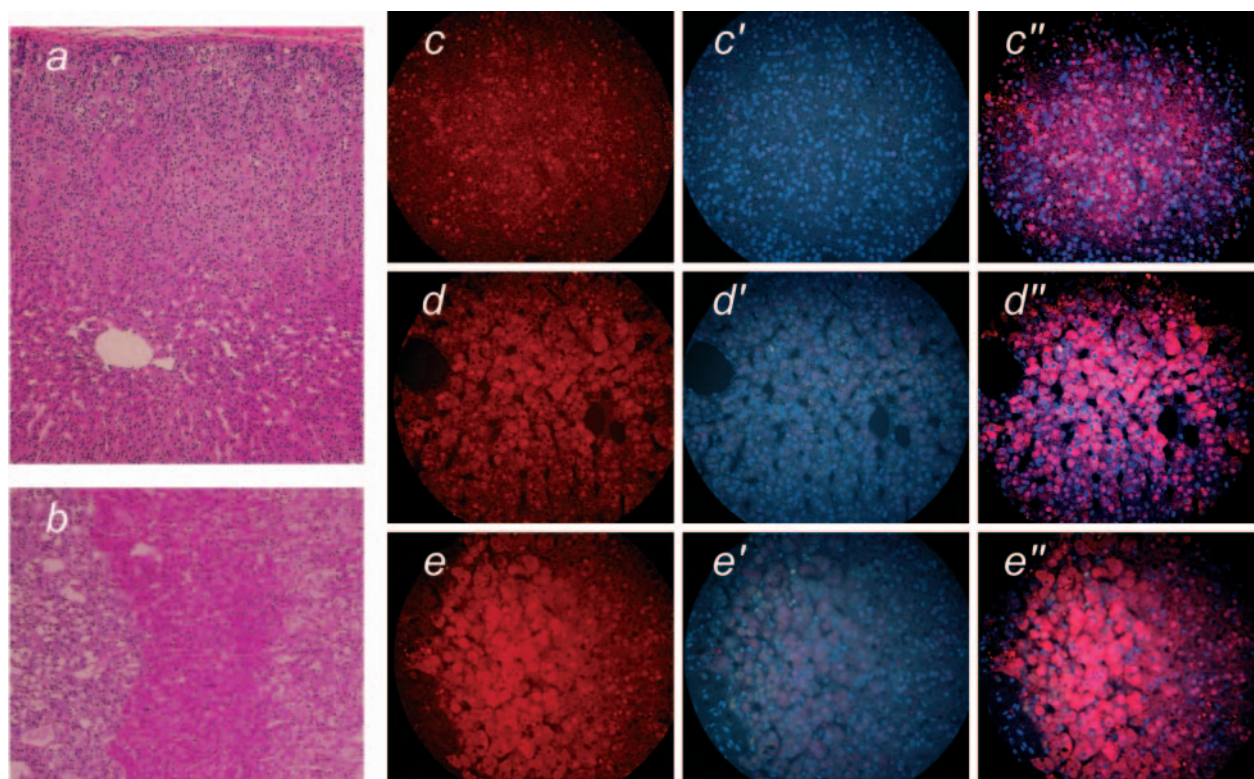


Figure 8 Immunocytochemical investigation of the distribution of α -enolase in the human adrenal cortex. (a, b) Hematoxylin- and eosin-stained sections of the adrenal cortex from different donors. The section in (a) shows a part of the gland where two opposing layers of cortex meet at the alar raphe (Dobbie and Symington 1966) and no medulla is present. The section in (b) shows medulla on the left, ZR in the center and ZF on the right. The fluorescence images in (c) and (d) are from sections adjacent to that shown in (a) and the images in (e) are from a section adjacent to that shown in (b). (c) is located in the middle of the ZF; (d) centers on the two facing ZR layers on either side of the alar raphe, and includes some ZF cells at both the top and bottom of the image; (e) centers on the ZR, with medulla on the left and part of the ZF on the right. Sections were incubated with α -enolase antibody, which was visualized with a red quantum dot conjugate. Sections were counterstained with DAPI (blue; c'–e') to show the nuclei. In (c''), (d'') and (e'') the red and blue images have been merged.

cytoplasmic in ZR cells. α -Enolase is a multifunctional protein, having several distinct molecular activities. Apart from its role in glycolysis, it is a surface receptor for the binding of plasminogen, a lens crystallin, a hypoxic stress protein and an autoimmune antigen (Pancholi 2001). As a protein involved in glycolysis it is expected to be cytoplasmic, but it is found in the nucleus in several cell types. Western blotting shows it to be present in both nucleus and cytoplasm of endothelial cells (Aaronson *et al.* 1995) and HeLa cells (Subramanian and Miller 2000). Immunohistochemistry shows a nuclear location for α -enolase in astrocytes (Langley and Ghandour 1981), type II neurons of the spiral ganglia (Dechesne and Keller 1996), bronchial epithelial cells, type I and type II alveolar cells and endothelial cells (Chang *et al.* 2003). During muscle regeneration α -enolase changes from a cytoplasmic to a perinuclear location (Merkulova *et al.* 2000). Proteomic studies have shown α -enolase to be found in the nuclei of Burkitt lymphoma BL 60 cells (Muller *et al.* 1999) and HEK-293 kidney cells (Schirle *et al.* 2003).

α -Enolase and its alternate translational form MBP-1 bind and negatively regulate the major (P2) promoter of the *c-myc* gene (Feo *et al.* 2000, Subramanian and Miller 2000, Ray and Miller 1991). These proteins bind in the minor groove surface of the TATA-box motif of DNA together with TATA-box-binding protein (TBP; Chaudhary and Miller 1995). Although both α -enolase/MBP-1 and TBP bind to the TATA box, α -enolase/MBP-1 does not bind to all promoters, and the precise sequence requirements for binding other than the TATA box element are not established. A plant α -enolase was shown to bind to a TATA-like element in the ZAT10 gene (Lee *et al.* 2002). Figure 10 compares the α -enolase-binding sequences of the *c-myc* promoter, the element in the ZAT10 gene, and the element used here from the first intron of HSD3B2. The central sequence in the HSD3B2 element, TAAAAAA, is a TBP-binding element but does not act as a promoter (Bernues *et al.* 1996, Patikoglou *et al.* 1999, Mishra *et al.* 2003).

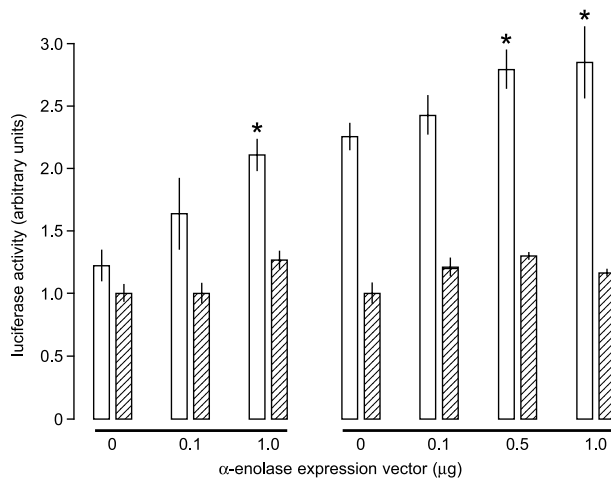


Figure 9 Effect of α -enolase on HSD3B2 promoter activity. NCI-295R cells were transfected with luciferase reporter constructs containing either nucleotides $-1019/+203$ of the human HSD3B2 gene (open bars) or $-1019/+13$ (striped bars). Firefly luciferase activity was normalized to an internal control (*Renilla* luciferase plasmid). Two separate experiments are shown here. The amounts (μ g) of co-transfected α -enolase expression vector are indicated. Each bar shows the mean \pm s.e. for normalized luciferase activity from four wells. Asterisks indicate values significantly different ($P < 0.05$) from the corresponding cells not transfected with α -enolase.

The impetus for examining proteins binding to the HSD3B2 gene was to find transcription factors that could be involved in the differential regulation of this gene in the zones of the adrenal cortex. The discovery of α -enolase as a DNA-binding protein in the nuclei of ZF cells raises the question of whether α -enolase could be involved in the regulation of 3β -HSD. In co-transfection experiments an α -enolase expression vector increased the activity of the HSD3B2 promoter, but only when the promoter contained the first intron of the gene, i.e. only when the α -enolase-binding site was present. The degree of stimulation of promoter activity by α -enolase was variable but statistically significant. Although the cause of the variability is unknown, it may result from the presence of endogenous α -enolase in the adrenocortical cell line used.

GTAAAAAATGGGGT	HSD3B2
GTATAAAAGCCGGT	c-myc
ATATAAAATCCGTA	ZAT10

Figure 10 Comparison of the α -enolase-binding elements in HSD3B2, *c-myc* (Ray and Miller 1991) and ZAT10 (-763 to -748 in the ZAT10 gene; Lee *et al.* 2002). Nucleotides in *c-myc* and ZAT10 that are common to HSD3B2 are shown with a gray background.

Levels could vary due to the growth status or other properties of the cells; this could blunt the effects of overexpression of the protein. It is also possible that α -enolase may play a role in the regulation of *c-myc* expression in the adrenal cortex; it is of interest that *c-myc* was previously identified as a gene with higher expression in ZR cells (Wang *et al.* 2001). The significance of the present experiments is that they add to a growing appreciation that the ZR cell is a molecularly distinct cell type within the human adrenal cortex, despite the fact that the biological significance of its major product, DHEA(S), remains largely unknown (Hornsby 1995).

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