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Promoter Analysis of Human Glutamate Carboxypeptidase II

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Abstract

The expression of glutamate carboxypeptidase II (GCP II) is reduced in selective brain regions in schizophrenic patients. To investigate transcriptional mechanisms regulating the human GCP II gene, a 3460 bp DNA fragment comprised of the proximal 3228 bp of 5' untranscribed sequence and first 232 bp of 5' UTR portion of this gene was cloned into the mammalian luciferase reporter gene vector pGL3-Basic. Transfection assays in human astrocyte-derived SVG and human prostate tumor-derived LNCaP cells demonstrated that constructs with 3460, 1590 and 761 bp portions of 5' region of human GCP II gene were able to drive the luciferase reporter gene. Additional deletion constructs showed that in the SVG cell line, constructs with 511 and 411 bp of GCP II gene fragments yielded highest transcriptional activity, with declining activity upon further removal of 5' sequences. Fifteen bp of the promoter 5' to a 225 bp GCP II fragment were essential for luciferase expression. Thus, in the SVG cells, the proximal 240 bp of the human GCP II promoter (232 bp of the 5' UTR and 8 bp of 5' untranscribed sequences) may represent the core promoter. Further, while a LyF-1 site lies within and overlaps a transcription start site in the 15 bp sequence, site-directed mutagenesis shows that LyF-1 is not the transcription initiator for the "TATA and CAAT" box lacking GCP II gene in the SVG cells. Finally, pattern differences in GCP II gene promoter expression in SVG and LNCaP cells suggest that sequences beyond 240 bp may be important for tissue-specific GCP II expression.

Keywords

Glutamate carboxypeptidase II; prostate-specific membrane antigen; regulation of gene transcription; promoter; Lymphoid transcription factor 1 (LyF-1); schizophrenia

1. Introduction

Glutamate carboxypeptidase II (GCP II), also known as NAALADase (N-acetylated- α -linked acidic dipeptidase), was first identified as the enzyme that hydrolyzes the neuropeptide N-acetyl-aspartyl glutamate (NAAG) to N-acetyl aspartate (NAA) and glutamate (Blakely et al., 1988; Robinson et al., 1986). NAAG is a highly abundant peptide neurotransmitter in the mammalian central nervous system. It is an antagonist of the NMDA receptor (Sekiguchi et

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al., 1989; Grunze et al., 1996; Bergeron et al., 2005) and an agonist of the metabotropic glutamate receptor 3, an inhibitor of glutamate release (Wroblewska et al., 1997; Neale et al., 2000). The aggregate effect of NAAG is thus to attenuate NMDA receptor activation. In the brain, GCP II is primarily, if not exclusively, expressed in astrocytes (Slusher et al., 1992; Berger et al., 1999; Ghose et al., 2004; Sacha et al., 2007). Enzymatic and molecular cloning studies further suggest that GCP II expressed in the human cerebellum, prostate specific membrane antigen (PSMA) expressed on hormone refractory metastatic prostate cancer cells (Israeli et al., 1993), and folate hydrolase I (FOLH1) in the small intestine are identical proteins (Pinto et al., 1996; Luthi-Carter et al., 1998; Halsted, et al., 1998; Bacich et al., 2001; Watt et al., 2001). However, their biological functions may significantly differ. Serum levels of PSMA or its DNA increase as prostate cancer progresses; consequently, it is useful as a clinical marker for diagnosis and prognosis of prostate malignancy (Missiades et al., 2004; Chen et al., 2005; Papadopoulou et al., 2006). FOLH1 hydrolyzes the polyglutamate side chain from folic acid permitting dietary folate absorption into the small intestine.

Mounting evidence supports the hypothesis that the endophenotype of schizophrenia includes hypofunction of cortico-limbic NMDA receptors (Coyle 2006). Reduced GCP II expression in brain regions relevant to schizophrenia would reduce NMDA receptor function by interfering with the catabolism of the endogenous NMDA receptor antagonist, NAAG (Tsai et al., 1995; Hakak et al., 2001). Since GCP II regulates both folate absorption and NMDA receptor activation via NAAG hydrolysis, it may also play an important role in the pathophysiology of schizophrenia. Thus, if maternal folate is low and homocysteine levels high, the potential for teratogenic effects may increase the risk for developing schizophrenia (Goff et al., 2004; Picker and Coyle 2005).

At present, little is known about the regulation of the expression of GCP II. With malignant transformation of prostate cells, GCP II expression markedly increases; for example, 30% of membrane protein is comprised of GCP II/PSMA in the prostate cancer cell line LNCaP (Israeli et al., 1994). Kindled seizures also up-regulate GCP II expression in the rat hippocampus (Meyerhoff et al., 1989). In contrast, reduced expression of GCP II has been reported in selective brain regions in post-mortem studies of patients with schizophrenia (Tsai et al., 1995) while administration of antipsychotic medications and NMDA receptor antagonists increases GCP II expression in rat cerebral cortex (Flores and Coyle 2003).

GCP II is a class II membrane glycoprotein with an apparent molecular mass of 94 to 100 kDa. O'Keefe et al. (1998), reported that a 1.2 Kb portion of the 5' region of the GCP II gene was able to drive reporter gene expression in prostate but not breast-derived cell lines (O'Keefe et al., 1998). A 2-Kb human genomic fragment containing the 5' untranscribed region of the GCP II gene was identified in prostate and non-prostate cell lines (Good et al., 1999). At the 3' end of this fragment, 614 bp appears to represent the core promoter required for transcriptional activity. A PSMA enhancer has also been identified within the third intron of FOLH1 (Watt et al., 2001), which apparently confers prostate specific expression of GCP II.

Thus, while GCP II, PSMA and FOLH1 may be identical proteins encoded by the gene now designated as FOLH1, sequences within the untranslated regions of their genes may confer specificity and/or differential tissue specific expression. Our interests focus on the potential role of down-regulation of GCP II in the pathophysiology of schizophrenia. In the present study, we provide the first evidence that GCP II gene promoter-driven transcription occurs in brain astrocytes. Transient transfection assays with nested GCP II promoter deletion-luciferase reporter gene constructs show differential activation of the GCP II promoter in the human astrocyte-derived SVG cell line versus the human prostate cancer cell line LNCaP. In the astrocyte cell line, a core promoter of 240 bp has been identified along with a 15 bp sequence harboring a potential transcription initiation site for the GCP II gene. The latter corresponds

to a previously identified transcription initiation site for PSMA/GCP II/FOLH1 and contains a LyF-1 binding motif. While LyF-1 can initiate transcription in genes lacking TATA and CAAT boxes and is expressed at higher levels in the SVG cells, site directed mutational analysis of the GCP II promoter suggests that LyF-1 is not the apparent transcription initiator for the GCP II gene in astrocytes.

2. Results

2.1. Human library screening and analysis

By PCR screening of a lambda genomic library with human GCP II specific primers, we identified a ~15 kb genomic clone containing GCP II exon 1 at the 3' end. Restriction mapping and sequencing confirmed identity to the GCP II gene (O'Keefe et al, 1998 GenBank Accession No. [AF007544](#); Good, 1999, GenBank Accession No. [AF061571](#)).

2.2. Expression of GCP II in human cell lines

To show that GCP II gene expression occurs in the cells to be used in the transient transfection assays with GCP II promoter plasmids, total RNA from SVG, CRL-1718, and LNCaP cells was examined for the presence of GCP II mRNA by RT-PCR. GCP II cDNA amplicons were detected in all three cell lines (Fig. 1A). Highest expression of GCP II mRNA was observed in the LNCaP cells and lowest in the SVG cells. Notably, HeLa cells do not express GCP II as would be predicted by upon regional expression studied (Slusher et al., 1992). Based on densitometric scanning, the relative ratio of GCP II mRNA in the three cell lines, LNCaP:CRL-1718:SVG, was 39:5:1, respectively.

Western blot analysis was also performed on membrane protein from the cells using a polyclonal antibody against the extracellular region of human GCP II. An approximately 100-kDa band was present in the prostate cancer cells LNCaP and an ~120-kDa band in the human brain astrocyte-derived cells SVG and the astrocyte cells CRL-1718 (Fig. 1B). Interestingly, GCP II protein levels showed a similar trend of expression as mRNA with highest and lowest abundance in the LNCaP and SVG cells (LNCaP:CRL-1718:SVG, 42:3:1, respectively).

2.3. Construction of GCP II promoter deletion constructs

In order to examine GCP II gene promoter transcriptional functionality, a 3460 bp Sac I/Sma I fragment from the isolated ~15 kb human GCP II clone was inserted into Sac I/Sma I restriction sites in the reporter plasmid vector pGL3-Basic to create the plasmid pGL3-hGCP3.4K. This plasmid contains a 3228 bp fragment of 5'-untranscribed region upstream of the transcription start site of the human GCP II gene as previously identified (nt 2062 to nt 5289) and the distal 5' 232 bp of transcribed but untranslated sequence (nt 5290 to nt 5521) of exon 1 (numbering according to Gene Bank Accession No. [AF061571](#), Good et al., 1999). The structure of plasmid pGL3-hGCP3.4K is shown in Fig. 2A.

The GCP II deletion constructs, pGL3-hGCP1590, pGL3-hGCP511, pGL3-hGCP225, and pGL3-hGCP202, were generated by unidirectional deletion using Exo III and S1 nuclease. Sequence analysis confirmed that these constructs spanned nt 3932-5521, nt 5011-5521, nt 5297-5521 and nt 5320-5521, respectively, of the GCP II promoter. By PCR amplification we constructed an additional series of clones with progressively truncated sequences upstream of the putative transcription start site for the GCP II gene (Israel et al., 1993). They are as follows: pGL3-hGCP761, nt 4761-5521; pGL3-hGCP561, nt 4961-5521; pGL3-hGCP411, nt 5111-5521; pGL3-hGCP321, nt 5201-5521; pGL3-hGCP281, nt 5241-5521; pGL3-hGCP240, nt 5282-5521. In addition, a 1602 bp fragment comprised of 1370 bp 5' untranscribed sequence and the distal 5' 232 bp of untranslated exon I (bp 3920 to 5521 in the GCP II gene) was excised from plasmid pGL3-hGCP3.4K by Nsi I/Sma I digestion, and a new construct pGL3-

hGCP1858* was created by re-ligation of the rest part of the linear pGL3-hGCP3.4K plasmid. This construct lacks the proximal 1370 bp of 5' untranslated region and the 232 bp of 5' UTR of hGCP II gene present in pGL3-hGCP3.4K. Construction of the latter was for the purpose of further defining essential promoter sequences for GCP II promoter transcriptional activity.

2.4. Human GCP II promoter function

The effects of sequential 5' deletion of promoter sequences on GCP II promoter function was examined in transient transfection assays in the SVG and LNCaP cells. The pGL3-hGCP3.4K elevated luciferase expression well above the pGL3-Basic promoterless control vector. Relative luciferase activity was 3.78-fold in the LNCaP cells and 10.3-fold in the SVG cells. The truncated plasmids, pGL3-hGCP1590 and pGL3-hGCP761, (1358 bp and 529 bp of 5' untranscribed fragments upstream of transcriptional start site, respectively) showed higher levels of luciferase activity than pGL3-3.4K in the SVG cells ($p \leq 0.001$). By contrast, these same plasmids activated luciferase activity to the same extent as pGL3-hGCP3.4K in LNCaP cells (Fig. 3). Both pGL3-hGCP225 (lacks 5' untranscribed region upstream of transcriptional start site and 7 bp of transcribed sequence) and pGL3-hGCP II 1858 bp* (1858 bp fragment of 5' region upstream from nt 2062 to 3919) showed no significant luciferase expression in either the SVG or LNCaP cells. The results suggest that promoter sequences for the hGCP II gene contained in the proximal 529 bp of 5' untranscribed sequence upstream of transcriptional start site and the distal 232 bp of the 5' end of exon I (total of 761 bp) may be essential for maximal GCP II gene promoter activity. The 3-fold higher expression of luciferase observed for pGL3-hGCP1590 and pGL3-hGCP761 in the SVG cells further suggest that there may be tissue specific elements in this region of the promoter which enhance expression in the astrocyte cell line.

To define the minimal promoter region sufficient for GCP II promoter-driven gene transcriptional activity, transient transfection assays were executed in the SVG and LNCaP cells using the shorter GCP II promoter-reporter gene constructs that were generated by progressive truncation of the pGL3-hGCP761 plasmid. Significant levels of transcriptional activity were apparent for all plasmids harboring >240 bp of GCP II promoter sequence (pGL3-hGCP561, pGL3-hGCP511, pGL3-hGCP411, pGL3-hGCP321, pGL3-hGCP281, pGL3-hGCP240 with 329bp, 279 bp, 179 bp, 89 bp, 49 bp, 7 bp of 5' untranscribed sequence, respectively). In contrast, the pGL3-hGCP225 and pGL3-hGCP202 bp did not significantly increase transcriptional activity above that of the promoterless pGL3-Basic control vector in either cell line (Fig. 4). As with the longer constructs above, GCP II promoter-driven luciferase expression was markedly higher with these shorter constructs in the SVG cells than in the LNCaP cells. The rise in luciferase activity in SVG cells derived from plasmids harboring 511 and 411 bp of 5' region was about 2-fold higher than those harboring 761, 561 or 240 bp of GCP II promoter with no significant differences in luciferase induction between the pGL3-hGCP761, pGL3-hGCP561 and pGL3-hGCP240 ($p > 0.05$). Removal of an additional 5'-15 bp from the pGL3-hGCP240 (pGL3-hGCP225) leads to a virtual complete loss of expression in both the SVG and LNCaP cells, suggesting that the 15 bp of nucleotides from nt 5282 to nt 5296 contain sequences essential for GCP II promoter-driven transcriptional activity in the SVG and LNCaP cells and further, that 240 bp of sequence may constitute the core promoter for the hGCP II gene in the astrocyte cell line.

2.5. Western blot and gel mobility shift analysis

Sequence analysis identified a potential LyF-1 binding motif within the apparent "essential 15 bp" of hGCP II promoter sequences (AGGCGCCTCTCAAAA) suggesting that the LyF-1 binding element might be a site for transcription initiation in the SVG astrocytic cell line (Fig. 5). It has previously been shown that LyF-1 subsumes such a role for the terminal deoxynucleotidyltransferase (TdT) gene and other lymphocyte specific genes (Hahm et al.,

1994;Lo et al., 1991). For other genes that lack TATA and CAAT boxes, Sp1 frequently serves as a transcription initiator as well (Blake and Azizkhan JC., 1989), and there is a GC-rich region that extends from the 5' end of the putative LyF-1 site to more distal 5' sequences. However, when this GC-rich region was subjected to transcription factor consensus binding site analysis using either the GenomeNet databases or Transfac databases, no match to an Sp1 binding site was identified. Therefore, only LyF-1 was further pursued as a potential transcriptional initiator for GCP II gene in the astrocyte cell lines.

LyF-1 protein expression was first assessed in nuclear extracts isolated from the SVG, CRL-1718 and LNCaP cells using western blot analysis. As shown in Fig. 6, Antibody IK H-100 (sc-13039, Santa Cruz Biotechnology, INC., Santa Cruz, CA), a polyclonal rabbit antibody specific to the C-terminal sequence common to all isoforms of LyF-1, detected 5-6 protein bands in the SVG, CRL-1718 and LNCaP nuclear extracts. LyF-1 belongs to the Ikaros gene family of proteins (Lo et al, 1991;Hahm et al., 1994;Molnar and Georgopoulos 1994;Sun et al., 1996). When the molecular weights of these bands were calculated based on electrophoretic mobility compared to protein molecular weight standards (data not shown), four of the proteins corresponded to Ikaros isoforms, IK1, IKx, IK2/3 and IK6. Correction of the densitometric signals for each of the protein bands for loading variation relative to the densitometric signal for the TATA binding protein (TBP) standard, showed that IK1 and IK_x were the predominant isoforms in the nuclear extracts of the three cell lines although IK_x was less abundant in the CRL-1718 nuclear extracts. A faint IK2/3 band was observed across all cell lines. Levels of the LyF-1 isoform, IK6, was also very low in the LNCaP cell nuclear extracts. In contrast, the CRL-1718 and SVG cell nuclear extracts showed high expression of this isoform, with levels ~1.57 fold in CRL-1718 cell nuclear extracts compared to the SVG cell nuclear extracts.

To assess the ability of LyF-1 to bind to the putative LyF-1 site within the 15 bp sequence essential for transcriptional activity, gel mobility shift assays were executed with the same nuclear protein extracts from the SVG, CRL-1718 and LNCaP cells (Fig. 7). Protein-DNA binding complexes were observed for each of the three cell lines. Major and minor complexes were apparent. The major complex was a slower migrating, i.e. higher molecular weight binding protein, and present at ~ 4-fold greater abundance than the faster migrating, minor complex. Both protein-DNA complexes were more prevalent with the SVG and LNCaP nuclear extracts by comparison to the CRL-1718 nuclear extracts. Inclusion of unlabeled, duplex LyF-1 consensus oligonucleotide competitor DNA in respective binding reactions, markedly suppressed total protein-DNA complex formed. However, despite a 100-fold excess of competitor DNA, residual protein-DNA complex remained.

2.6. Site-directed mutagenesis studies

Site-directed mutagenesis was used to further evaluate the contributions of the 15 bp sequence from nt 5281 to nt 5296 (AGGCGCCTCTCAAAA) to core promoter activity as the LyF-1 binding site (CCTCTCAAA) overlaps one of several proposed transcription start sites for the GCP II gene (Israel et al., 1993; O'Keefe et al., 1999) (Fig. 8). The LyF-1 site in the putative core promoter of the GCP II gene represents an 8/9 bp match to the human consensus LyF-1 site (CCTCCCAA, Lennon et al., 1997; Kawai et al., 2003). Six pairs of mutagenic primers were designed to either alter nucleotides within the LyF-1 site to prevent LyF-1 binding (mut1, mut3, and Mut4), create a perfect match LyF-1 site (mut2) or retain the original GCP II gene LyF-1 binding site (mut5 and mut6) but alter nucleotides 5' to the transcription start site. Mutant 2 (Mut2, 9/9 bp match to the LyF-1 binding motif, AGGCGCCTCCCAAAA) and Mutant 4 (Mut4, 5/9 bp match, AGGCGGAGCTCAAAA) expressed markedly increased luciferase activity compared to the pGL3-hGCP240 plasmid construct in LNCaP cells (2.1- and 4.5-fold, respectively, $p \leq 0.001$). Mut4 also showed significantly higher luciferase expression in the

SVG cells by comparison to pGL3-hGCP240 (1.2-fold, $p \leq 0.001$) while luciferase activity expressed by the Mut2 construct was significantly less by comparison to pGL3-hGCP240 (0.8-fold, $p \leq 0.001$). Similarly, the remaining four mutant constructs showed reduced luciferase activity relative to pGL3-hGCP240 ($p \leq 0.001$). While mutants 5 and 6 retain the original LyF-1 consensus sequence, mutations were introduced 5' to the putative LyF-1 site. It is possible that these alterations change DNA structure, and thereby potential promoter interactions, to decrease LyF-1 transcriptional activation. In all cases where LyF-1 binding site mutation reduced GCP II promoter activity, the decrement was only partial. Taken together, these results suggest that if the transcription initiation site is contained within this 15 bp sequence of nucleotides, LyF-1 likely does not act as a transcriptional initiator of the GCP II gene in the astrocyte cells or the prostate cancer cells.

3. Discussion

Human GCP II appears identical to hPSMA and FOLH1 based on cDNA sequence, enzymatic activity and immunoreactivity (Pinto et al., 1996; Luthi-Carter et al., 1998; Halsted et al., 1998; Bacich et al., 2001; Watt et al., 2001). Previous studies reported that 2 Kb and 1.2 Kb of the 5' promoter of the PSMA gene were effective in driving reporter gene expression in prostate-derived cell lines and non-prostate cell lines but not in CNS-derived cell lines (O'Keefe et al., 1998; Good et al., 1999). However, GCP II is expressed in the brain, with particularly high abundance in astrocytes (Berger et al., 1999; Sacha et al., 2007). Furthermore, reduced expression of GCP II appears to associate with a variety of illnesses, including behavioral and neurological disorders. In particular, we are interested in the role of decreased GCP II expression in schizophrenia (Tsai et al., 1995; Hakak et al., 2001) as we have also shown that antipsychotic medications can stimulate GCP II in the brain (Flores and Coyle, 2003).

In the preceding studies, we provide the first functional evidence that nucleotide sequences 5' to the originally identified translation initiation site (Israeli et al., 1993) in the hGCP II promoter can drive transcriptional activity in the astrocyte cell line SVG. Deletion mutation analysis further shows that GCP II promoter driven-reporter gene expression in the astrocyte cell line exhibits a different activation profile than in prostate cancer cells. The core promoter in the case of the former appears to consist of 240 bp comprised of the proximal 8 bp of 5' untranslated region and 232 bp of 5' UTR in exon 1. Within these sequences lies a LyF-1 binding motif overlapping the mRNA start site. The site resides within a 15 bp stretch of nucleotides that are essential for GCP II promoter-driven transcription in the SVG cells. However, while LyF-1 can act to initiate transcription (Hahm et al., 1994; Lo et al., 1991), site directed mutational analysis shows that it is unlikely the transcription initiator for the hGCP II gene in astrocytes or the prostate cancer cells LNCaP.

To investigate the transcriptional regulation of the GCP II gene in human brain astrocytes, a 15 Kb clone was isolated from a human lambda genomic library established from whole blood. Restriction mapping and sequencing showed that the clone shared sequence identity with the human PSMA gene (O'Keefe et al., 1998; Good et al., 1999) and spanned the 3' extreme of exon 1 through upstream flanking regions in the 5' untranslated sequences of GCP II gene. Based on the published cDNA sequence for the PSMA gene (Israeli et al., 1993), the transcription start site should be located at nt 5290 and the translation start site (ATG) at nt 5551. Thus, the present clone, starting at its 3' extreme, includes nucleotide sequences encoding a translated region consisting of the intracellular domain and the transmembrane domain as well nucleotide sequences representing the 5' UTR and 5' untranslated sequences of the GCP II gene.

As described earlier, GCP II protein is expressed in the human brain and most abundantly so in brain astrocytes associated with the temporal lobe (Sacha et al., 2007). We demonstrate that the human astrocyte cell lines, SVG and CRL-1718, express GCP II mRNA (RT-PCR) and GCP II protein (western analysis), indicating that the GCP II gene is actively expressed in these astrocyte-derived cells (Fig. 1). The relative abundance of both mRNA and protein was lowest in the SVG cells by comparison to their expression in the prostate cancer cell line, LNCaP, where the endogenous gene is also expressed. Transient transfections assays with hGCP II promoter-luciferase constructs confirm that GCP II transcriptional activation occurs in astrocytes. GCP II promoter-driven luciferase activity for all constructs was higher in the SVG astrocyte cell line than in the prostate cancer cell line. A different profile of GCP II promoter-driven luciferase expression from the nested deletion constructs was observed in the SVG cells by comparison to the LNCaP cells. In the SVG cells, a minimum of 240 bp, consisting of 232 bp of the 5' UTR in exon 1 and 8 bp of 5' untranslated sequence are necessary to drive transcriptional activity, suggesting that this extent of 5' untranslated portion and exon 1 represents the core promoter for the hGCP II gene. Elimination of an additional 15 bp of 5' sequence completely prevents GCP II promoter-driven gene activation. Hence, these 15 bp containing 8 bp of 5' untranslated portion and 7 bp of transcribed but untranslated sequences within exon 1 of the GCP II gene may constitute sequences essential for transcriptional activation in astrocytes.

Good et al. (1999) showed that 614 bp of hPSMA promoter sequence in hPSMA-luciferase reporter gene constructs was necessary for driving luciferase expression in prostate cell lines (LNCaP, human PSMA-expressing prostate cancer cell line, and DU145, prostate cancer cell line) as well as non-prostate cell lines (HeLa, human cervical cancer cell line, and 911, human retinoblastoma cell line). A longer 2-Kb construct compared with the 614-bp fragment provided higher expression levels when using prostate derived cell lines (DU 145 and LNCaP). The increased transcription using the 2-Kb fragment was not great in non-prostate cell lines. Little or no transcription over basal levels was seen with 232-bp fragment. The lower PCR primer site using to generate the 232 bp construct lies distal to the 3' Sma I restriction site that we used, thereby eliminating 47 additional bp of 3' nt by comparison to our construct. O'Keefe et al. (1998), using 5' RACE, identified several additional transcription start sites in the hPSMA gene promoter; two at +67 and +27 bp relative to the originally identified transcription start site and two at -195 and -235 bp relative to the translation start site, all of which lie within the non-coding sequences of the hPSMA gene.

This study provides the first demonstration of hGCP II promoter-driven expression in the SVG cell line. While we can not exclude the possibility of alternative transcription initiation sites, the deletion mutation findings, with nested deletions clustered in closer proximity to the 15 bp sequence of nucleotides essential for transcription (761, 561, 511, 411, 321, 281, 240, 225, 202) certainly present a strong case of a single transcription initiation site for the hGCP II gene in the astrocyte cells. Finally, our results also show that expression of hGCP II gene promoter driven-luciferase expression is markedly higher (as much as ~3-fold, $p \leq 0.001$) in the SVG cell line than in the LNCaP cell line and further, that the promoter sequences required for transcriptional activity and/or maximal transcriptional activity may also differ for astrocyte versus prostate cells. Within the 761 bp region of hGCP II promoter stimulating highest luciferase expression, an incremental rise in hGCP II promoter driven-luciferase activity was apparent with the addition of up to 171 bp 5' to the 240 bp core promoter (as much as a 2-fold with the 411 bp promoter construct) in the SVG cells, followed by a decline in activity thereafter. In contrast, highest hGCP II promoter driven-luciferase activity in the LNCaP cells was observed with constructs harboring 240, 281 and 321 bp of hGCP II promoter, followed by a decline (411 and 511 bp of promoter) and subsequent increase (561 and 761 bp of promoter). The differences in levels of expression and expression profiles are striking, and suggest the possibility of tissue specific regulated hGCP II promoter activity within this

segment of 5' region of the GCP II gene. Further microdissection and analysis of the sequences within this region are currently being pursued to examine this possibility. Watt et al. (2001) have previously identified a sequence within intron 3 of the FOLH1 gene, which confers tissue-specific expression of PSMA in the prostate. Our recent findings would represent the first identification of tissue-specificity directed by untranslated sequences in the hGCP II promoter, if corroborated.

Consensus sequence matching for transcription factor binding motifs shows a number of potential binding sites for transcription factors present between nt 4761 to nt 5296 in the hGCP II gene promoter, including sites for YY1, specificity protein 1 (Sp1), lymphoid transcription factor 1 (LyF-1) and GATA-binding factors (Fig. 5). As described earlier, the LyF-1 site is of particular interest since it lies within the previously identified transcription initiation site (Israel et al., 1993) and the 15 bp sequence in the hGCP II gene essential for transcriptional activity in the SVG astrocyte cells, a gene with neither TATA nor CAAT boxes. LyF-1 protein is encoded by specific mRNAs derived from alternative splicing of the Ikaros gene (Hahm et al., 1994). It was first identified (Lo et al., 1991) as regulating transcription of the TdT gene in lymphocytes and is especially elevated during B and T lymphocyte development. At least nine of the Ikaros/LyF-1 isoforms are found in mice and humans. While all nine Ikaros/LyF-1 isoforms possess at least two C-terminal Krüppel-like zinc fingers (Sun et al., 1996), their total N-terminal zinc finger content determines whether they act as positive and negative regulators of transcription (Molnar and Geogopoulos 1994). Isoforms with three or more N-terminal ZnFs, IK1-3 and IK_x, bind with high affinity to the GGGA motif in the promoters of target genes to activate transcription while those with fewer than three N-terminal ZnFs are DNA non-binding isoforms and suppress transcription (Sun et al., 1996). Furthermore, the expression of LyF-1 is not limited to the lymphocyte (Payne et al., 2003; Masumoto et al., 2002). In this study, IK1 and IK_x were the predominant LyF-1 isoforms in the three cell lines utilized. However, in CRL-1718 and SVG cells, the dominant negative isoform Ik6 was also abundant and highest in the CRL-1718 cells. In contrast, very low expression was observed in the LNCaP cells. At present, it is unclear how the relative abundance of different Ikaros/LyF-1 isoforms in our cell lines may affect hGCP II promoter activity either in regulating GCP II expression in developing, differentiating or mature cells. We show here that LyF-1 protein expression predicts LyF-1 binding to the LyF-1 consensus site in the GCP II promoter for the SVG, CRL-1718, and LNCaP cells. However, site-directed mutation analysis revealed that introduction of point mutations into the LyF-1 binding site (mut1-3) residing within the 15 bp sequence required for transcriptional activity reduces (~ 50%), but does not eliminate, in hGCP II promoter-driven gene expression in the SVG cells. For two constructs (mut5 and mut6) where point mutations were introduced 5' to the LyF-1 site, the decline in promoter activity suggests that sequences at the 5' end of the 15 bp stretch of essential transcription sequence may also be critical to transcriptional activity. In any case, our results do not support the hypothesis that LyF-1 serves as a transcription initiator for the hGCP gene in the SVG cells. Interestingly, the mut2 and mut4 constructs harboring point mutations within the LyF-1 site lead to significantly increased hGCP II promoter driven-luciferase expression in the LNCaP cells. As described earlier, the original 240 bp construct, prior to mutation, showed elevated luciferase expression in the LNCaP cells.

Previous post-mortem studies on brains of schizophrenic individuals showed reduced GCP II mRNA and enzyme activity in the frontal cortex, consistent with GCP II down-regulation in this region of the brain (Tsai et al, 1995; Hakak et al., 2001). The temporal cortex, is another brain region associated with schizophrenia. While GCP II expression is low in this brain region, it is highly expressed in temporal white matter (astrocytes) by comparison to temporal gray matter as well as frontal cerebral cortex, the caudate nucleus, brainstem and spinal cord (Sacha et al., 2007). Thus, identifying the transcriptional regulatory elements in the core and extended

promoter sequences of the human GCP II gene, particularly in astrocytes, appears critical to understanding the role of GCP II under-expression in schizophrenia.

4. Experimental procedures

4.1. Isolation of human genomic clones

A human whole blood lambda genomic library (Stratagene@Lambda DASH@Library, #945203) was screened using PCR-based methodology (Israel, 1993). Primers were derived from the published human GCP II gene sequence (GenBank accession No. [AF007544](#)): sense, 5'-CTACTCAGCTGGCCCATGGC-3'; and antisense, 5'-AGGCCCAGACAGGCGGATC-3'. PCR was executed using 40 ng of each primer, 0.2 mM dNTPs and 0.4 U of Taq polymerase (Promega Inc., Madison, WI) in a total volume of 20 µl in the buffer supplied by the manufacturer. Initial denaturation was 5 min at 94°C followed by 35 cycles of amplification (94°C for 30 sec, 60°C for 3sec, and 72°C for 1 min).

The ~15 kb genomic clone obtained, containing GCP II exon I, was digested with NotI and subcloned into the bacterial plasmid vector pBluescript® SK(-) (Stratagene, La Jolla, CA). The plasmid was then transformed into competent DH-5α cells, amplified and purified using a QIAGEN plasmid midi kit (QIAGEN Sciences, Maryland). DNA sequencing was performed using an Applied Biosystems Taq DyeDeoxy cycle sequencing kit on an ABI 3700PRISM automated sequencer (Applied Biosystems, Foster City, CA) to verify the identity of the clone.

4.2. RNA extraction and RT-PCR

Total RNA from SVG, CRL-1718 and LNCaP cells was prepared using Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA) and 1 µg of RNA reverse transcribed into single stranded cDNA using the Transcriptor First Strand cDNA Kit (Roche Applied Science, Mannheim, Germany) according to manufacturer's direction. PCR amplification was executed using 25 bp primers from the human GCP II cDNA sequences, 5'-CAGCGTGGAAATATCCTAAATCTGA -3' and 5'-TTGGATGAACAGGAATA CTTGGAA -3' (Burger et al., 2002). Samples were incubated for 5 min at 94°C (hot start), followed by 35 cycles of 15 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C.

4.3. Construction of pGL3-hGCP II deletion plasmids

A 3460 bp fragment consisting of proximal 3228 bp of 5' untranslated sequence and first 232 bp of 5' UTR portion of this gene beginning at -3228 bp upstream of the translation start site identified for the human GCP II gene (O'Keefe et al, 1998; Good, 1999) was excised by Sac I/Sma I digestion of the 15 kb GCP II genomic DNA cloned pBluescript® SK(-) vector, and sub-cloned into the pGL3-Basic vector (Promega Inc., Madison, WI) containing the firefly luciferase reporter gene restriction cut with the same enzymes. From the resulting plasmid, designated pGL3-hGCP3.4K, a series of 5' nested deletion constructs were generated using Exo III and S1 nuclease digestion or PCR technology.

To generate deletion constructs by PCR, 5' and 3' primers with NheI (5'...g/ctagc...3') and Bgl II (5'...a/gatct...3') restriction sites were designed. For PCR amplification, the 5' primers, hGCP 761, 5'-gcggttagccctccccggttcaagc-3'; hGCP 561, 5'-gcggttagccacgccccggttcaaaa-3'; hGCP 511, 5'-gcggttagctacatgtttattaat-3'; hGCP 411, 5'-gcggttagcttttctgctctgctt-3'; hGCP 321, 5'-gcggttagccagagaggagagctc-3'; hGCP 281, 5'-gcggttagccagcaagagctggaca-3' or hGCP 241, 5'-gcggttagccagcgctctcaaaa-3' were paired with the 5' primer 5'-gcgagatctaccgcgcctgtgc-3'. Thermal cycling consisted of an initial denaturation step (94°C for 5 min), 35 cycles of amplification (94°C for 1 min, 57°C for 1 min, 72°C for 1 to 1.5 min), and a final extension step (72°C for 10 min) using Platinum® Taq DNA Polymerase High Fidelity (Invitrogen Life Technologies, Carlsbad, CA). The resultant PCR products were cut

with Nhe I and Bgl II, and then cloned into Nhe I and Bgl II restriction sites of the pGL3-Basic vector. All constructs were confirmed by restriction enzyme mapping and sequencing.

4.4. Site-directed mutagenesis

The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to insert point mutations into the pGL3-hGCP241 plasmid according to the manufacturer's specifications. Mutagenic primers were designed with 1 to 3 bp mismatches targeting specific elements within the core promoter sequence. The mutant sequences were as follows: Mut1: 5'-gcggctagAGGCGCCTCCTAAAA -3'; Mut2: 5'-AGGCGCCTCCCAAAAggggccgg -3'; Mut3: 5'-gcggctagAGGCGCCTCTTTTAaggggccgg -3'; Mut4: 5'-cgcggttagAGGCGGAGCTCAAAAggggccg -3'; Mut5: 5'-cgcggttagCTTCGCCTCTCAAAAggggccg -3'; Mut6: 5'-cgcggttagAGTTACCTCTCAAAAggggccg -3'. Introduction of mutations into the reporter gene constructs were confirmed by sequencing.

4.5. Cell culture and transient transfection assays

Human fetal glial cell line SVG (P12), human astrocytoma cell line CRL-1718 and human prostate tumor cell line LNCaP were purchased from the American Type Culture Collection (Manassas, VA). The SVG cell line was maintained in Eagle's Minimum Essential Medium with Earles Balanced Salt Solution containing 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (SVG medium). CRL-1718 and LNCaP cell lines were grown in RPMI 1640 medium containing 2 mM L-glutamine, 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin (CRL-1718/LNCaP medium). Cells were sustained at 37°C in an atmosphere of 5% CO₂-balance air.

Transfection assays in SVG and LNCaP cells were carried out in 24-well culture plates using Lipofectamine™ 2000 (Promega Inc., Madison, WI) according to the manufacturer's instructions. Briefly, 1.4×10^5 SVG cells and 1.6×10^5 LNCaP cells were plated per well and incubated in 0.5 ml of antibiotic-free CRL-1718/LNCaP medium. When the cells were approximately 90% confluent (24 hours after plating), they were overlaid with plasmid DNA/lipofectamine complexes (1 µg of total plasmid DNA per well consisting of 0.7 µg of GCP II promoter-reporter gene plasmid plus 0.3 µg of pSV-β-galactosidase normalization plasmid) in a total volume of 0.1 ml serum-free CRL-1718/LNCaP medium and incubated at 37°C in a humidified incubator in an atmosphere of 5% CO₂-balance air. The medium was replaced after 6 hours with complete medium and cells incubated an additional 42 h. Cells were then washed with Phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, pH ~ 7.3) and harvested in 150 µl of 1X lysis buffer. Cellular debris was removed by centrifugation at 800 g. Protein concentrations were determined using Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA). To determine luciferase activity, 80–150 µl of lysate supernatant was mixed with 50 µl luciferase substrate (Promega Inc., Madison, WI) and luminescence measured using a MLX Microtiter® plate Luminometer (DYN-EX Technologies, Chantilly, VA). To correct for variation in transfection efficiency, β-galactosidase activity was also determined using the β-Galactosidase Enzyme Assay System (Promega Inc., Madison, WI). Data are expressed as relative luciferase activity, defined as luciferase activity normalized to the luciferase activity of the pGL3-Basic vector control and β-galactosidase. A pGL3-control-promoter vector was included in all assays. At least three replicates were included in each sample group, and experiments repeated three times.

4.6. Western blot analysis

To isolate membrane protein, cells were homogenized in 5 mM Tris-HCl, pH 7.4 containing 2 mM EDTA and protease inhibitor cocktail (10 mg/ml benzamidine, 5 mg/ml leupeptin, and 5 mg/ml trypsin inhibitor, Sigma-Aldrich, St. Louis, MO) and centrifuged at 500 g for 15 min

at 4°C. The resulting supernatant was centrifuged at 45,000 g for 15 min at 4°C. Membrane pellets were centrifuged twice in the homogenization buffer and resuspended in 75 mM Tris-HCl, pH 7.4-12.5 mM MgCl₂-5 mM EDTA. Nuclear extracts were prepared using a CellLytic™ NuCLEAR™ Extraction Kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's direction. Protein concentrations were determined as described above. Ten to twenty-five µg of membrane or nuclear protein extracts were separated on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Millipore Co., Bedford, MA). GCP II and LyF-1 were detected by chemiluminescence using rabbit antiserum against human GCP II (made by our lab, unpublished) or anti-Ikaros antibody (sc-13039, Santa Cruz Biotechnology, Santa Cruz, CA) respectively as primary antibodies, horseradish peroxidase-conjugated goat anti-rabbit IgG H& L (ab6721, Abcam Inc. Cambridge, MA) as secondary antibody and Western Lightning Chemiluminescence Reagent plus (PerkinElmer LAS, Inc, Boston, MA). Semi-quantitative assessment of protein bands from the fluorograms was executed by computerized densitometry on a MacIntosh G4 computer (Apple, Cupertino, CA) using Quantity One Quantitation Software (Bio-Rad, Hercules, CA).

4.7. Gel mobility shift assay

Nuclear protein was prepared as described for Western blot assay. Complementary oligonucleotides for the LyF-1 (TTTGAGAGG) binding site identified within GCP II promoter by means of the Motif Search program from Genome Net (<http://motif.genome.jp>) were synthesized, and double-stranded oligonucleotides generated by annealing complementary nucleotides after end-labeling with [γ -³²P]ATP. The complementary LyF-1 single-stranded probes were as follows: LyF-1, 5'-ttaaggcgCCTCTCAAaaggggcc -3' and 5'-ggccctTTTGAGAGGgccttaa -3'.

Probe was labeled using [γ -³²P]ATP and T4 polynucleotide kinase (Promega Inc., Madison, WI). Protein-DNA binding reactions were carried out at room temperature in a total volume of 25 µl containing 5% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5 (binding buffer), 50 µg/ml poly (dA-dT):poly (dA-dT), 2 × 10⁵ cpm of a [γ -³²P]-labeled duplex probe and 10 µg of nuclear protein. For competition experiments, 100 to 200-fold excess of unlabeled probe was added to the reaction mixture. After incubation for 30 min, the samples were separated on a 5% polyacrylamide gel in 0.5 × TBE electrophoresis buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, PH 8.0). The gel was dried and exposed to X-ray film (Molecular Technologies, St. Louis, MO) at -80°C. Relative intensities of the protein-DNA bands on the resulting autoradiograms were quantified as described for western blotting.

4.8. Statistical analysis

Data are presented as the mean ± S.E.M. with n = 3 for each experimental group and three replicate experiments. Statistical significance with respect to control was determined by one-way analysis of variance followed by post hoc comparison using the Student-Newman-Keuls multiple comparisons test to compare all values against each other. A p value ≤ 0.05 was considered statistically significant.

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Fig. 1.

Human GCP II expression in cultured cells. A. RT-PCR analysis for human GCP II mRNA was performed on total RNA isolated from LNCaP, SVG and CRL-1718 cells using specific primers as described in "Experimental procedures". The expected 130 bp PCR amplicon was detected in all of the cell lines. PCR amplicons generated from total RNA from non-expressing HeLa cells and an RNA blank (H₂O) were used as negative controls, and human GCP II cDNA as a positive control. B. Western blot analysis was executed on membrane protein isolated from the LNCaP, SVG and CRL-1718 cells using rabbit antiserum against human GCP II (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) to demonstrate the presence of GCP II. A band at approximately 100 kDa was detected in the LNCaP cell line whereas a 120 kDa band appeared in the SVG and CRL-1718 cell lines.

Figure 2.
pGL3-hGCP II plasmids. A. Schematic of the pGL3-hGCP3.4K plasmid. B. Schematic of the deletion strategy and deletion constructs generated by restriction enzyme digestion, Exo III/S1 nuclease and PCR strategies. A 3460 bp fragment from the 15 kb human GCP II clone consisting of the proximal 3228 bp of 5' upstream untranscribed sequence and the first 232 bp of 5' UTR in exon 1 was cloned into the Sac I/Sma I restriction sites of the pGL3-Basic vector upstream of the firefly luciferase reporter gene (Luc⁺). The extent of 5'-untranscribed region and exon I (232 bp) sequences of the GCP II gene inserted into the pGL3-Basic vector are depicted.

Figure 3.

Expression of luciferase reporter gene constructs in SVG and LNCaP cell lines. pGL3-Basic is the promoterless vector. The pGL3-hGCP plasmids consist of that vector harboring sequential 5' deletions of the untranscribed sequence and the distal 232 bp of 5' UTR from hGCP II gene. Lipo designates the untransfected control. Luciferase activity was determined as described in "Experimental procedures". Controls included promoterless pGL3-Basic and no plasmid. Luciferase activity is expressed relative to control, normalized to beta-galactosidase to correct for transfection efficiency. The mean $\pm$ S.E.M. is presented based on three experiments with 3 replicates per experiment. ***, significantly different from control, $p \leq 0.001$; c, significantly different from comparator construct, $p \leq 0.001$.

Figure 4.

GCP II promoter activity in SVG and LNCaP cells. SVG and LNCaP cells were transfected with GCP II promoter deletion-luciferase reporter gene plasmids as described in “Experimental procedures”. Controls included promoterless pGL3-Basic and no plasmid (Lipo only). Results are expressed as the mean $\pm$ S.E.M. from three experiments in which each construct was assayed in triplicate. *, significantly different from control, $p \leq 0.05$; ***, significantly different from the control, $p \leq 0.001$; a, significantly different from comparator constructs, $p \leq 0.05$; b, significantly different from comparator construct, $p \leq 0.01$; c, significantly different from comparator construct, $p \leq 0.001$.

4761 C
pGL3-hGCP761 SP1 GATA-1
4801 gtagctggga ctacaggagc cgcaccaccac gcccaagctaa tttttgtatt ttttagtagag
4861 atgggtttca ccatgttggc GATA-1
caggatggtc GATA-1
tcgatttctc gacttcggtga tccgcctgtc
4921 tgggcctccc aaagtgtctg gattaccggc gtgagccacc SP1 YY-1
Acgcccggct ttaaaaaatg
pGL3-hGCP561
4981 C/EBP
gttttgtaat gtaagtggag gataataccc Tacatgttta ttaataacaa taatattctt
5041 LyF-1 SP1 pGL3-hGCP511
taggaaaaag ggcgcggtgg tgatttacac tgatgacaag cattcccgac tatggaaaaa
5101 aagcgcagct Ttttctgtctc tgctttttatt cagtagagta ttgtagagat tgtatagaat
pGL3-hGCP411
5161 ttcagagttg aataaaagt ctcataatt ataggagtgg Agagaggaga gtctctttct
pGL3-hGCP321
C/EBP
5221 tcctttcatt tttatatttta Agcaagagct ggacattttc caagaaagt tttttttttt
pGL3-hGCP281
LyF-1 GATA-1
5281 aAggcgcctc tcaaaaGggg cgggatttcc ttctctgga ggcagatgtT gcctctctct
pGL3-hGCP240 pGL3-hGCP225 pGL3-hGCP202
5341 ctgcctcgga ttggttcagt gcaactctaga aacactgctg tgggtggagaa actggacccc
5401 aggtctggag cgaattccag cctgcagggc GATA-1
tgataagcga ggcattagtg agattgagag
5461 agactttacc SP1
ccgccgtggg ggttggaggg cgcgcagtag agcagcagca caggcgcggg
5521 t
↑

Figure 5.

pGL3-hGCP761 nucleotide sequences and putative regulatory elements. From plasmid pGL3-hGCP761, additional GCP II promoter-reporter gene deletion constructs were generated by PCR as described in “Experimental procedures”. The 5' end of each deletion construct is indicated by boldface, uppercase letters and the 3' end (identical for all constructs) by a white arrow with the construct name designated below the nucleotide sequence. The transcription start site of the GCP II gene is located at nt 5290 (Israel et al., 1993). Putative transcription factor binding sites, as determined by consensus sequence match, are designated by boldface, lowercase, underscored letters with the transcription factor designated above the nucleotide sequence.

Fig. 6.

Gel mobility shift assay with LyF-1 consensus sequences in the GCP II promoter. Single-stranded complementary oligonucleotides encoding the LyF-1 binding element were end-labeled with [γ - 32 P] dATP and annealed to form double-stranded probe as described in "Experimental procedures". The radiolabeled duplex probe was combined with 10 μ g of nuclear extracts from the SVG, CRL-1718, or LNCaP cells. Lane 1, 4, and 7: nuclear extract and [γ - 32 P]-labeled LyF-1 probe; Lane 2, 5 and 8: nuclear extract, [γ - 32 P]-labeled LyF-1 probe and unlabeled LyF-1 probe (specific competitor); Lane 2, 6, and 9: nuclear extract, [γ - 32 P]-labeled LyF-1 probe and unlabeled beta-Actin probe (non-specific competitor).

Fig. 7.

LyF-1 protein in astrocyte and prostate cell lines. Western blot analysis was performed to examine endogenous LyF-1 protein expression in the astrocyte (SVG and CRL-1718) and prostate (LNCaP) cells lines as described in “Experimental procedures”. Ten μ g of nuclear protein extract was used per sample. The blot was probed with polyclonal anti-IKars antibody (Santa Cruz Biotechnology, Inc.). Equal protein sample loading was monitored by hybridizing the same membrane filter with anti-TATA binding protein (TBP) antibody. The molecular weights of LyF-1 are indicated on the right of the panels.

A. MUTATION OF 15 bp GCP II PROMOTER SEQUENCES

Human LyF-1 9 bp consensus sequences:

C C T C C C A A A

15 bp sequences of GCP II gene promoter:

A G G C G C C T C T C A A A A

Mut1:	A G G C G	C C T C	C T	A A A A
Mut2:	A G G C G	C C T C	C C	A A A A
Mut3:	A G G C G	C C T C T	T T T	A A
Mut4:	A G G C G	G A G	C T C A A A A	
Mut5:	C T T	C G	C C T C T C A A A A	
Mut6:	A G T T A	C C T C T C A A A A

Fig. 8.

Effects of site-directed mutagenesis on GCP II transcriptional activity. Mutations were introduced into sequences 5' to the LyF-1 binding site and within the site itself in the pGL3-hGCP240 construct. A. Human LyF-1 consensus sequence, GCP II LyF-1 consensus sequence and mutations introduced into pGL3 hGCP240 to generate pGL3-hGCP240mut1, pGL3-hGCP240mut2, pGL3-hGCP240mut3, pGL3-hGCP240mut4, pGL3-hGCP240mut5 and pGL3hGCP240mut 6. The substituted nucleotides are in boldface type. B and C. The mutant constructs were transfected into the SVG and LNCaP cells and luciferase activity determined after 42 hr as described in "Experimental procedures". Luciferase activity is expressed relative to the promoterless control (pGL3-Basic) and normalized to beta-galactosidase activity to correction for transfection efficiency. *, significantly different from control, $p \leq 0.05$, ***, significantly different from control, $p \leq 0.001$; c, significantly different from comparator construct, $p \leq 0.001$.