HNF1\(\alpha\) upregulates the human AE2 anion exchanger gene (\(SLC4A2\)) from an alternate promoter

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Received 8 September 2003

Abstract

The human AE2 gene (\(SLC4A2\)) is transcribed in a widespread fashion from the upstream promoter, the resultant full-length transcript AE2a being encountered in most tissues. Moreover, alternate promoter sequences within intron 2 may drive tissue-restricted expression of variants AE2b\(_1\) and AE2b\(_2\), mainly in liver and kidney. AE2b\(_2\) proximal promoter sequences are highly active in transfected liver-derived HepG2 cells and contain an HNF1 motif. Mutation-disruption of this motif dramatically decreased alternate promoter activity in HepG2 cells but not in prostate-derived PC-3 cells. Electromobility shift and supershift assays indicated that HNF1\(\alpha\) from HepG2 nuclear extracts binds the HNF1 sequence. Transactivation studies in PC-3 cells showed enhanced activity of the wild-type construct upon cotransfection with an HNF1\(\alpha\) expression plasmid, while activity of the HNF1-mutated construct remained unaffected. Since liver AE2 is putatively involved in the biliary secretion of bicarbonate, HNF1\(\alpha\) may have a role in increasing bicarbonate secretion in response to certain stimuli.

Keywords: Human AE2; Alternate promoter; Chloride/bicarbonate exchange; Hepatocyte nuclear factor 1\(\alpha\); Liver-derived HepG2 cells; Prostate-derived PC-3 cells; Tissue-specific expression

Na\(^+\)-independent anion exchangers (AE) are membrane proteins that mediate electroneutral and reversible exchange of Cl\(^-\) and HCO\(_3\)\(^-\) across the plasma membrane [1,2]. AE polypeptides usually load Cl\(^-\) into the cell and extrude HCO\(_3\)\(^-\), promoting intracellular acidification. In cooperation with other ion carriers, they are involved in the regulation of intracellular pH, cell volume homeostasis, and transepithelial acid/base transport. To date, four members of the AE family (AE1, AE2, AE3, and AE4) have been characterized in several species [2–5]. The corresponding genes show similarities in size, sequence, and exon/intron organization, although they are located in different chromosomes. A common feature among AE genes is the usage of alternative promoters, which lead to the expression of alternative transcripts, most often in a tissue-specific manner [1]. Thus, a 5'-truncated variant of the erythroid AE1 mRNA [6–9] is expressed in the kidney, while an AE3 transcript is found in cardiac muscle as a 5'-truncated variant of the complete form, which is mainly expressed in brain [10–13]. Concerning AE2, the complete message transcribed from the upstream promoter (AE2a) has been encountered in all human tissues that have been explored (liver, stomach, thyroid, prostate, and kidney), while expression of two 5'-truncated AE2 mRNAs (AE2b\(_1\) and AE2b\(_2\)) was only found in liver and kidney [14]. Alternative exons 1b\(_1\) and 1b\(_2\) are transcribed from AE2b\(_1\)/AE2b\(_2\) overlapping promoter sequences within intron 2 [14], each one splicing to exon 3 in corresponding 5'-truncated variants. As a result of the 5'-diversity of human AE2 mRNAs, the first 17 amino acids of AE2a are replaced by three initial residues (MTQ) in AE2b\(_1\) and by eight initial residues (MDFLLRPQQ) in AE2b\(_2\). While former experiments...
showed the capability of the AE2a message to be functionally expressed in human cells [15,16], recent experiments provide similar evidence for the alternative messages AE2b1 and AE2b2.

AE2 is the member of the AE family putatively involved in the biliary secretion of bicarbonate. Immunohistochemical studies in human liver using a monoclonal antibody against a peptide common to all three AE2 isoforms showed immunoreactivity at the luminal surface of the hepatobiliary tree only [17]. Therefore, it seems that all three AE2 isoforms have an apical location, each being able to participate in the secretion of bicarbonate to bile. In this context, the use of alternate promoter sequences may result in differentially regulated expression of AE2 isoforms in hepatobiliary cells. Previous dual-luciferase reporter gene assays of AE2b2/AE2b1 overlapping promoter regions in transiently transfected HepG2 cells determined that a 310-bp region corresponding to the proximal promoter of AE2b2 is highly active [14]. This region contains several potential binding motifs for liver-enriched factors, including the sequence TCTTAATGATTAACC, which is a 1-bp variant (underlined) of the 15-bp consensus sequence for hepatocyte nuclear factor-1 (HNF1) [18]. There are two liver-enriched transcription factors, HNF1α and HNF1β, that may bind the HNF1 element as homo- or heterodimers [19–21]. Both HNF1α and HNF1β are expressed mainly in liver and kidney, but also in intestine and pancreas [22], while HNF1β alone is found in lung and gonads [23,24]. Liver HNF1α has been recently reported to be a master player in regulating bile acid and cholesterol metabolism [25,26]. Here, we determined that HNF1α can: (i) bind the HNF1-motif variant of the human AE2 alternate promoter region and (ii) transactivate alternative transcription of the AE2 gene. Our findings suggest that HNF1α may have a major role in upregulating alternative transcription of the human AE2 gene in the liver, and therefore it may contribute to the biliary secretion of bicarbonate in response to certain stimuli.

### Materials and methods

**Human cell lines.** HepG2 cells (from human hepatoblastoma) were grown in DMEM with glutamax, while PC-3 cells (from human prostate cancer) were grown in RPMI 1640 with glutamax (Gibco). Both media were supplemented with 10% fetal calf serum (Gibco), penicillin/streptomycin (BioWhittaker), and 10 mM Hepes (Sigma), pH 7.4, at 37°C.

**Isolation of total RNA and real-time polymerase chain reaction.** Total RNA was isolated from each cell line with the TRI Reagent (Sigma). Reverse transcription of total RNA (2 μg) and real-time polymerase chain reaction (PCR) were performed as described [27], using specific primers (Sigma-Genosys) (cf. Table 1 and Fig. 1).

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**Figure 1.** Comparisons between HepG2 and PC-3 cells in the expression of mRNA isoforms for AE2 and HNF1. After reverse transcription of total RNA, expression of mRNA isoforms was measured by real-time PCR using specific primer pairs (cf. Table 1). Data were normalized with GAPDH. The levels of transcript expressions in PC-3 cells are given as percentage (means ± SEM of at least three samples) of those in HepG2, which are each noted as 100%.

**Table 1**

<table>
<thead>
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<tbody>
<tr>
<td>GAPDH forward</td>
<td>CCAGGTCTCATTGACACAC</td>
</tr>
<tr>
<td>GAPDH reverse</td>
<td>TGGCTAATGAGAAGAGG</td>
</tr>
<tr>
<td>AE2a forward</td>
<td>TCCAGGCGAGCCGAGTTATG</td>
</tr>
<tr>
<td>AE2b1 forward</td>
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<td>AE2 reverse</td>
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<td>(common for all isoforms)</td>
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<td>HNF1α forward</td>
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<td>HNF1α reverse</td>
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<td>HNF1β forward</td>
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<tr>
<td>HNF1β reverse</td>
<td>GTGCTGAGTGACTGAC</td>
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</tr>
<tr>
<td>AE2a forward</td>
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</tr>
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<td>AE2b1 forward</td>
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<td>AE2b2 forward</td>
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<td>TGCCCAAAGCCTCAACACCT</td>
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<td>GTGCTGAGTGACTGAC</td>
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construct and 2 ng of pRL-SV40 per well, using the Tfx-50 Reagent (Promega). For cotransfection assays with PC-3 cells, 0.2 μg per well of either pBJ5-HNF1z or the empty vector pBJ5 (kindly provided by Dr. Gerald Crabtree [30]) was added to the transfection reaction. Forty-two hours after transfections, dual-luciferase activities were assayed in a Lumat LB 9507 luminometer (EG&G Berthold).

For electromobility shift assays (EMSA), the double-stranded probe 5'-CCTACCTGAGCCTTGGCATGA (HNF1 motif underlined) was produced by annealing of partially complementary oligonucleotides HNF1 forward and HNF1 reverse (Table 3), containing the same changes as the promoter construct with a disrupted HNF1 motif (cf. above). Each probe (0.2 pmol) was incubated in a 20-μl mixture with 1 μg of poly(dI-dC) (Amersham), 8–16 μg of nuclear extract from non-transfected cells, 12 mM Hepes, pH 7.8, 0.1 M KCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, and 50 ng BSA. For competition assays, an excess of unlabelled probe (1-, 10- or 30-fold) was added. As non-specific DNA-binding competitor an unlabelled probe with an Oct2A-binding site was used. Supershift experiments included the addition of 4 μg of anti-HNF1α or anti-HNF1β antibodies (Supershift reagent sc-6548 X or sc-7411 X, respectively, Santa Cruz Biotechnology). Binding reactions were incubated for 15 min at room temperature before electrophoresis on a 4% polyacrylamide gel. After electrotransference to a positively charged nylon membrane (Roche), bands were detected non-isotopically with the Dig Gel Shift Kit and exposed to an autoradiography film for at least 30 min.

When using nuclear extracts from PC-3 transfected cells, a lower amount was prepared and isotopic EMSA was performed to increase the sensitivity. The HNF1 probe was labeled with [32P]ATP (Amersham) and T4 polynucleotide kinase (Promega). Binding buffer and conditions were the same as for non-isotopic EMSA, but only 0.02 pmol of probe and 2 μg of nuclear extract were used. After PAGE, the gel was dried and exposed to an autoradiography film for at least 42 h.

Western blot. Eight micrograms of nuclear extracts in 66 mM Tris, 100 mM DTT, 2.6% SDS, 4.8 M urea, 10% glycerol, and 10 mg/ml of bromophenol blue was run in 0.1% SDS–6% PAGE and electrotransferred to a BioTrac NT membrane ( Pall). Sequential incubations were carried out with anti-HNF1α (sc-6548 X diluted 1/8000) and anti-goat IgG-HRP (a donkey antibody from Santa Cruz Biotechnology, diluted 1/100,000). Western Lightning (Perkin-Elmer) and Hyperfilm ECL (Amersham) were used for visualization of the bands.

Results

Comparative studies between HepG2 and PC-3 cells for the expression of AE2 and HNF1 mRNA isoforms

Real-time quantitative PCR showed that the steady-state levels of AE2α mRNA were higher (144 ± 18%) in prostate-derived PC-3 cells than in liver-derived HepG2 cells, whereas the levels of both alternative messages AE2b1 and AE2b2 were significantly lower (14 ± 2% and 18 ± 3%, respectively) in prostate cells (Fig. 1). The low expression of alternative transcripts in PC-3 cells was associated with low levels of HNF1 transcripts in these cells compared to HepG2 cells. In fact, expression of HNF1α mRNA was undetectable in prostate cells and the levels of HNF1β mRNA were significantly lower (23 ± 1%) in these cells than in HepG2 (Fig. 1).

Table 2

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Orientation</th>
<th>Sequence (5' to 3')</th>
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</thead>
<tbody>
<tr>
<td>−471b2</td>
<td>Forward</td>
<td>CCTACCTGAGCCTTGGCATGA</td>
</tr>
<tr>
<td>−286b2</td>
<td>Forward</td>
<td>TAGAGGCGACGTCGAAATCT</td>
</tr>
<tr>
<td>+24b2</td>
<td>Reverse</td>
<td>TGAAGGCGACGGAAGGCTCCAT</td>
</tr>
<tr>
<td>−143b2</td>
<td>Reverse</td>
<td>CAAAAGGGGACATGGAGGC</td>
</tr>
<tr>
<td>Int-Mut-HNF1</td>
<td>Forward</td>
<td>AGGGCTTTAGGCCTGACAAATCAGCTCC</td>
</tr>
<tr>
<td>Int-Mut-HNF1</td>
<td>Reverse</td>
<td>GGCTTGACGGCTAAAGCTGAGAAAATC</td>
</tr>
</tbody>
</table>

Underlined oligonucleotides indicate mutations that disrupt the HNF1 binding site.

Table 3

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<tr>
<td>HNF1 forward</td>
<td>CCTACCGGCTTATGTTAACCCC</td>
</tr>
<tr>
<td>HNF1 reverse</td>
<td>GGAACGGGCCATATGCTGAGTTC</td>
</tr>
<tr>
<td>Mutated-HNF1 forward</td>
<td>CCTAACCGGCTTATGTTAACCCC</td>
</tr>
<tr>
<td>Mutated-HNF1 reverse</td>
<td>GGAACGGGCCATATGCTGAGTTC</td>
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Involvement of the HNF1 motif in the alternate transcriptional activity of AE2

We assessed the functionality of the 1-bp variant of the HNF1 consensus sequence spanning positions –127b2 to –113b2 in the minimal AE2b2 promoter [14]. Thus, we carried out transfections of HepG2 cells with promoter constructs either lacking the region with the HNF1 sequence (see the 3′-deleted construct –286b2/+143b2 in Fig. 2) or containing a disrupted HNF1 motif with six mutations of the consensus sequence (–286b2/+24b2Mut). Dual-luciferase assays with transfected HepG2 cells showed that transcriptional activities were markedly attenuated versus cells transfected with the wild-type construct –286b2/+24b2 (28 ± 1% and 32 ± 3%, respectively; both p < 0.001, cf. Fig. 2). In PC-3 cells, luciferase activities were much lower (approximately 50-fold) than in HepG2 cells. In PC-3 cells, however, HNF1-motif disruption in –286b2/+24b2Mut had no effect (101 ± 9% versus wild-type construct –286b2/+24b2; data not shown), indicating that the HNF1 motif is not functional in these cells.

Binding of HNF1α to the HNF1 motif

EMSA with HepG2 nuclear extracts and a DIG-labeled 31-bp probe containing the HNF1 sequence (and flanking bases in the promoter) resulted in two shifted bands (Fig. 3A). Only the upper shifted band was specific, since it could be competed with unlabeled specific probe, but not with an unlabeled probe having a disrupted HNF1 motif or with the non-specific oligonucleotide Oct2A (Fig. 3A). Furthermore, the upper shifted band was not obtained when performing EMSAs with the labeled probe containing the disrupted version of the HNF1 site (Fig. 3B). These data show that our 1-bp variant of the HNF1 element is able to bind nuclear proteins from HepG2 extracts. Interestingly, EMSA using a labeled probe with the C at the second nucleotide (the 1-bp variant of the HNF1 consensus sequence) mutated to G (the consensus nucleotide) showed a similar binding than the wild-type HNF1 probe (Fig. 3B).

When supershift assays with antibodies to HNF1α and HNF1β were carried, a supershift of the specific shifted band was only observed with the HNF1α antibody but not with the HNF1β antibody (Fig. 3C). Therefore, in HepG2 cells the HNF1 element appears to be bound by HNF1β/HNF1α homodimers.

No specific shifted band was detected in EMSA with PC-3 nuclear extracts instead of HepG2 extracts (Fig. 4A). This was expected because HNF1α is not expressed in PC-3 cells, as indicated by real-time PCR experiments (Fig. 1). Western blot performed with nuclear extracts confirmed that HNF1α is present in HepG2 but not in PC-3 cells (Fig. 4B). On the other hand, immunoblot analysis of PC-3 cells transfected with an HNF1α-expressing vector showed expression of

Fig. 2. Luciferase activities of constructs with sequences of the human alternate overlapping promoters AE2b2/AE2b1. The upper diagram shows the promoter regions from the distal part of intron 1 to the end of exon 1b1. Dual-luciferase assays of HepG2 cells transiently transfected with the corresponding constructs were carried out in triplicate and repeated several times (indicated by the numbers inside the bars). For each experiment, relative luciferase units were normalized with values obtained for the reference construct –286b2/+24b2; results are given as means ± SEM. Statistical significance (*) of differences to the reference construct was determined by Student’s t test with the Bonferroni correction (α = 0.01). Black arrows indicate the location of the HNF1 motif; x indicates the mutagenesis-disrupted HNF1 site.
HNF1α in these cells (Fig. 4B). EMSA and supershift studies using nuclear extracts from PC3 cells transfected with the HNF1α vector demonstrated the occurrence of HNF1α binding activity in these transfected cells (Fig. 4C).

Transactivation studies in PC-3 cells

To demonstrate that HNF1α may transactivate the human alternate AE2 promoter, wild-type construct −286b2/+24b2 and the HNF1α-mutated counterpart −286b2/+24b2mut (Fig. 2) were each cotransfected with either the pBJ5-HNF1α vector or the empty vector pBJ5 into PC-3 cells (Fig. 5), which do not express HNF1α (cf. Figs. 1 and 4B). Luciferase activity of cells transfected with the wild-type construct was markedly increased when cotransfected with the expression vector for HNF1α (231 ± 18%, p < 0.001; see Fig. 5), whereas no increase was observed in cells cotransfected with the empty vector pBJ5 (96 ± 9%, not significant). On the other hand, the
activity of mutated construct \(-286b1/24b2\) was not affected by any cotransfection, remaining similar to that of the wild-type construct transfected alone into PC-3 cells (109 ± 10% and 108 ± 10% for pBJ5 and pBJ5-HNF1\(\alpha\) cotransfections, respectively, both not significant; Fig. 5). Thus, cotransfection experiments in PC-3 cells demonstrate that HNF1\(\alpha\) is able to transactivate the AE2\(\alpha\) proximal promoter.

**Discussion**

The human AE2 anion exchanger gene is widely expressed from its upstream promoter AE2\(\alpha\). In the liver as well as in the kidney, this gene may also be alternatively expressed from overlapping promoter sequences AE2\(\alpha\)/AE2\(\beta\) within intron 2 [14]. In the present study, we have determined that liver-enriched nuclear factor HNF1\(\alpha\) plays an important role for transactivating the alternate promoter AE2\(\beta\) in liver-derived HepG2 cells, and that HNF1\(\alpha\) exerts this function through its binding to the HNF1 motif in the alternate promoter AE2\(\beta\) [14]. Moreover, our data from real-time PCR and luciferase studies show that this is not the case in prostate-derived cell line PC-3, and indicate that HNF1\(\alpha\)-mediated alternative transcription of the AE2 gene in humans is rather cell specific, similar to what has been recently reported in rabbit gastric mucosa for the different cell types [31].

The HNF1 motif located at position \(-113b2\) in the overlapping alternate promoter sequences AE2\(\beta\)/AE2\(\beta\), follows the most stringent criteria reported for bona fide HNF1 sites [18]. Thus, it locates in the minimal AE2\(\beta\) promoter region and is particularly surrounded by other transcription factor motifs [14]. However, our HNF1 motif contains a C at the second nucleotide position in the consensus sequence that, to our knowledge, has never been found among hundreds of them [18]. EMSA experiments showed that nuclear extract from HepG2 cells binds similarly either HNF1-probes with C or G at the second nucleotide position (Fig. 3B).

The HNF1 element is known to be able to interact with homo- or heterodimers of HNF1\(\alpha\) and HNF1\(\beta\) [19–21]. They both are factors included in the wide HNF network [32,33] that may regulate tissue-restricted expression of some genes [34–40]. Hypothetically, either HNF1\(\alpha\) and/or HNF1\(\beta\) could be responsible for the transcriptional activation of the AE2 alternate promoter in liver-derived HepG2 cells. However, supershift assays indicate that the HNF1 element is mainly bound by HNF1\(\alpha\)/HNF1\(\alpha\) homodimers in these liver cells (Fig. 3C). In prostate cells, the lack of HNF1\(\alpha\) binding activity (Fig. 4A) correlates well with the absence of HNF1\(\alpha\) expression in these cells (Figs. 1 and 4B). PC-3 cells transfected with an HNF1\(\alpha\) vector to express HNF1\(\alpha\) (Fig. 4B) showed HNF1\(\alpha\) binding activity as determined by EMSA and supershift studies (Fig. 4C). Cotransfection experiments in these prostate cells definitively demonstrated that HNF1\(\alpha\) can transactivate the AE2\(\beta\) proximal promoter (Fig. 5). These experiments also suggest that cell-specific alternative AE2 expression is largely influenced by the presence or absence of HNF1\(\alpha\), although other transcription factors are most probably involved as well. Thus, cells lacking HNF1\(\alpha\), such as PC-3, show low but detectable expression of alternative transcripts (Fig. 1), and disruption of the HNF1 motif does not fully eliminate transcriptional activity of the AE2\(\beta\) proximal promoter in any of the two cell lines tested.

Former studies have demonstrated that, in basal conditions, expression of alternative messages AE2\(\alpha\) and AE2\(\beta\) accounts each for about 10% of the full-length AE2\(\alpha\) expression [14], which is driven from upstream promoter sequences that resemble house-keeping gene promoters [41]. The physiological significance of an alternative expression of the AE2 gene in the liver regulated by HNF1\(\alpha\) might be related to the possibility for liver cells to increase the expression of the AE2 gene when needed for bicarbonate secretion. Biliary secretion of bicarbonate occurs through a Na\(^+\)-independent anion exchanger along the apical membrane of hepatobiliary cells. All three AE2 isoforms may be involved in this exchange, as recent studies in HEK293 cells transfected with AE2-isoform-expressing vectors indicate that each isoform is able to display AE activity at a similar degree (V. Aranda, S.M., and J.F.M., unpublished).

The role of HNF1\(\alpha\) in directing the expression of genes involved in bile acid transport has been recently highlighted. The livers of Hnf1\(\alpha\) (Tcfl) knockout mice show decreased expression of several basolateral membrane bile acid transporter genes such as Scl10a1 (Ntcp or sodium taurocholate cotransporting polypeptide gene), Scl21a3 (Oatp1 or organic anion transporting polypeptide-1 gene), and Scl21a5 (Oatp2), leading to impaired portal bile acid uptake [25]. Moreover, the promoter regions of Oatp4 and its human ortholog OATP-C have been shown to be responsive to HNF1\(\alpha\) [42]. These and other studies [26,43,44] provide support for an important role of HNF1\(\alpha\) as a key regulator of liver-specific organic anion transporter genes and hence for the production of the bile acid-dependent bile flow. Likewise, our present report provides evidence that HNF1\(\alpha\) is able to regulate a gene involved in biliary secretion of bicarbonate, and thus in the generation of the bile acid-independent bile flow.

**Acknowledgments**

We are grateful to E. Sáez for excellent technical assistance, A. Izal, J. M. Bañales, Drs. M. Zaratiegui, and R. Hernández for their help,
and J. Salas, Drs. C.D. Funk, and F. Lecanda for valuable advice on the manuscript. Also, we thank Dr. G. Crabtree for kindly providing pBl5 vectors. This work was supported by grants from the Spanish Fondo de Investigaciones Sanitarias (Project 99/0647) and from the Instituto de Salud Carlos III (C03/02), and by the FIMA foundation. R. Malumbres was a recipient of a predoctoral fellowship from the Spanish Ministry of Education and Science.

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