Transformed but not normal hepatocytes express UCP2

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Abstract Uncoupling protein 2 (UCP2) expression in liver is restricted to non-parenchymal cells. By means of differential display screening between normal rat liver and H4IIE hepatoma cells we have isolated a cDNA clone encompassing part of UCP2 cDNA. Northern blot analysis revealed that UCP2 is expressed in some hepatocarcinoma cell lines, while it is absent in adult hepatocytes. UCP2 mRNA in H4IIE cells was downregulated when cells were cultured for 36 h in 0.1% serum and its expression was restored upon addition of 10% serum or phorbol esters. Hypomethylation of *UCP2* was observed in transformed UCP2 expressing cells. Our results indicate that UCP2 is expressed in some hepatocarcinoma cell lines and that serum components may participate in maintaining elevated UCP2 levels.

Key words: Uncoupling protein; Hepatoma; Mitochondrion; Tumor marker; Hypomethylation

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent gastrointestinal cancers in the world population [1]. Neoplastic transformation of the liver is a complex and multistep process which involves multiple genetic abnormalities. The inactivation of tumor suppressor genes and the activation of oncogenes and cell growth related genes [2,3] are among the genetic changes which may participate in the development of the neoplastic phenotype. However, a better understanding of the molecular biology of HCC is still needed in order to facilitate the development of new therapeutic strategies and diagnostic procedures. With this aim we have performed differential display (DD) analysis [4] between normal rat liver and the rat hepatoma cell line H4IIE as a model of transformed hepatic cell.

One of the genes identified as overexpressed in the H4IIE cells with respect to normal liver was the recently cloned uncoupling protein 2 (UCP2), a member of a family of proton channels located in the inner mitochondrial membrane [5]. UCP1 was the first member of this family of mitochondrial uncouplers to be identified [6], and is exclusively expressed in brown adipose tissue, where it mediates heat generation and calorie burning through the dissipation of the electrochemical gradient across the inner mitochondrial membrane [7]. UCP2, which also alters mitochondrial potential when expressed in yeast [5], is more widely expressed in rat and human tissues [5,8], however the low levels of UCP2 mRNA detected in liver are restricted to non-parenchymal cells [9,10]. In the present work we have evaluated *UCP2* expression in human HCC cell lines and studied possible mechanisms behind UCP2 induction, such as serum dependence and potential signaling systems involved. Given the prominent role that altered DNA methylation may play in the regulation of gene expression during carcinogenesis [11], we also studied *UCP2* methylation status in normal and transformed cells in order to identify the potential molecular mechanisms leading to UCP2 mRNA induction.

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Abbreviations: UCP2, uncoupling protein 2; DD, differential display; HCC, hepatocellular carcinoma; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; AFP, α-fetoprotein

2. Materials and methods

2.1. Cell lines and culture conditions

The rat H4IIE hepatoma cells and the human HepG2, Hep3B, SK-HEP-1 and HuH-7 HCC derived cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.2. DD analysis

Total cellular RNA was isolated by extraction with guanidinium thiocyanate followed by centrifugation in a cesium chloride solution as previously described [12]. 25 μg of total RNA was incubated for 30 min at 37°C with 10 units of RNase-free DNase I (Boehringer Mannheim, Mannheim, Germany) in 10 mM Tris pH 7.5, 10 mM $MgCl_2$, phenol-chloroform extracted, ethanol precipitated, and redissolved in sterile nuclease-free water.

Differential display was performed using oligo(dT) anchored primers [4,12] with Hieroglyph mRNA Profile Kit (Genomyx, Beckman Instruments, Fullerton, CA) following the manufacturer's instructions, in a Perkin Elmer GeneAmp 2400 thermal cycler. Reactions were performed with each cDNA solution in duplicate. Control reactions were set using sterile nuclease-free water or each DNase-treated RNA instead of the cDNA solution.

Following DD-PCR, radiolabeled cDNA fragments were electrophoretically separated on 4.5% polyacrylamide gels under denaturing conditions in a Genomyx LR DNA sequencer (Genomyx). Bands of interest were excised from the gel, and the gel slides were placed directly into PCR tubes. Reamplification was performed using T7 promoter 22-mer and M13 reverse 24-mer primers. Amplified DNA fragments were cloned into the plasmid vector pCR2.1-TOPO using TOPO-TA Cloning Kit (Invitrogen, De Schelp, The Netherlands) and sequenced in both directions using M13 reverse (−24) primer and M13 forward (−20) primer. Nucleotide sequence homology search analyses of the EMBL [13] and GenBank [14] databases were performed using the program FASTA [15].

2.3. Northern blot analysis

Total RNA was isolated from hepatocytes and hepatoma cell lines by the guanidinium thiocyanate method [16]. Aliquots (20 μg) of total RNA were size fractionated by electrophoresis in a 1% agarose gel under denaturing conditions. RNAs were then blotted and fixed to Nytran membranes (Schleicher and Schuell, Keene, NH). Prehybridization and hybridization were performed as previously described [17]. UCP2 mRNA was detected with the 0.6 kb cDNA probe obtained in our DD analysis. The rat α-fetoprotein (AFP) probe was a generous

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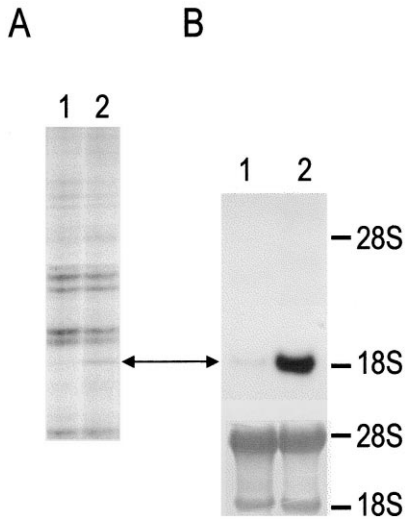

Fig. 1. DD analysis between normal rat liver and H4IIE cells. A: Sequencing gel electrophoresis of PCR amplified cDNAs from normal rat liver (1) and H4IIE cells (2). A differentially displayed fragment (arrow) was detected, isolated, reamplified and upon sequencing was identified as part of UCP2 cDNA. B: Northern hybridization analysis of total RNAs from normal rat liver (1) and H4IIE cells (2), performed with the previously isolated cDNA fragment, revealed a 1.7 kb band which was markedly induced in the transformed cell line. Methylene blue staining of the blots was performed as loading control. The migration of the ribosomal markers is indicated.

gift of Dr. J.L. Danan (Centre de Recherche sur L'Endocrinologie, Meudon, France). Probes were labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham, Little Chalfont, UK) by random priming using the Megaprime DNA labeling system (Amersham). Specific activity was usually approximately 5×10^8 cpm/μg of DNA. Quantitation was performed by scanning densitometry of the X-ray films. The amount and quality of the loaded RNA samples were evaluated by methylene blue staining of the 28S and 18S rRNAs, and by hybridization with the 36B4 cDNA [18].

2.4. DNA isolation, methylation analysis and Southern blot

High molecular weight DNAs from normal human liver and human hepatoma HepG2 and Hep3B cells were prepared as previously described [19]. 10 μg of DNA was digested to completion with 5 U/μg *Msp*I or *Hpa*II (Boehringer Mannheim) by incubation at 37°C for a total of 6 h, enzyme was added in two aliquots at 3 h intervals. Samples were then electrophoresed on 1% agarose gels and then transferred to Nytran membranes. Prehybridization and hybridization with UCP2 cDNA probe were carried out as described [19]. Membranes were then exposed to X-ray films.

3. Results

3.1. DD analysis

DD analysis was carried out between rat liver and the rat hepatoma cell line H4IIE as described in Section 2. Several bands were differentially expressed in hepatoma cells as compared to normal liver. One of the bands selected for analysis, preferentially overexpressed in H4IIE cells (Fig. 1A), was excised from the gel, amplified and sequenced. Sequencing of 0.3 kb of the total 0.6 kb of this clone revealed 100% identity to the rat UCP2 cDNA (nucleotides 502–865) [8], and could hybridize 1.7 kb mRNA in total cellular RNA from rat liver tissue and H4IIE cells (Fig. 1B). The band detected using Northern analysis corresponded in size to that reported for the UCP2 transcript in rat liver [8], and was induced about 14-fold in the rat hepatoma H4IIE cells.

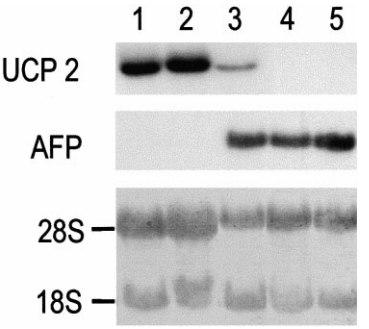

Fig. 2. Expression of UCP2 in H4IIE cells (1) and the human HCC cell lines HepG2 (2), Hu-H7 (3), Hep3B (4) and SK-HEP-1 (5) analyzed by Northern blotting of total RNA. The same blot was also probed with AFP. Methylene blue staining of the blots was performed as loading control.

3.2. Expression of UCP2 in human HCC cell lines

We next examined whether UCP2 mRNA was also present in human HCC cell lines. Using our UCP2 cDNA clone as a probe we carried out Northern blot analysis in four human HCC cell lines. UCP2 mRNA was detected in two of four HCC cell lines, HepG2 and HuH-7, while it was absent in Hep3B and SK-HEP-1 cells (Fig. 2). Highest levels of UCP2 mRNA were detected in HepG2 cells and were comparable to those present in the H4IIE cell line.

It is known that transformed hepatic cells may express a number of genes which are characteristic of embryonic tissues,

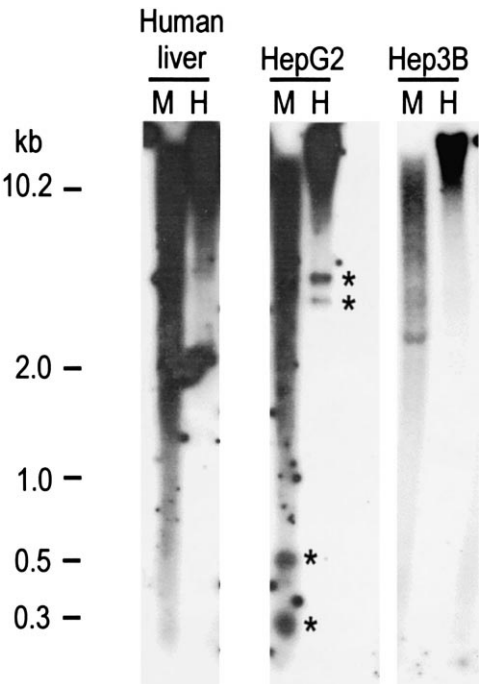

Fig. 3. Methylation status of UCP2 in normal human liver, HepG2 and Hep3B cells. DNA was extracted and digested to completion with *Msp*I (M) and *Hpa*II (H) as described in Section 2. Asterisks indicate the location of new bands detected in *Msp*I and *Hpa*II digested DNA from HepG2 cells.

such as the AFP [20]. We have tested AFP expression in the HCC cell lines mentioned above. Northern blot analysis demonstrated a high level of AFP mRNA in SK-HEP-1, Hep3B and HuH-7 cells, while no signal was detected in H4IIE or HepG2 (Fig. 2).

3.3. UCP2 methylation status in normal liver and HCC cells

Possible changes in the methylation status of the *UCP2* gene in transformed hepatic cells were examined. DNA isolated from normal human liver and the human HCC cell lines HepG2 and Hep3B was subjected to Southern blot analysis after complete digestion with the restriction enzymes *Msp*I and *Hpa*II. Both enzymes recognize the same sequence (CCGG) but display differential sensitivity to the presence of 5-methyl-C [21,22]. *Hpa*II cannot cleave if the internal C is methylated, while methylation of the external C prevents cleavage by both enzymes [23]. New bands were detected in the *Hpa*II and *Msp*I treated DNA from the tumor cell line HepG2 (Fig. 3), which may indicate hypomethylation of the *UCP2* gene. However, a similar restriction pattern was observed between normal human liver DNA and Hep3B cells which do not express UCP2 mRNA.

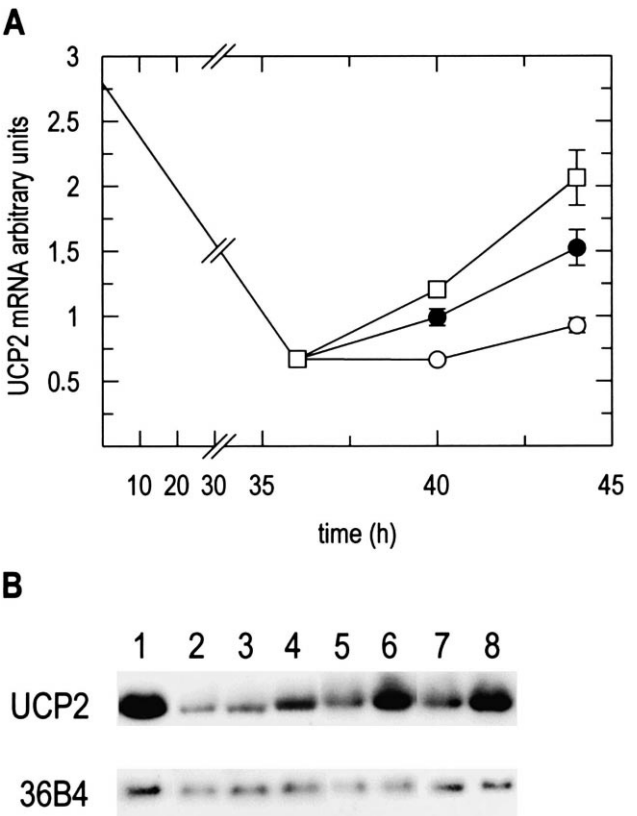

Fig. 4. Serum and phorbol esters modulate UCP2 mRNA levels in H4IIE cells. A: Cells growing in 10% serum were washed and refed medium containing 0.1% serum (time point designated zero). After 36 h in low serum, cells were treated with fresh medium containing 10% serum (open squares), 0.1% serum plus 10 μM PMA (closed circles) or 0.1% serum (open circles). UCP2 mRNA levels were determined in each case, values represent the mean ± S.E.M. of three independent experiments. B: Representative Northern blot assay showing UCP2 mRNA in the various conditions mentioned above; (1) cells growing in 10% serum, (2) cells after 36 h in 0.1% serum, cells after 4 h (3) and 8 h (4) in fresh medium with 0.1% serum, cells after 4 h (5) and 8 h (6) of treatment with 10% serum, cells after 4 h (7) and 8 h (8) in the presence of fresh medium containing 0.1% serum plus 10 μM PMA. Blots were probed with 36B4 cDNA as loading control.

3.4. Serum levels modulate UCP2 expression in H4IIE cells

We next examined whether UCP2 mRNA levels in H4IIE could respond to changes in the culture conditions in order to identify possible environmental signals which may contribute to UCP2 induction. For this purpose, cells growing in 10% serum were shifted to medium containing 0.1% serum for 36 h with the aim of depleting serum derived factors without challenging cellular viability. After this time we evaluated UCP2 mRNA content by Northern blotting. As shown in Fig. 4, serum deprivation induced a significant decrease in UCP2 mRNA levels. To test whether this response was reversible, fresh medium containing 0.1% or 10% serum was added back to the cells. Serum refeeding resulted in the elevation of UCP2 mRNA, reaching values close to those observed in control cells (10% serum) after 8 h of treatment. In order to gain more insight into the mechanisms behind UCP2 mRNA induction we examined the possible implication of some signaling pathways commonly modulated by serum components. For this purpose, serum starved cells were treated with the phorbol ester PMA (10 μM), the cAMP inducing drug forskolin (10 μM) or the glucocorticoid triamcinolone (1 μM). Upregulation of UCP2 mRNA levels was only observed in response to PMA (Fig. 4), forskolin and triamcinolone treatment did not have an effect (data not shown).

4. Discussion

By means of DD analysis between normal rat liver tissue and the HCC cell line H4IIE, we have isolated a 0.6 kb clone that was preferentially expressed in the transformed hepatic cells. Upon sequencing it was found that this clone corresponded to part of the recently described *UCP2* gene [5]. UCP2 is a member of the mitochondrial carrier protein family which includes the 2-oxoglutarate/malate, the citrate and the phosphate carriers, the ATP/ADP translocator and UCP1 [24]. The rat UCP2 cDNA has recently been cloned together with UCP3, another new member of this family of proteins [8]. UCP2 amino acid sequence shows 99% and 95% identity with the mouse and human proteins respectively [5]. *UCP2* expression has been detected in a wide variety of tissues [5,8] including fetal liver [25], while UCP1 is confined to brown adipose tissue which is scarce in adult humans. The high degree of identity between UCP2 proteins from different species, its ability to alter mitochondrial potential and its wide tissular distribution suggest that this gene may play an important role in cellular energetics [26]. In spite of the wide distribution of UCP2, its presence in liver has been demonstrated to occur only in non-parenchymal cells, mainly in Kupffer cells [9,10]. Our initial observation of the presence of UCP2 mRNA in rat H4IIE cells led us to examine whether HCC cells of human origin could also express this gene. We have found that UCP2 mRNA was present in two out of four HCC cell lines examined, demonstrating that UCP2 induction may also take place in transformed hepatic cells of human origin. Interestingly, *UCP2* expression was detected in cell lines such as HepG2 which did not express AFP mRNA, a common marker of hepatocellular transformation [20,27].

It is known that transformed cells display many metabolic traits characteristic of the embryonic and fetal stages of development [28,29]. In the case of liver cells the similarities between the fetal liver and the tumor cells are also evident at the mitochondrial level [30,31]. The induction of UCP2 mRNA in some HCC cell lines may be regarded as more evidence of the reversion to a fetal phenotype in terms of energy metabolism. In this sense, it has been proposed that mitochondria of tumor cells are undifferentiated and behave in many senses like fetal mitochondria [30,31].

Alterations in the pattern of gene expression participate in the multistep process of carcinogenesis [12,32,33]. It is known that changes in DNA methylation take place during neoplastic transformation and may facilitate the aberrant profile of genes expressed in tumor cells [11,34]. In an effort to identify the possible molecular mechanisms behind the induction of UCP2 gene expression in HCC cells, we have studied the methylation status of this gene in normal human liver, HepG2 and Hep3B cells. Our results show alterations in the methylation pattern of *UCP2* in HepG2 which are consistent with DNA hypomethylation. However, in Hep3B cells, which do not express UCP2, the methylation pattern was similar to that observed in normal liver. It is interesting to note that the induction of AFP expression in human HCC has also been associated with hypomethylation of this gene [27], supporting a role for DNA methylation in gene expression during hepatic carcinogenesis.

In a search for other mechanisms which may contribute to the presence of UCP2 transcripts in hepatocarcinoma cells, we have explored the influence of environmental factors inherent in the culture conditions of our cells which may participate in this phenomenon. In this sense we have shown that UCP2 expression was substantially reduced in H4IIE cells when cultures were serum starved. This effect may indicate the involvement of factors present in serum in the induction of UCP2 mRNA at least in this cell line. UCP2 mRNA levels were restored shortly after reexposure to serum containing medium. Interestingly, when serum starved cultures were treated with the phorbol ester PMA UCP2 mRNA levels were also induced, suggesting a role for PKC in the regulation of UCP2 expression in hepatocarcinoma cells. In this sense, it is worth noting that an AP1 binding site has recently been described in the strong positive regulatory region of the UCP2 promoter [35]. Conversely, treatment with the cAMP inducing drug forskolin or the glucocorticoid triamcinolone did not have an effect on UCP2 mRNA levels, although glucocorticoid and cAMP response elements have been also reported in the UCP2 promoter [35].

Although the metabolic implications of UCP2 expression in HCC cells remain to be determined, our observations contribute to the understanding of the biology of hepatic tumors. Additionally the assessment of the presence of UCP2 mRNA in hepatocytes from liver biopsies may be of diagnostic interest.

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