Anisoosmotic regulation of the Mi-2 autoantigen mRNA in H4IIE rat hepatoma cells and primary hepatocytes

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Abstract Using the differential display polymerase chain reaction (DDRT-PCR) a 169 bp cDNA product, which is 88.8% homologous to the human Mi-2β autoantigen, was identified in H4IIE rat hepatoma cells. At protein level 100% homology was found. The Mi-2 mRNA was downregulated after hypoosmotic exposure and upregulated after hyperosmotic exposure in H4IIE cells and rat hepatocytes. The human Mi-2 is an autoantigen in dermatomyositis and is a member of the SNF/RAD 54 helicase family. Accordingly, Mi-2 may not only be a target of osmosignalling but could also be involved in the osmosignalling pathway towards gene expression in H4IIE and liver parenchymal cells.

Key words: Cell volume; Differential display polymerase chain reaction; Gene expression; Helicase; Liver cell

1. Introduction

Anisoosmotic changes in cellular hydration have been recognized as important modulators of metabolic cell function and gene expression (for review see [1–3]). This involves not only the regulation of osmoregulatory genes, such as the expression of aldose reductase [4,5] or specific sodium-coupled transporters for *myo*-inositol [6–8], betaine [9,10] and taurine [11], but also the expression of genes encoding proteins that are not linked to osmoregulation. Examples of the latter are the rapid increases of mRNA levels for β-actin [12], tubulin [13], ornithine decarboxylase [14], cyclooxygenase-2 [15], c-jun [16] and phosphoenolpyruvate carboxykinase [17]. Furthermore, CD9, a cell surface glycoprotein belonging to the tetraspan superfamily [18] and with multiple roles in cell activation, adhesion, cell motility functions, as well as cell-cell interactions [19], was shown to be upregulated by hypertonicity in Madine Darby canine kidney (MDCK) cells [20,21].

In mammals, little is known about the signal transduction pathways leading to the transcriptional regulation of genes in response to osmotic stress, however, mitogen-activated protein kinase cascades appear to play an important role [22–24]. Recently an osmosensitive transcriptional regulation of h-sgk, a serum and glucocorticoid-regulated serine/threonine protein kinase, has been identified [25].

In the present study, the differential mRNA display polymerase chain reaction (DDRT-PCR) method [26,27] was applied in order to identify osmoregulated genes, which might participate in the osmosignalling pathway in mammalian cells. The study identified an osmosensitive regulation of Mi-2 mRNA levels. Because the Mi-2 protein belongs to the SNF/RAD 54 helicase family, the possibility has to be considered that Mi-2 mRNA expression is not only a target, but also a component of the osmosignalling pathway towards gene expression.

2. Materials and methods

2.1. Cell culture

H4IIE rat hepatoma cells (American Type Culture Collection [ATCC] CRL 1600, Rockville, Maryland, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM)-Ham's F12/5% CO_2/5 mM glucose/2.5 mM L-glutamine at 37°C, pH 7.55, supplemented with 10% fetal bovine serum. When cells had almost reached confluence, they were washed twice with Dulbecco's PBS and the culture was continued in serum-free medium for an additional 16–24 h. Thereafter, cells were maintained under various test conditions.

For isolation of liver parenchymal cells (PC), male Wistar rats (300–400 g body weight) were raised in the local institute for laboratory animals and held, fed ad libitum on stock diet, according to the rules of the local ethical guidelines. PC were prepared by collagenase perfusion as described previously [28]. Cells were plated on collagen-coated dishes at a density of about 1×10^6 cells/ml and were then cultured in DMEM/37°C, 5% CO_2, pH 7.4/glucose (5 mM) supplemented with 100 nM insulin, 100 nM dexamethasone, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 5% fetal bovine serum for 24 h. Thereafter, cells were maintained under various test conditions.

The osmolarity changes were performed by appropriate changes of the NaCl concentration.

2.2. Northern blot analysis

Total RNA from near-confluent H4IIE hepatoma cell cultures or liver parenchymal cells in cluster 6 dishes (Costar, Cambridge, MA, USA) was isolated using the RNeasy Total RNA Kit (Qiagen, Hilden, Germany). Liver, small intestine, spleen, testis and kidney were isolated from Wistar rat and about 1 g of tissue was immediately homogenized in 10 ml of guanidine thiocyanate solution on ice with an Ultra-Turrax instrument at high speed and total RNA was isolated as described in [29]. RNA samples were electrophoresed in 0.8% agarose/3% formaldehyde and then blotted onto Duralon-UV membranes (Stratagene, Heidelberg, Germany) with 20×SSC (3 M NaCl, 0.3 M trisodium citrate). Following the blotting procedure the membranes were UV-crosslinked (Hoefer UV-crosslinker 500, Hoefer, San Francisco, CA, USA) and observed under UV illumination to determine RNA integrity and location of the 28S and 18S rRNA bands. After brief rinsing with 2×SSC (300 mM NaCl, 30 mM trisodium citrate) the blots were subjected to a 2 h pre-hybridization at 43°C in 50% deionized formamide in sodium phosphate buffer (250 mM, pH 7.2), containing 250 mM NaCl, 1 mM EDTA, 7% (w/v) SDS and 100 μg/ml salmon sperm DNA. Hybridization was carried out in the same solution with approximately 10^6 cpm/ml [α-^{32}P]dCTP-labelled Mi-2 or GAPDH cDNA probes. The probes were labelled by random priming under the conditions recommended by the supplier (Pharmacia, Freiburg, Germany). Membranes were washed three times in 2×SSC/

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Abbreviations: DDRT-PCR, differential display polymerase chain reaction method; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PC, liver parenchymal cells

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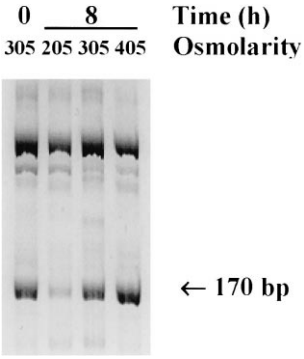

Fig. 1. Separation of [α^{33}P]dATP radiolabelled DDRT-PCR products on a native 6% polyacrylamide sequencing gel. H4IIE cells were grown in normoosmolar medium (305 mosmol/l) and they were switched at time point 0 (0 h) for 8 h (8 h) to the same or hypoosmolar (205 mosmol/l) or hyperosmolar (405 mosmol/l) medium. Total RNA was extracted, reverse transcribed and amplified by PCR with the primer pair D4/U1 from the primer kit GeneExScreen (Biometra).

0.1% SDS for 15 min, once in sodium phosphate buffer (25 mM, pH 7.2)/EDTA (1 mM)/0.1% SDS, and once in sodium phosphate buffer (25 mM, pH 7.2)/EDTA (1 mM)/1% SDS for 20 min. Blots were then exposed to Kodak X-Omat AR or Kodak BIOMAX film at −70°C with intensifying screens. Densitometric evaluation was performed with the E.A.S.Y. RH-system (Herolab, Wiesloch, Germany).

2.3. Differential mRNA display PCR

Almost confluent H4IIE cells were exposed for 8 h to hypoosmotic (205 mosmol/l), normoosmotic (305 mosmol/l) and hyperosmotic (405 mosmol/l) media. Total RNA was extracted using the RNeasy Total RNA Kit (Qiagen, Hilden, Germany). Differential mRNA display [26,27] was performed using the primer kit for mRNA display amplification (GeneExScreen Primer Kit, Biometra, Göttingen, Germany) [28], AMV reverse transcriptase (Promega, Mannheim, Germany) and Taq DNA polymerase (Goldstar DNA Polymerase, Eurogentec, Seraing, Belgium) as per manufacturers' descriptions. Briefly, 0.2 μg of each sample was reverse transcribed and a 1:10 dilution of these probes was amplified by PCR in the presence of [α-^{33}P]dATP (3000 Ci/mmol, ICN, Eschwege, Germany) [31]. Radiolabelled products were resolved on native 6% polyacrylamide DNA sequencing gels. For analyzing differential mRNA display products the dried gels were exposed to Kodak X-Omat AR films. cDNA bands that appeared on the autoradiogram to be markedly different under anisoosmolar conditions compared with normoosmolar controls were cut from the gel. DNA was extracted and reamplified by PCR with use of the same primer pairs (GeneExScreen Primer Kit) and cloned in pGEM-T vector (Promega, Mannheim, Germany) before sequencing. The complete sequence of both cDNA strands was determined by cycle sequencing with the DNA sequencing kit ABI Prism Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Weiterstadt, Germany).

3. Results and discussion

The differential display polymerase chain reaction (DDRT-PCR) method was used for the identification of osmoregulated genes. After culture of H4IIE cells in normoosmolar medium (305 mosmol/l) the cells were exposed to hypoosmo-

A

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HSMi-2    3709-GACAACAAAGAGGGAGAAGATAGCAGTGTTATCCACTACGATGATAAGGC-3758
                ***** * ********** ******** ***** ** ** ********
RNMi-2       1-TACAACGAGGAGGGAGAAGACAGCAGTGTCATCCATTATGACGATAAGGC-  50

HSMi-2    3759-CATTGAACGGCTGCTAGACCGTAACCAGGATGAGACTGAAGACACAGAAT-3808
               ********* ***** ** ** ** ************************
RNMi-2      51-CATTGAACGACTGCTGGATCGAAATCAGGATGAGACTGAAGACACAGAAT- 100

HSMi-2    3809-TGCAGGGCATGAATGAATATTTGAGCTCATTCAAAGTGGCCCAGTATGTG-3858
               ************************* ************** *********
RNMi-2     101-TGCAGGGCATGAATGAATATTTGAGTTCATTCAAAGTGGCTCAGTATGTG- 150

HSMi-2    3859-GTACGGGAAGAAGAAATGG-3877
               ********* ** ***
RNMi-2     151-GTACGGGAAAAAAAAAAAA- 169
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B

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Mi-2_human    1240-EGEDSSVIHYDDKAIERLLDRNQDETEDTELQGMNEYLSS-1279
                   ****************************************
Mi-2_rat         1-EGEDSSVIHYDDKAIERLLDRNQDETEDTELQGMNEYLSS-  40

Mi-2_human    1280-FKVAQYVVRE-1289
                   **********
Mi-2_rat        41-FKVAQYVVRE-  50
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Fig. 2. A: The anisoosmolar regulated PCR product (RNMi-2) found by DDRT-PCR with the primer pairs D4/U1 from the primer kit GeneExScreen (Biometra) was aligned with the human sequence for the Mi-2 autoantigen (HSMi-2; accession number X86691). Identical nucleotides are shown by asterisks. B: Alignment of the protein sequence (Mi-2_rat), translated from the DDRT-PCR product (RNMi-2; positions 10–158), to the corresponding protein sequence of the human Mi-2 autoantigen (Mi-2_human, positions 1240–1289). Identical proteins are shown by asterisks.

lar (205 mosmol/l) and hyperosmolar (405 mosmol/l) medium for 8 h. Following the isolation of total RNA, DDRT-PCRs were performed with different sets of primers from a primer kit established by Bauer and co-workers [30]. Among several DDRT-PCR products obtained, a 169 bp cDNA product amplified with the upstream primer 1 (U1; 5′-TACAACGAGG-3′) and the downstream primer 4 (D4; 5′-TTTTTTT-TTTTCC-3′) was of particular interest. This PCR product appeared to be induced in an osmosensitive manner (Fig. 1). The sequence of the 169 bp cDNA product was determined after subcloning into pGEM-T vector. A search with the BLAST Search Result service of the National Center for Biotechnology Information (NCBI) yielded 91% identity with the human mRNA for the 218 kDa Mi-2 protein. In an attempt to characterize the major antigenic component of the dermatomyositis-specific Mi-2 autoantigen, Ge et al. [32] and Seelig et al. [33] independently applied a similar molecular biologic approach by screening lambda phage human expression libraries using Mi-2 autoantibodies [34] as probes. Ge et al. [32] found a partial coding sequence of 1591 bp (accession number U08379), and suggested a 240 kDa protein by reaction of Mi-2 autoantibody, calling it Mi-2α, while Seelig et al. [33] described a full length sequence of 5736 bp (accession number X86691) identified as Mi-2β, which encodes a new protein of 218 kDa. Alignment of the two protein sequences showed that the Mi-2α sequence was 83.3% similar to the N-terminal region of the Mi-2β protein, with 68% identical amino acids [35]. Our isolated and sequenced cDNA product of 169 bp from rat H4IIE hepatoma cells, called RNMi-2, showed 88.8% identity to the human sequence of Mi-2β (HSMi-2; position 3709–3877) (Fig. 2A). At the protein level the two sequences (Mi-2_human and Mi-2_rat; nomenclature in accordance to the SwissProt database) are even 100% homologous (Fig. 2B), indicating that the isolated PCR product is the rat Mi-2β autoantigen. This PCR product was used for testing the anisoosmolar regulation of the Mi-2 autoantigen in H4IIE and rat liver PC. After switching H4IIE cells or PC from normoosmolar medium to hypoosmolar or hyperosmolar medium, the mRNA abundance was tested by Northern blotting. Fig. 3 shows that in H4IIE cells as well as in PC the Mi-2 mRNA levels under the influence of a 6 h anisoosmotic exposure were strongly dependent on the osmolarity of the medium. Detectable are two transcripts with sizes of 4.2 and 8.5 kb respectively. Ge et al. [32] identified a single 7.5–8.0 kb mRNA of HeLa cells by Northern blot analysis. Keeping the fact in mind that Mi-2α and Mi-2β are not completely identical, the largest transcript of 8.5 kb seems to be the one of interest. When compared with the normoosmotic conditions, significant changes in Mi-2 mRNA levels were detectable after 3 h of hyperosmotic exposure and after 6 h of hypoosmotic exposure in H4IIE cells (Fig. 4). The Mi-2

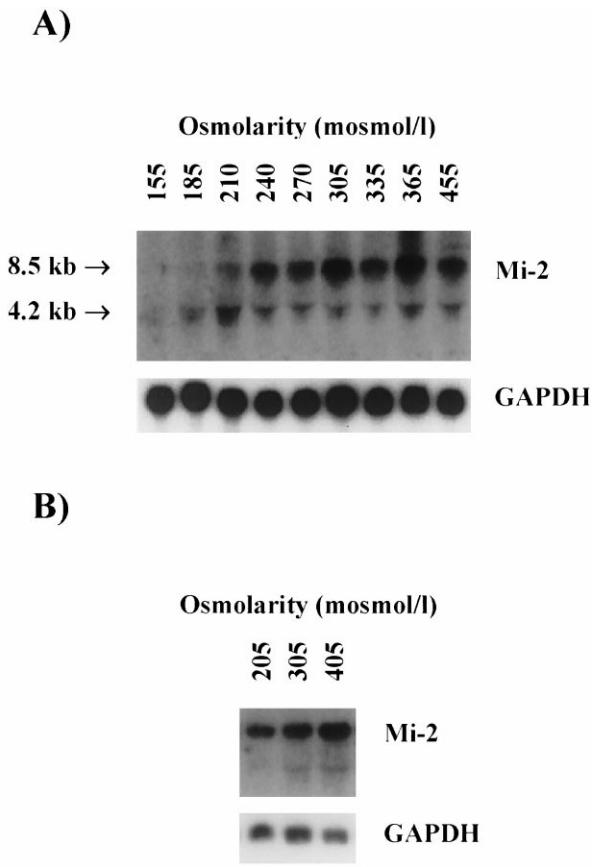

Fig. 3. Effect of anisoosmotic exposure on Mi-2 and GAPDH mRNA levels in H4IIE rat hepatoma cells and liver parenchymal cells. H4IIE (A) or liver parenchymal cells (B) were incubated for 6 h in media with different osmolarities. The osmolarity changes were performed by appropriate changes of the NaCl concentration. Thereafter, the cells were harvested for RNA isolation and subjected to Northern blot analysis for Mi-2 and GAPDH (20 μg total RNA per lane). The size of the mRNA bands was calculated from the running distance for the 18S and 28S rRNAs and is shown on the left. This experiment is representative of three seperate experiments.

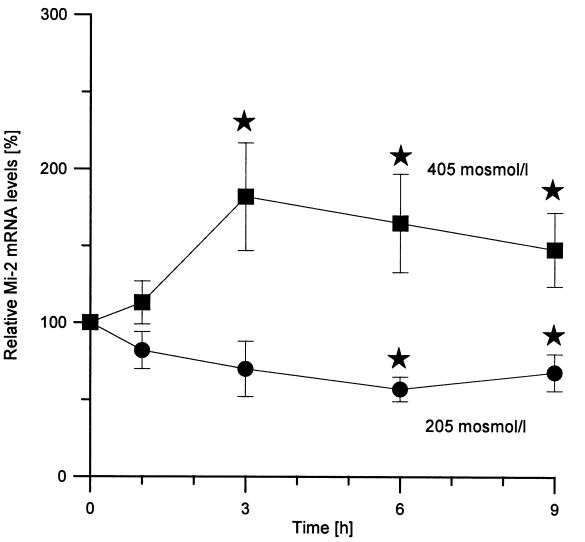

Fig. 4. Time course of the anisoosmolarity effects on relative Mi-2 mRNA levels in rat H4IIE hepatoma cells. H4IIE cells were incubated for the time periods indicated in normoosmotic (305 mosmol/l), hypoosmotic (205 mosmol/l) or hyperosmotic (405 mosmol/l) medium. The osmolarity changes were performed by increasing or lowering the NaCl concentration by 50 mM. At the time points indicated, cells were harvested for RNA isolation and subjected to Northern blot analysis for Mi-2 and GAPDH. Mi-2 mRNA levels were normalized for GAPDH mRNA levels, and the relative Mi-2 mRNA lvels found in normotonic incubations (305 mosmol/l) at each time point were set at 100% and the relative Mi-2 mRNA levels found in hypoosmotic (●) or hyperosmotic (■) media were expressed as percentages thereof. Data are given as means ± S.E.M. and are from three or four different experiments for each condition.

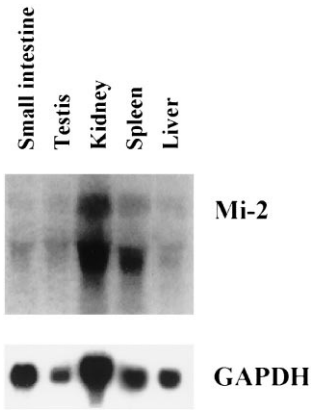

Fig. 5. Detection of Mi-2 and GAPDH mRNA levels in rat tissues. Liver, testis, small intestine, spleen and kidney from male Wistar rats were isolated for RNA preparation and total RNAs were then subjected to Northern blot analysis for Mi-2 and GAPDH (20 μg total RNA per lane).

mRNA bands were also detected in rat small intestine, testis, spleen and kidney (Fig. 5).

Analysis of protein sequence similarities revealed that Mi-2 belongs to the SNF/RAD 54 helicase family [36] and both sequences (Mi-2α and Mi-2β) contain a zinc-finger-like motif (residues 366–417) with a Cys_4-His-Cys_3 pattern (PHD motif) that is found in more than 40 nuclear proteins [37] and is thought to represent a DNA (or RNA) binding domain. The protein family of PHD fingers may recognize a set of similar nuclear targets related to chromatin structure and chromatin regulation, such as the differentially modified tails of the nucleosomal histones. This suggests their involvement in the regulation of transcriptional activity. Therefore it is interesting to speculate that Mi-2 could be part of the signal transduction pathway which triggers the cellular response towards osmotic stress. Perhaps Mi-2 is a downstream part of the osmosignal transduction pathway by regulating transcriptional activation in the nucleus. Further investigations are necessary to elucidate the function of the Mi-2 protein and the regulation of Mi-2 mRNA after exposure to stress factors.

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