Application of the cDNA-array-technology for the identification of zinc-responsive genes in mammalian cells

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Introduction

Zinc is an essential trace element with cofactor functions in a large number of proteins of intermediate metabolism, hormone secretion pathways and immune defense. Despite numerous experimental findings, mainly obtained in experimental animals, the zinc status is difficult to assess, since specific, sensitive and reliable indicators are still lacking [1,2]. As a cofactor of transcription factors, zinc is involved also in control of gene expression [3] and therefore, zinc-sensitive genes could be used as biomarkers for the determination of the zinc status *in vivo*. We used the intestinal epithelial carcinoma cell line HT29 as a reporter cell system to investigate the effects of an altered cellular zinc concentration on gene expression *in vitro*. The screening method was based on the cDNA-array-technology, which allows to monitor the expression profile of thousands of individual genes simultaneously in a single experiment [4, 5].

1

Methods

Cell culture
HT29 cells were cultured in 25 cm^2 T-flasks and incubated for 72 hours in media containing either a normal zinc concentration (0.25 ppm Zn, control) or a high zinc concentration (10 ppm Zn, supplementation).

Measurement of intracellular zinc concentration ($c(Zn)_i$)
Plated onto 24-well plates, HT29 cells were cultured until they reached 80-90 % confluency. Cultures were loaded with the zinc sensitive dye Newport Green™ DCF Diacetat (Molecular Probes) [6]. Control and zinc-supplemented media were added and the increase in the fluorescence emission intensity due to the binding of intracellular free Zn^{2+} was measured for 48 h (Fluoroscan, Labsystems).

RNA Isolation, Probe Labeling, Hybridization and Analysis of cDNA-arrays
Isolation of total RNA was performed using a modified phenol-chloroform extraction according to the manufacturers protocol (Clontech). ^{33}P-labeled cDNAs were synthesized using gene specific primers as described by Clontech. After hybridization of the cDNA-nylon-arrays (Atlas™ Human 1.2 Array III, Clontech) with the radiolabeled cDNA probes, the arrays were washed and exposed to the same phosphor screen for 96 h. For the quantitative analysis the signal intensity of each gene represented on the array (1176) was normalized to the total array intensity. Genes were identified as significantly modulated in expression, when the ratio in signal intensities of supplementation to control experiment was reproducibly > ±1.3 (n=3).

Northern analysis
Equal quantities of total RNA were separated on a formaldehyde-agarose gel (5 µg and 10 µg rsp. per lane) and transferred to a nylon membrane (Amersham). Gene specific probes were radiolabeled with ^{32}P-dATP using a random-primed DNA-labeling-Kit (Amersham) and hybridized onto the nylon membranes. After washing, the membranes were exposed to a phosphor screen.

Real-time RT-PCR
Total RNA was reverse transcribed according to a standard protocol and the resulting cDNAs were applied to real-time PCR. The sequence information for the gene specific PCR primers was obtained from Clontech. Real-time PCR reactions were performed with SYBR Green I (Molecular Probes) chemistry in a LightCycler (Roche). The authenticity of the PCR products was verified by melting curve analysis and agarose gel electrophoresis. cDNA quantities were normalized to GAPDH quantities obtained from the same sample.

2

Results I

To investigate the effects of altered zinc concentrations in HT29 cells, cells were cultured for 72 h in two different media (control: 0.25 ppm zinc; supplementation: 10 ppm zinc). The conditions proved to change the intracellular zinc concentration shown by increased intracellular free zinc and by the zinc-modulated expression of metallothionein 1 (MT-1) (Fig. 1/2).

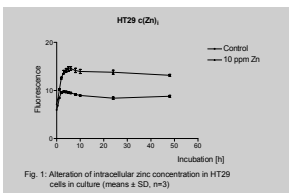

Fig. 1: Alteration of intracellular zinc concentration in HT29 cells in culture (means ± SD, n=3)

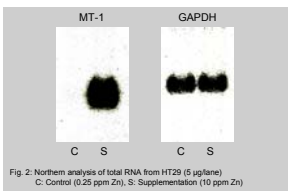

Fig. 2: Northern analysis of total RNA from HT29 (5 µg/lane)
C: Control (0.25 ppm Zn), S: Supplementation (10 ppm Zn)

3

Results II

The screening for zinc-sensitive genes was performed using arrays, which were hybridized with radioactive labeled cDNAs from cells with either normal or high zinc concentration (Fig. 3). The arrays were analyzed using phosphor screens. In HT29 cells grown under high zinc conditions, 17 genes with altered mRNA levels were identified (Tab. 1). For selected genes the altered mRNA levels were verified by Northern analysis (Fig. 4) and quantitative RT-PCR (data not shown). The investigated genes are summarized in Table 2.

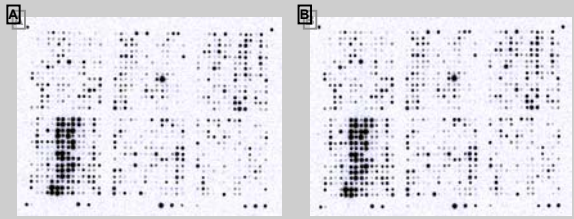

Fig. 3: Phosphor images of cDNA-arrays (Clontech), hybridized with ^{33}P-labeled cDNAs from treated HT29 cells
A: Control (0.25 ppm Zn), B: Supplementation (10 ppm Zn)

Tab. 1: List of identified genes

Gene name	GenBank acc. #	Position	Regulation in high-zinc cells	Function
Zinc Finger Protein HSAL2 (Fragment)	X98834	A08h	1,73x	Transcription
Dead Box Protein 3, Y-Chromosomal	AF000985	B02d	3,37x	Transcription
Heterogeneous Nuclear Ribonucleoprotein M	L03532	B02g	1,41x	Transcription
Cleavage Stimulation Factor, 64 kD Subunit	M85085	B03a	1,48x	Transcription
Thymopoietin Alpha	U09086	B05i	1,83x	Hormone metabolism
Complement Component C7 Precursor	J03507	B07i	1,50x	Immune system
Microfibril-Associated Glycoprotein Precursor	U19718	C02a	1,60x	Matrix protein
Nuclear Autoantigenic Sperm Protein	M97856	C04b	1,69x	Trafficking
Ferritin Light Chain	M11147	C06a	1,59x	Iron metabolism
Lamin B Receptor	L25931	E02i	2,04x	Nuclear lamina
RAS-Related Protein R-RAS3	AF022080	E08g	1,46x	Signal transduction
26S Proteasome Regulatory Subunit S14 (P31)	D38047	E13k	1,42x	Protein degradation
Hypothetical 40.0 kD Protein	D29810	F09l	1,61x	Unknown
Hepatitis A Virus Cellular Receptor 1	AF043724	A01f	-1,34x	Cell surface antigen
Creatine Kinase, Ubiquitous Mitochondrial Precursor	J04469	D01e	-1,52x	Energy metabolism
Gastrointestinal Peptide	AF048700	F01c	-1,64x	Insulin secretion
Antigen NY-CO-37 (NY-CO-38)	AF039699	F14m	-1,49x	Unknown

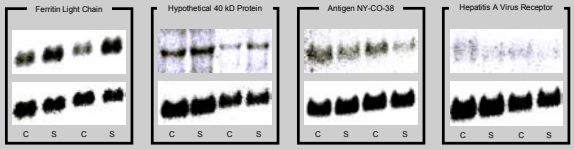

Fig. 4: Verification of identified genes by Northern analysis with total RNA from HT29 cells (5 bzw. 10 µg/lane);
the upper row shows the regulation of several zinc-sensitive genes, the lower row shows control hybridizations with a GAPDH cDNA probe to verify equal loading
C = Control (0.25 ppm Zn), S = Supplementation (10 ppm Zn)

Tab. 2: List of verified genes

Gene name	verified by	Regulation in high-zinc cells
Zinc Finger Protein HSAL2 (Fragment)	Real-time PCR	1,62x
Heterogeneous Nuclear Ribonucleoprotein M	Real-time PCR	1,29x
RAS-Related Protein R-RAS3	Real-time PCR	2,39x
Ferritin Light Chain	Northern Blot	1,45x
Hypothetical 40.0 kD Protein	Real-time PCR	1,41x
	Northern Blot	1,35x
Hepatitis A Virus Cellular Receptor 1	Real-time PCR	-2,86x
	Northern Blot	-1,29x
Gastrointestinal Peptide	Real-time PCR	-5,13x
Antigen NY-CO-37 (NY-CO-38)	Northern Blot	-1,28x

4

Summary

Searching for zinc-sensitive genes which may serve as biomarkers for the assessment of the zinc status *in vivo*, the condition of zinc excess was simulated *in vitro*. The screening using cDNA-arrays has shown, that the experimental conditions caused altered expression of about 1 % of the represented genes on the array. The modulated expression of eight genes was verified independently by Northern analysis and quantitative polymerase chain reaction. The identified zinc-sensitive genes are members of different functional classes, which indicates, that increased cellular zinc levels may influence several metabolic functions simultaneously. In summary, the established reporter cell system and the cDNA-array-technology may provide a tool for the identification of zinc-modulated genes, which may be useful to assess the zinc status of mammalian cells.

5

References

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6