IDENTIFICATION OF REGULATORY ELEMENTS IN STRESS INDUCIBLE EPIMERASE PROMOTER FROM FILAMENTOUS FUNGUS *Rhizopus nigricans*

Tanja Vilfan, Boštjan Černila, Bronislava Črešnar,* and Katja Breskvar

Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Vrazov trg 2, 1000 Ljubljana, Slovenia

Received 06-06-2003

Abstract

Our previous studies revealed that exposure of filamentous fungus *Rhizopus nigricans* (*R. nigricans*) to different stressors up-regulates the expression of cytosolic hsp70 and epimerase genes. Analysis of the epimerase gene promoter showed that it includes putative binding sites for transcriptional activators and repressors, which could be involved in the control of gene expression. We proposed that two types of regulatory elements could regulate the epimerase gene induction during heat shock: two putative heat shock elements (HSE) and possibly STRE, present in many promoters of yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) genes which are induced in different stress conditions, including heat shock. The aim of the present study was to investigate *in vitro* the two types of promoter regulatory elements, possibly involved in activation of the epimerase gene during heat shock. The results obtained with electrophoretic mobility shift assay (EMSA) showed that in the fungus *R. nigricans* the epimerase gene is induced during heat shock via HSF-independent mechanism and that heat shock activates transcription factors capable of binding to putative STRE regulatory element.

Introduction

The fungus *Rhizopus nigricans* is a primitive eukaryote from the phylum *Zygomycota*. In previous studies it was shown that addition of progesterone, testosterone, or deoxycorticosterone to growth medium induces multienzyme steroid hydroxylating system.[1] The system has been well studied on the protein level and it was suggested it has detoxifying function as it converts the above mentioned steroid hormones into more soluble products, which are less toxic for the organism.[2] Furthermore, Northern analyses showed that all three steroid hormones induce the expression of a gene coding for a sugar epimerase, an enzyme involved in the metabolism of galactose,[3] and three genes for cytosolic Hsp70s.[4,5] Moreover, higher level of transcription of the epimerase gene was also observed under conditions of heat shock and osmotic stress, while higher levels of studied Hsp70 mRNAs were detected after heat shock or oxidative stress, and in the presence of ethanol or heavy metals. The

induction of the epimerase and the induction of hsp70 genes therefore are triggered by different sets of stressors. In addition, regulatory elements possibly involved in the epimerase gene up-regulation with the studied stressors can be distinguished from those found in promoters of fungal hsp70 genes. We report here the results of preliminary *in vitro* studies of putative heat shock elements (HSE) and general stress response element (STRE), possibly involved in the epimerase gene induction after exposure of *R. nigricans* to heat shock, for their specific protein binding capacity. The electrophoretic mobility shift assay (EMSA) showed that both putative HSE are not capable of binding proteins specifically and that STRE on the other hand could be responsible for the gene induction during heat shock.

Results and discussion

Filamentous fungus *R. nigricans* is able to respond to different stressors, including progesterone, testosterone and deoxycorticosterone with induction of a specific set of proteins, such as an epimerase and cytosolic Hsp70 that are involved in defense mechanisms of this primitive eukaryotic organism. At present time less is known about regulation of these inducible genes. The purpose of this work was to elucidate which of the putative promoter elements could be responsible for the epimerase gene up-regulation during heat shock.

For this purpose we analysed the epimerase gene promoter. The results of this analysis revealed the presence of three putative regulatory elements, possibly involved in the epimerase gene induction upon heat shock. From the data presented in Table 1 it can be seen that we identified two possible heat shock elements HSE1 and HSE2, and a putative regulatory element STRE. The nucleotide sequence of HSE1 contains three highly conserved pentanucleotides 5'-nGAAn-3' in alternating orientation, typical for functional HSEs.[9] Two repeats completely match the conserved pentanucleotide, and in the third one found in HSE1 only two nucleotides out of three conserved are present. HSE2 shows even greater deviation from the consensus HSEs as it contains only two repeats typical for functional HSEs among which only the one present at 5' end completely matches the above mentioned pentanucleotide. In contrast to the HSEs, the identified putative STRE in the epimerase promoter is totally in accordance with STRE,

which is present in promoters of many *Saccharomyces cerevisiae* genes inducible with several stressors, including heat shock.[10]

Table 1. Putative regulatory elements in the epimerase promoter, possibly involved in up-regulation of the gene during heat shock.

Putative regulatory element	Regulatory element orientation and position
HSE1: TGAAACTTCGAGACT	(+), -1938 to -1924
HSE2: CGAAAGTTTGATTCA	(+), -2001 to -1987
STRE: AGGGG	(+), -1119 to -1115

From the results presented in Figure 1 it is possible to speculate that both of the identified putative heat shock elements HSE1 and HSE2 are not functional. This may not be surprising due to their deviations from otherwise very well conserved consensus nucleotide sequence of HSE among different organisms.[9]

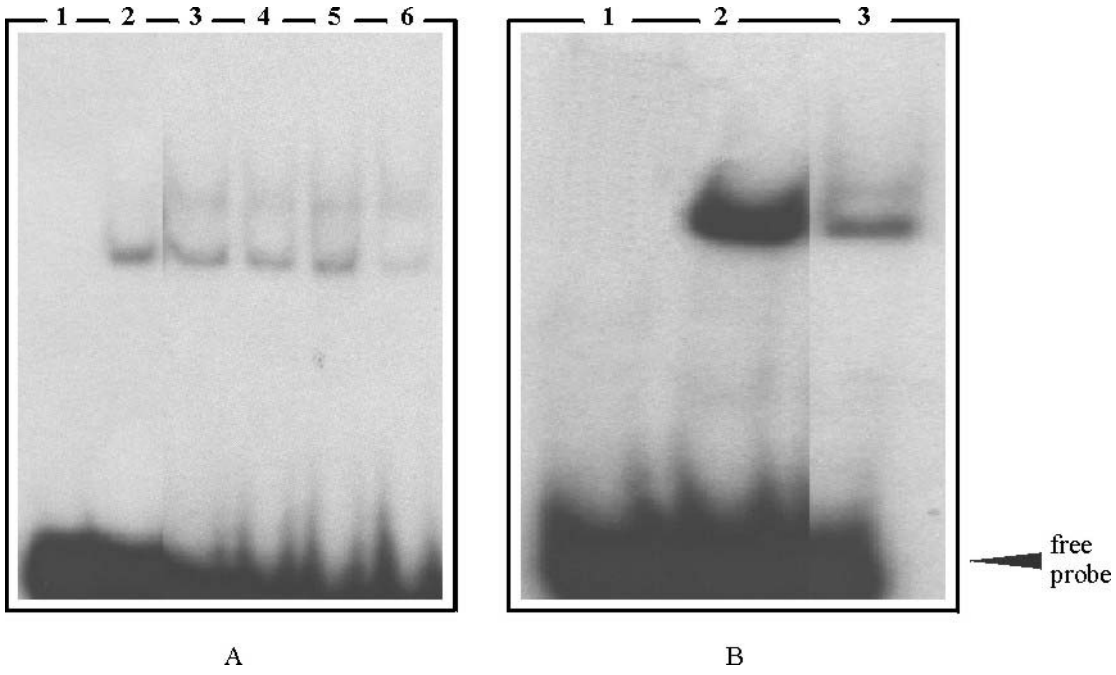

Figure 1. Gel mobility shift analyses of both putative HSE of the epimerase gene. The protein-binding experiments and subsequent separation of DNA-protein complexes were performed as described in Experimental. Lanes in A: 1- HSE1 without protein extract; 2- HSE1 with protein extract from nontreated mycelia; 3- HSE1 with protein extract from mycelia exposed to heat shock at 32 °C for 20 min; 4, 5, 6- HSE1 with protein extract as in lane 3 with nonlabelled specific competitor added in 10-, 100-, 1000- fold molar excess, respectively. Lanes in B: 1- HSE2 without protein extract; 2- HSE2 with protein extract from nontreated mycelia; 3- HSE2 with protein extract from mycelia exposed to elevated temperatures.

From the results obtained with EMSA experiments in which double-stranded oligonucleotide synthesized according to the nucleotide sequence of STRE-like regulatory element found in the epimerase promoter region was used it is possible to suppose that the induction of the epimerase gene with heat shock might be achieved through the binding of specific protein factors to this regulatory element (Figure 2). Since our preliminary studies also revealed a possible role of STRE in regulation of the epimerase gene expression during osmotic stress (data not shown) we can speculate that the epimerase gene regulation is the same as regulation of many *S. cerevisiae* genes, which include STRE binding site in their promoters.[10] Therefore results obtained with *in vitro* studies of STRE binding capacity support the idea that STRE could be conserved among different organisms due to its role in gene regulation during versatile growing condition.[11]

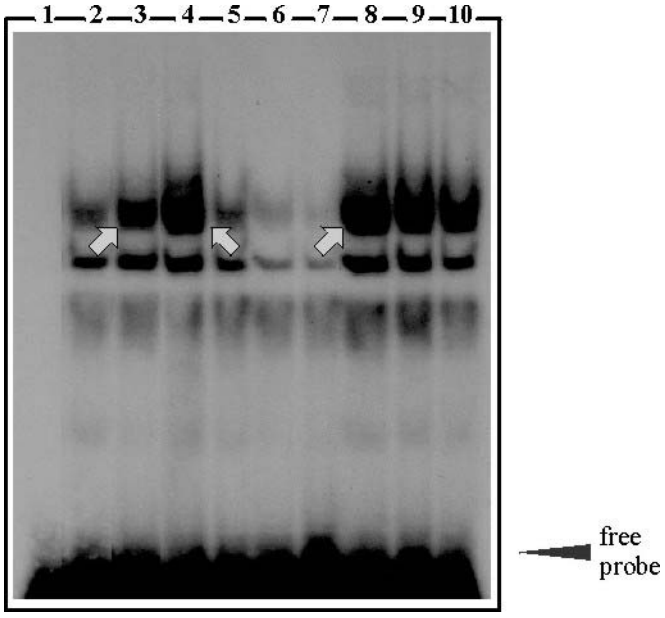

Figure 2. Gel mobility shift analysis of putative STRE of the epimerase gene. The protein-binding experiments and subsequent separation of DNA-protein complexes were performed as described in Experimental. Lanes in the figure: 1- STRE without protein extract; 2- STRE with protein extract from nontreated mycelia; 3- STRE with protein extract from mycelia exposed to 32 °C for 20 min; 4- STRE with protein extract from mycelia exposed to 32 °C for 40 min; 5, 6, 7- STRE with protein extract as in lane 4 with nonlabelled specific competitor added in 10-, 50- and 100- fold molar excess, respectively; 8, 9, 10- STRE with protein extract as in lane 4 with nonlabelled unspecific competitor consHSE added in 10-, 50- and 100- fold molar excess, respectively.

Experimental

Organism and growth conditions. The filamentous fungus *Rhizopus nigricans*, strain B233 was cultivated in a liquid medium (0.5% yeast extract, 0.5% soya bean, 0.5% NaCl, 0.5% K_2HPO_4, 2% glucose, pH 6.5) as described previously.[6] After 18 h of growth mycelia were transferred from 28 °C to 32 °C for 20 or 40 min. Nontreated mycelia were incubated in growth medium at 28 °C discontinuously.

Preparation of whole-cell protein extract. 10 mL of ice-cold extraction buffer (20 mM HEPES/KOH pH 7.9, 100 mM KCl, 5 mM DTT, 2 mM EDTA, 1 mM PMSF, antipain, chymostatin, leupeptin, pepstatin - 10 μg/mL each) were added to 2 g of mycelia powder prepared as described previously.[5] The suspension was homogenized with Potter S homogenizer (B. Braun), incubated on ice for 30 min, and centrifuged in Beckman J2-21 centrifuge twice for 10 min at 4 °C and 10000 rpm using JA20 rotor (Beckman). After centrifugation protein extract was divided into 100 μL aliquotes and stored at - 80 °C. Protein concentration of cellular extract was determined by the Lowry method immediatly before use.[7]

Analysis of epimerase gene promoter. Promoter regulatory elements were identified using MatInspector accessible at www.gdf.de/diodv/matinspector.html.

Electrophoretic mobility shift assay (EMSA). Synthetic oligonucleotides were designed according to putative regulatory elements present in the promoter region of the epimerase gene (Črešnar, unpublished data) and were purchased from Genset, France. Oligonucleotides HSE1*fwd*: 5'-ggCATCgTTgAAACTTCgA-3' and HSE1*rev*: 5'-gTAgTCTCgAAgTTTCAAC-3'; HSE2*fwd*: 5'-ggCTTTTTCgAAAgTTTgATTC-3' and HSE2*rev*: 5'-CACTACATCggATgAATCAAACTTTC-3'; STRE*fwd*: 5'-gTTgAACTAggggTgAAAACAAgAgg-3' and STRE*rev*: 5'-CCCACggCCCTCTTgTTTTCACCCCTA-3'; consHSE*fwd*: 5'-CTAgAAgCTTCTAgAAgC-3' and consHSE*rev*: 5'-AgAAgCTTCTAgAAgCTT-3' were synthesised as complementary oligonucleotide pairs and after annealing labelled with Klenow fragment of DNA polymerase using [α^{32}P]-dCTP.[8] Unincorporated nucleotides were removed from labelled oligonucleotides with purification columns NucTrap Probe (Stratagene, USA) according to producer's instructions. The binding reactions were carried out in 20 μL reaction mixtures containing 10 μM polydI/dC, 25 mM HEPES/KOH pH 7.9, 60 mM KCl, 2 mM DTT, 5 mM EDTA, 10% glycerol, 10 μg

proteins and radiolabelled oligonucleotide (5000-20000 cpm). Reaction mixtures were incubated at room temperature for 15 min, then loaded onto 5% non-denaturing polyacrylamide gels and electrophoresed at 200V for 3-4 h in 0.5 × TBE buffer. For competition assays nonlabelled specific or unspecific oligonucleotides were added to reaction mixture 30 min prior the addition of labelled oligonucleotide.

Acknowledgements

This work was financially supported by the Slovene Ministry of Education, Science and Sport.

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Povzetek

Predhodne raziskave so pokazale, da različni stresni pogoji v filamentozni glivi *Rhizopus nigricans* (*R. nigricans*) povečajo izražanje genov za citosolne Hsp70 in epimerazo. Z analizo epimeraznega promotorja smo ugotovili, da promotor vsebuje morebitna vezavna mesta za transkripcijske dejavnike, ki bi lahko uravnavali izražanje gena za epimerazo. Izražanje gena za epimerazo med toplotnim stresom bi bilo lahko uravnavano preko dveh vrst regulatornih elementov: dveh morebitnih elementov HSE in preko elementa STRE, ki je prisoten v številnih promotorjih genov kvasovke *Saccharomyces cerevisiae* (*S. cerevisiae*) in se izražajo v različnih stresnih pogojih, vključno med toplotnim stresom. Cilj naše raziskave je bil proučiti obe vrsti regulatornih elementov in njihovo vlogo med toplotnim stresom v pogojih *in vitro*. Rezultati poskusov premikov kompleksov DNA-proteini v električnem polju (EMSA) so pokazali, da je v glivi *R. nigricans* mehanizem povečanega izražanja gena za epimerazo neodvisen od dejavnikov HSF in da toplotni stres aktivira transkripcijske dejavnike, ki se vežejo na morebitni regulatorni element STRE.