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Crosstalk between transcription factors in regulation of the human glutathione S-transferase P1 gene expression in Me45 melanoma cells

A. M. Slonchak, A. Chwieduk[1], J. Rzeszowska-Wolny[1], M. Yu. Obolenskaya

Institute of Molecular Biology and Genetics NAS of Ukraine
150, Zabolotnogo str., Kyiv Ukraine, 03680

[1]M. Sklodowska-Curie Memorial Cancer Center and Institute of Oncology in Gliwice
Wybrzezhe Armiji Krajowej 15, 44-101, Gliwice, Poland

elephass@gmail.com

Aim. The human GSTP1 is a major enzyme of phase II detoxification in the most cell types. Aberrant expression of GSTP1 is associated with carcinogenesis and development of multidrug resistance. The GSTP1 gene expression is regulated at multiple levels including transcriptional, post-transcriptional and post-translational. We concentrated our attention on the transcriptional level of regulation. **Methods**. *Transient transfection of Me45 melanoma cells with constructs containing the luciferase gene under the control of complete and truncated GSTP1 promoter was utilized to identify a role of different promoter regions in regulation of the gene transcription in Me45 cells. To identify the transcription factors, interacting with the GSTP1 promoter sites, the competitive EMSA and super shift assay were applied.* **Results**. *GSTP1 transcription in Me45 cells is positively regulated by binding NF- B to the cognate site and ER in complex with unknown protein to the ARE site; the complex of ER with c-Fos negatively regulates the gene expression via CRE site. The interaction of c-Fos/ER with GSTP1 CRE site and indirect interaction of ER with GSTP1 ARE were identified.* **Conclusions**. *The positive regulation of the human GSTP1 gene in Me45 melanoma cells is exerted via NF- B and ARE sites and the negative one via CRE site of the promoter. ER is indirectly involved in the regulation of GSTP1 transcription. It is bound via c-Fos with CRE site and via unknown protein with ARE site.*

Keywords: glutathione S-transferase, promoter, transcription factors, NF- B, estrogen receptor, melanoma, transcription regulation.

Introduction. Glutathione S-transferases comprise a multigene superfamily of enzymes that catalyze the conjugation of electrophilic toxic compounds with glutathione, playing a key role in phase II of detoxification [1]. The human GSTP1 isoform is a major GST isoenzyme in most cell types, except hepatocytes [2]. Besides its typical role in detoxification it possesses other functions, including a ligandin function [3], modulation of signaling pathways [4], conjugation and transport of steroid hormones [5], and participates in dinitrosyl-diglutathionyl-iron complex storage and metabolism [6]. Aberrant expression of *GSTP1* is associated with carcinogenesis and development of multidrug resistance (MDR).

The regulation of the *GSTP1* gene expression is in the focus of researchers and clinicians interests because the stimulation of *GSTP1* expression is expected to be used as a preventive approach against cancer while its down-regulation is in need to counteract the development of MDR. The *GSTP1* gene expression is regulated at multiple levels including transcriptional, post-transcriptional and post-translational [7]. We concentrate our attention on the transcriptional level of regulation.

Despite the vast literature devoted to GSTP1 enzyme the functional characteristics of responsive elements in gene promoter and tissue-specific peculiarities of their regulation are poorly understood. Moreover the previous investigations of molecular mechanisms involved in the *GSTP1* regulation were focused mainly on breast cancer, leukemia and prostate cancer cells. In present research we performed a functional analysis of *GSTP1* promoter in human melanoma cells Me45. We utilized truncated promoter constructions to compare the functional role of different *cis*-acting promoter elements and identified transcription factors binding the responsive elements by competitive EMSA (electrophoretic mobility shift assay) and supershift assay.

Matherials and methods. *Cell culture.* Human melanona cell line Me45 was obtained from Polish Cell Bank and propagated in DMEM/F12 medium («Sigma», USA) supplemented with 588 µg/ml L-glutamine, 0.16 % $NaHCO_3$, 10 % heat inactivated fetal calf serum («Gibco», USA) and 100 µg/ml gentamicine. Cells were grown at 37 °C in an atmosphere of 95 % air and 5 % CO_2.

Promoter deletion constructs. Fragments of *GSTP1* gene promoter were prepared by PCR. The oligonucleotide 5'-ACTCACTGGTGGCGAAGACT-3' (position +15 to +35) was used as the downstream primer for all constructions. Each of the following oligonucleotides was used as upstream primers to amplify promoter fragments: 5'-CATAAACACCA- ACCTCTTCCCC-3' (position –1379 to –1357) for *pGSTP1415*, 5'-ATAGC-CTAAGGCACAGCCAC-3' (position –1162 to –1142) for *pGSTP1197*, 5'-TTTCCTTTCCTCTAAGCGGC-3' (position –405 to –385) for *pGSTP440*, 5'-AGTCCGC-G GGACCCTCCAGA-3' (position –105 to –85) for *pGSTP140* and 5'-AGAGCGGCCGGCGCCGTGAC-3' (position –85 to –64) for *pGSTP120*. The amplified products were subcloned into pCR®2.1-TOPO® vector («Invitrogen», USA). The recombinant plasmids were sequenced and the orientation of inserts was determined. Plasmids with directly oriented inserts were submitted to digestion with *KpnI* and *XhoI*. Excised inserts were religated into *pGL3*-basic (plasmid with Glo™ Lu- ciferase 3 basic) plasmid («Promega», USA). Resulted constructs were named *pGSTPX*, were *X* corresponds to the length of the inserted promoter fragment and *GSTP* is the gene name. Sequences of relevant regions of the final constructs were confirmed by sequencing in both directions in OIigo.pl DNA IBB PAN Service (Poland).

Transient transfection assay. Me45 cells were grown in 24-well plates to 60 % confluence and transfected with 500 ng of *pGSTP* together with 25 ng of *pRL-TK* (plasmid with Renilla Luciferase and Th midine kinase Promoter) plasmid («Promega», USA) per well using Lipofectamine™ LTX and PLUS™ reagents («Invitrogen», USA). After 20 h the firefly and renilla luciferase activities were assessed using Dual Luciferase® Reporter Assay System («Promega», USA).

Electrophoretic mobility shift assay. Nuclear extracts from Me45 cells were prepared by modified method of Dignam et al. [8]. The following oligonucleotides and their complementary sequences were used as probes in EMSA experiment: ARE (Antioxidant Response Element of human *GSTP1* promoter) 5'-CGCCGTGACTCAGCACTGGG-3', NF- B-like (Nuclear Factor B-like site of human *GSTP1* promoter) 5'-TCCGCGGGACCCTCCAGAAG-3', NF- B (Nuclear Factor B site of human *GSTP1* promoter) 5'-CTTAGGGAATTTCCCCCCGC-3', CRE (Cyclic AMP Response Element site of human *GSTP1* promoter) 5'-GAGACTACGTCATAAAATAA-3', GATA (GATA-1 binding site of human *GSTP1* promoter) 5'-GAGATCAATATCTAGAAATAA-3'. Probes (10 pmoles) were labeled with 20 pmoles [-^{32}P]-ATP 6000 Ci/mmole («Hartmann Analytic», Deutschland) by polynucleotide kinase («Roche», Switzerland).

Unincorporated nucleotides were removed by gel-filtration through Bio-gel® P-30 («Bio-Rad», USA). Electrophoretic mobility shift assay was performed using Electropforetic mobility shift assay kit («Promega»). Consensus oligonucleotides for AP-1 (Activator Protein 1), NF- B (Nuclear Factor B), CREB (Cyclic AMP Response Element Binding protein), GATA, ER (Estrogen Receptor) and RAR (Retinoic Acid Receptor), antibodies against human c-Jun, c-Fos, MafF/G/K, ER , Nrf3 (Nuclear erythroid 2 p45 related factor 3), NF- B p50, NF- B p65 and normal rabbit IgG were from «Santa Cruz Biotechnology» (USA).

Results and discussion. *Functional analysis of the GSTP1 promoter regions in Me45 cells.* The structure of *GSTP1* promoter is summarized in fig. 1. To identify
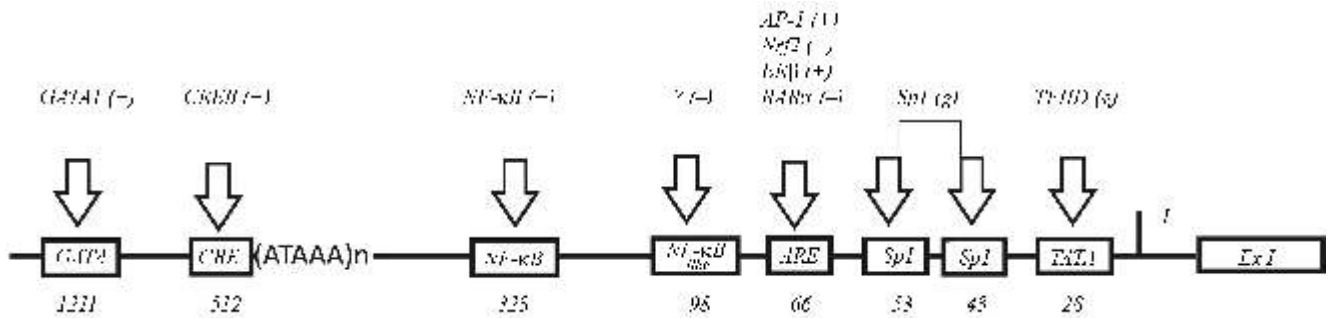
Fig. 1. Structure of the human *GSTP1* gene 5'-regulatory region and potential transcription factors interacting with it [9–13]: «+» – positive regulation; «–» – negative regulation; *g* – general transcription factors
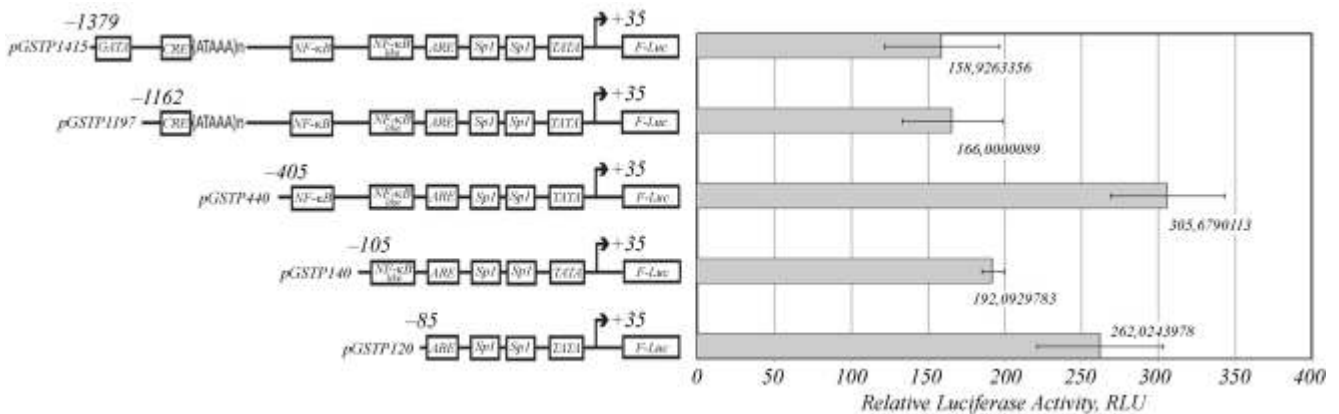

Fig. 2. Schematic representation of the reporter constructs and their activities in transfected Me45 cells. Relative luciferase activity was calculated as a ratio of firefly to renilla luciferase light emission. Cells cotransfected with *pGL3*-basic and *pRL-TK* vectors were as a negative control

the role of *GSTP1* promoter regions in regulation of *GSTP1* transcription in Me45 cells we utilized transient transfection assay with reporter constructs containing complete or truncated *GSTP1* promoter fused to the firefly luciferase gene. For this purpose we designed the reporter constructs each lacking the DNA fragment with one transcription factor binding site (fig. 2). The diagram in fig. 2 represents relative firefly luciferase activities in lysates of Me45 cells transfected with reporter constructs. Each bar in the graph represents the average of 3 independent experiments with triplications in each.

Transfection of the largest vector (*pGSTP1415*) containing the *GSTP1* promoter fragment from –1379 to +35 resulted in relatively high level of *f-luc* gene expression in Me45 cells. Deletion of the *GSTP1*-flanking region between –1379 and –1162, containing GATA-binding site, did not influence significantly the expression of the reporter gene. Deletion of the region from –1162 to –405, which contains CRE and ATA-AA-repeat, resulted in increase of f-luc expression approximately 1.8-fold in comparison with previous construct. Further deletion of the region from –405 to –105, containing NF- B site, reduced the reporter gene expression 1.6-fold. Deletion of the region from –105 to –85, known as an NF- B-like element, resulted in 1.5-fold increase of f-luc expression.

Thereby, the results of the transient transfection experiments suggest the presence of the negative regulatory elements located in the regions from –1162 to –405 and from –105 to –85. Also it provided the evidence for the presence of the strong positive regulatory element located from –405 to –105. The similar role of promoter sequences in the regulation of
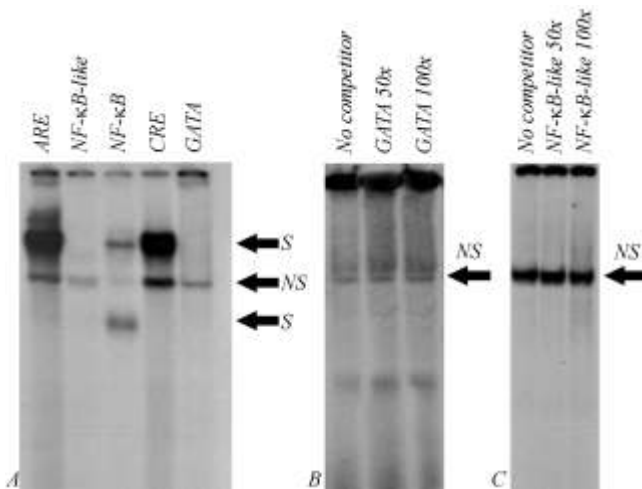
Fig. 3. *In vitro* binding of Me45 nuclear proteins to *GSTP1* promoter sites: *A* – electrophoretic mobility shift assay, demonstrating the ability of Me45 nuclear proteins to form complexes with ARE, NF-κB, NF-κB-like, CRE and GATA sites; *B* – results of competitive EMSA demonstrating, that protein binding to NF-κB-like site is nonspecific; *C* – results of competitive EMSA demonstrating, that protein binding to GATA site is nonspecific; *S* – specific complex; *NS* – nonspecific complex

GSTP1 gene transcription was identified by Jhaveri and Morrow [14] for breast cancer cells.

The study of ARE, NF-κB-like, NF-κB, CRE and GATA binding sites interactions with nuclear proteins from Me45 cells. For identification of the transcription factors interacting with the *GSTP1* promoter the electrophoretic mobility shift assay (EMSA) was applied. The ability of 20 bp promoter fragments, containing ARE, NF-κB-like, NF-κB, CRE and GATA sites to bind nuclear proteins from Me45 cells was examined in this experiment. Fig. 3 shows that all oligonucleotides form complexes with nuclear proteins. Specificity of the protein binding was assessed in a competition experiment, in which nuclear proteins were preincubated in 50- and 100-fold molar excess of unlabeled probe. In this experiment we determined, that ARE, NF-κB and CRE sites specifically bind nuclear proteins while NF-κB-like and GATA sites do not. One band observed in all elrctrophoregrams was non-specific because it was not eliminated in competitive experiments (fig. 3, *A*, *B*, *C*).

Surprisingly, we did not find any proteins interacting with NF-κB-like element which was identified as a negative regulator of *GSTP1* transcription in the transient transfection experiment. We suppose that

the «negative» role of the NF-κB-like element in *GSTP1* transcription may be connected with the presence of palindrome GGGACCCtc in the region that may hinder an enchanceosome formation.

The region spanning nucleotides from –85 to +35 which is shown to be able to support the transcription of the reporter gene in Me45 cell at the level even higher than the full-length promoter is known to be a minimal promoter essential for the *GSTP1* gene expression. This minimal promoter region contains ARE site which interacts with different transcription factors – AP-1 [15], Nrf2 [16], ERβ [17] and RARα [18], depending on cell type. To identify the transcription factors acting on this site in Me45 cells we performed competitive EMSA with consensus oligos for AP-1, Maf (the DNA-binding component of Nrf2), ERβ and RARα and supershift assay with antibodies for these transcription factors. Consistent with results shown in fig. 4, *A*, a 50- and 100-fold molar excess of unlabeled consensus oligonucleotides for AP-1, Maf, estrogen receptor beta (ERβ) and retinoic acid receptor (RAR) were not able to compete for the nuclear proteins binding to the ARE site. It means that AP-1, Maf, ERβ and RARα do not interact with ARE site through their DNA-binding domains. To clarify these results the supershift experiment with polyclonal antibodies to c-Jun (cross-reactive to JunB and JunD), c-Fos (cross-reactive to FosB, Fra1 and Fra2), MafF/G/K, ERβ and Nrf3 (the placenta-specific homolog of Nrf2) was performed. As indicated in fig. 4, *A*, neither transcription factors Jun, Fos nor Maf and Nrf3 prevent the formation of specific complex of ARE site with a nuclear protein. Only ERβ antibody prevents the whole complex formation resulting in appearance of a new complex with higher electrophoretic mobility. This result clearly indicates that in Me45 nuclear extracts ERβ binds to the *GSTP1* ARE site through another yet unknown protein and DNA-binding domain of ERβ is not involved in these interactions.

The region of *GSTP1* promoter from –405 to –105 contains NF-κB site and positively regulates the reporter gene transcription in Me45 cells. This site binds NF-κB in K562 leukemia cells and mediates the gene induction by TNFα [11]. The results of the *GSTP1* promoter NF-κB site binding assay are summarized in fig. 4, *B*. Two specific bands were
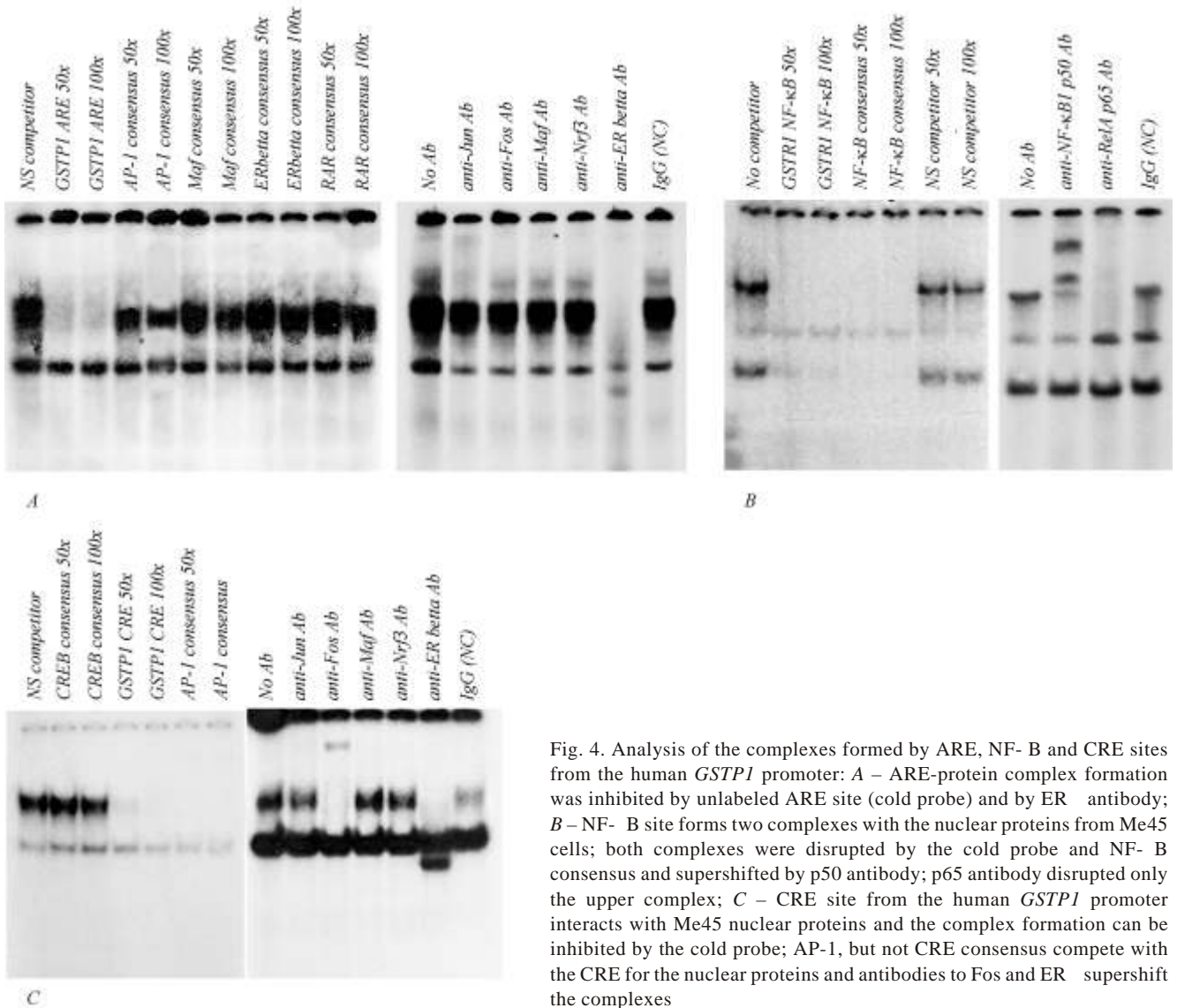
Fig. 4. Analysis of the complexes formed by ARE, NF-κB and CRE sites from the human *GSTP1* promoter: *A* – ARE-protein complex formation was inhibited by unlabeled ARE site (cold probe) and by ERβ antibody; *B* – NF-κB site forms two complexes with the nuclear proteins from Me45 cells; both complexes were disrupted by the cold probe and NF-κB consensus and supershifted by p50 antibody; p65 antibody disrupted only the upper complex; *C* – CRE site from the human *GSTP1* promoter interacts with Me45 nuclear proteins and the complex formation can be inhibited by the cold probe; AP-1, but not CRE consensus compete with the CRE for the nuclear proteins and antibodies to Fos and ERβ supershift the complexes

observed in the reaction of genuine NF-κB site containing oligo with nuclear extract. The unlabeled NF-κB consensus was able to efficiently compete for the nuclear proteins from both specific complexes leading to suggestion that NF-κB binds to *GSTP1* NF-κB site in this cell line. To clarify the matter, nuclear extract was incubated with polyclonal antibodies to p50 and p65 subunits of NF-κB before the probe was added to the EMSA reaction. In the supershift assay of NF-κB site two new bands were observed after the incubation with p50 antibody – one originated from the lower and one from the upper complex, providing the evidence that both complexes contain p50. The upper complex of nuclear proteins and

NF-κB site contains the p50/p65 heterodimer, while the lower complex observed in binding reaction is the p50/p50. These data together with the results of transient transfection assay strongly suggest that NF-κB interacts with the human *GSTP1* NF-κB site and up-regulates gene transcription in Me45 cells.

The negative regulatory element –1162 … –404 contains a CRE site and ATAAA-repeated sequence. It was previously reported that CRE site of *GSTP1* mediates gene response to cAMP by interacting with CREB in Calu-6 lung cancer cells [19]. Competitive EMSA was also conducted to determine which protein is a part of the DNA-protein complex formed by CRE site in Me45. Regarding the ability of CRE sites in

different genes to interact with CREB [19] and AP-1[20] proteins, consensus oligonucleotides for both transcription factors were utilized in the competitive EMSA. A representative autoradiograph in fig. 4, *C*, shows, that CREB consensus oligonucleotide could not compete with *GSTP1* promoter CRE for protein binding, however genuine oligonucleotide CRE and AP-1 consensus competed successfully. This suggests that CRE site forms the complex with AP-1 in Me45 cells. The supershift experiment with antibodies against the transcription factors known to interact directly or indirectly with CREs of other genes was performed to verify the results. Antibodies to c-Jun (cross-reactive to JunB and JunD), c-Fos (cross-reactive to FosB, Fra1 and Fra2), MafF/G/K, ER and Nrf3 were utilized in this assay. The supershifted bands were observed after the incubation of nuclear extracts with Fos and ER antibodies. The supershift analysis indicates that ER together with Fos protein interacts with the human *GSTP1* CRE in Me45 cells and this interaction has a negative regulatory effect.

The phenomenon that protein binding sites can be shared between different transcription factors is called a transcription factor crosstalk [20]. It can be realized by interaction of a «noncanonical» transcription factor directly with a DNA sequence which has a partial homology to the binding sites of this and another transcription factors [20] or by protein-protein inter-actions of «noncanonical» transcription factor with a «genuine» protein bound to its recognition site. In case of the human *GSTP1* promoter both types of crosstalk are present – noncanonical c-Fos together with ER crosstalks with CREB at CRE site and ER together with an unknown protein crosstalks with AP-1 at ARE site. In both cases CREB and Fos/Jun has an opposite effect on gene transcription. In case of the *GSTP1* promoter this negative effect is seems to be potentiated by ER binding which is known to repress Fos-driven transcription [21]. In the present finding we identified ER indirectly interacting with two promoter ele-ments – CRE and ARE sites. It evidences for the importance of this protein for the formation of the enchanceosome on *GSTP1* promoter.

The ER signaling mechanisms discussed until now provide an explanation for the regulation of genes lacking estrogen response element and requiring a second DNA-binding transcription factor to mediate ER association with the DNA. ER and ER have been shown to act in opposite ways at Fos/Jun-binding sites. In the presence of E2 ER activates transcription via its AF-1 and AF-2 transactivating domains while ER -E2 which lacks a functional AF-1inhibits the Fos/Jun-dependent transcription [22]. We suggest that ER exerts the similar inhibitory effect at CRE site of *GSTP1* promoter. The role of ER associated with an unknown protein at ARE site is different and may activate transcription. The dual function of ER in regulation of different promoter elements may be considered in context of enchanceosome formation.

Conclusions. In the present research the transcriptional mechanisms controlling the basal level of *GSTP1* expression in Me45 cells have been analyzed for the first time. The obtained data indicate that the *GSTP1* transcription in this cell type is positively regulated by binding of NF- B to –323 site and ER in complex with unknown protein binding to the ARE site; the complex of ER with c-Fos at CRE site nega-tively regulates the gene expression. The interaction of c-Fos/ER with *GSTP1* CRE site and indirect inter-action of ER with *GSTP1* ARE site have been discovered.

The regulation of *GSTP1* transcription in Me45 melanoma cells has been examined in details also for the first time. Several transcription factors – NF- B in p50/p50 homodimer and p50/p65 heterodimer, ER and c-Fos regulate *GSTP1* transcription in these cells. Positive regulation is exerted via NF- B and ARE sites and negative via CRE site. ER is indirectly involved in regulation of *GSTP1* transcription. It is bound via c-Fos with CRE site and via unknown protein with ARE site.

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