УДК 577.152.28

# TRANSCRIPTION REGULATION IN DIFERENTIAL EXPRESSION OF THE HUMAN *GSTP1* GENE IN BREAST AND CHORIOCARCINOMA CELLS

*A. M. SLONCHAK[1], A. CHWIEDUK[2], J. RZESZOWSKA-WOLNY[2], M. Yu. OBOLENSKA[1]*

*[1]Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine, Kyiv;*
*[2]M. Sklodowska-Curie Memorial Cancer Center and Institute of Oncology in Gliwice;*
*e-mail: elephass@gmail.com*

*Glutathione S-transferase P1 is a major phase II detoxification enzyme in most cell types. Aberrant expression of GSTP1 is associated with carcinogenesis and development of multidrug resistance. GSTP1 gene transcription is regulated by promoter methylation and by transcription factors. To elucidate the mechanisms responsible for the different levels of GSTP1 expression observed in Hbl-100 and BeWo cells we utilized truncated promoter constructs to compare the functional role of different promoter elements. We also identified transcription factors binding the responsive elements by electrophoretic mobility shift assay. The applied approaches provided the evidence that binding of transcription factors to ARE, CRE and NF-κB sites are responsible for the cell specific levels of GSTP1 expression in Hbl-100 and BeWo cells. It was also indicated that partial promoter methylation occurs in BeWo cells.*

*K e y  w o r d s: glutathione S-transferase P1, gene expression, carcinogenesis, promoter, DNA methylation, response element, transcription factors, transcription regulation.*

Glutathione S-transferases is a family of the phase II detoxification enzymes that catalyze the conjugation of electrophilic toxic compounds with glutathione [1]. The human Pi class isoform of GSTs is a major GST isoenzyme in most cell types [2] and besides its typical role in detoxification it possesses other functions, including a ligand binding [3], modulation of signaling pathways [4], conjugation and transport of steroid hormones, storage and metabolism of dinitrosyl-diglutathionyl-iron complex [5]. Aberrant expression of *GSTP1* is associated with carcinogenesis [6] and development of multidrug resistance (MDR) [7].

*GSTP1* gene expression is regulated at multiple levels including transcriptional, post-transcriptional and post-translational [8]. This finding is focused on transcriptional level of regulation where methylation of CpG-dinucleotides in 5′-flanking region of the gene and transcription factors play the main role. 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. This gene is known to be regulated by inducible binding of NF-κB [9], AP-1 [10], Nrf2 [11], CREB [12] and GATA1 [13] to corresponding sites in the 5′-regulatory region. In our previous work [14] we demonstrated that *GSTP1* is also regulated by the estrogene receptor beta (ERβ) which indirectly interacts with CRE and ARE sites of the gene promoter (Fig. 1).

The present research was conducted to gain a better understanding of the transcriptional mechanisms responsible for cell-specific levels of basal *GSTP1* expression. We examined *GSTP1* expression in several cell lines and selected two cell types of different origin possessing different levels of *GSTP1* expression – Hbl-100 breast cancer cells and choriocarcinoma cells BeWo.

Hbl-100 cell line originates from primary cell culture derived from an early lactation sample of human milk. These cells exhibit several characteristics of transformed cells including ability to form colonies in soft agar, an aneuploid chromosome complement, and continuous growth. They also retain some features of normal mammary cells, e.g. they synthesize hyaluronic acid, glycosaminoglycan associated with normal cell adherence, at a level significantly higher than in the breast cancer MCF-7 cells. The free saccharides synthesized by HBL-100 cells contain specific milk sugar, lactose. Like nonmalignant breast cells they do not respond to the cell growth inhibitor from plasma-derived human serum [15]. In contrast to BeWo cells Hbl-100 cells do not synthesize estradiol from testosterone. Due to their specific characteristics Hbl-100 cells are frequently used with malignant cells in comparative studies.

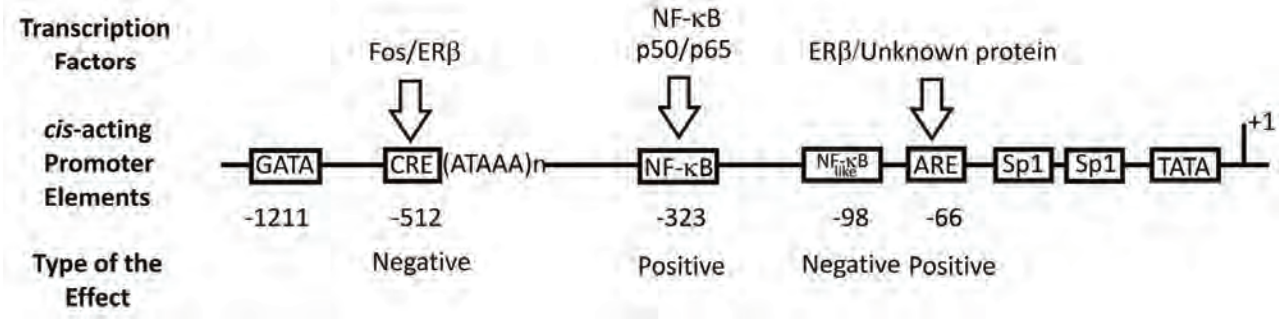


*Fig. 1. Structure of the human GSTP1 gene 5′-regulatorey region and its interactions with transcription factors: (+) – positive regulation, (-) – negative regulation, (g) – general transcription factors*

BeWo cell line is the first human trophoblastic endocrine cell line to be maintained in continuous culture [16]. It was initiated from a malignant gestational choriocarcinoma of the fetal placenta. BeWo is a typical malignant cell line, which is invasive, polyploid and capable for continuous growth in culture. It also retains some morphological and biochemical characteristics of normal trophoblast, e.g. they form syncytia and secrete placental hormones including lactogen, progesterone, estrone, estradiol and choriogonadotropin. These cells are frequently used in studies of placental transport, detoxification, hormone production and syncytia formation [17]. Both types of cells, Hbl-100 and BeWo, possess the features of normal and malignant cells.

To clarify the mechanisms responsible for different levels of *GSTP1* expression observed in these cells we assessed the CpG methylation of the promoter region and utilized truncated promoter constructs to compare the functional role of different promoter elements. We also identified transcription factors binding the responsive elements by competitive electrophoretic mobility shift assay (EMSA) and supershift analysis.

## Materials and Methods

### *Cell culture*

Choriocarcinoma cell line BeWo was obtained from ATCC and propagated in DMEM/F12 medium (Sigma, USA) supplemented with 1x MEM Nonessential amino acids (Gibco, USA), 588 μg/ml L-glutamine, 0.16% $NaHCO_3$, 10% heat inactivated fetal calf serum (Gibco, USA) and 100 μg/ml gentamicine. Breast cancer cell line Hbl-100 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% $CO_2$.

### *Quantitative RT-PCR*

Total RNA was isolated from cells grown to 80% confluence in 6-well plates using Total RNA Mini kit (A&A Biotechnology, Poland). RNA (1 μg/lane) was analyzed by electrophoresis. First strand cDNA was synthesized from 1 μg of total RNA using iScript cDNA Synthesis kit (Bio-Rad, USA) and 1 μl of RT-mixture was used as a template for quantitative PCR. Quantitative real-time PCR was performed in a Chromo4 thermal cycler (Bio-Rad, USA) using Real Time PCR Master Mix SYBR Set A (A&A Biotechnology, Poland). Primers for *GSTP1* were gstpl: 5′-CCCAAGTTCCAGGACGGAGA-3′ and gstpr 5′-GCCCGCCTCATAGTTGGTGT-3′. Samples were denatured at 94 °C for 10 min, and cDNA products were amplified with 50 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 15 s. Calculations of the initial amounts of mRNA were performed according to the cycle threshold method using Opticon Monitor 3 Software (Bio-Rad, USA). The amount of RNA was normalized to that of RPL41 ribosomal protein mRNA quantified with RT-PCR performed under the same conditions.

### *Semi-quantitative Western blot analysis*

BeWo and Hbl-100 cells grown to 80% confluence in 6-well plates were lysed in 1 ml of RIPA Buffer (Thermo Scientific, USA) supplemented with Complete Proteinase Inhibitor Cocktail (Roche, Switzerland). Protein concentration was determined using Bradford reagent (Bio-Rad, USA). Total proteins (25 μg) of each cell lysate were resolved in 12% SDS-PAGE according to Laemmli method [18], and electroblotted onto Immobilon-P Transfer Membrane (Millipore, USA). Immunodetection of GSTP1 protein was accomplished with rabbit polyclonal anti-GSTP1 antibody (StressGen, USA) diluted 1 : 1000 with 2% BSA in TBS following the procedure recommended by antibodies manu-

facturer. To detect the immunoreactive proteins, horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, USA) diluted 1 : 10000 with 2% BSA in TBS, and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, USA) was used. Visualization of the immunoreactive bands was achieved by exposing the membrane to the X-ray film. Films were developed and scanned. Band intensities were quantified with image analysis software GelPro Analyzer 4.5 (Media cybernetics, USA). The amount of protein was normalized by Ponceau S staining of the membranes after electrotransfer.

### *Promoter deletion constructs and transient transfection assay*

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 were used as upstream primers to amplify promoter fragments: 5′-CATAAACACCAACCTCTTCCCC-3′ (position -1379 to -1357) for pGSTP1415, 5′-ATAGCCTAAGGCACAGCCAC-3′ (position -1162 to -1142) for pGSTP1197, 5′-TTTCCTTTCCTCTAAGCGGC-3′ (position -405 to -385) for pGSTP440, 5′-AGTCCGCGGGACCCTCCAGA-3′ (position -105 to -85) for pGSTP140 and 5′-AGAGCG GCCGGCGCCGTGAC-3′ (position -85 to -64) for pGSTP120. Primers were designed by Vector NTI Advance 10.0 software (Invitrogen, USA) using *GSTP1* gene sequence AY324387. PCR was performed in 25 µl of reaction mixture containing 1x QUIAGEN PCR Buffer, 1.5 mM $MgCl_2$, 200 µM each dNTP, 10 pmoles of each primer, 1x Q-solution, 500 ng DNA and 2.5 units of Taq-DNA polymerase. DNA was denatured at 94 °C for 10 min, and promoter fragments were amplified with 30 cycles of denaturation at 94 °C for 30 s, annealing and extension at 60 °C for 120 s (for 1415bp fragment), 90 s (for 1197 bp fragment), 60 s (for 440 bp fragment) and 30 s (for 140 and 120 bp fragments). Final extension step was performed at 72 °C for 15 min. The amplified products were gel-purified using GelOut Kit (A&A Biotechnology, Poland) and subcloned into pCR2.1-TOPO vector (Invitrogen, USA), excised by KpnI and XhoI and religated into KpnI and XhoI linearized and dephosphorylated pGL3-basic plasmid (Promega, USA). DNA template for PCR was isolated from human peripheral blood using Genomic Mini Kit (A&A Biotechnology, Poland). All PCR reagents and kits for plasmid isolation were obtained from Quigen Inc (USA). Final plasmids for transfection were isolated using EndoFree Plasmid Maxi Kit (Quiagen, USA). All enzymes were from MBI Fermentas (Lithuania). Sequences of relevant regions of the final constructs were confirmed by sequencing in both directions in OIigo.pl DNA IBB PAN Service (Poland).

BeWo and Hbl-100 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 (Promega, USA) per well using Lipofectamine LTX and PLUS reagents (Invitrogen, USA). After 20 h the culture medium was removed, cells were washed with PBS and lysed in 250 µl of passive lysis buffer (Promega, USA). Firefly and renilla luciferase activities were assessed in 5 µl of the lysates using Dual Luciferase Reporter Assay System (Promega, USA). Light emission resulting from luciferase activity was measured in Lumat LB 9506 luminometer (Berthold technologies, USA) by integration of peak light emission during 10 s at 25 °C. The ratio between arbitrary firefly and renilla luciferase light units was calculated for each probe. Each experiment was repeated three times with triplications in each one.

### *Electrophoretic mobility shift assay (EMSA)*

BeWo and Hbl-100 cells were grown in T225 flasks to 80% confluence. After removal of culture medium cells were washed with PBS, harvested and resuspended in 5 volumes of Buffer A (10 mM HEPES (pH 7.9) 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT) and incubated on ice for 10 min, then Nonidet P-40 was added to the cell suspension to the final concentration 0.1%. Cells were vortexed for 10 s and nuclei were pelleted by centrifugation for 10 min at 650 g. The pellet was resuspended in 1.5 V Buffer B (20 mM HEPES (pH 7.9), 0.2 mM EDTA, 1.5 mM $MgCl_2$, 25% glycerol, 1 mM DTT) and an equal volume of Buffer C (20 mM HEPES (pH 7.9), 840 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 1 mM DTT) was added. Nuclear proteins were eluted by shaking at 4 °C for 45 min, cell debris was separated from the lysates by centrifugation at 15000xg for 15 min and supernatant was then aliquoted and stored at − 80 °C. All steps were performed at 4 °C and Complete Proteinase Inhibitor Cocktail (Roche, Switzerland) was added to all solutions extemporaneously. A Bradford Bio-Rad protein assay was used to determine protein concentration.

The following oligonucleotides and their complementary sequences were used as probes in EMSA experiment: ARE (ARE site of human GSTP1 promoter) 5′-CGCCGTGACTCAGCACTGGG-3′, NF-κB-like (NF-κB-like site of human GSTP1 promoter) 5′-TCCGCGGGAC-

CCTCCAGAAG-3′, NF-κB (NF-κB site of human GSTP1 promoter) 5′-CTTAGGGAATTTC-CCCCCGC-3′, CRE (CRE site of human GSTP1 promoter) 5′-GAGACTACGTCATAAAATAA-3′, GATA (GATA-1 binding site of human GSTP1 promoter) 5’-GAGATCAATATCTAGAAATAA-3′. Probes were prepared by denaturation of complementary oligonucleotides for 2 min at 95 °C, annealing for 20 min at 60 °C and 30 min at 22 °C. Probes (10 pmoles) were incubated for 30 min with 20 pmoles [γ-P32]-ATP 6000 Ci/mmole (Hartmann Analytic, Deutschland) in 20 μl of reaction mixture containing 10 units polynucleotide kinase (Roche, Switzerland) and PNK (polynucleotide kinase) Buffer. Unincorporated nucleotides were removed by gel-filtration through Biogel P-30 (Bio-Rad, USA).

Cell nuclear extracts (5 μg) were preincubated for 10 min at 25 °C with 1 μg sonicated *E. coli* DNA and with or without 5-10 pmoles unlabeled competitor in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol, 1 mM DTT. Then 0.1 pmole of 32P-γ-ATP labeled probe was added to the mixture and incubated for further 30 min at 25 °C. The reaction mixture was then loaded onto a prerun (200 V for 1 h at 4 °C) 6% native polyacrylamide gel (29 : 1 cross-linking ratio) containing 1 x TBE. Electrophoresis was performed at 20 mA for 3 h at 4 °C and the gel was then dried and radiographed. In supershift experiment the reaction mixture was preincubated for 20 min at a room temperature with 2 μg of antibody before the addition of radiolabeled probe. Consensus oligonucleotides for AP-1, NF-κB, CREB, GATA, ER and RAR, antibodies against human c-Jun (sc-44X crossreactive to JunB and JunD), c-Fos (sc-253X crossreactive to FosB and Fra2), MafF/G/K (sc-22831X), ERβ (sc-8974X), Nrf3 (sc-15460X), NF-κB1 p50 (sc-1191X), NF-κB RelA/p65 (sc-7151) and normal rabbit IgG (sc-2027) were from Santa Cruz Biotechnology, USA.

### *Methylation-specific PCR*

Genomic DNA from BeWo and Hbl-100 cells was isolated using Genomic Mini Kit (A&A Biotechnology, Poland) and bisulphite − modified using CpGenome Fast DNA Modification Kit (Millipore, USA). Combinatorial MSP analysis was performed with 2 μl of modified DNA as described by Gonzalgo et al. [19]. Modified CpGenome Universal Methylated DNA (Millipore, USA) and CpGenome Universal Unmethylated DNA (Millipore, USA) were used as positive and negative control, respectively.

## Results and Discussion

### *Expression of* GSTP1 *in different cells*

To select the cell types with different levels of *GSTP1* expression we estimated the *GSTP1* mRNA level in melanoma cells ME-45, breast cancer cells MCF7, normal breast cells Hbl-100, bronchioalveolar carcinoma cells H358, alveolar carcinoma cells A549, myelogenous leukaemia cells K562, hepatocarcinoma cells HepG2, lung cancer cells Hct116, immortalized human bronchial epithelial cell BEAS2B, adrenal carcinoma cells FN-H296 and choriocarcinoma cells BeWo. For this purpose quantitative RT-PCR was applied. Normalized levels of *GSTP1* mRNA expression in analyzed cells are represented in Fig. 2. For the further experiment we selected Hbl-100 as a cell line with the highest level of GSTP1 expression and BeWo cells as a line with a low, but not zero level of *GSTP1* mRNA.

We observed at the average 2.7-fold higher level of GSTP1 protein (Fig. 3) and 2.1-fold higher level of *GSTP1* mRNA in Hbl-100 cells compared to BeWo (Fig. 2).

### *Functional analysis of GSTP1 promoter regions in Hbl-100 and BeWo cells*

To elucidate the functions of *GSTP1* promoter regions in regulation of *GSTP1* transcription in Hbl−100 and BeWo cells we utilized transient transfection assay with reporter constructs containing the firefly luciferase gene under the control of complete or truncated *GSTP1* promoter. We designed the reporter constructs each lacking the DNA fragment with one transcription factor binding site (Fig. 4). The graph in Fig. 4 represents relative firefly luciferase activities in lysates of Hbl-100 and BeWo cells transfected with reporter constructs. Each bar in the graph represents the average of 3 independent experiments with triplications in each.

Transfection of the vector (pGSTP1415) containing the complete *GSTP1* promoter fragment (from − 1379 to +35) resulted in relatively high level of f-luc gene expression in both cell lines. 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 ATAAA-repeat, resulted in a increase of f-luc expression approximately 1.7-fold in BeWo and 1.3-fold in Hbl-100 cells in comparison with the previous construct. Deletion of the next region (from − 405 to − 105), which contains NF-κB site, reduced the reporter

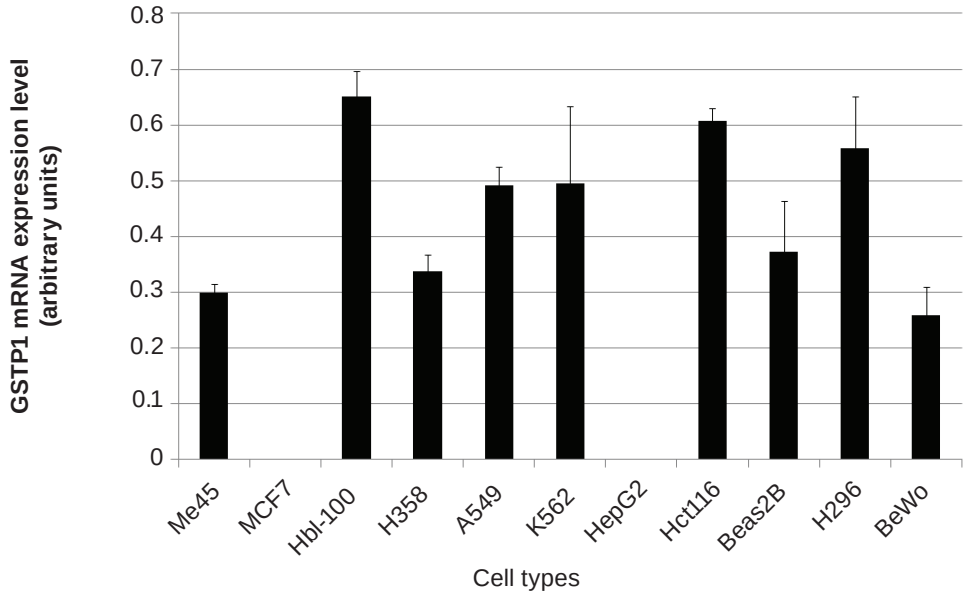


*Fig. 2. Quantitative RT-PCR analysis of mRNA from different cells. Data are shown as a ratio of the GSTP1 mRNA to the ribosomal protein RPL41 mRNA*

gene expression 2.5-fold in BeWo and 2.2-fold in Hbl-100 cells. Deletion of the region from − 105 to − 85, known as an NF-κB-like element, elicited the f-luc expression 1.7-fold in BeWo and 1.,5-fold in Hbl-100 cells.

Hence, this approach identified two negative regulatory elements in the regions from − 1162 to − 405 and from − 105 to − 85 and the strong positive regulatory element located from − 405 to − 105. The role of these elements is similar in both cell types, however in BeWo cells the influence of each element is more pronounced than in Hbl-100 cells. These elements act in the same way in both cell types analyzed in these experiments as well as in Me45 cell analyzed recently.

### *The study of ARE, NF-κB-like, NF-κB, CRE and GATA binding sites interactions with nuclear proteins from BeWo and Hbl-100 cells*

To identify the transcription factors interacting with the *GSTP1* promoter the electrophoretic mobility shift assay (EMSA) was performed. The ability of 20bp promoter fragments, containing ARE, NF-κB-like, NF-κB, CRE and GATA sites to bind nuclear proteins from Hbl-100 and BeWo cells was examined in this experiment. Fig. 5 indicates that all oligonucleotides form complexes with Hbl-100 and BeWo nuclear proteins. The protein binding specificity was examined in a competition experiment, in which nuclear proteins were preincubated in 50- and 100-fold molar excess of unlabeled probe. This approach provided the evidence that

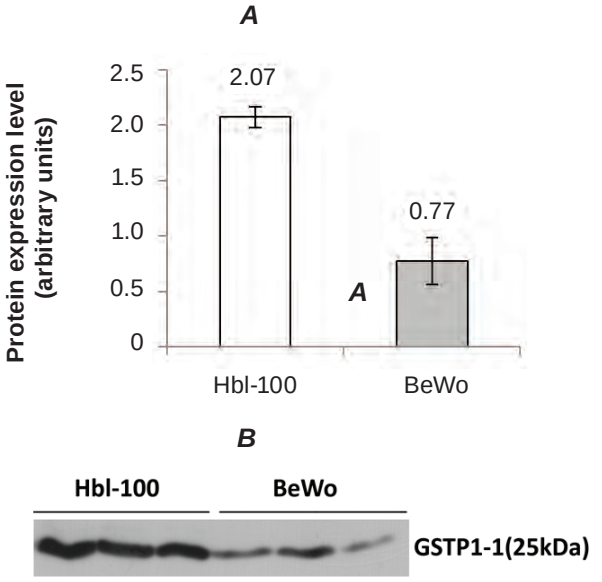


*Fig. 3. Western blotting analysis of GSTP1-1 protein level in Hbl-100 and BeWo cells. The Ponceau S-stained membrane was used for normalization. The graph (a) shows the normalized densitometric results as a ratio of GSTP1-1 band optical density to the optical density of the whole line on Ponceau S-stained membrane. The graphs represent the mean data of three separate experiments. The lower panel (b) shows the representative blot probed with anti-GSTP1-1 antibodies*

CRE, NF-κB and ARE, but not NF-κB-like and GATA sites bind specifically nuclear proteins from Hbl-100 and BeWo cells. One of the complexes

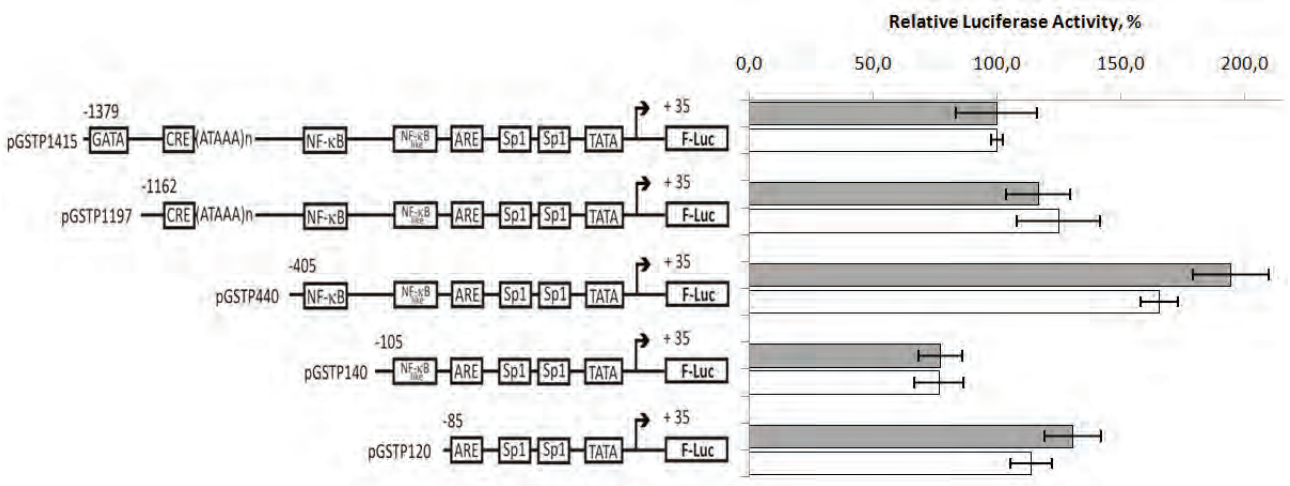


*Fig. 4. Reporter constructs and their activities in transfected Hbl-100 and BeWo cells. Data were normalized to expression of pRL-TK vector cotransfected to the cells together with the reporter constructs. Relative luciferase activity was calculated as a ratio of firefly to renilla luciferase light emission. Expression of various vectors was represented as a percentage relative to an average of the largest construct, containing full GSTP1 promoter. Gray bars indicate the level of reporter gene expression in BeWo and white bars in Hbl-100 cells*

observed in all binding reactions was non-specific because it was not eliminated in competitive experiments. (Fig. 5, *A*, *B*, *C*).

### *Identification of transcription factors interacting with* GSTP1 *promoter in Hbl-100 and BeWo cells*

The region of the *GSTP1* gene from - 85 to + 35 is known to be a minimal promoter essential for the gene expression. It was able to support the transcription of the reporter gene in Hbl-100 and BeWo cell at the level even higher than the full-length promoter. This minimal promoter region contains ARE site which can interact with AP-1 [20], Nrf2 [11] and ERβ [21] in different cell types. To identify the transcription factors interacting with ARE site of GSTP1 promoter in Hbl-100 and BeWo cells we have used consensus oligonucleotides for potential transcription factors. Fig. 6,*A* indicates that a 50- and 100-fold molar excess of unlabeled consensus oligonucleotides for AP-1, Maf, estrogen receptor β (ER beta) and retinoic acid receptor (RAR) do not compete for the nuclear proteins binding to ARE site. It means that AP-1, Maf, ERβ and RAR do not interact with ARE site through their DNA-binding domains. To confirm 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. 6,*A*, neither Jun, Fos nor Maf and Nrf3 transcription factors are involved in formation of the specific complex with ARE site. However, ERβ antibody disrupts this complex in BeWo and Hbl-100 cells and results in formation of the new complex with a higher electrophoretic mobility. This result clearly indicates that in Hbl-100 and BeWo nuclear extracts ERβ bind to the *GSTP1* ARE site through another yet unknown protein as it was previously demonstrated for Me45 cells. DNA-binding domain of ERβ is not involved in these interactions. The observed complexes were identical in both cell types and therefore not responsible for the cell-specific differences in *GSTP1* expression between Hbl-100 and BeWo.

The same approach of competitive EMSA was used for identification of the transcription factor binding to CRE site. Regarding the ability of CRE sites to interact with CREB and AP-1 proteins, consensus oligonucleotides for both transcription factors were utilized in the competition. An autoradiograph in Fig. 6,*C* indicates that CREB consensus oligonucleotide cannot compete with *GSTP1* promoter CRE for protein binding, while genuine oligonucleotide Cre and AP-1 consensus compete successfully. It is quite plausible that CRE site forms the complex with AP-1 in Hbl-100 and BeWo cells. The supershift experiment with antibodies to Jun, Fos, MafF/G/K, ERβ and Nrf3 was performed to verify the suggestion. The supershifted bands appeared after the incubation of Hbl-100 and BeWo nuclear extracts with Fos and ERβ antibodies. Therefore ERβ in a complex with Fos protein interacts with CRE site in both types of cells.

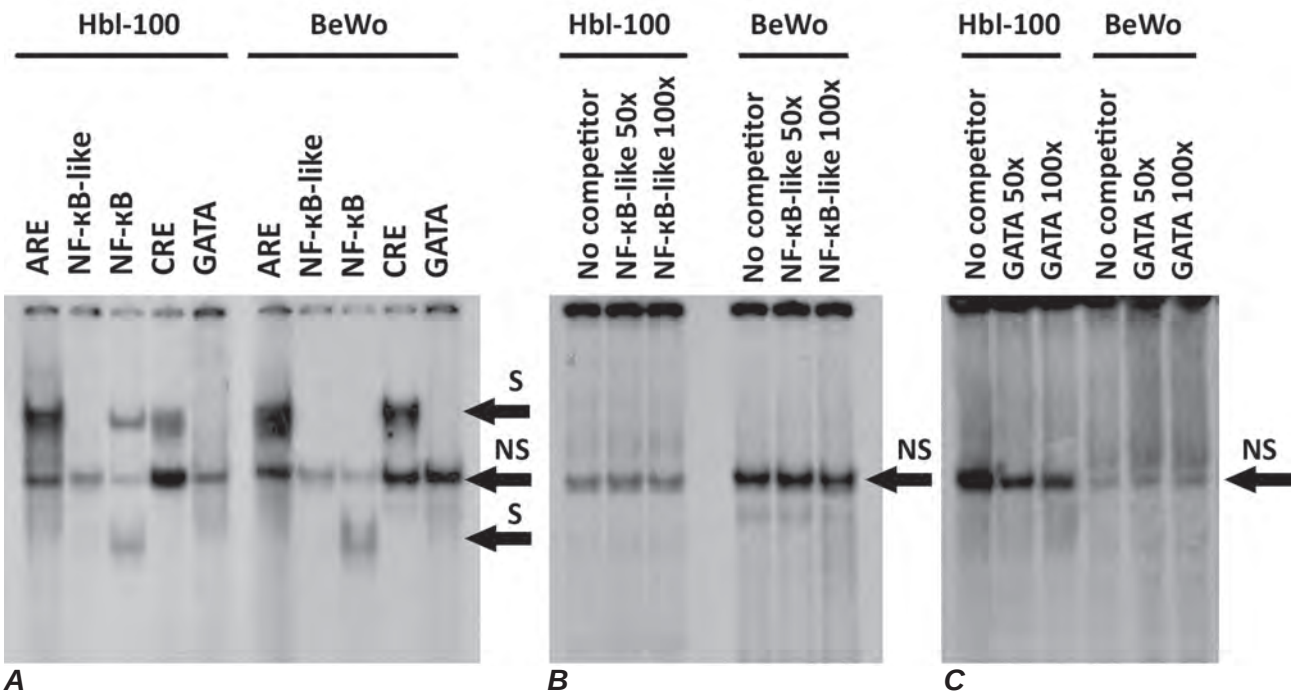


*Fig. 5. In vitro binding of Hbl-100 and BeWo nuclear proteins to the GSTP1 promoter sites. (A) Electrophoretic mobility shift assay, demonstrating the BeWo and Hbl-100 nuclear protein complexes formed with ARE, NF-κB, NF-κ-like, CRE and GATA sites. (B) Results of competitive EMSA demonstrating, that 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*

Different patterns of supershifted bands observed in the experiment with ERβ antibody and nuclear extracts from BeWo and Hbl-100 cells point to different mode of the transcription factors interaction at CRE site in both types of cells. In BeWo cells after antibody-mediated removal of ER only Fos protein binds to CRE site and this complex moves faster than the triple DNA/Fos/ERβ complex. The fact, that the antibody prevents formation of triple complex indicates that the epitopes, recognized by the antibody to ERβ, participate in the interaction of ERβ and Fos. In Hbl-100 cells these epitopes are not involved in the ERβ and Fos interactions because the antibody does not prevent complex formation, but attaches to it and slows down its electrophoretic mobility. The structural differences between the complexes at CRE site in Hbl-100 and BeWo cells may refer to the peculiarities of GSTP1 transcription regulation in these cells. BeWo is an estrogen-producing cell line which secretes the estradiol into the cell culture medium, while Hbl-100 does not produce it. ERβ is known to bind Fos either in liganded with E2 or nonliganded state. We suppose, that E2 binding to ERβ in BeWo cells, but not in Hbl-100 cells induces the conformational transition of the receptor and changes the way it interacts with Fos. ERβ is known for its inhibitory effect on Fos-driven transcription and we also suppose that E2 binding to ERβ could potentiate this ER-dependent transcription suppression in BeWo cells in comparison with Hbl-100 cells.

The results of the *GSTP1* promoter NF-κB site binding assay are represented in Fig. 6,*B*. Two specific bands were observed in the reaction of NF-κB site containing oligo with Hbl-100 nuclear proteins and only one lower band was observed after the incubation with the nuclear proteins from BeWo cells. The unlabeled NF-κB consensus efficiently competed for the nuclear proteins from both specific complexes leading to suggestion that NF-κB binds to *GSTP1* NF-κB site in both cell lines but the subunit composition of bound NF-κB differs between these cell lines. To clarify this matter, nuclear extracts were incubated with polyclonal antibodies to NF-κB1 p50 and RelA p65 subunits of NF-κB before the probe was added to the EMSA reaction. In the assay performed with nuclear proteins from BeWo cells in the presence of p50 antibody one supershifted band was observed, while incubation with p65 antibody had no effect on electrophoretic mobility of the complex providing

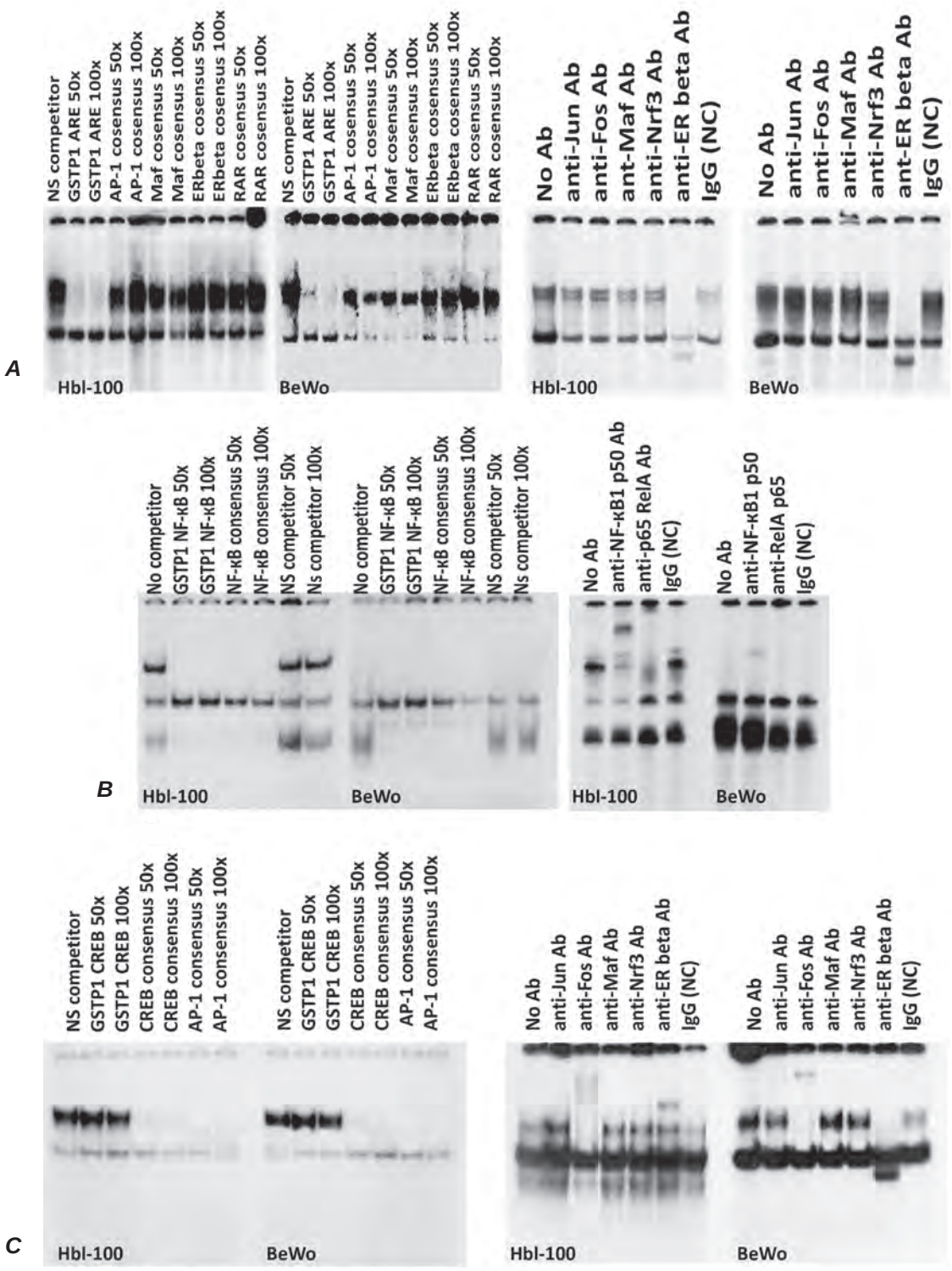


*Fig. 6. EMSA of the complexes formed by ARE, NF-kB and CRE sites from the human GSTP1 promoter. (A) ARE-protein complex formation was inhibited by unlabeled ARE site (cold probe) and by ERβ antibody; (B) NF-κB-site forms different complexes with the nuclear proteins from Hbl-100 and BeWo cells; both complexes were disrupted by the cold probe and NF-κB consensus and supershifted by p50 antibody; p65 antibody disrupted only the upper complex with Hbl-100 proteins; (C) CRE site from the human GSTP1 promoter interacts with Hbl-100 and BeWo 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*

the evidence that in BeWo cells the NF-κB site interacts only with p50 subunit of NF-κB. In the experiment with Hbl-100 nuclear extracts two supershifted bands were observed with p50 antibody, one similar to that in BeWo cells and other with higher molecular mass. It gives the evidence that both complexes observed in "no competition" binding reaction interact with the p50 antibody and thus contain p50. In the presence of p65 antibody only the upper complex of Hbl-100 proteins with NF-κB-containing oligo disappears. The ability of the lower complex, observed in both cells to interact only with p50 antibody indicates that it is a p50/p50 homodimer. The upper complex, formed only by Hbl-100 proteins interacts with p50 and p65 antibodies and thus is a p50/p65 heterodimer.

Hence, the transcription factor binding analysis indicated that in BeWo cells NF-κ-binding site of *GSTP1* promoter interacts only with p50/p50 NF-κB1 homodimer, while in Hbl-100 cells it also interacts with p50 NF-κB1/p65 RelA heterodimer. NF-κB is a dimeric transcription factor composed of the five mammalian Rel proteins p65/RelA, c-Rel, RelB, NF-κB1/p50, and NF-κ/p52 in almost any combination. Only RelA, c-Rel, and RelB, but not p50 or p52, possess C-terminal transactivation domains. Instead, p50 and p52 contain the strong DNA-binding domain which is absent in RelA, RelB and c-Rel. In this regard only the heterodimers of RelA, RelB and c-Rel with either p50 or p52 are active transcription transactivators. In the present research the active form of NF-κB (p50/p65) was identified in a complex with *GSTP1* promoter only in Hbl-100 cells. According to the results of the transient transfection assay, the region of *GSTP1* promoter from − 405 to − 105 which contains NF-κB acts as a strong positive regulatory element. Regarding this data we can assume that the absence of an active NF-κB in BeWo cells is a considerable factor responsible for the lower *GSTP1* promoter activity in comparison with Hbl-100 cells.

### *GSTP1 promoter methylation*

Methylation of the promoter region of *GSTP1* gene in Hbl-100 and BeWo cells was examined by MSP. To confirm the specificity of this approach commercially available methylated and unmethylated human DNA were used as controls for MSP with every assay. Representative results of the gel analysis of bisulfite-treated DNA samples amplified with methylated- and unmethylated-specific primers are shown in Fig. 7. Both cell lines were found to have the sequence amplifiable with unmethylated DNA-specific primers demonstrating the presence of unmethylated allele of *GSTP1* gene in each cell type. However, PCR with both sets of methylated DNA-specific primers generated the products with DNA extracted from BeWo cells, providing the evidence for methylation of promoter region in at least two CpG sequences in any of *GSTP1* alleles. With Hbl-100 DNA PCR with

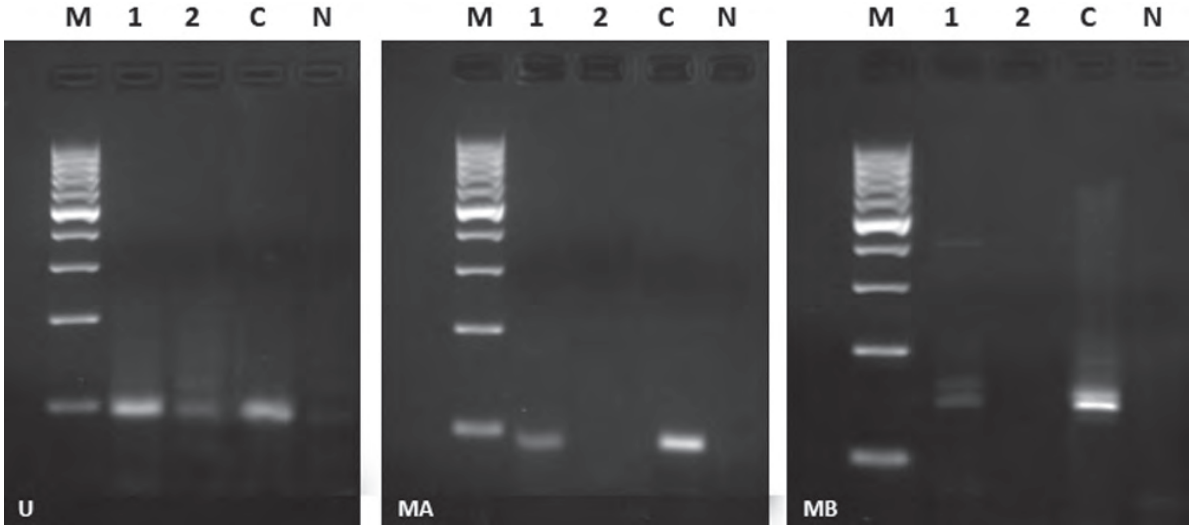


*Fig. 7. Methylation analysis of GSTP1 promoter in Hbl-100 and BeWo cells. Bisulfite-treated DNA was used for PCR amplification with primer set designed for identification of unmetylated (U) and two primer sets (A and B) for identification of methylated GSTP1 DNA (MA and MB). M − molecular weight marker (Gene ruller™ 100 bp DNA Ladder, MBI Fermentas); 1 − DNA from BeWo cells; 2 − DNA from Hbl-100 cells; C − positive controls with methylated (MA and MB) and unmethylated (U) commercially available DNA; N − «no template» control*

methylated DNA-specific primers did not result in generation of any product, providing the evidence for the complete unmethylation of *GSTP1* promoter in these cells.

Thus, the combinatorial MSP analysis revealed the presence of methylated and unmethylated alleles of *GSTP1* gene in choriocarcinoma cells BeWo and the unmethylated state of *GSTP1* in breast cancer cells Hbl-100. Methylation of CpG dinucleotides in *GSTP1* promoter is the most common alteration responsible for malignancy-associated *GSTP1* repression. It is thought that methylation occurs in promoters of genes repressed by transcription factors and accounts for further switching them off. Thus promoter methylation in BeWo cells may result from transcription factor-mediated down-regulation of the gene.

Initiation of the eukaryotic gene transcription is a complex process which includes DNA demethylation, enchanseosome formation, chromatin remodeling and assembly of the preinitiatory complex. Each event in this process is strictly controlled by the cell- and state-specifically coordinated interactions between transcription factors, coactivator and corepressors with chromatin and transcription machinery components. In this finding we analyzed the transcriptional mechanisms controlling the cell-specific levels of *GSTP1* gene expression in Hbl-100 and BeWo cells. We provide the evidence that p50/p50 NF-κB homodimer in BeWo cells contrasts with p50/p65 NF-κB heterodimer in Hbl-100 cells. These differences between both promoters together with different structures of the complexes in CRE sites may contribute to correspondingly lower and higher content of GSTP1 mRNAs in BeWo and Hbl-100 cells. Our data also indicate that ERβ is involved in transcriptional crosstalk with CREB and AP-1 corresponding in CRE and ARE sites in BeWo and Hbl-100 and may influence the cell-specific *GSTP1* transcription in these cells. We also identified that partial promoter methylation occurs in a cell with down-regulated *GSTP1* which may contribute to further locking the gene in inactive state during carcinogenesis.

**Acknowledgement**

The experimental part of this work was carried out in the Institute of Oncology in Gliwice and was financed by the Polish Ministry of Education and Science, grant number N 401 157 32/3043.

## РЕГУЛЯЦІЯ ТРАНСКРИПЦІЇ В ДИФЕРЕНЦІАЛЬНІЙ ЕКСПРЕСІЇ ГЕНА *GSTP1* ЛЮДИНИ У КЛІТИНАХ МОЛОЧНОЇ ЗАЛОЗИ ТА ХОРІОКАРЦИНОМИ

*А. М. Слончак[1], А. Кведук[2], Дж. Жешовска-Вольни[2], М. Ю. Оболенська[1]*

[1]Інститут молекулярної біології та генетики НАН України, Київ;
[2]Раковий центр ім. М. Склодовської-Кюрі та Інститут онкології у Глівіце;
e-mail: elephass@gmail.com

Глутатіон S-трансфераза P1 є головним ферментом II фази детоксикації в більшості типів клітин. Аберантна експресія *GSTP1* пов'язана з канцерогенезом та з формуванням множинної стійкості пухлин до хіміотерапії. Транскрипція гена *GSTP1* регулюється за рахунок метилювання промотору та дії транскрипційних факторів. Щоб дослідити механізми, які відповідають за різні рівні експресії гена, що спостерігаються у клітинах Hbl-100 та BeWo нами було застосовано люциферазний тест для з'ясування функціональної ролі промоторних елементів та дослідження електрофоретичної рухливості ДНК-протеїнових комплексів для ідентифікації транскрипційних факторів. Використані підходи дозволили встановити, що за клітинно-специфічний рівень експресії гена *GSTP1* у клітинах Hbl-100 та BeWo відповідає зв'язування транскрипційних факторів із сайтами ARE, CRE та NF-κB. Також було встановлено, що у клітинах BeWo відбувається часткове метилювання промотору.

Ключові слова: глутатіон S-трансфераза P1, експресія гена, канцерогенез, промотор, метилювання ДНК, елемент відповіді, транскрипційний фактор, регуляція транскрипції.

## РЕГУЛЯЦИЯ ТРАНСКРИПЦИИ В ДИФФЕРЕНЦИАЛЬНОЙ ЭКСПРЕССИИ ГЕНА *GSTP1* ЧЕЛОВЕКА В КЛЕТКАХ МОЛОЧНОЙ ЖЕЛЕЗЫ И ХОРИОКАРЦИНОМЫ

*А. М. Слончак[1], А. Кведук[2], Дж. Жешовска-Вольни[2], М. Ю. Оболенська[1]*

[1]Институт молекулярной биологии и генетики НАН Украины, Киев;
[2]Раковый центр им. М. Склодовской-Кюри и Институт онкологии в Гливице;
e-mail: elephass@gmail.com

Глутатион S-трансфераза P1 является основным детоксикационным ферментом в большинстве типов клеток. Абберантная экспрессия гена *GSTP1* связана с онкогенезом и формированием у опухолей множественной лекарственной устойчивости. Транскрипция гена *GSTP1* регулируется за счет метилирования и действия транскрипционных факторов. Чтобы исследовать механизмы, отвечающие за разные уровни экспрессии гена *GSTP1*, наблюдаемые в клетках Hbl-100 и BeWo, был использован люциферазный тест для определения функциональной роли промоторных элементов и определение электрофоретической подвижности ДНК-протеиновых комплексов для идентификации транскрипционных факторов. Использованные подходы позволили установить, что за клеточно-специфический уровень экспрессии гена *GSTP1* в клетках Hbl-100 и BeWo отвечает связывание транскрипционных факторов с сайтами ARE, CRE и NF-κB. Также было установлено, что в клетках BeWo происходит частичное метилирование промотора.

Ключевые слова: глутатион S-трансфераза P1, экспрессия гена, канцерогенез, промотор, метилирование ДНК, элемент ответа, транскрипционные факторы, регуляция транскрипции.

1. *Hayes J. D., Flanagan J. U., Jowsey I. R.* // Annu. Rev. Pharmacol. Toxicol. – 2005. – **45**. – P. 51–88.
2. *Moscow J. A., Fairchild C. R., Madden M. J. et al.* // Cancer Res. – 1989. – **49**, N 6. – P. 1422–1428.
3. *Oakley A. J., Lo B. M., Nuccetelli M. et al.* // J. Mol. Biol. – 1999. – **291**, N 4. – P. 913–926.
4. *Zhao X., Fan Y., Shen J. et al.* // Mol. Cells. – 2006. – **21**, N 3. – P. 395–400.
5. *Turella P., Pedersen J. Z., Caccuri A. M. et al.* // J. Biol. Chem. – 2003. – **278**, N 43. – P. 42294–42299.
6. *Song J. Z., Stirzaker C., Harrison J. et al.* // Oncogene. – 2002. – **21**, N 7. – P. 1048–1061.
7. *Diah S. K., Smitherman P. K., Townsend A. J. et al.* // Toxicol. Appl. Pharmacol. – 1999. – **157**, N 2. – P. 85–93.
8. *Moffat G. J., McLaren A. W., Wolf C. R.* // Biochem. J. – 1997. – **324** (Pt 1). – P. 91–95.
9. *Morceau F., Duvoix A., Delhalle S. et al.* // Biochem. Pharmacol. – 2004. – **67**, N 7. – P. 1227–1238.
10. *Morrow C. S., Goldsmith M. E., Cowan K. H.* // Gene. – 1990. – **88**, N 2. – P. 215–225.
11. *Nishinaka T., Ichijo Y., Ito M. et al.* // Toxicol. Lett. – 2007. – 170, N 3. – P. 238–247.
12. *Lo H. W., li-Osman F.* // J. Cell Biochem. – 2002. – **87**, N 1. – P. 103–116.
13. *Schnekenburger M., Morceau F., Duvoix A. et al.* // Biochem. Biophys. Res. Commun. – 2003. – **311**, N 4. – P. 815-821.
14. *Slonchak A. M., Cwieduk A., Rzerzowska-Wolny J. et al.* // Біополімери і клітина. – 2009. – **25**, № 3. – С. 210–217.
15. *Gaffney E. V.* // Cell Tissue Res. – 1982. – **227**, N 3. – P. 563–568.
16. *Pattillo R. A., Gey G. O.* // Cancer Res. – 1968. – **28**, N 7. – P. 1231–1236.
17. *Rahnama F., Shafiei F., Gluckman P. D. et al.* // Endocrinology. – 2006. – **147**, N 11. – P. 5275–5283.
18. *Laemmli U. K.* // Nature. – 1970. – **227**, N 5259. – P. 680-685.
19. *Gonzalgo M. L., Nakayama M., Lee S. M. et al.* // Urology. – 2004. – **63**, N 2. – P. 414–418.
20. *Duvoix A., Schmitz M., Schnekenburger M. et al.* // Biofactors. – 2003. – **17**, N 1–4. – P. 131–138.
21. *Montano M. M., Deng H., Liu M. et al.* // Oncogene. – 2004. – **23**, N 14. – P. 2442–2453.

Received 30.03.2009