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Intracellular Localization and Content of YB-1 Protein in Multidrug Resistant Tumor Cells

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Abstract—The multifunctional mammalian protein YB-1 is a member of the large DNA- and RNA-binding protein family with an evolutionarily ancient cold-shock domain. YB-1 is involved in multiple DNA- and mRNA-dependent events and regulates gene expression at various levels. It can be found both in the nucleus and the cytoplasm. Bound to DNA in the cell nucleus, YB-1 functions as a transcription factor interacting with inverted CCAAT-box (Y-box) in promoters and enhancers of multiple genes. In particular, YB-1 regulates activity of the multidrug resistance (MDR) genes *MDR1* and *LRP*. In tumors, YB-1 has been suggested to be an early and global marker of MDR. In this study, we compared amounts of *YB-1* mRNAs and intracellular localization of YB-1 protein in six pairs of drug sensitive and drug resistant sublines of diverse tumors. We have shown that neither great increase in the level of *YB-1* mRNA nor substantial increase in the number of cells with nuclear localization of YB-1 are obligatory traits of drug resistant tumor cell populations. However, the cells with highest amounts of *YB-1* mRNA also demonstrated increased quantities of *MDR1*, *MRP1*, *BCRP*, and *LRP* mRNAs encoding different MDR proteins. Transfection of two different populations of drug-sensitive cells with *YB-1* cDNA led to increase in the amount of *YB-1* mRNA. The quantities of *MRP1* and *LRP* mRNAs increased in both populations. Introduction of *YB-1* small hairpin RNA (shRNA) resulted in decreased amounts of *YB-1* mRNA, as well as *MRP1*, *LRP*, and *MDR1* mRNAs (in three different cell lines). Our data suggest that although YB-1 regulates several *MDR* genes, it could not be regarded as a global marker of already formed drug resistant tumor cell populations. It is most likely that at the first steps of MDR development YB-1 activity is necessary for propagation of resistant cell populations rather than for maintenance of drug resistance.

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Key words: YB-1 protein, mRNA, tumor cell, multidrug resistance, resistant cell, protein localization

Mammalian YB-1 protein is a member of multifunctional DNA/RNA-binding protein family with an evolutionarily ancient cold-shock domain [1-4]. Bacterial members of this family are known as the main cold-shock proteins. Both bacterial members [2] and mammalian YB-1 [5] are involved in adaptation of cells to growth at low temperature. YB-1 via its interaction with DNA acts as a transcription factor regulating expression of genes containing Y-box element in their enhancers and/or promoters, particularly expression of the main histocompatibility complex II gene [6], multidrug resistance gene *MDR1* [7], and genes encoding cyclins A and B1 [8]. YB-1 also participates in DNA repair, recombination, and replication [1]. This protein interacts with mRNA and is involved in its splicing in the nucleus [9, 10], it is the main packing protein for mRNA in cytoplasm [11], and it also regulates lifetime [12, 13] and template activity of mRNA in protein synthesis [14-20]. YB-1 has been shown to elevate cell resistance to ionizing radiation or chemical agents damaging DNA [21]. It is supposed to play a role of universal early marker of multidrug resistance of tumors [1, 22, 23].

Multidrug resistance (MDR) of tumor cells is a serious obstacle for chemotherapy of malignant tumors. MDR is the system of tumor cell defense against multiple drugs varying in chemical structure and mechanism of their action on cells [24-26]. Elevated activity of the

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MDR protein P-glycoprotein (Pgp; ABCB1) was the first discovered mechanism determining MDR. In succeeding years, other molecular mechanisms of MDR have been found, and the drug resistance of tumor cells has been recognized as a complex phenomenon based on a number of molecular changes, and several mechanisms modulating sensitivity of tumors to treatment can function simultaneously in the same cell [26]. The most studied mechanisms important for clinical practice are: (i) activation of transmembrane proteins excreting various chemical substances out of the cell; (ii) activation of enzymes involved in a detoxification system utilizing glutathione; (iii) alterations in genes and proteins involved into the control of apoptosis [24, 26].

Both elevation of YB-1 amount in tumor cells and its translocation from cytoplasm into the nucleus might play a role in the MDR phenomenon [1, 23]. In various tumors (breast cancer, lung cancer, osteosarcoma, and some others) the number of cells with nuclear localization of YB-1 is elevated in comparison with normal tissues, and this elevation was found to correlate with unfavorable progress of the diseases and resistance to chemotherapy [22, 27-31]. A number of chemotherapeutics were found to induce translocation of YB-1 from cytoplasm to the cell nucleus [7, 21]. The elevation of YB-1 content in breast cancer cells can result in overexpression of the *MDR1* gene encoding Pgp and in appearance of MDR [22]. However, the role of YB-1 in appearance and development of MDR remains unclear. In particular, systematized studies of resistant tumor cells are absent. A few works comparing individual resistant tumor cell lines with corresponding wild-type cells demonstrate that the amount of YB-1 protein is elevated in nuclei of the cultivated resistant cells of colon cancer HCT15, ovary cancer KFr, and breast cancer MCF-7/ADR compared with their sensitive variants [32, 33]. After introduction of genetic suppressor elements (GSE) into the cultivated cells with following selection in a medium with cytostatic, the culture acquired MDR accompanied by the elevation of mRNA expression level of various genes: the genes involved in DNA repair and replication as a stress response and the genes encoding transcription factors including *YB-1* [34]. Nonetheless, it is unclear how frequently the link between overexpression and/or nuclear localization of YB-1 and elevation of expression level of MDR genes and proteins occurs, and whether the YB-1 overexpression in resistant tumor cells simultaneously influences the expression of several different MDR genes. Such evidence would elucidate the involvement of YB-1 in regulation of complex multifactor MDR. Nothing is known about any link between YB-1 and resistance extent of tumor cells of various tissue origin. Results of the experiments would enable evaluation of the extent of YB-1 participation in MDR regulation and the stages of MDR development at which the participation of YB-1 is necessary. To answer these questions, we have studied the interrelationship between the content and localization of YB-1 and amounts of several MDR mRNAs/proteins, as well as levels of drug resistance in six pairs of sensitive and resistant cell lines of different histogenesis. We have shown that in resistant cell lines of various origin the number of cells with nuclear localization of YB-1 is elevated in comparison with parent variants; however, this amount can differ significantly. The content of mRNA/protein YB-1 in resistant cell variants is elevated sometimes, but not always, and does not correlate with the level of drug resistance in the cells. The elevated content of YB-1 can correlate with the elevated amounts of several mRNAs/proteins involved in MDR. We have also found that increase (as a result of stable transfection of cells with *YB-1*-containing construct) or decrease (under the influence of shRNA/*YB-1*) is accompanied, respectively, either by elevation or by decrease in contents of some mRNAs/proteins involved in MDR.

MATERIALS AND METHODS

Cell lines. The list of cell lines used in the study is given in the table [35-40]. The cells grew in the DMEM or RPMI1640 media containing 10% fetal calf serum at 37°C, 5% CO_2. The cells resistant to cytostatics were grown in 2-3 passages after defrost with a selective agent added into the medium. The selective agent was excluded from the medium no later than two weeks before experiments.

Isolation of RNA and RT-PCR. Total RNA was isolated using the TRI Reagent (Sigma, USA) according to the manufacturer's protocol. Reverse transcription was carried out with random primers. The housekeeping genes of β-actin and GAPDH were amplified for matching the cDNA levels in various samples. The following specific primers were used for actin, GAPDH, YB-1, MDRl, MRP1, BCRP, and LRP for amplification of corresponding length products:

actin (540 bp) GTGGGGCGCCCCAGGCACCA (forward)
CTCCTTAATGTCACGCACGATTTC (reverse)

GAPDH (513 bp) CCCCTGGCCAAGGTCATCCATGACAACTTT (forward)
GGCCATGAGGTCCACCACCCTGTTGCTGTA (reverse)

YB-1 (476 bp) ACAAGAAGGTCATCGCAACGAAG (forward)
GGTTGGAATACTGTGGTCGACG (reverse)

MDR1 (167 bp) CCCATCATTGCAATAGCAGG (forward)
GTTCAAACTTCTGCTCCTGA (reverse)

MRP1 (180 bp) ATCAAGACCGCTGTCATTGG (forward)
GAGCAAGGATGACTTGCAGG (reverse)

BCRP (172 bp) TGCCCAGGACTCAATGCAACAG (forward)
ACAATTTCAGGTAGGCAATTGTG (reverse)

LRP (405 bp) CCCCCATACCACTATATCCATGTG (forward)
TCGAAAAGCCACTGATCTCCTG (reverse).

Amplification conditions: 30 sec at 94°C followed by 25 cycles of 10 sec at 94°C, 10 sec at 60°C, and 10 sec at 72°C; then 1 min at 72°C. The amplification products in 20-μl volume of the reaction mixture were separated by electrophoresis in 2% agarose gel with ethidium bromide. The gel was photographed using a digital camera.

Immunocytochemical quantification of MDR proteins. To determine the contents of Pgp, MRP1, BCRP, and LRP proteins the cells were fixed by 4% formaldehyde and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. Then the cells were incubated with monoclonal antibodies (mABs) UIC2 (against Pgp), MRP1 mABs, BCRP mABs, and LRP mABs (Chemicon, USA) at 32°C for 30 min. After triple washing with PBS, the cells were incubated with FITC-conjugated goat antibodies against mouse IgG (Chemicon) for 1 h at 32°C. The cells were washed free of the second antibodies three times with PBS. The number of cells stained by the antibodies and cell fluorescence intensity were evaluated using a Becton Dickinson FACScan flow cytofluorometer (USA).

Immunocytochemical determination of cellular localization of YB-1. To determine intracellular localization of YB-1 the cells were grown in Petri dishes, 30 mm in diameter, on coverslips, fixed with 4% formaldehyde, washed three times with PBS, and the cellular membranes were permeabilized with 0.1% Triton X-100. Then the cells were incubated with rat polyclonal antibodies against YB-1 (the antibodies to YB-1 kindly provided by Dr. S. P. Domogatsky were raised by immunization of rats with synthetic peptide corresponding to 15 C-terminal amino acid residues of YB-1 "AENSSAPEAEQGGAE"). After the incubation with antibodies, the cells were washed three times with PBS. Then the cells were incubated with the second antibodies against the rat IgG labeled by TRITC (Sigma). The cells with cytoplasmic and nuclear localization of YB-1 were counted using a fluorescence microscope (Carl Zeiss, Germany). Each experimental point corresponds to at least 200 cells.

Immunoblotting. To determine the intracellular localization of YB-1, nuclear and cytoplasmic extracts were prepared from KB3-1 cells as described previously [41]. The concentrated lysing buffer (×5) was added to the extracts and incubated at 100°C for 5 min. The samples were stored at −20°C. The proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane with 0.2 μm pore diameter (Whatman) for 1 h at 300 mA in the following buffer: 25 mM TRIS-base, 90 mM glycine, 10% isopropanol, and 0.1% SDS, pH 8.5. To avoid nonspecific sorption of antibodies, the membrane was incubated for 16 h at 4°C in 5% solution of dry defatted milk dissolved in TBS: 10 mM Tris-HCl, pH 7.6, containing 140 mM NaCl, and then incubated with the antibodies to YB-1 at working dilution 1 : 5000 in the same buffer for 1.5 h at room temperature with gentle shaking. Unbound antibodies were washed off three times, 10 min each, in TBS with 0.05% Tween-20. Immunocomplexes were detected by incubation with rat anti-IgG conjugated with alkaline phosphatase (Promega, USA) for 1.5 h at room temperature. Unbound antibodies were washed off three times, 10 min each, in TBS with 0.05% Tween-20. Nitro Blue Tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were used as substrates for alkaline phosphatase. Chromogenic agents dissolved in 70% dimethylformamide were added into the buffer for alkaline phosphatase containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$ up to the final concentrations of 0.033% NBT and 0.0166% BCIP. The color was developed for 15 min, and the reaction was stopped by washing of the membrane in H_2O.

Transfection of KB3-1 and HCT116 cells with *YB-1* cDNA. The *YB-1* cDNA was cloned by the *Eco*RI restriction site into the vector pEGFP-N3 (Clontech, USA). Both KB3-1 and HCT116 cells ($3 \cdot 10^5$) were transfected with the plasmid pEGFP-N3/YB-1 or pEGFP-N3 (1 μg/ml medium) using Escort IV (2 μl/μg plasmid, Sigma-Aldrich, USA) according to the manufacturer's protocol. Both plasmids contain the gene *neo*. Eight hours after transfection, the serum-free medium was substituted by medium containing 10% serum. Stabile clones were selected in medium containing 400 μg/ml of G418 (Invitrogen, USA). The clones were isolated 14 days after transfection, which were resistant to G418 and expressed green fluorescent protein (GFP). After 5-6 passages in the presence of G418, the cells were tested using antibodies against the studied proteins or used for preparation of RNA.

Synthesis and transfection of shRNA/YB-1. shRNA was produced by transcription with DNA. DNA-oligonucleotides chemically synthesized by Sintol (Russia) were used as a template. The template (complementary) DNA strand contained a sequence of one of two chains of phage T7 RNA polymerase promoter and a sequence complementary to shRNA (5′- TAGTGAGAGTGGGGAAAA-GAATAGGTGGCATTCTTTTCCCCACTCTCACG-GTACCCTATAGTGAGTCGTATTA-3′). This DNA strand was annealed with the second chain of T7 RNA polymerase promoter (5′-TAATACGACTCACTATA-GGG-3′). Following transcription, the shRNA was purified by electrophoresis in polyacrylamide gel and anion-exchange chromatography. Thus prepared shRNA had

the following sequence: 5′-ggguaccGUGAGAGUGG-GGAAAAGAAugccaccuaUUCUUUUCCCCACU-CUCACua-3′ and was targeted to the segment 616-634 bp of YB-1 mRNA (GenBank Accession Number OCU16821). Transfection with shRNA was carried out using Escort IV (Sigma-Aldrich) according to the manufacturer's protocol. Cells ($5 \cdot 10^5$) were transfected with either 1 or 4 μg of shRNA/YB-1 and 8 μl of Escort IV. The amount of YB-1 mRNA was determined by RT-PCR in both initial cell population and 24 and 48 h after transfection.

Examination of cell sensitivity to cytostatics. The sensitivity of each cell line to various chemotherapeutic drugs was determined by the standard technique via growth inhibition. The cells were sown in 24-well plates, $2 \cdot 10^4$ cells per well, in a medium with various dosages of drugs. The active cells were counted in seven days by phase contrast microscopy. All the experiments were replicated twice.

RESULTS

Characteristics of studied cell lines. Twelve cultures of malignant cells of various histogenesis were used in the work. Pairs of the cell lines are listed in the table—parental cells (wild-type cells) in which the drug resistance index is unity, and their resistant derivatives. The resistant cells varied significantly in their levels of drug resistance. Since most reports consider YB-1 as Pgp-dependent MDR regulator, we have used cell lines that are usually considered as cells with Pgp-mediated MDR (pairs of cell lines from No. 1 to No. 5 in the table). One of the studied cell lines, K562/i-S9, was produced as a result of stable transfection of the parental cells with the *MDR1* gene. Other resistant cell lines were produced by selection in media containing various chemotherapeutic drugs. One of the cell lines, COR-23L/R, possessed MRP1-mediated MDR (No. 6 in the table). *YB-1* mRNA was reliably detectable in the sensitive variants of the studied cells by RT-PCR (Fig. 1a). *MRP1* mRNA was detected in all the sensitive variants (Fig. 1a) at various, but always satisfactorily high levels. *LRP* mRNA was detected in most cases (Fig. 1a). *LRP* mRNA was not detected only in hemoblastosis cells (cell line K562), this fact possibly reflecting tissue specificity of *LRP* gene expression. *MDR1* mRNA was found far more rarely (in three from six cell lines), yet rarely *BCRP* was detected. Thus, various patterns of MDR providing gene expression are noteworthy in parental cells suggesting possible links

Cell strains used in the study

No.	Cell strain	Drug resistance	Tumor*	Index of resistance** (selective agent***)	Reference
1	K562 K562/i-S9	— +	chronic myeloid leukemia	1 29 (vinblastine)****	[35]
2	KB3-1 KB8-5	— +	oral carcinoma	1 6 (colchicine)	[36]
3	MCF-7 MCF-7/ADR	— +	breast cancer	1 60 (adriablastine)	[37]
4	mS mS-0.5	— +	melanoma	1 130 (colchicine)	[38]
5	LIM 1215 LIM 1215/Act	— +	intestinal cancer	1 20 (actinomycin D)	[39]
6	COR-23L/P COR-23L/R	— +	lung cancer	1 23 (adriablastine)	[40]

* Neoplasm from which the parent cell culture was originated.

** Resistance index: ratio of LD_{50} for selective agent for resistant cells to LD_{50} for this agent for parental cells. LD_{50} is the dose of a substance causing death of 50% of the cells.

*** Selective agent: drug used for production of resistant cell sublines.

**** K562/i-S9 cells were produced without resorting to selective agent (via introduction of *MDR1* gene). Vinblastine was used for determination of resistance index.

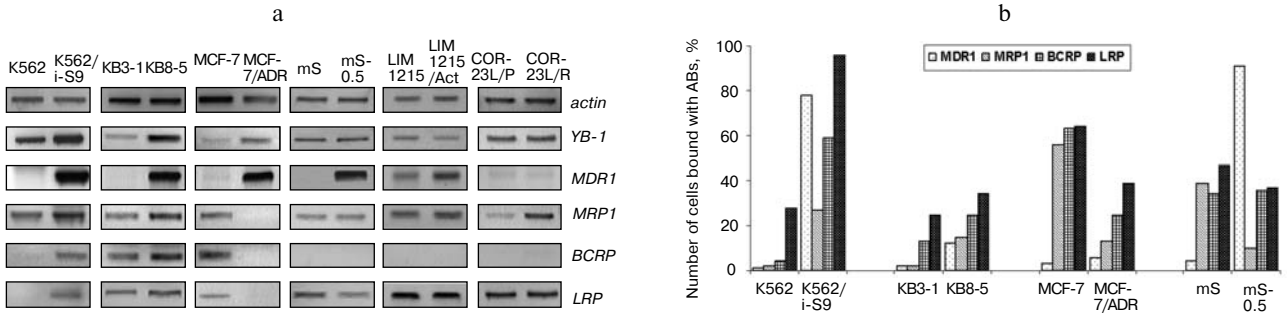

Fig. 1. Comparison of *YB-1* mRNA amount with amounts of mRNAs and proteins providing multiple drug resistance in antineoplastic drug sensitive and resistant tumor cell populations. a) mRNA amounts for *YB-1*, *MDR1*, *MRP1*, *BCRP*, *LRP* and *actin* (as a standard) determined by RT-PCR. b) Percentage of cells with detectable MDR proteins determined by flow cytofluorimetry after staining with corresponding antibodies.

between constitutive expression of these genes and histogenesis of tumor cells. The data on the resistant variants of the described cells with various extent of resistance against cytostatics is presented below.

***YB-1* mRNA content is elevated in some variants of tumor cells resistant to chemotherapeutic drugs.** Elevated amount of *YB-1* mRNA was observed in three of six studied resistant cell lines (KB8-5, MCF-7/ADR, and K562/i-S9) compared with sensitive variants (KB3-1, MCF-7, and K562) (Fig. 1a). In three other pairs of cell lines the level of *YB-1* mRNA was the same in sensitive (mS, COR-23L/P, and LIM1215) and resistant (mS-0.5, COR-23L/R, and LIM1215/Act) cell variants (Fig. 1a). Obviously, change in *YB-1* mRNA levels in resistant cells is not a necessary sign of drug resistance. It is obvious that the alteration of *YB-1* mRNA level does not correlate with the levels of drug resistance of the cells (compare MCF-7/ADR and KB8-5 possessing indexes of drug resistance equal to 60 and 6, respectively, see the table).

Elevation of *YB-1* mRNA content and change in MDR mRNAs/proteins in resistant cells. The mRNA contents were compared in sensitive and resistant cell pairs by RT-PCR for various *MDR* genes, including *MDR1*, *MRP1*, *BCRP*, and *LRP* (Fig. 1a). Numbers of cells containing Pgp, MRP1, BCRP, and LRP proteins were determined by flow cytofluorimetry after cell fixing and staining using specific antibodies (Fig. 1b).

Elevated amounts of all the studied MDR mRNAs/proteins (*MDR1*, *MRP1*, *BCRP*, and *LRP*) were found in two cell lines, K562/i-S9 and KB8-5, in which the content of *YB-1* mRNA was especially high. No alteration was found in *YB-1* mRNA in three resistant lines (mS-0.5, COR-23L/R, and LIM1215/Act) compared with sensitive parental cells. In two of them (mS-0.5 and LIM1215/Act), the content of *MDR1* mRNA only was elevated, and in one of them (COR-23L/R), the content of *MRP1* mRNA only was elevated.

The resistant breast cancer cell line MCF-7/ADR stands out because the elevated *YB-1* mRNA content is combined with elevation of mRNAs for *MDR1* and Pgp protein, but is accompanied by the decrease in mRNAs for *MRP1*, *BCRP*, and *LRP* and their protein contents (Fig. 1, a and b).

In general, an impression is formed of the lack of direct interrelationship between the expression levels of *YB-1* and *MDR* genes. Such interrelationship cannot be excluded for tumors of distinct type only. Our data suggest that *YB-1* expression is more prominent in those resistant cells in which the contents of several MDR mRNA/proteins are elevated.

The number of cells with nuclear localization of YB-1 is elevated in resistant cell lines. Localization of YB-1 protein was studied in three pairs of sensitive and resistant cells. Immunofluorescence microscopy demonstrated elevated numbers of cells with nuclear YB-1 localization in all three studied resistant cell lines (KB8-5, MCF-7/ADR, and mS-0.5) compared with their sensitive variants (Fig. 2b). The resistant melanoma cell line mS-0.5 is noteworthy because it contains the same *YB-1* mRNA level as its parental cell line. The results of staining of mS and mS-0.5 cells with antibodies against YB-1 are shown in the Fig. 2a. The number of cells with nuclear localization of YB-1 has grown substantially in the population of this culture (Fig. 2b). In KB8-5 culture, the number of cells with nuclear localization of YB-1 has grown weakly. To confirm this elevation, we evaluated the amount of YB-1 protein in cytoplasmic and nuclear fractions of sensitive and resistant cell variants of cell line KB by immunoblotting. We revealed some decrease in YB-1 amount in cytoplasmic extracts and increase of its amount in nuclear extracts in resistant cells KB8-5 in comparison with parental cells KB3-1 (Fig. 2c). Thus, the number of cells with YB-1 nuclear localization is elevated in populations of various resistant cell lines, although the number of these cells substantially varies in different cultures.

Introduction of cDNA of *YB-1* into sensitive cells KB3-1 and HCT116 increases the expression levels of *MRP1* and *LRP* genes. The sensitive cells KB3-1 and

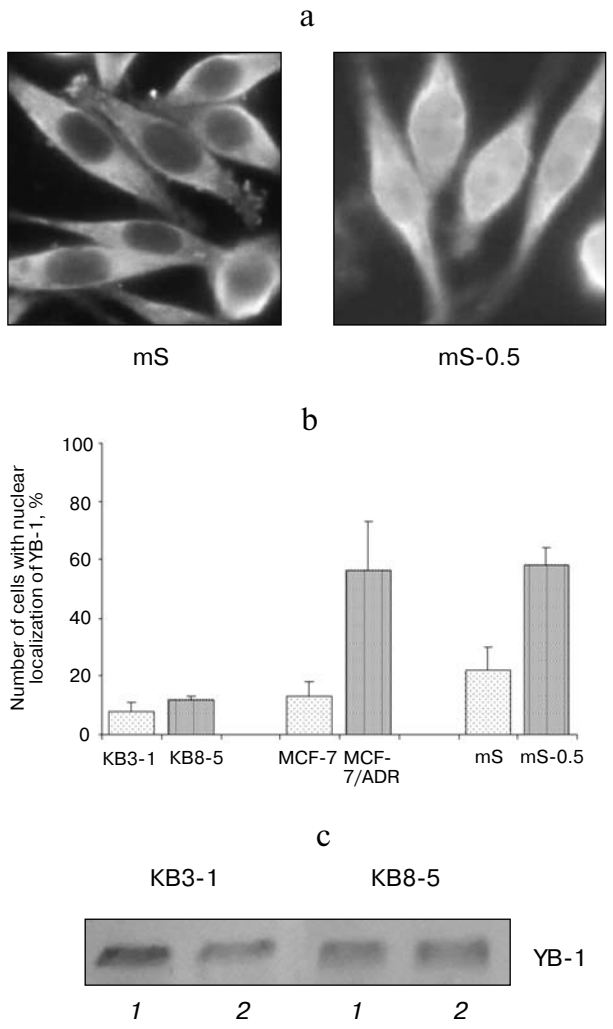

Fig. 2. Nuclear and cytoplasmic distribution of YB-1 in cell populations of sensitive and resistant lines. a) Immunofluorescence microscopy of human melanoma cells mS (parental line) and mS-0.5 (resistant subline) (magnification ×400). The cells were stained by polyclonal antibodies against YB-1 and TRITC-labeled second antibodies. b) Percentage of cells with nuclear localization of YB-1 in populations of tumor cells of various histogenesis: sensitive (KB3-1, MCF-7, and mS) and drug-resistant (KB8-5, MCF-7/ADR, and mS-0.5) cell lines. Cells were stained by polyclonal antibodies against YB-1 and second fluorescent antibodies and analyzed by fluorescence microscopy. c) Determination of YB-1 protein amount in cytoplasmic (*1*) and nuclear (*2*) extracts of sensitive (KB3-1) and resistant (KB8-5) variants of KB cells by immunoblotting.

HCT116 (human intestine adenocarcinoma cells) were transfected with either the experimental plasmid carrying a chimeric transgene *YB-1/GFP* or with the control plasmid carrying a transgene *GFP*. The clones of G418-resistant cells were selected after the incubation in the medium with G418. The elevated content of *YB-1* mRNA in clones transfected with *YB-1/GFP* in comparison with the control clone transfected with *GFP* is demonstrated by RT-PCR (Fig. 3, a and b). Increased levels of *MRP1* and *LRP* mRNAs, but not *MDR1* and *BCRP* mRNAs were observed in both clones of two different cell types with elevated level of *YB-1* mRNA (Fig. 3, a and b). These experiments may suggest that additional introduction of the *YB-1* gene into the cells influences differently the expression of various *MDR* genes.

Suppression of *YB-1* expression using shRNA is accompanied by decrease in the levels of *MRP1*, *LRP*, and *MDR1* mRNAs. The resistant cell lines MCF-7/ADR and mS-0.5, as well as sensitive cell line HCT116, were transfected with shRNA/*YB-1*. RNA was isolated from the initial and transfected cells 24 and 48 h after the transfection, and the contents of *YB-1* mRNA and various MDR mRNAs were determined by RT-PCR. It turned out that the decrease in *YB-1* mRNA amount under the action of shRNA/*YB-1* on the sensitive HCT116 cells induced a decrease in *MRP1* mRNA amount, but not *MDR1* or *LRP* mRNA amounts at 48 h (Fig. 4a). Inhibition of *YB-1* expression under the action of shRNA/*YB-1* in the resistant cells MCF-7/ADR is accompanied by a decrease in *MRP1* and *LRP* mRNA contents already in 24 h (Fig. 4b). The decrease in *YB-1* mRNA content under the action of shRNA/*YB-1* was accompanied by a decrease in *MDR1* mRNA content in melanoma cells mS-0.5, which are characterized by high level of drug resistance caused by *MDR1*/Pgp (Fig. 4c). Thus, the inhibition of *YB-1* expression under the action of shRNA/*YB-1* influences the contents of mRNA of various *MDR* genes, and this influence can differ in various cell types.

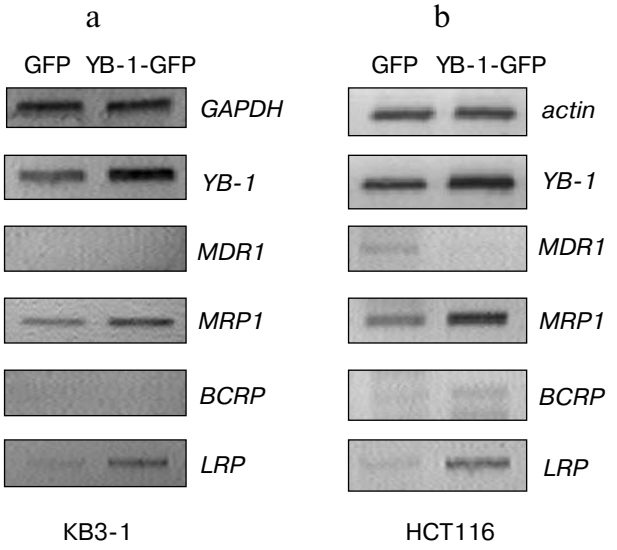

Fig. 3. Contents of *YB-1*, *MDR1*, *MRP1*, *BCRP*, and *LRP* mRNAs determined by RT-PCR in clones of KB3-1 (a) and HCT116 (b) cells stably transfected with *YB-1/GFP* transgene in comparison with the cell clone stably transfected by the control transgene *GFP*.

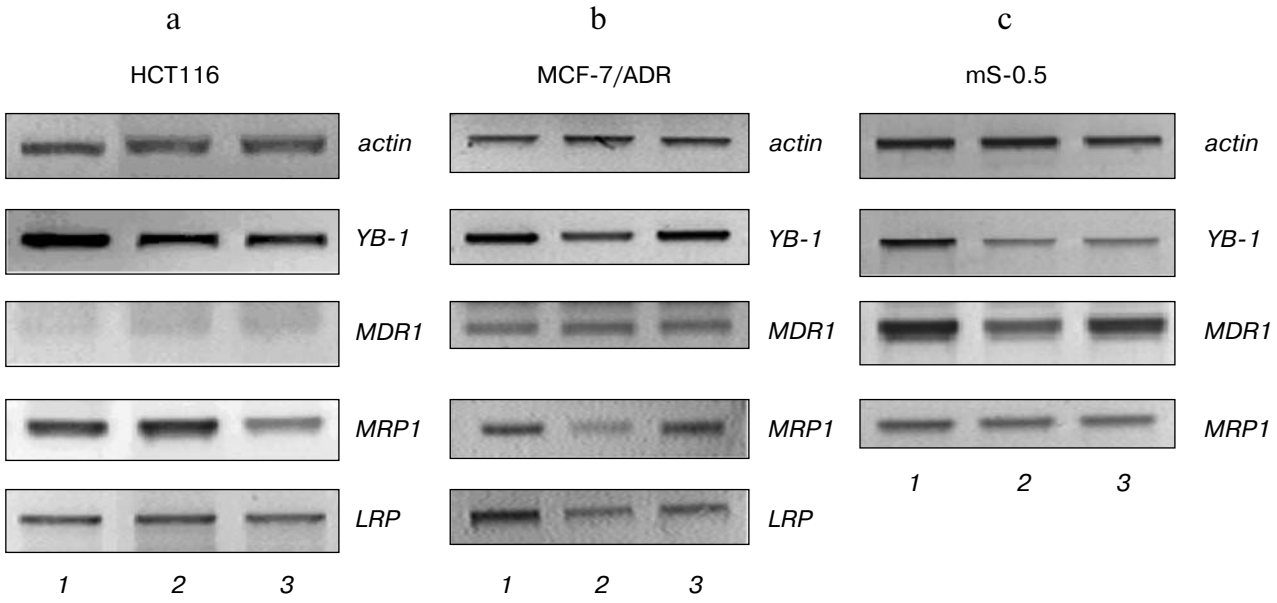

Fig. 4. Contents of *YB-1*, *MDR1*, *MRP1*, *BCRP*, and *LRP* mRNAs determined by RT-PCR in HCT116 cells (a), MCF-7/ADR cells (b), and mS-0.5 cells (c) after 0 (control) (*1*), 24 (*2*), and 48 h (*3*) of treatment with shRNA/*YB-1*.

DISCUSSION

The goal of this work was to study the role of YB-1 protein in regulation of multifactor MDR in tumor cells. We studied 12 tumor cell cultures including six pairs of sensitive and resistant variants to reveal a link between this protein and the content of mRNAs encoded by several *MDR* genes and the content of their proteins. The resistant lines were thought to be resistant due to overexpression of *MDR1*/Pgp (five lines) and *MRP1* (one line) (table). However, not only constitutive (basal) levels of mRNAs of genes putatively determining the drug resistance in these cells, but also levels of mRNA of some other genes and levels of proteins encoded by these genes were elevated in the resistant cells (Fig. 1). The elevation of expression of several *MDR* genes (*MDR1*, *MRP1*, and *BCRP*) in drug-resistant cells has been described earlier (for example, see [42, 43]). Thus, our data suggests that the resistant cells under our study possess multifactor MDR determined by several proteins of drug resistance.

Using the semiquantitative method of RT-PCR, we have shown the higher content of *YB-1* mRNA in three of six studied resistant cell variants compared with their parent drug-sensitive variants. More prominent differences between the parent and resistant cells were found in the cell lines KB8-5 (resistance index 6) and K562/i-S9 (resistance index 29) (Fig. 1a). The differences in *YB-1* mRNA content in sensitive and resistant variants were not large in the cell line MCF-7/ADR (Fig. 1a). The resistance index for these resistant cells was 60. These results demonstrate that *YB-1* mRNA content and the drug resistance indexes of tumor cells do not correlate. Substantially elevated *YB-1* mRNA content was observed at low level of drug resistance, which is comparable with that revealed in the clinical material (KB8-5). The absence of elevation of *YB-1* mRNA content in the resistant cells mS-0.5, COR-23L/R, and LIM1215/Act suggests that the *YB-1* overexpression often, but not always, occurs in drug resistant cells (Fig. 1a).

The studied cells were derived from tumors of different tissue origin (table). The elevation of *YB-1* mRNA was observed in both resistant cells of solid tumors and resistant cells of hemoblastosis, i.e., the elevation of *YB-1* mRNA in the resistant cells does not depend on their adhesion to substrate. The elevation of *YB-1* mRNA in resistant cells depends neither on the method of their production nor on selective agent: it was observed in cells produced via introduction of *MDR1* gene (K562/i-S9) and in cells selected using two different substances: colchicine (KB8-5) and adriablastine (MCF-7/ADR). The resistance obtained with the same selective agents in other cell lines (mS-0.5 and COR-23L/R, respectively) was not accompanied by elevation of *YB-1* mRNA. The elevation of *YB-1* mRNA is most probably associated with cell context. It is to be elucidated what changes of signaling pathways in tumor cells determine the "cell context" in the given case. It is clear, however, that the elevation of *YB-1* mRNA in the resistant cells is not a necessary feature of drug resistance and is not indicative for extent of drug resistance of cells.

It has been shown earlier that YB-1 can regulate at transcriptional level the expression of genes *MDR1*, *MRP1*, and *LRP*, whose protein products are involved in the phenomenon of MDR development [7, 32, 44]. We have studied the interrelation between the elevated *YB-1* mRNA level in resistant cells and the content of some mRNAs and proteins mediating MDR. The content of *YB-1* mRNA was most prominently elevated in two resistant cell lines with elevated mRNA contents for all the studied *MDR* genes (*MDR1*, *MRP1*, *BCRP*, and *LRP*), that is, in cell lines KB8-5 and K562/i-S9. The elevated content of *MDR1* mRNA and Pgp protein was found often, but not always, correlated with the elevated amount of *YB-1* mRNA in drug resistant tumor cells (in three of five cell lines). However, we also found that some elevation of *YB-1* mRNA may be associated not only with elevation, but also with decrease in *MRP1*, *BCRP*, and *LRP* mRNAs and their encoded protein contents (this was observed in MCF-7/ADR cell line). Obviously, the link between the contents of *YB-1* mRNA and MDR mRNA and proteins in resistant tumor cells is not so unequivocal, as it was believed earlier [1, 23].

To confirm the anticipation on possible involvement of YB-1 in MDR regulation, we have transfected sensitive cells KB3-1 and HCT116 with plasmid carrying cDNA of *YB-1/GFP* transgene. Then cell clones were selected demonstrating stable elevation of *YB-1* mRNA content in comparison with the cells transfected with control plasmid. The elevation of *YB-1* expression in KB3-1 and HCT116 cells proved to be accompanied by elevation of *MRP1* and *LRP* mRNA content. The treatment of sensitive HCT116 cells and resistant MCF-7/ADR cells with small hairpin RNA (shRNA) against *YB-1* mRNA resulted in a decrease in *YB-1* mRNA in various cell types at 24 or 48 h. This decrease in *YB-1* mRNA content was accompanied by a decrease in *MRP1* and *LRP* mRNA (in MCF-7/ADR cells) and by a decrease in *MRP1* mRNA content in HCT116 cells. Thus, the change in *YB-1* mRNA amount was accompanied by respective alteration in amounts of *MRP1* mRNA in various cell types. These data suggests that YB-1 can participate in transcriptional regulation of the *MRP1* gene. The amount of *MDR1* mRNA, which was several times higher than *MRP1* and *LRP* mRNA contents in MCF-7/ADR cells, was unchanged. The different mode of regulation of *MDR1*/Pgp and other genes and MDR proteins by *YB-1* in cells selected for Pgp-mediated MDR cannot be excluded. Several mechanisms may underlie the substantial elevation of mRNA and *MDR1*/Pgp protein contents in these cells, including transcriptional gene activation, its amplification, and mRNA stabilization, whereas other *MDR* genes are activated due to the elevation of transcription regulated by YB-1. However, experiments with mS-0.5 cells demonstrate that the decrease in *YB-1* mRNA under the influence of shRNA/*YB-1* can be accompanied by a change in *MDR1* mRNA amount (Fig. 4c). These results are evidence for the contemporary views on YB-1 participation in regulation of *MDR1* gene and suggest that this regulation is most probably dependent on cell type. Our data also demonstrates that an additional target for YB-1 is the gene encoding MRP1, another transport protein.

It is known that the translocation of YB-1 into the nucleus is necessary for exhibition of its transcriptional activity. A number of works are known where the nuclear localization of YB-1 is linked to appearance of resistant phenotype in tumor cells [21, 22, 33]. Therefore, we studied YB-1 localization in three pairs of sensitive and resistant cell lines of tumors of various histogenesis. The more frequent nuclear localization of YB-1 was observed in all the studied resistant cell lines compared with sensitive variants; however, the extent of translocation of YB-1 from cytoplasm into the nucleus was different in various cell lines (compare the KB8-5 cells with two other cell lines, Fig. 2b). Note that the portion of cells with nuclear localization of YB-1 was substantially elevated in the population of cell line mS-0.5, which did not differ in *YB-1* mRNA content from sensitive cell population. These data and the data on correlation between the amount of *YB-1* mRNA and the contents of various MDR mRNA/proteins suggest that the nuclear localization of YB-1 can be a virtual marker of resistance in tumor cells determined by various MDR proteins. This suggestion is now actively discussed [23]. However, in the development of a test for MDR using YB-1 as a marker, it is important to determine the amount of this protein (or its mRNA amount) in cells. Especially clear evidence for this necessity is the resistant cell line KB8-5, in which the number of cells with nuclear YB-1 location is only moderately increased (Fig. 2b), whereas the amount of *YB-1* mRNA is elevated substantially. Contrariwise, the amount of *YB-1* mRNA was not increased in mS-0.5 population, but the number of cells with nuclear *YB-1* location was increased significantly.

To summarize our data, it can be concluded that they suggest the involvement of YB-1 in regulation of the complex multifactorial phenomenon of MDR in tumor cells. However, its participation in regulation of various *MDR* genes in various cells may be associated with different mechanisms (at least it can be associated with increase in amount of YB-1 and its translocation into the nucleus from the cytoplasm). Our results also demonstrate the dependence of YB-1 influence on MDR of tumor cells on their peculiar features, that is, on cell context. Although our data suggests that YB-1 regulates the activity of the *MDR* gene group, this regulation can be scarcely regarded as a universal feature of already existing cell populations resistant to chemotherapeutic drugs. The activity of YB-1 would be expected to be necessary for the formation of new resistant cell populations rather than for maintenance of drug resistance.

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