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Regular paper

E2F site in the essential promoter region does not confer S phase-specific transcription of the *ABCC10* gene in human prostate cancer cells

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ABCC10 (MRP7) plays a role in cellular detoxification and resistance to anticancer drugs. Since *ABCC10* gene transcription in human prostate cancer CWR22Rv1 cells was found dependent on *E2F* binding sequence motif, *ABCC10* expression in G_1 and S phases of the cell cycle of CWR22Rv1 cells, was analyzed. The cells were synchronized in G_1 phase by double thymidine block and in S phase by thymidine/mimosine double block. *ABCC10* mRNA level was found to be similar in S phase-synchronized and asynchronous cell populations. In G_1 phase it decreased by 2.4- to 3-fold. It is thus inferred, that *ABCC10* expression in CWR22Rv1 cells is not S phase-specific but is primarily associated with cell proliferation.

Key words: ABCC10, MRP7, E2F, p107, RBL1, cell cycle, non-classical E2F target gene

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Abbreviations: ABCC10, ATP-binding cassette transporter C family member 10; MRP7, multidrug resistance protein 7

INTRODUCTION

ABCC10 is a member of the C family of ATP-binding cassette (ABC) transport proteins, also known as multidrug resistance protein 7 (MRP7). Its substrate specificity was determined to include amphiphiles: glucuronate conjugates (17β-estradiol-(17-β-D-glucuronide) and glutathione S-conjugates (leukotriene C4) (Chen *et al.*, 2003). As such, it is involved in cellular extrusion of toxic compounds. ABCC10 was also shown to be associated with resistance to a broad range of anticancer agents, taxanes, epothilone B, vinca alkaloids, antifolates and cistplatin (unpublished), daunorubicine, etoposide, irinotecan and nucleoside analogues (Bessho *et al.*, 2009; Hopper-Borge *et al.*, 2009, 2011; Oguri *et al.*, 2008). Among human organs, the *ABCC10* gene was found to be relatively highly expressed in testis (Hopper *et al.*, 2001).

E2F transcription factor binding site in cooperation with two *Sp1* factor binding sites, were identified as *cis* elements supporting basal *ABCC10* gene promoter activity in human prostate cancer CWR22Rv1 cells (Dabrowska & Sirotnak, 2004). E2Fs are traditionally known as key regulators of cell cycle progression into S phase. E2F-effector genes with cell cycle functions are referred to as traditional E2F targets (Dimova & Dyson, 2005). E2Fs are also known to control transcription of genes referred to as non-classical targets whose functions are not directly involved in cell cycle progression (e.g. p21-activated protein kinase, prolyl isomerase Pin1, neogenin), (Andrusiak *et al.*, 2011; Julian & Blais, 2015; Julian *et al.*, 2016; Ryo *et al.*, 2002; Sosa-Garcia *et al.*, 2015). According to a classical cell cycle regulation model by retinoblastoma RB/E2F pathway, E2F1-3 factors released from pocket protein pRB upon its phosphorylation by cyclin G_1-dependent kinase complexes (cyclin D/cdk4 and cyclin E/cdk2), transactivate transcription of target genes that mediate S phase entry (e.g. cyclin E, cyclin A2, phosphatase cdc25), and DNA replication (*e.g.* dihydrofolate reductase, thymidine kinase, thymidylate synthase, ribonucleotide reductase, DNA α/δ polymerases), (Bracken *et al.*, 2004). E2Fs may also act as transcription repressors mediating transition into G_1 and G_0 phases of the cell cycle. This function is ascribed mainly to constitutively expressed E2F-4 and E2F-5 factors complexed with different from pRB, pocket proteins, i.e. retinoblastoma-like protein 1 (RBL1, p107) and retinoblastoma-like protein 2 (RBL2, p130), (Dimova & Dyson, 2005). The pocket protein binding at *ABCC10* promoter *E2F* site in the nuclear extract of asynchronously growing CWR22Rv1 cells was identified as RBL1 (Dabrowska & Sirotnak, 2004).

ABCC10 gene is not apparently a cell cycle controlling factor. Nevertheless, its transcriptional regulation during the cell cycle of CWR22Rv1 cells was followed in the present study.

MATERIALS AND METHODS

Cell culture and synchronization. CWR22Rv1 cell line (ATCC) was maintained in RPMI1640 medium supplemented with 25 mM HEPES, 10% fetal bovine serum (Atlanta Biologicals, GA), 100 units/ml penicillin and 100 μg/ml streptomycin. Cell synchronization protocols were designed according to Spector and coworkers (1998). G_1 phase synchronization was attained by double-thymidine block. The cells were exposed twice, for 18 h each time, to 2 mM thymidine (SigmaAldrich), with 16 h culture in the regular medium in between. The S phase synchronization was attained by thymidine/mimosine double-block. The cells were exposed to 2 mM thymidine for 18 h, subsequently maintained for 16 h in the regular medium and treated with 400 μM L-mimosine (SigmaAldrich), for 18 h. After synchrony procedures, the cells were harvested by trypsinization at the indicated time points over 24 h, and fixed in absolute ethanol at –20°C for cell cycle analysis. The cells grown in parallel were harvested into Invitrogen TRIzol reagent, and stored at –80°C for RNA isolation.

FACS analysis. The fixed cells were rehydrated by double wash in Phosphate Buffered Saline, then stained

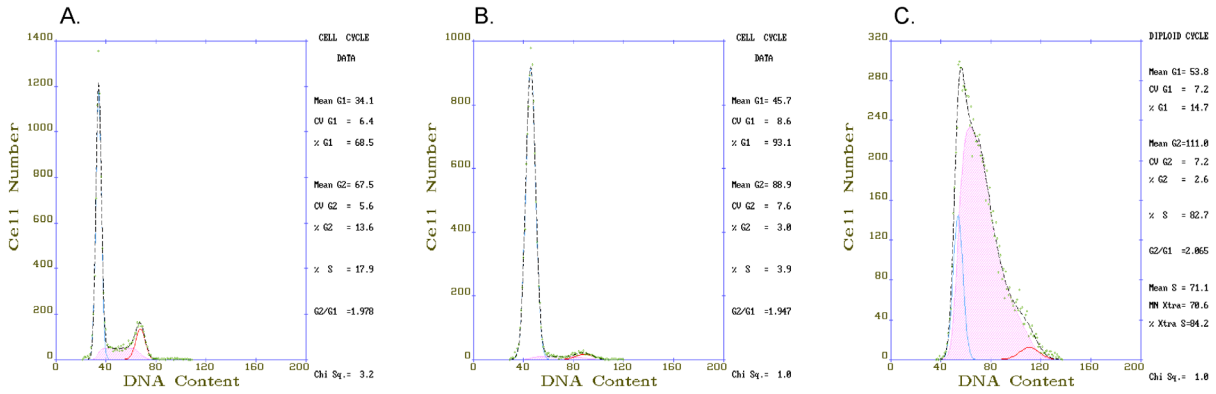

Figure 1. Histograms showing cell cycle distribution of CWR22Rv1 cell populations growing asynchronously (A), as well as synchronized in G_1- (B) and S- (C) phases.
The histograms correspond to the samples marked with asterisks in Tables 1 and 2.

with 50 μg/ml propidium iodide (SigmaAldrich) solution in 3.8 mM sodium citrate pH 7.0 containing 100 μg/ml boiled RNase A (Invitrogen). DNA level of 10 000 events per sample was measured on BD FACS Calibur flow cytometer. Cell cycle distribution was analyzed using MultiCycle AV, DNA analysis software (Phoenix Flow Systems, CA).

Quantitative RT-PCR. The cells frozen in TRIzol reagent served for RNA isolation followed by cDNA synthesis, as described by Dabrowska and Sirotnak (2004). Quantitative PCR was performed applying TaqMan probe-based assays, designed according to Khokhar and coworkers (2001), using β-actin (ACTB) as an endogenous reference gene. The reactions were run on ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). The results were calculated applying comparative threshold cycle (C_T) method according to ABI Prism 7700 SDS user bulletin#2, and are expressed as $2^{-\Delta CT}$ where $\Delta C_T = C_T$ (*ABCC10*) – C_T (*ACTB*).

Statistical analysis. The significance of differences in the *ABCC10* mRNA levels in synchronized *vs.* asynchronous cell population, was assessed in Statistica 12.5 software, using non-parametric Kruskal-Wallis test with $p<0.05$ considered significant.

RESULTS AND DISCUSSION

Cell synchronization by thymidine block relies on DNA synthesis inhibition caused by depletion in the deoxynucleotide pool, occurring as a result of ribonucleotide diphosphate reductase inhibition due to dTTP accumulation (Spector *et al.* 1998). Exposure to thymidine drives the cells into a block at G_1/S phase border. Plant amino acid mimosine also inhibits DNA synthesis by affecting nucleotide synthesis and blocks the cells at late G_1 or S phase (Chung *et al.*, 2012; Krude, 1999; Rosner *et al.*, 2013). 1 hour after release from the block the cells are considered to synchronously enter S phase (Spector *et al.* 1998). The synchrony approaches that allowed to obtain CWR22Rv1 cells blocked at G_1- and S-phases, were double-thymidine block and thymidine/mimosine double-block, respectively. CWR22Rv1 cell population consisting of 92–93% of cells in G_1 phase (*vs.* 69% in asynchronous log-phase culture), was obtained right af-

Table 1. Cell cycle distribution of CWR22Rv1 cells after G_1 phase synchronization. Asynchronous log-phase cell population is given as control. Asterisks indicate the samples used for *ABCC10* level quantification.

	Cell cycle phase		
Time	G_0/G_1 (%)	S (%)	G_2/M (%)
0	92.1	5.5	2.4
30 min	92.5	5.4	2.1
1 h*	93.1	3.9	3.0
2 h	89.2	9.5	1.3
4 h	31.0	67.7	1.3
6 h	33.0	62.1	4.8
8 h	32.3	23.3	44.4
12 h	28.6	26.1	45.3
16 h	33.3	23.9	42.8
20 h	48.6	21.7	29.8
24 h	58.3	19.2	22.5
Control*	68.5	17.9	13.6

Table 2. Cell cycle distribution of CWR22Rv1 cells after S phase synchronization. Asynchronous log-phase cell population is given as control. Asterisks indicate the samples used for *ABCC10* level quantification.

	Cell cycle phase		
Time	G_0/G_1 (%)	S (%)	G_2/M (%)
0	19.5	76.9	3.6
30 min*	14.7	82.7	2.6
1 h	17.8	72.5	9.7
2 h	15.3	51.5	33.2
4 h	19.2	35.8	45.0
6 h	25.5	13.8	60.6
8 h	49.4	11.2	39.4
12 h	73.6	10.4	16.0
16 h	80.1	10.0	9.8
20 h	81.0	10.5	8.4
24 h	80.9	13.7	5.4
Control*	68.5	17.9	13.6

Table 3. *ABCC10* expression in various CWR22Rv1 cell populations, assayed by quantitative RT-PCR and given as $2^{-\Delta CT}$ ± S.D. for N=3. *p<0.022.

CWR22Rv1 cell population	*ABCC10* level
asynchronous	2.00±0.00
G_1 phase-synchronized	0.67±0.06*
S phase-synchronized	1.60±0.10

ter the block release (Table 1, Fig. 1). After 4 h those cells significantly progressed into S phase (68% *vs.* 6% at time 0). The population containing 83% of cells in S phase (*vs.* 18% in asynchronous log-phase culture), was obtained 30 min after release from the mimosine block (Table 2, Fig. 1). After 2 h, a considerable fraction of cells (33% *vs.* 4% at time 0) progressed into G_2/M phase. The applied herein G_1 phase synchronization protocol differed from that applied in the case of LNCaP prostate cancer cells, by 7-hour longer growth in the regular media (Wang *et al.*, 2016). The protocol applied in the case of S phase synchronization was analogous to those used by others (Chung *et al.*, 2012; Li *et al.*, 2014).

The *ABCC10* transcript was found to be expressed at a nearly identical level in asynchronous log-phase and S phase-synchronized CWR22Rv1 cell populations (Table 3). Its expression diminished by 3-fold in the cells blocked in G_1 phase *vs.* asynchronous cell population. Eventually, it was 2.4-fold higher during S than G_1 phase. A complex containing RBL1 (p107), the pocket protein associated with repression of target gene transcription in G_1 phase (Henley & Dick, 2012), was previously identified in asynchronously growing CWR22Rv1 cells to bind *ABCC10* promoter *E2F* site (Dabrowska & Sirotnak, 2004). This is apparently the interaction exerting control of *ABCC10* transcription in asynchronous cell population and it could also be responsible for downregulation of *ABCC10* level in G_1 phase-synchronized cells. In accordance with a traditional model of RB/E2F-controlled transcription, *ABCC10* expression in S phase-synchronized cells was higher than in G_1 phase-synchronized cells. However, since it was unchanged in comparison to asynchronous cell population, *ABCC10* is apparently not an S phase-specific gene. The *trans* factors occupying *E2F* site of its promoter in S phase remain to be verified but in the light of findings challenging a traditional RB/E2F regulatory model, by proving redundancy in E2F functions and binding patterns (Xu *et al.*, 2007), it cannot be excluded that RBL1 is also involved in such an interaction at the *ABCC10* promoter during S phase. This reasoning remains in accord with E2F-4 factor, the main RBL1 binding partner, found to activate transcription of mitochondrial transporter *ABCB10* gene in human myelogenous leukemic cells (Karwaciak *et al.*, 2014). It is doubtful that lack of *ABCC10* upregulation in S phase-synchronized *vs.* asynchronous CWR22Rv1 cell population is evoked directly by the mimosine treatment, since the upregulation should be exactly expected under those conditions, as demonstrated for prototypic multidrug resistance protein, P-glycoprotein in the prostate cancer spheroids (Wartenberg *et al.*, 2002). Precise delineation of *ABCC10* gene transcriptional regulation appears reasonable in view of elaboration of perspective treatment options for multidrug-resistant cancers (Kathawala *et al.* 2015).

It can thus be concluded that functional *E2F* site in the essential promoter region of non-classical E2F target, *ABCC10* gene, does not confer an S phase-specific expression in CWR22Rv1 cells. *ABCC10* expression is primarily correlated not with the cell cycle progression but with regular growth of the cell population.

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