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FOS Promoter is Overactive Outside of Genome Context and Weakly Regulated by Changes in the Na^+_i/K^+_i Ratio

Andrey M. Gorbunov[1], Dmitrii A. Fedorov[1], Olga E. Kvitko[1], Olga D. Lopina[1], and Elizaveta A. Klimanova[1,a]*

[1]*Faculty of Biology, Lomonosov Moscow State University, 119234 Moscow, Russia*
[a]*e-mail: klimanova.ea@yandex.ru*

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Abstract—Changes in the Na^+ and K^+ intracellular concentrations affect expression of the *FOS* gene. Here, we obtained a genetic construct coding for the TurboGFP-dest1 protein under control of the human *FOS* promoter (−549 to +155) and studied its expression in HEK293T cells exposed to monovalent metal cations. Amplification of the *FOS* promoter sequence from genomic DNA was efficient only in the presence of Li^+ ions. Incubation of cells with ouabain or in a medium containing Li^+ ions instead of Na^+ ions caused intracellular accumulation of Na^+ and Li^+ ions, respectively. In addition, both stimuli increased the levels of endogenous *FOS* mRNA and the average fluorescence intensity of TurboGFP-dest1 in transfected cells. The mRNA levels of TurboGFP-dest1 were significantly higher than the *FOS* mRNA levels and were little affected by the stimuli.

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INTRODUCTION

The gradient of the Na^+ and K^+ ions between the extracellular medium and cytoplasm of animal cells is essential for the regulation of various cellular functions and is strictly maintained mainly by the activity of Na,K-ATPase [1]. However, some physiological and pathological conditions might cause changes in the intracellular concentrations of these cations, which could affect expression of certain genes [2], in particular, the early response gene *FOS* coding for the c-Fos transcription factor [3]. The baseline *FOS* expression is extremely low, but can increase dramatically in response to external stimuli. Transcription of *FOS* is controlled by multiple regulatory elements, including serum-response element (SRE), cAMP-responsive element (CRE), and activator protein 1 (AP-1) binding site [4].

As demonstrated in various types of mammalian cells, the effect of changes in the Na^+_i/K^+_i ratio on the gene expression is not mediated by the Ca^{2+}-dependent signaling or alterations in the membrane potential, intracellular pH, or cell volume [4-6]. Most of these genes, including *FOS*, are early response genes encoding transcription and translation factors [7]. The product of the *FOS* gene is the AP-1 family pioneer transcription factor c-FOS involved in the chromatin remodeling [8]. The study aimed at establishing the nature of the Na^+_i/K^+_i-sensitive regulation of *FOS* expression have shown that the promoter regulatory elements, such as SRE, CRE, and AP-1 binding site, are not involved in the transcriptional activation of gene expression driven by the *Fos* promoter in response to ouabain (specific inhibitor of Na,K-ATPase) [5]. By using genetic constructs carrying the human *FOS* gene with deletions in the promoter, Nakagawa et al. [9] were able to identify two sequences responsible for the ouabain-induced activation of transcription: the SRE element and a region between −222 and −123 bp upstream of the transcription start site (TSS) [9]. However, the authors did not investigate the mechanism of such regulation. It is important to note that according to the bioinformatic analysis, the identified region (−222 to −123) contained a sequence potentially capable of forming a G-quadruplex. G-quadruplexes are non-canonical secondary DNA structures formed

* To whom correspondence should be addressed.

by guanine-rich sequences [10] that can bind monovalent cations. The ability of monovalent metal ions to stabilize G-quadruplexes decreases in the following order: $K^+ > Rb^+ > Na^+ > Cs^+ > Li^+$ [11]. The presence of G-quadruplexes in the coding sequences and promoters of some Na^+_i/K^+_i-sensitive genes, including *FOS*, was predicted [12]. We hypothesize that these structures may be involved in the Na^+_i/K^+_i-dependent regulation of transcription.

To further investigate the role of G-quadruplexes in the regulation of *FOS* transcription, we obtained a genetic construct encoding TurboGFP-dest1 (a destabilized variant of TurboGFP) under control of the human *FOS* promoter and studied the effects of ouabain and Li^+ ions on the TurboGFP-dest1 expression in transfected HEK293T cells. Introduction of plasmid DNA encoding TurboGFP-dest1 into the cells caused Na^+_i accumulation, resulting in the hyperactivation of the human *FOS* promoter (−549 to +155 bp relatively to the TSS) and reduction of its sensitivity to monovalent metal cations.

MATERIALS AND METHODS

Genetic constructs. pFOS-TurboGFP and pRPLP0-TurboGFP reporter vectors were constructed that encoded TurboGFP-dest1 under control of the *FOS* or *RPLP0* promoters, respectively. The *RPLP0* promoter was chosen as a control because *RPLP0* belongs to housekeeping genes and its promoter is not sensitive to changes in the Na^+_i/K^+_i ratio, as well as lacks predicted G-quadruplexes. The promoter regions of *FOS* (−549 to +155 bp) and *RPLP0* (−700 to +295 bp) were amplified by PCR with an Encyclo Plus PCR kit (Eurogen, Russia) using genomic DNA from HeLa cells as a template and primers carrying *Hind*III and *Asi*GI restriction sites (Online Resource 1). The *FOS* promoter was amplified in a buffer proposed by Chashchina [13] in which KCl was replaced by LiCl (60 mM Tris-HCl, pH 9.5, 20 mM LiCl, 2.5 mM $MgCl_2$, 5% DMSO). PCR products were analyzed by sequencing. The *FOS* and *RPLP0* promoters were cloned into the promoterless peTurboGFP-PRL-dest1 vector (Eurogen) using *Hind*III and *Asi*GI restriction endonucleases (SibEnzyme, Russia). Full sequences and maps of the used plasmids are presented in the Online Resource 1.

Culturing and transfection of HEK293T cells. HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; PanEco, Russia) containing 4.5 g/L glucose, 0.58 g/L glutamine (PanEco, Russia), 10% fetal bovine serum (FBS) (Cytiva, USA), 100 unit/mL streptomycin and penicillin (Life Technologies, USA) at 37°C in a humidified atmosphere with 5% CO_2. Cell viability was determined using alamarBlue reagent (Thermo Fisher Scientific, USA) according to the manufacturer's recommendations. One day before the transfection, the cells were seeded in 24-well plates (10^5 cells in 500 µl of culture medium per well) pre-coated with poly-D-lysine (MP Biomedicals, USA) and cultured for 24 h. The next day, 2 h before the transfection, the cells were placed in the culture medium of the same composition but without antibiotics (500 µl per well), and then transfected with a mixture of 0.8 µg of plasmid DNA (pFOS-TurboGFP or pRPLP0-TurboGFP) and 2 µl of Lipofectamine 2000 (Invitrogen, USA) in 50 µl of Opti-MEM (Gibco, USA) per well and incubated for 24 h at 37°C in a humidified atmosphere and 5% CO_2. The medium was then replaced with low-serum DMEM (4.5 g/L glucose, 0.58 g/L glutamine, 0.1% FBS) and incubated for 24 h.

Cell treatment. For studying the effect of ouabain on the Na^+_i/K^+_i ratio, the cells were incubated in the presence of 1 µM ouabain for 3 h. For the intracellular accumulation of Li^+ ions, the cells were incubated for 5 h in the Li-medium containing 10 mM HEPES-LiOH (pH 7.4), 130 mM LiCl, 5.2 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 0.3 mM KH_2PO_4, and 6 mM glucose; Na-medium of the same composition, except containing 130 mM NaCl instead of 130 mM LiCl, was used as a control (the composition of the Na-medium mimicked the inorganic salt composition of DMEM).

Assessment of the intracellular content of Na^+, K^+, and Li^+ by atomic absorption spectrometry. The intracellular ion content was determined according to the previously described method [14] with some modifications. Briefly, at the end of incubation, the plates were placed on ice. The medium was discarded, after which the cells were washed three times with 3 ml of ice-cold 0.1 M $MgCl_2$. Next, 1.5 ml of 5% trichloroacetic acid (TCA) was added to the wells and incubated for several hours at 4°C. The contents of the wells were scraped off, transferred into microcentrifuge tubes, and centrifuged for 10 min at 18,000*g*. The supernatant was collected into separate tubes, the residual TCA was aspirated, and the precipitate was dissolved in 0.1 M NaOH containing 0.1% sodium deoxycholate. The amount of protein was determined by the Lowry method [15]. The intracellular content of Na^+, Li^+, and K^+ ions was measured by atomic absorption spectrometry using a Kvant-2m1 spectrometer (Kortek, Russia) in a propane-air mixture at 589.6, 670.8, and 766.5 nm, respectively. NaCl (0.05-2 mg/L Na^+), LiCl (0.05-2 mg/L Li^+), and KCl (0.5-2 mg/L K^+) solutions were used as standards. The contents of Na^+, Li^+, and K^+ in each sample were normalized to the protein content.

Laser scanning confocal microscopy and image analysis. HEK293T cells were transfected with pFOS-TurboGFP or pRPLP0-TurboGFP and subjected to experimental treatments as described above.

Cell microscopy was performed with an Olympus IX83P2ZF microscope (Japan) in the confocal imaging mode (diameter of confocal membrane aperture, 130 μm) at 37°C in 5% CO_2 in a live-cell incubation chamber (Tokai Hit, Japan) using a 10× objective with a numerical aperture of 0.4. Fluorescence emission was excited with a 488-nm laser (absorption maximum of turboGFP-dest1, 482 nm) and registered at 490-540 nm. The images acquired were processed with the Fiji/ImageJ 1.54f program. Individual cells were selected using "masks" by setting a critical intensity level to visually distinguish them from the background, and the average fluorescence intensity within the selected cells was calculated.

Analysis of pFOS-TurboGFP and pRPLP0-TurboGFP transcription by qPCR. The plates with the cells were placed on ice, the cells were washed with 1 ml of ice-cold Ca^{2+}- and Mg^{2+}-free Hank's salt solution, and lysed by adding 400 μl of Trizol reagent (Invitrogen) to each well. The lysates were treated with a chloroform-mixture; the aqueous phase was collected and used for RNA isolation with a Qiagen RNeasy kit (Netherlands). Reverse transcription was performed with an ImProm-II™ Reverse Transcription System kit (Promega, USA) according to the manufacturer's recommendations (0.5 μg of isolated RNA per reaction). qPCR was performed with a Bio-Rad Real-Time PCR System (Bio-Rad, USA) in the following regime: denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 10 s, primer annealing at 58°C for 10 s, and elongation at 72°C for 20 s. The primers were selected using the BLAST NCBI database and synthesized by Syntol (Russia). The primer sequences are given in the Online Resource 1. Gene expression levels were calculated by the ΔΔCt method using the *RPLP0* gene as a reference [16]. The average level of *FOS* expression in control samples was taken as 100%. PCR products were analyzed by sequencing.

Statistical analysis. The Origin software package (OriginLab Corporation, USA) was used for statistical data processing. The samples were tested for the normality of distribution using the Shapiro–Wilk test. To compare two independent groups, the Student's *t*-test for normally distributed samples and the Mann–Whitney U-test for non-normally distributed samples were used. Multiple comparisons for normally distributed data were performed using the one-factor analysis of variance (ANOVA) followed by the Tukey's test. The differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISSCUSSION

To investigate the mechanism of Na^+_i/K^+_i-dependent regulation of transcription, we constructed the pFOS-TurboGFP and pRPLP0-TurboGFP reporter vectors encoding TurboGFP-dest1 under control of the human *FOS* promoter (−549 and +155) and *RPLP0* promoter (−700 and +295 bp, no predicted G-quadruplexes), respectively (Fig. 1a). The sequences of the *FOS* and *RPLP0* promoters were amplified using genomic DNA from HeLa cells as a template. Importantly, we were able to amplify the *RPLP0* promoter using commercial buffer solution for Encyclo polymerase, while amplification of the *FOS* promoter under these conditions was unsuccessful. For this reason, we used a buffer for GC-rich sequences for the *FOS* promoter amplification; however, the length of the resulting PCR product was shorter than the expected one by 78 bp (Fig. 1b), presumably, due to the presence of G-quadruplexes in the *FOS* promoter sequence. G-quadruplexes are known to disrupt PCR in the presence of K^+ ions, and most commercial buffers for DNA polymerases contain K^+ ions. Hence, we used a modified buffer solution suggested by Chashchina et al. [13] containing Li^+ ions instead of K^+ ions, to successfully amplify the *FOS* promoter sequence.

Next, we evaluated the effect of transfection itself on the cell intracellular ion composition. Figure 2a shows that lipofection with pFOS-TurboGFP or pRPLP0-TurboGFP (but not the treatment with Lipofectamine 200 alone) was accompanied by a 40-60% increase in the Na^+_i concentration even 48 h after transfection. There are at least two factors that may be responsible for this effect. First, ion transport could be affected by the induction of interferon expression by foreign AT-rich DNA via an RNA polymerase III-dependent pathway [17]. On the other hand, expression of TurboGFP-dest1 could influence various intracellular functions. For example, TurboGFP-dest1 was found to affect the NF-κB signaling pathway in T cells, HEK293 cells, and HeLa cells [18]. It is possible that TurboGFP-dest1 expression can have an impact on the ion transport as well. However, the precise mechanisms of this phenomenon remain unclear.

To compare the effects of Li^+ and Na^+ ions on the *FOS* promoter activity, the cells were incubated in DMEM containing 0.1% FBS in the presence of 1 μM ouabain or in the Li-medium containing 135 mM Li^+. Figure 2b shows that incubation in the presence of 1 μM ouabain for 3 h caused an 11.5-fold decrease in the K^+_i concentration and an 11.8-fold increase in the Na^+_i concentration in the cells. Incubation in the Li-medium for 5 h led to the intracellular accumulation of Li^+ (to 740 nmol/mg protein), while the concentration of Na^+_i and K^+_i decreased by 85 and 90%, respectively, compared to the cells incubated in the Na-medium (Fig. 2c). Exposure to ouabain or Li-medium did not affect the cell viability (data not shown), although the morphology of cells in the Li-medium was slightly altered (Fig. 2d).

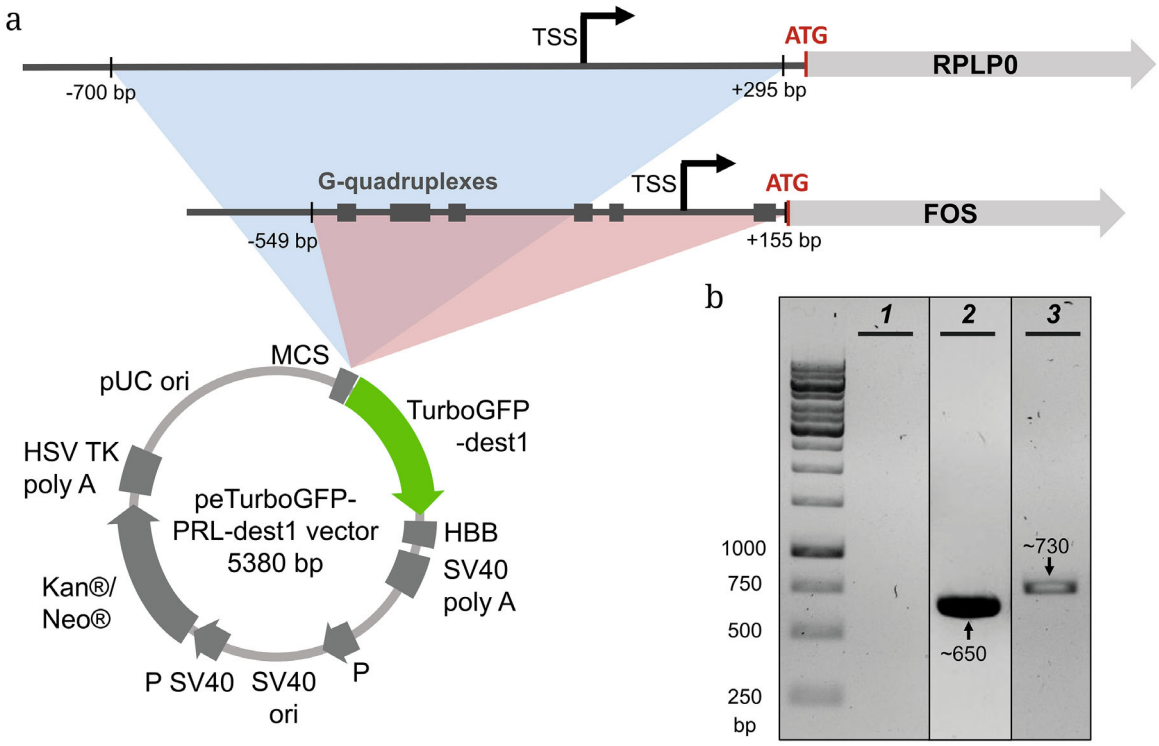

Fig. 1. Reporter vectors used to study the *FOS* promoter activity. a) pFOS-TurboGFP and pRPLP0-TurboGFP vectors encoding TurboGFP-dest1 under control of the *FOS* promoter (−549 to +155 bp) and *RPLP0* promoter (−700 to +295 bp), respectively. b) Electrophoresis of the *FOS* promoter amplification products obtained by PCR with different buffer solutions in 1.5% agarose gel stained with ethidium bromide: *1*, commercial buffer solution for Encyclo polymerase; *2*, commercial buffer solution for GC-rich sequences; *3*, modified buffer solution with K^+ ions substituted by Li^+ ions. The amplified DNA fragments contained *Hind*III and *Asi*GI restriction nuclease sites for subsequent cloning; expected length of PCR product, 728 bp; TSS, transcription start site.

To assess the activity of the *FOS* promoter in transfected cells, we measured the fluorescence intensity of TurboGFP-dest1 using confocal microscopy, as well as determined the levels of TurboGFP-dest1 mRNA by qPCR. Figure 3 shows that the median fluorescence intensity in the cells transfected with pFOS-TurboGFP and incubated in the presence of 1 μM ouabain or Li^+ (Li-medium) increased by 21 and 11%, respectively, while no significant changes in the fluorescence intensity were observed for the cells transfected with pRPLP0-TurboGFP. However, contrary to our expectations, the fluorescence intensity of cells transfected with pFOS-TurboGFP and pRPLP0-TurboGFP was very similar, despite the fact that the levels of endogenous *RPLP0* mRNA (Ct = 18.4 ± 0.12) were orders of magnitude higher compared to the levels of endogenous *FOS* mRNA (Ct = 28.3 ± 0.07). No significant differences were found between the TurboGFP-dest1 gene transcription levels (control *vs.* ouabain-treated cells and Na-medium *vs.* Li-medium samples) in case of both vectors. Incubation of cells transfected with pFOS-TurboGFP in the presence of ouabain and in the Li-medium increased the content of endogenous *FOS* mRNA 17 and 10 times, respectively (Fig. 4). It is important to note that introduction of plasmid DNA into the cells was accompanied by the increase in the content of Na^+_i ions, but had no effect on the amount of endogenous *FOS* mRNA (data not shown). This indicates that the elevated expression of TurboGFP-dest1 under the *FOS* promoter was not related to the experimental conditions, but rather resulted from the dysregulation of the *FOS* promoter activity and/or the presence of the TurboGFP-dest1 coding sequence in the used constructs. The levels of TurboGFP-dest1 mRNA were 1000-3000 times higher than the levels of endogenous *FOS* mRNA in all experimental and control samples. We believe that these results indicate overactivation of the *FOS* promoter in the pFOS-TurboGFP vector, so that its activity was comparable to that of promoters of the housekeeping genes (e.g., *RPLP0).* This might be the reason why the observed effect of the experimental treatments on the activity of the *FOS* promoter in the content of the reporter vector was insignificant.

Taking into account the above data, we can conclude that the plasmid carrying the coding sequence for TurboGFP-dest1 under control of the *FOS* promoter is not suitable for studying the role of G-quadruplexes

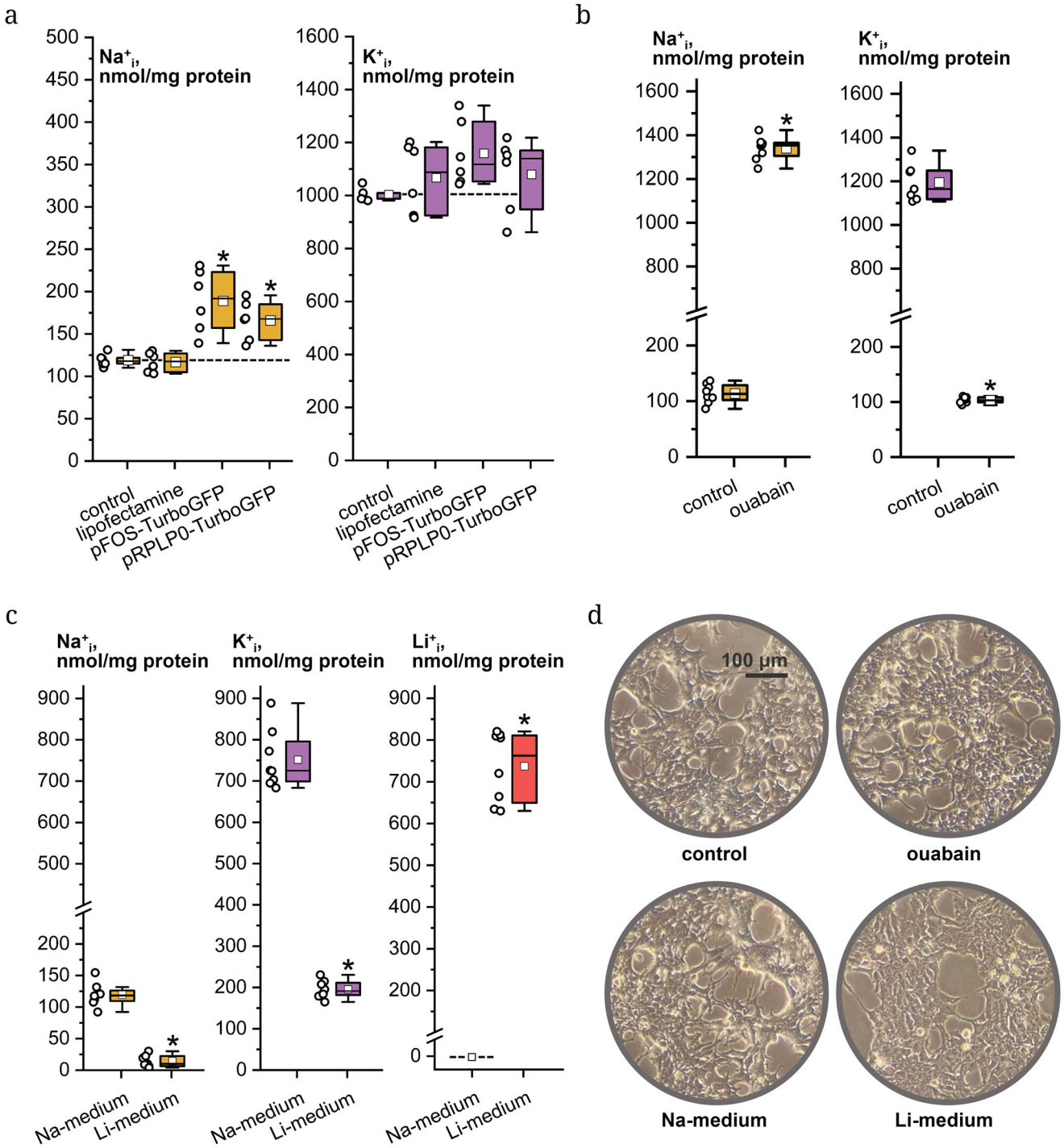

Fig. 2. Effect of different experimental treatments on the intracellular content of monovalent metal cations in HEK293T cells. a) Transfection of cells with the pFOS-TurboGFP and pRPLP0-TurboGFP vectors increased the content of Na^+_i by 60 and 40%, respectively, but had no significant effect on the content of K^+_i. Transfection with Lipofectamine 2000 alone did not affect the Na^+_i and K^+_i content. b) Incubation of cells in the presence of 1 μM ouabain for 3 h resulted in ~12-fold increase in the Na^+_i content, but significantly decreased the content of K^+_i. c) Incubated of cells in the Li-medium (135 mM Li^+, 5.5 mM K^+) for 5 h led to a considerable intracellular accumulation of Li^+ (up to 740 nmol/mg protein) and reduction in the Na^+_i and K^+_i content compared to cells incubated in the Na-medium (135 mM Na^+ and 5.5 mM K^+). d) Phase-contrast images of cells incubated in the presence of 1 μM ouabain for 3 h, in Na-medium for 5 h, or in Li-medium for 5 h. The data are presented as individual experimental values (n = 6-8) and box plots with whiskers equal to 1.5 interquartile range. Statistically significant differences were identified using the t-test or ANOVA followed by the Tukey's test (* $p < 0.05$).

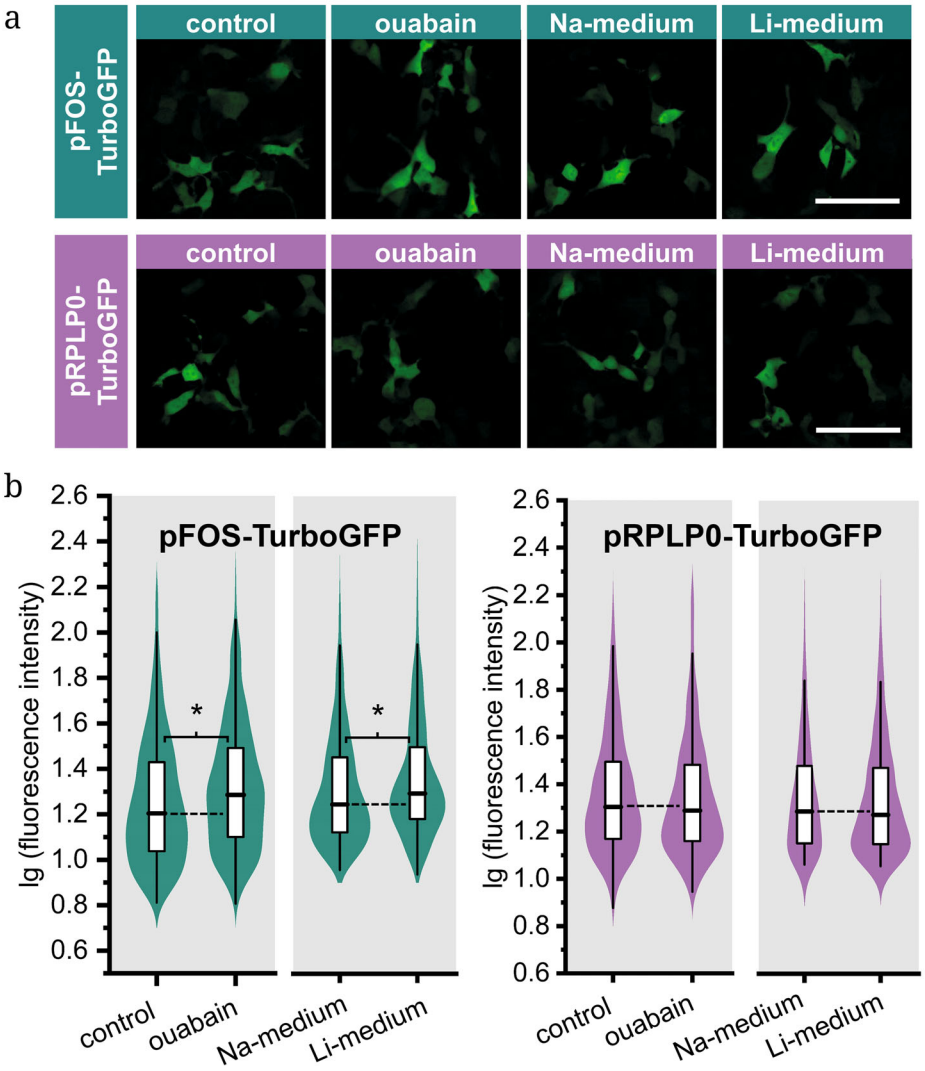

Fig. 3. Expression of the TurboGFP-dest1 reporter protein under control of the *FOS* or *RPLP0* promoters. a) Confocal images of cells transfected with pFOS-TurboGFP and pRPLP0-TurboGFP. Two days after transfection, the cells were incubated in the presence of 1 μM ouabain for 3 h, in Li-medium for 5 h, or in Na-medium for 5 h. Scale bars: 100 μm. b) Distribution of TurboGFP-dest1 mean fluorescence intensity in individual cells (n = 500-1000). Statistical analysis revealed a small but significant increase in the reporter protein fluorescence in response to ouabain compared to the control (DMEM). A similar effect was observed in the cells incubated in the Li-medium compared to the Na-medium. Statistically significant differences were detected using the Mann–Whitney U-test (* $p < 0.05$).

in the regulation of *FOS* expression by monovalent cations. Apparently, it is necessary to create a genetic construct that, in addition to the *FOS* promoter and 5′-UTR, would include the *FOS*-coding sequence and the 3′-UTR rather than the reporter gene.

The effect of Na^+_i/K^+_i ratio on the *FOS* promoter activity has been studied before. Using a vector containing the firefly luciferase gene under control of the *FOS* 5′-UTR (−1264 to +103 bp), Haloui et al. found that ouabain did not affect the expression of luciferase [4], which is consistent with our results. In another study, ouabain caused a significant increase in the *FOS* transcription level (in this case, the full-length human *FOS* gene was used) [9]. Based on the results of these studies and our findings, we hypothesize that regulatory elements in the *FOS* coding sequence may deactivate transcription during the cell resting state. The first intron of the *FOS* gene contains a strong transcription elongation block (+363 to +387 bp) [19-22], which suppressed the basal expression of not only the *FOS* promoter, but also of the "strong" metallothionein-2 gene promoter placed in the vector together with the *FOS* coding sequence [21]. It is likely that the high activity of the *FOS* promoter in plasmid DNA can be explained by the absence of regulatory elements normally located at a distance from the *FOS* gene in the genome or by the differences in the modification of plasmid and genomic DNAs.

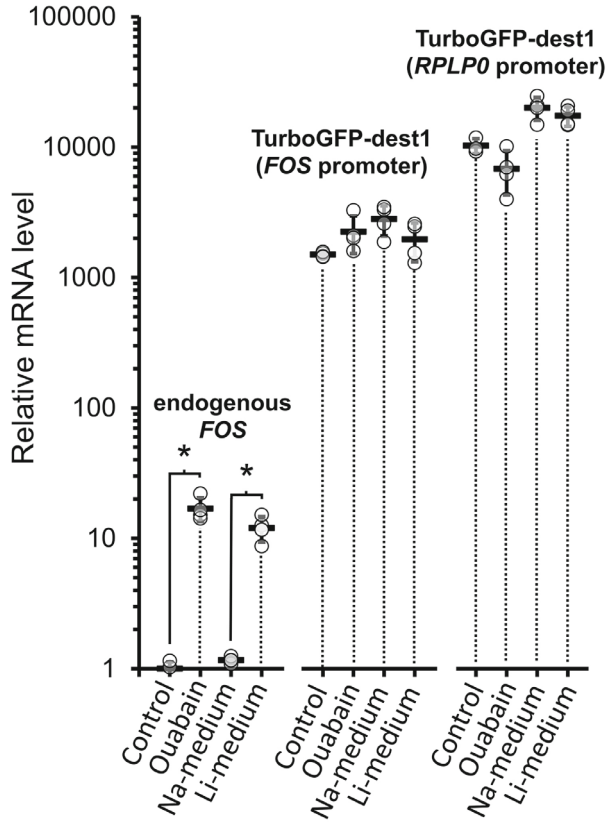

Fig. 4. Expression of endogenous *FOS* mRNA and TurboGFP-dest1 mRNA under control of the *FOS* and *RPLP0* promoters in cells transfected with the pFOS-TurboGFP or pRPLP0-TurboGFP vectors. Two days after transfection, the cells were incubated in the presence of 1 μM ouabain for 3 h, in Li-medium for 5 h, or in Na-medium for 5 h. The levels of endogenous *FOS* and TurboGFP-dest1 mRNAs were measured in cell lysates by qPCR. The data are presented as geometric mean (GM) with whiskers from GM×SD to GM/SD and individual experimental values (overlapping; n = 4). Statistical analysis revealed a significant increase in the levels of endogenous *FOS* mRNA in response to ouabain and Li-medium compared to the control and Na-medium, respectively. Statistically significant differences were detected using the t-test (* $p < 0.05$).

It is also important to take into account possible differences in the post-transcriptional regulation of the TurboGFP-dest1 and FOS expression, for example, different stability of the reporter protein mRNA. However, these assumptions need further investigation.

CONCLUSIONS

Using a genetic construct encoding TurboGFP-dest1 under control of the *FOS* promoter, we demonstrated that ouabain increased the activity of the *FOS* promoter in HEK293T cells and caused intracellular accumulation of Li^+ ions. The levels of the TurboGFP-dest1 gene expression in the cells transfected with the obtained construct were extremely high and insensitive to changes in the intracellular ion composition. It is likely that repression of the *FOS* expression during the cell resting state requires regulatory elements located outside of the *FOS* promoter region. We also found that lipofection of HEK293T cells with the obtained genetic constructs resulted in the Na^+_i accumulation, which may be due to the activation of cellular response to foreign DNA or a nonspecific effect of TurboGFP-dest1 on the ion transport.

Supplementary information. The online version contains supplementary material available at https://doi.org/10.1134/S0006297925600371.

Contributions. E.A.K. developed the concept, supervised the study, and edited the manuscript; A.M.G., D.A.F., and O.E.K. planned and performed experiments and prepared the manuscript; O.D.L. developed the study conception and edited the manuscript.

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Ethics approval and consent to participate. This work does not contain any studies involving human and animal subjects.

Conflict of interest. The authors of this work declare that they have no conflicts of interest.

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