ISSN 0006-2979, Biochemistry (Moscow), 2010, Vol. 75, No. 6, pp. 777-783. © Pleiades Publishing, Ltd., 2010.
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Mitochondrial DNA Transcription in Mouse Liver, Skeletal Muscle, and Brain Following Lethal X-Ray Irradiation

N. E. Gubina*, O. S. Merekina, and T. E. Ushakova

Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, ul. Institutskaya 3, 142290 Pushchino, Moscow Region, Russia; fax: +7(4967)330-553; E-mail: windhavenn@gmail.com

Received September 22, 2009
Revision received November 5, 2009

Abstract—Using quantitative real-time PCR, the levels of mitochondrial DNA transcripts in murine tissues (skeletal muscle, liver, and brain) were determined at different time points (1, 5, and 24 h) following X-ray irradiation at the dose of 10 Gy. One hour after irradiation the levels of mitochondrial transcripts *ND2*, *ND4*, *CYTB*, and *ATP6* dramatically decreased by 85-95% and remained at the same minimum level for 24 h in all analyzed tissues. This decrease was not associated with depletion of mtDNA as a matrix for transcription, since mtDNA copy number increased after irradiation in all tissues. The decrease in mitochondrial transcription in liver, brain, and skeletal muscle did not generally result from the damage of cell transcription apparatus, because the transcription level of nuclear housekeeping gene *BETA-ACTIN* remained virtually unchanged after irradiation. The mitochondrial gene transcription decreased after irradiation in the same manner as that of the nuclear gene *TFB2M* encoding mitochondrial transcription factor, whose regulatory role under normal conditions is well understood.

DOI: 10.1134/S0006297910060131

Key words: mtDNA, transcription, X-ray irradiation, real time PCR, murine tissue

Mitochondria are eukaryotic cell organelles whose major role is ATP supply. Their important feature is their own genetic apparatus, mitochondrial DNA (mtDNA). Mammalian mtDNA is a circular double-stranded molecule, about 16 kb in size, localized in the mitochondrial matrix [1]. Complementary mtDNA strands differ in GC composition and are called H-strand (heavy, with preponderance of purines) and L-strand (light, with preponderance of pyrimidines), respectively. From one point of view, the mtDNA in association with proteins is attached to the inner mitochondrial membrane as a "mitochondrial nucleoid" [2].

Mammalian mtDNA contains 37 genes encoding 13 transcripts for electron transport chain proteins, two rRNAs (12S and 16S), and 22 tRNAs required for autonomous synthesis of these proteins [3]. Both mtDNA strands carry genetic information, but its major part encoding 12 mRNAs, 2 rRNAs, and 12 tRNAs is in the H-strand (the L-strand encodes only one mRNA and 10 tRNAs) [4]. Genomic organization of mammalian mtDNAs is characterized by high compactness: introns are absent, and coding sequences are separated by a few bases or even overlap each other (*ND4* and *ND4L*, *ATP6* and *ATP8*) [1].

Unlike most eukaryotic genes, each representing an individual transcription unit, the mtDNA genes are transcribed as two polycistronic products of H- and L-strand, respectively [5]. Promoters for mtDNA transcription—HSP (heavy strand promoter) and LSP (light strand promoter)—are functionally independent and localized in the area of so-called D-loop, the major mtDNA noncoding region [5]. Mitochondrial transcription processes are coupled with replication of mtDNA. A small RNA fragment synthesized from the LSP remains in association with DNA in the GC-rich area upstream from the H-strand replication origin. The products of specific processing of this transcript serve as primers for H-strand replication [6].

Transcription of mtDNA requires mitochondrial RNA-polymerase and at least three transcription factors: mtTFA (TFAM), TFB1M and/or TFB2M for initiation, and mTERF for termination of transcription. Mitochondrial RNA-polymerase (mtRNApol) of mammals is a protein with M_r about 120 kDa that has homology with yeast and bacteriophage RNA-polymerases [7]. The mtRNA-polymerase possesses either low or unspe-

* To whom correspondence should be addressed.

cific activity in the absence of transcription factors. The key role in mammalian mtDNA transcription initiation is attributed to the mtTFA protein (24 kDa), which specifically binds to mtDNA to bend and untwist it [8]. Both TFB1M and TFB2M interact with the mtTFA *C*-terminal domain and are necessary for recognition of the promoter by mtRNA-polymerase [9]. Lastly, the mtDNA transcription termination is implemented by the 34-kDa protein mTERF [10]. It was supposed earlier that it acts as a physical barrier, but researchers recently incline to an opinion on specific interaction between mTERF and mtRNApol [4].

The mitochondrial transcript processing is synchronized with transcription, as it occurs with nuclear mRNA precursors [11]. The recognition signal for processing endonucleases is a characteristic "clover leaf" structure of tRNA that is already formed at the stage of transcription [1]. The final products of mtDNA transcription are featured by a series of structural peculiarities. In particular, mitochondrial rRNAs are smaller than their cytoplasmic or bacterial homologs, both rRNAs undergo methylation, and 16S rRNA contains a short poly-A tail of one to ten residues length [12]. Unlike cytoplasmic ones, the mitochondrial mRNAs do not undergo capping and do not contain 5′- and 3′-untranslated regions, and their poly-A tails averaging 55 nucleotides begin directly after the stop-codon [13]. Polyadenylation fulfilled by the mitochondrial poly-A polymerase is necessary for RNA stabilization [14].

Thus, both the structure and general characteristics of normal mtDNA transcription are now known.

Mitochondria are implicated in progression of many pathological processes such as neurodegenerative and neuromuscular disorders, ischemia, carcinogenesis, diabetes, and premature ageing [15-17]. The background for these processes is attributed to mtDNA lesions caused by genotoxic agents and high doses of free radicals [17, 18]. Mitochondrial protein mutants cause uncoupling of the respiratory chain leading to production of reactive oxygen species, increase in number of mtDNA breaks, and, finally, to the state called mitochondrial dysfunction [19]. Mitochondrial dysfunction can result not only from disturbance of mtDNA structure, but also from some events at the level of mitochondrial gene transcription, but it remains unclear how mtDNA transcription occurs under oxidative stress.

In this work we present data from studies on transcription of mitochondrial genes *ND2*, *ND4*, *CYTB*, and *ATP6* and mtDNA transcription initiation factor gene *TFB2M* in brain, liver, and skeletal muscle cells after X-ray irradiation of mice at the lethal dose of 10 Gy.

MATERIALS AND METHODS

Animals and irradiation. Male Balb/c mice were from the experimental animal nursery of the Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences (Pushchino, Moscow Region).

The animals were irradiated using a RUT-250-15-1 (X-ray therapeutic unit with movable beam) at an exposure rate of 2 Gy/min; the final dose was 10 Gy. Following irradiation, mice were kept under standard conditions of the ITEB RAS vivarium. A certain times after irradiation (1, 5, 24, and 72 h) mice were sacrificed by neck dislocation, and tissues isolated (brain, liver, and femoris) were washed with cold saline for experiment.

Liver mitochondria from the unirradiated control mice were isolated by differential centrifugation [20]. Thus prepared mitochondria were used for isolation of mtDNA.

Total and mitochondrial DNAs were isolated by phenol–chloroform separation followed by precipitation with ethanol; the pellet of DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

Isolation of RNA and synthesis of the first cDNA strand. Total RNA was isolated from each tissue by phenol–chloroform separation using guanidine thiocyanate [21]. Before reverse transcription, RNA samples were treated with DNase I (Sileks, Russia). Following incubation with the enzyme for 30 min at 37°C, DNase I was inactivated by heating for 5 min at 65°C. The RNA samples were tested for purity (absence of mtDNA) by real-time PCR with mitochondrial gene-specific primers.

cDNA was synthesized using the RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas, Lithuania) according to the manufacturer's protocol. Oligo-d$(T)_{18}$ was used as primer. The single-stranded cDNA products were used as templates for real-time PCR. All PCR experiments were performed on the same cDNA sample for each mouse.

Real-time PCR. The number of cDNA and mitochondrial and nuclear gene fragments was evaluated by real-time PCR (RT-PCR) using a 7500 Real-Time PCR System (Applied Biosystems, USA) and TaqMan probes (Sintol, Russia). Mitochondrial cDNAs and cDNA of the reference *BETA-ACTIN* gene were amplified separately (in individual tubes).

The RT-PCR reaction mixture contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.25 mM dNTPs, 2.5 mM Mg^{2+}, 250 nM of each primer and probe, 1.5 U of Taq-polymerase (Fermentas), and 2 μl of either cDNA or DNA as a template. The samples were heated for 10 min at 95°C followed by 45 cycles of amplification of cDNA or gene fragments: 95°C for 15 sec and 62°C for 1 min. Reporter fluorescence was recorded at the elongation stage (62°C).

The primers and probes for amplification of cDNAs and fragments of mitochondrial and nuclear genes *ND2*, *ND4*, *CYTB*, *ATP6*, *BETA-ACTIN*, and *TFB2M*, as well as *D-LOOP* fragment, are shown in the table. Primers were constructed using Primer Express 2.0 software such that the amplified fragment was localized near the 3′-end of the mRNA.

Primers and probes for RT-PCR

Oligonucleotide	5′-3′ nucleotide sequence
ND2 FOR	CAC GAT CAA CTG AAG CAG CAA
ND2 REV	ACG ATG GCC AGG AGG ATA ATT
ND2 PROBE	**FAM**-AAA TAC TTC GTC ACA CAA GCA ACA GCC T-**BHQ1**
ND4 FOR	ATT ATT ATT ACC CGA TGA GGG AAC C
ND4 REV	ATT AAG ATG AGG GCA ATT AGC AGT
ND4 PROBE	**ROX**-ACG CCT AAA CGC AGG GAT TTA TTT CCT A-**BHQ2**
CYTB FOR	GCC ACC TTG ACC CGA TTC T
CYTB REV	TTG CTA GGG CCG CGA TAA T
CYTB PROBE	**ROX**-CGC TTT CCA CTT CAT CTT ACC ATT-**BHQ2**
ATP6 FOR	AAT TAC AGG CTT CCG ACA CAA AC
ATP6 REV	TGG AAT TAG TGA AAT TGG AGT TCC
ATP6 PROBE	**Cy5**-AAA AGC TCA CTT GCC CAC TTC CTT CCA-**BHQ2**
D-LOOP FOR	AAT CTA CCA TCC TCC GTG AAA CC
D-LOOP REV	GCC CGG AGC GAG AAG AG
D-LOOP PROBE	**R6G**-ACA ACC CGC CCA CCA ATG CC-**BHQ1**
TFB2M FOR	GCC GTT GCC TGA TTC TGA TTT
TFB2M REV	GCT CCG ATC GAT TCC TGG AT
TFB2M PROBE	**TAMRA** AGG AGT CGT CCC CGT GGA **BHQ2**
BETA-ACTIN FOR	AGC CAT GTA CGT AGC CAT CCA
BETA-ACTIN REV	TCT CCG GAG TCC ATC ACA ATG
BETA-ACTIN PROBE	**FAM** TGT CCC TGT ATG CCT CTG GTC GTA CCA C-**BHQ1**

Note: Fluorescence emitters and scavengers are drawn in bold.

Copy numbers for cDNAs and fragments of mitochondrial and nuclear genes were determined from calibration plots based on serial dilutions of mtDNA and total DNA, respectively. Initial nucleic acid concentrations (μg/μl) were determined by spectrophotometry, DNA weight (μg) was recalculated to copy number from known dependence of DNA molecular weight on size. All calculations were performed using the 7500 Real-Time PCR System Software (Applied Biosystems, USA).

RESULTS

Number of mitochondrial transcripts in different tissues of control mice. It is known that in all tissue types the mitochondrial DNA is transcribed to form two polycistronic RNA precursors encompassing all mitochondrial genes [5]. In this work, we determined the number of transcripts of four mitochondrial genes (*ND2*, *ND4*, *CYTB*, and *ATP6*) localized in different mtDNA regions in brain, liver, and skeletal muscle cells of control mice. Transcription level was expressed as the copy number of the examined gene mRNA per copy of mRNA of the nuclear reference gene *BETA-ACTIN*.

The data of measuring the number of mitochondrial *ND2*, *ND4*, *CYTB*, and *ATP6* gene transcripts in brain, liver, and skeletal muscle cells of control mice are shown in Fig. 1. In brain and liver cells the number of mitochondrial transcripts varies from 30 (*ND2*) to 300 (*ATP6*) copies per mRNA copy of the nuclear reference gene *BETA-ACTIN* (Fig. 1a). At the same time, in the cells of skeletal muscle the number of mitochondrial mRNAs is one order of magnitude higher, varying from 200 mRNA copies of *ND2* to 7000 mRNA copies of *ATP6* (Fig. 1b).

The numbers of *ND2* and *ND4* gene transcripts insignificantly differ from each other in all of the tissues and are the least in comparison to other genes. So, with their average number taken as unity, the ratio of transcripts (*ND2* + *ND4*)/2 : *CYTB* : *ATP6* would be 1 : 4 : 7 in brain, 1 : 2 : 10 in liver, and 1 : 4 : 20 in skeletal muscle cells. Thus, in mouse brain, liver, and skeletal muscle cells the ratios between mitochondrial transcripts are rather similar.

Change in mitochondrial gene transcription in different murine tissues following X-ray irradiation at dose of 10 Gy. Despite the difference in number of mitochondrial transcripts in tissues of control mice, the mitochondrial gene transcription alters in the same manner after lethal X-ray irradiation. Figure 2 shows the data on *ND2*, *ND4*,

CYTB, and *ATP6* gene transcription in murine brain (a) and skeletal muscle cells (b) 1, 5, and 24 h after X-ray irradiation at the dose of 10 Gy. In both tissues the number of mitochondrial transcripts decreased already 1 h after irradiation. By 5 h after irradiation the transcription level of these genes was 6-9% in skeletal muscle and 11-15% in brain cells. The same results were obtained for liver cells (unpublished data).

Determination of mtDNA copy number in different tissues of control and irradiated mice. Using real-time PCR, we determined the number of mtDNA copies in brain, liver, and skeletal muscle cells of control and irradiated mice per haploid set of nuclear DNA. It is worth noting that the copy number of mtDNA was defined as an average number of the same mtDNA genes as those evaluated upon determination of the number of *ND2*, *ND4*, *CYTB*, *ATP6*, as well as the regulatory mtDNA fragment *D-loop* transcripts, because we did not find significant difference between them. The following values were obtained for the control animals: 18,425 ± 1253 mtDNA copies in liver, 17,450 ± 5187 in skeletal muscle, and only one half (9021 ± 2971) in brain cells.

Unlike postradiation changes in mtDNA transcription, the number of mtDNA copies did not fall below the control level in any of the tested murine tissues after X-ray irradiation at the dose of 10 Gy. On the contrary, increase in number of mtDNA copies was observed in all tissues after irradiation. The mtDNA copy number was 350% of control one day after irradiation in liver cells, 450% of control 72 h after irradiation in skeletal muscle cells, and the most prominent sixfold increase over the control was observed in brain cells already 1 h after irradiation (see Fig. 3).

Determination of number of *TFB2M* gene transcripts in different tissues of control and irradiated mice. The nuclear gene *TFB2M* (mtDNA transcription factor), as

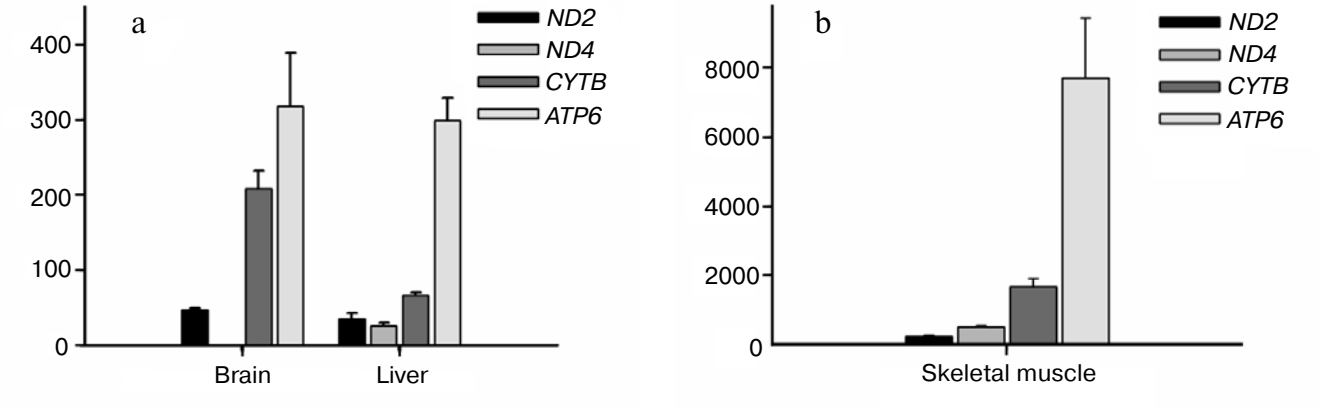

Fig. 1. Number of mitochondrial gene transcripts in tissues of control mice. The number of mitochondrial transcripts is expressed in mtRNA copies per mRNA of the reference gene *BETA-ACTIN*.

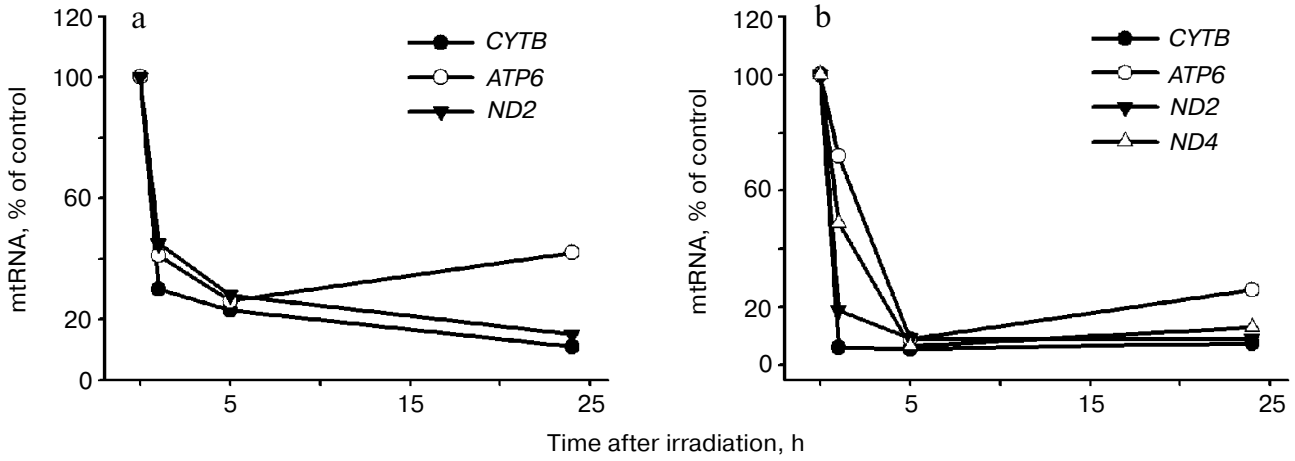

Fig. 2. Change in transcription of mitochondrial genes *ND2*, *ND4*, *CYTB*, and *ATP6* in murine brain (a) and skeletal muscle cells (b) following X-ray irradiation at the dose of 10 Gy. The number of mitochondrial transcripts in the cells of control mice per mRNA of the reference gene *BETA-ACTIN* is taken as 100%.

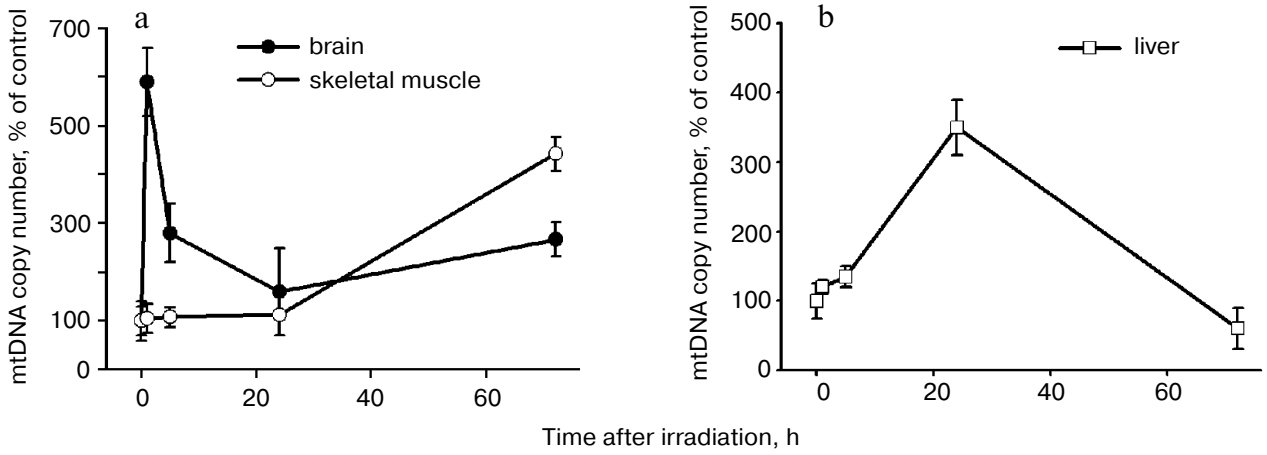

Fig. 3. Changes in mtDNA copy number in murine liver, skeletal muscle, and brain cells following X-ray irradiation at the dose of 10 Gy. The mtDNA copy number per haploid set of control mice is taken as 100%.

well as the nuclear gene *BETA-ACTIN*, has a single copy in the genome. Nevertheless, the transcription level of *TFB2M* in control mice is two orders lower than that of *BETA-ACTIN*. In particular, liver and skeletal muscle cells contain 30-40 copies of *TFB2M* mRNA per 1000 copies of *BETA-ACTIN* mRNA. One hour after X-ray irradiation of mice at the lethal dose of 10 Gy, transcription of this gene dramatically decreases and is not restored for three days after irradiation. In liver cells the number of transcripts decreases fivefold in comparison to the control level; by this time the *TFB2M* transcription level in skeletal muscle cells is only 4% of control value (Fig. 4).

DISCUSSION

More than two thousand proteins are required for maintenance of normal mitochondrial function [22]. Most of them are expressed in the nucleus, but the genes encoding 13 key protein subunits of the electron transport chain are localized in mitochondrial DNA [1].

This work presents data on transcription of four mitochondrial genes localized in the mtDNA H-strand. Expression products of the *ND2* and *ND4* genes comprise an integral part of the respiratory complex I [23], the gene *CYTB* encodes cytochrome *b*, one of catalytic subunits of the *bc*1 complex [24], and the gene *ATP6* encodes the ATPase subunit α comprising the proton channel of complex V of the electron transport chain [25].

Known data on normal mitochondrial transcription suggested equimolar ratio of mitochondrial transcripts because mitochondrial genes are transcribed as polycistronic blocks [5]. However, our data suggest significant differences in mtRNA amounts in each of the examined tissues. The transcript ratios (*ND2* + *ND4*)/2 : *CYTB* : *ATP6* are rather similar—1 : 4 : 7 in brain, 1 : 2 : 10 in liver, and 1 : 4 : 20 in skeletal muscle cells. The dominance of *ATP6* and *CYTB* transcripts among those of other mitochondrial genes under normal conditions was reported earlier [26]. The difference in number of mitochondrial transcripts might result from posttranslational processes, particularly, from different rates of degradation. In HeLa cells under normal conditions the *COX2* and *ATP6/8* transcripts are characterized by the longest half-life (5-8 h), *ND3* – the shortest (1 h), whereas *CYTB* and *ND2* are characterized by intermediate half-life (2 h) [27]. This circumstance seems to explain (at least partially) the difference in number of mitochondrial gene transcripts in the examined murine tissues.

Besides the difference in number of mitochondrial transcripts within one tissue, we have found that the total

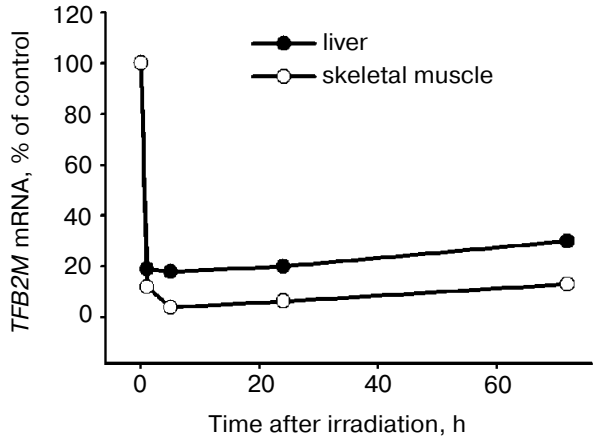

Fig. 4. Changes in transcription of nuclear gene *TFB2M* in murine liver and skeletal muscle cells following X-ray irradiation at the dose of 10 Gy. The number of *TFB2M* mRNA per 1000 mRNA molecules of the reference *BETA-ACTIN* gene of control mice is taken as 100%.

number of transcripts, and hence the total transcription level, varies depending on tissue type. In particular, high transcription level in skeletal muscle is likely associated with contractile activity of muscle fiber requiring extremely high energy consumption in the form of ATP. It should be mentioned that the higher level of mtRNA in skeletal muscle compared to other tissues was also evaluated from analysis of cDNA libraries [28].

Despite tissue-specific difference in transcription level under normal conditions, lethal irradiation of mice leads to drastic decrease in the number of all studied mitochondrial transcripts in the examined tissues. Since the genes *ND2*, *ND4*, *CYTB*, and *ATP6* are localized in different mtDNA regions, the uniform decrease in their transcription levels suggests that transcription of the whole mtDNA is decreased.

The most logical explanation of the decrease in mtDNA transcription might be either decrease in copy number or solution of continuity of mtDNA as template for transcription, especially in the D-loop area containing promoters. The idea is that the absence of introns and continual transcription activity make mtDNA extremely sensitive to genotoxic agents [29]. The mtDNA compared to nuclear DNA is several times more vulnerable to damaging factors, such as benzo(a)pyrene, *N*-methyl-*N*-nitrosourea, UV, and gamma rays [30-32].

Nonetheless, X-ray irradiation of mice at a lethal dose of 10 Gy resulted in several times increase in mtDNA copy number in all examined tissues. It is known that the mechanism of base excision repair (BER), the major component of nuclear DNA reparation that removes pyrimidine dimers and distorted fragments of double helix, does not act in mitochondria [33]. So, it is very likely that under conditions closing out a complete restoration of damaged molecules the observed activation of mtDNA replication after irradiation is necessary for preservation of the mitochondrial genome. In addition, the increase in mtDNA copy number can be observed in cells subjected to chemical oxidants, accompanies mitochondrial dysfunction appearing in the course of viral therapy, and is considered as a compensatory reaction of the cell in response to energy deficit following radiation-induced mtDNA lesions [34-36]. Some way or other, our data do not support the idea that the decrease in mitochondrial transcription results from decrease in mtDNA copy number.

Despite relative autonomy of the mitochondrial genome, its transcription, translation, and replication are under the control of the nuclear genome. As mentioned above, the following nuclear proteins are implicated in mtDNA transcription: mitochondrial RNA-polymerase, transcription initiation factors TFAM, TFB1M, and TFB2M, termination transcription factor mTERF, processing endonucleases RNases P and Z, and mitochondrial poly-A polymerase [4]. We studied transcription of the factor TFB2M because this protein seems to be the bottleneck in regulation of mitochondrial transcription. We have shown that change in transcription of the nuclear gene *TFB2M* after irradiation coincides with dynamics of change in transcription of all mitochondrial genes. These data could allow the conclusion that coincidence of transcription dynamics of mitochondrial genes and *TFB2M* reflects postradiation inhibition of both nuclear and mitochondrial transcription in the cell rather than regulatory processes; however, in liver cells we found a dramatic increase in number of *ENDOG* gene transcripts (unpublished data). This gene encodes mammalian endonuclease G, a protein with molecular mass of 29 kDa localized in the mitochondrial intermembrane space. This protein is implicated in programmed cell death [37]. In liver cells that are radioresistant, endonuclease G can participate in mtDNA splitting into removable fragments under lethal X-ray irradiation [38]. Postradiation increase in number of *ENDOG* gene transcripts and relatively constant transcriptional level of *BETA-ACTIN* are indicative of generally unimpaired transcription in the nucleus after lethal irradiation.

Thus, the observed decrease in the level of mitochondrial transcription in murine tissues after lethal irradiation is not a result of damage of the cell transcription apparatus. Also, this decrease is not a consequence of mtDNA template deficit, as follows from the presented data. In this connection, the most acceptable explanation of reduction in mitochondrial transcription under oxidative stress conditions is its regulatory inhibition via nuclear transcription initiation factors. Unlike nuclear DNA, mtDNA synthesis is not terminated by disturbance of its structure [29]. Considering this fact, the decrease in mtDNA transcription can be interpreted as a part of the mechanism directed to prevention of synthesis of defective proteins and development of mitochondrial dysfunction. It is also possible that the reduction of mitochondrial transcription provides the systems of replication and elimination with time to purify the mtDNA pool from mutant copies.

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