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Differentially expressed mRNAs as a consequence of oxidative stress in intact cells

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Abstract **Intracellular redox conditions influence the activity of several transcription factors leading to a modulation of the expression of the genes controlled by these factors. We examined the changes in cell transcription patterns after oxidative stress induced by diethylmaleate (DEM). Using the differential display technique we identified several differentially expressed sequence tags, four of which are identical or highly homologous to sequences contained in the human cDNAs encoding vimentin, *c-fos*, cytochrome oxidase IV and ribosomal protein L4; another one corresponds to a transcript of the mitochondrial genome of unknown function. The remaining five cDNAs are not recorded in any sequence data bank. One of these, named Rox3, lights up two mRNA species of ~3400 and 3600 bp, significantly increased after treatment with DEM or with other oxidizing agents. This increase appears precociously after exposure to DEM and it is completely prevented by pretreatment with *N*-acetylcysteine. The Rox3 fragment was used to screen a cDNA library; one fully sequenced clone showed 100% homology with the putative human guanine nucleotide regulatory protein nep1.**

Key words: Differential display; Oxidative stress; Diethylmaleate; Gene expression; Guanine nucleotide regulatory protein

1. Introduction

Modifications of intracellular redox conditions induced by reactive oxygen intermediates (ROIs) have long been known to cause severe cell damage and have been implicated in ageing, cancer and various other diseases [1]. More recent evidence suggests that changes in intracellular redox conditions could be also involved in the molecular basis of the regulation of several cell functions. In fact, oxidants provoke, at least in vitro, either a decrease of the DNA-binding efficiency of several transcription factors, such as USF [2] and AP1 [3], or an activation, as in the case of HoxB5 [4]. NFkB is activated in vivo by the exposure of the cell to oxidants, which trigger the disassembling of IkB-NFkB complex and the translocation of NFkB to the nucleus [5]. On the contrary, Sp1 is inactivated by oxidative stress both in vitro [6] and in vivo [7]. This phenomenon is in agreement with the impaired DNA-binding efficiency of Sp1 in aged tissues [8]. Similarly, the DNA-binding activity of the glucocorticoid receptor stimulated with dexamethasone decreases both in vitro [9] and in vivo [7] as a consequence of oxidative stress.

All these phenomena could lead to modification of the expression of many genes. Several mRNAs are induced by oxidative stress and most of them are known as 'DNA damage-inducible genes' [10]. Among these, the *gadd* genes may represent part of a novel regulatory pathway involved in the negative control of mammalian cell growth [11].

Diethylmaleate (DEM) induces oxidative stress by depleting reduced glutathione (GSH) in intact cells [12]. The GSH depletion causes an impairment of several oxygen free radical scavenging systems and in turn it provokes an increase of ROI concentration. We used this molecule to study in vivo the effect of oxidative stress on several transcription factors [7,13]. Here we report the identification of genes whose expression is modified as an early consequence of the modification of intracellular redox conditions caused by DEM.

2. Materials and methods

2.1 Cell cultures

HeLa, Hep3B and K562 cells were cultured at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% fetal calf serum (Hiclone), penicillin (50 IU/ml) and streptomycin (50 μg/ml). The amount necessary to reach the desired concentration of DEM (Sigma) was added directly to the medium in the plate, using a stock solution of 6 M DEM in dimethylsulfoxide (DMSO) [7]. Buthionine sulfoximine (Sigma) and *N*-acetylcysteine (Sigma) were added to the culture medium to obtain final concentrations of 5 and 30 mM, respectively [7].

2.2. mRNA differential display, cloning and DNA sequencing

100 ng of purified polyadenylated RNAs from untreated and DEM-treated HeLa cells were reverse transcribed using the T12GC- or T12CC-anchored primers. cDNAs were subsequently amplified by PCR using one of the above-mentioned anchored primers and different arbitrary primers as described elsewere [14]. The recovery and reamplification of cDNA fragments from a dried 6% 7 M urea sequencing gel were performed as described [14]. The re-amplified cDNA fragments were cloned in pGEM-T vector (Promega) and both strands were sequenced with the Sequenase kit (USB Biochemicals). The nucleotide sequences were analysed by searching for homologies with the GenBank and the EMBL Data Bank.

2.3 RNA isolation and Northern blot analysis

Total cellular RNAs from subconfluent cell cultures were prepared by the standard guanidinium isothiocyanate/CsCl gradient centrifugation method. 20 μg of total RNAs were size-fractionated by 1.5% agarose/formaldehyde electrophoresis and transferred to a Nytran membrane (Schleicher & Schuell). Poly A^+ RNAs were purified by using the Oligotex-dT mRNA kit (Qiagen). The cDNA fragments were excised from the vector, gel purified and labeled with [α-^{32}P]dATP with a random primer DNA-labeling kit (Boehringer Mannheim). They were used as probes in Northern blot experiments to confirm their differential expression.

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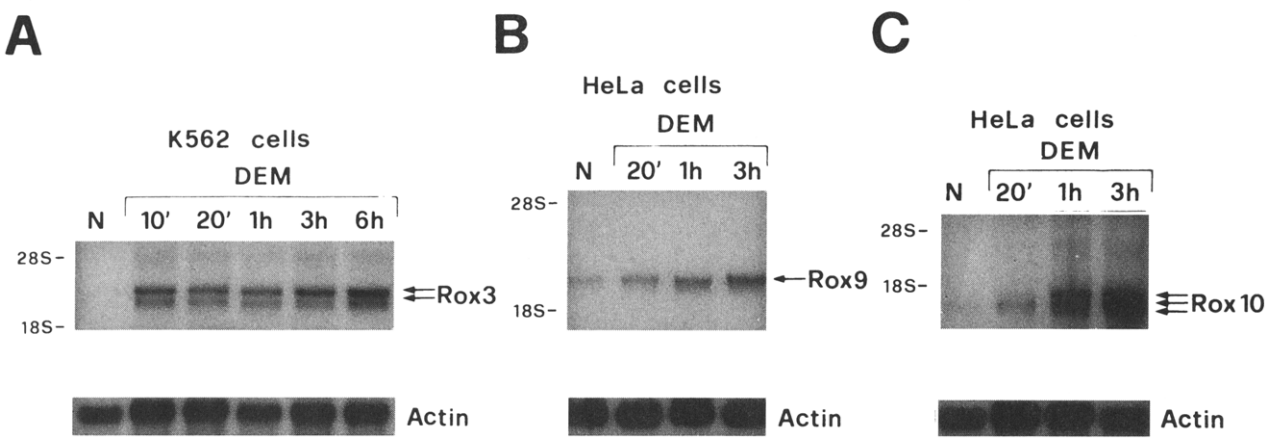

Fig. 1. Time course of DEM-induction of Rox3, Rox9 and Rox10 mRNAs. RNAs from K562 (Panel A) or HeLa (Panels B and C) cells treated with 1.2 mM DEM for the indicated times were hybridized to Rox3 (Panel A), Rox9 (Panel B) or Rox10 (Panel C) probes. The same filters were rehybridized with a fragment of the actin cDNA.

2.4 cDNA library screening

The expressed tag Rox3 was labeled and used as a probe to screen a K562 cell cDNA library in λgt11 in order to isolate the correspondent full-length cDNA. Two positive phages were isolated and their cDNA inserts subcloned in pGEM3Z vector (Promega). The larger insert (λRox3.1) was 1491 bp in size; it was sequenced in both directions and the nucleotide sequence was analysed with data banks. The 751-bp fragment and the 3′ of λRox3.1 (740 bp) were obtained by polymerase chain reaction using as a template the λRox3.1 clone.

3. Results

3.1. mRNA differential display reveals the presence of several genes induced by oxidative stress

To identify genes that are differentially expressed as a consequence of oxidative stress caused by DEM, we used the differential display technique to compare patterns of mRNA expression from control and HeLa cells treated with 1.2 mM DEM for different times. We identified 10 differentially expressed cDNA fragments in normal and in DEM-treated HeLa cells; they were recovered from the dried denaturing polyacrylamide gels and re-amplified by using the corresponding pair of primers. The re-amplified cDNAs were cloned, sequenced and used as probes in Northern blot experiments to confirm their differential expression (data not shown). The nucleotide sequences of these cDNAs were analysed by searching for homologies with the GenBank and the EMBL Data Bank and this allowed us to identify several known mRNA fragments and various unknown mRNAs. Table 1 shows the expressed tags we found that hybridize to the mRNAs, whose abundance is modified by the DEM treatment. The cloned fragment named Rox6 is 100% homologous with the human cDNA encoding the *c-fos* protooncogene, whose induction following an oxidative stress was already demonstrated [15]. The cDNA fragments Rox2 and Rox8 hybridize to mRNAs that decrease upon the treatment with DEM and their sequences are identical with he human cDNAs of vimentin [16] and of cytochrome oxidase gene subunit IV [17], respectively. Rox7 and Rox9 cDNAs are identical with the cDNAs encoding a transcript of the mitochondrial genome [18] and the human ribosomal protein L4 [19], respectively. In both these cases, an increase of the corresponding mRNAs was observed as a consequence of the DEM treatment. The other cDNA fragments did not show any homology with sequences present in nucleic acids data banks.

3.2. Effect of time of exposure to DEM on Rox3, Rox9 and Rox10 expression

Rox3, Rox9 and Rox10 cloned fragments were used as probes in Northern blot analysis to evaluate changes in gene transcript levels in cells exposed to DEM for different times. K562 cells were treated with 1.2 mM DEM for 10 and 20 min and 1, 3 and 6 h and RNAs were analysed by Northern blot (Fig. 1, Panel A). Rox3 fragment detected two transcripts of ~3400 and 3600 bp specifically expressed in K562 cells exposed to 1.2 mM DEM for 10 min and the level of the

Table 1
cDNA fragments corresponding to mRNAs whose expression was modified by DEM-treatment

Expressed tag	Size of mRNA lighted-up	Homology with known genes	Effect of DEM treatment
Rox1	2200 bp	No homology	↑
Rox2	2000 bp	Human vimentin gene	↓
Rox3	3400–3600 bp	No homology	↑
Rox4	1500 bp	No homology	↓
Rox5	1200 bp	No homology	↑
Rox6	2200 bp	Human *c-fos* protooncogene	↑
Rox7	700 bp	Mitochondrial tRNA gene custer	↑
Rox8	750 bp	Cytochrome oxidase IV	↓
Rox9	2500 bp	Human ribosomal protein L4	↑
Rox10	800/900–1000 bp	No homology	↑

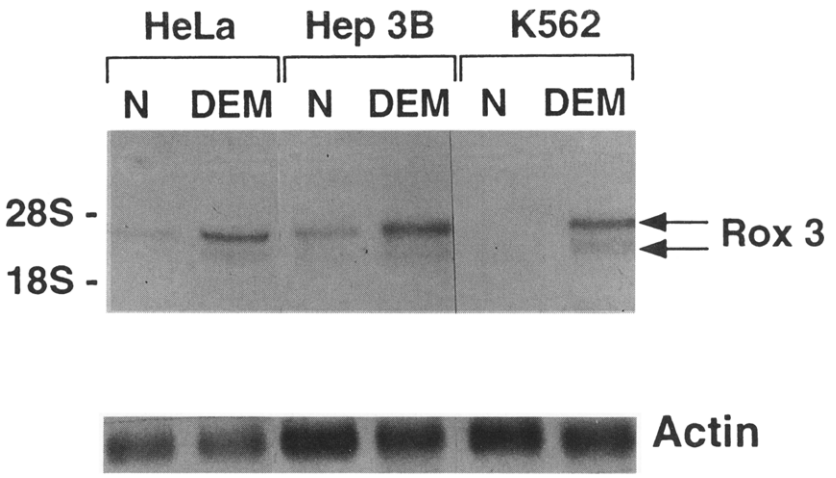

Fig. 2 Northern blot analysis of Rox3 mRNA. Rox3 amplicon lights up the same mRNAs in HeLa, Hep3B and K562 cells. Total RNAs from untreated (N) and 1.2 mM DEM-treated cells (DEM) are compared. The same membrane was hybridized with a fragment of the actin cDNA.

transcripts remained almost constant up to 6 h (Fig. 1, Panel A). Similarly, Rox9 cDNA hybridizes to ribosomal protein L4 mRNA and Rox10 to three transcripts of different sizes and their expression was progressively increased after exposure to DEM for 20 min and 1 and 3 h (Fig. 1, Panels B and C).

3.3. Rox3 is a housekeeping gene

We focused our attention on the cDNA named Rox3 and examined its expression in three different cell lines exposed to 1.2 mM DEM for 6 h. As shown in Fig. 2, Rox3 fragment detected two transcripts of ~3400 and 3600 bp in HeLa, Hep3B

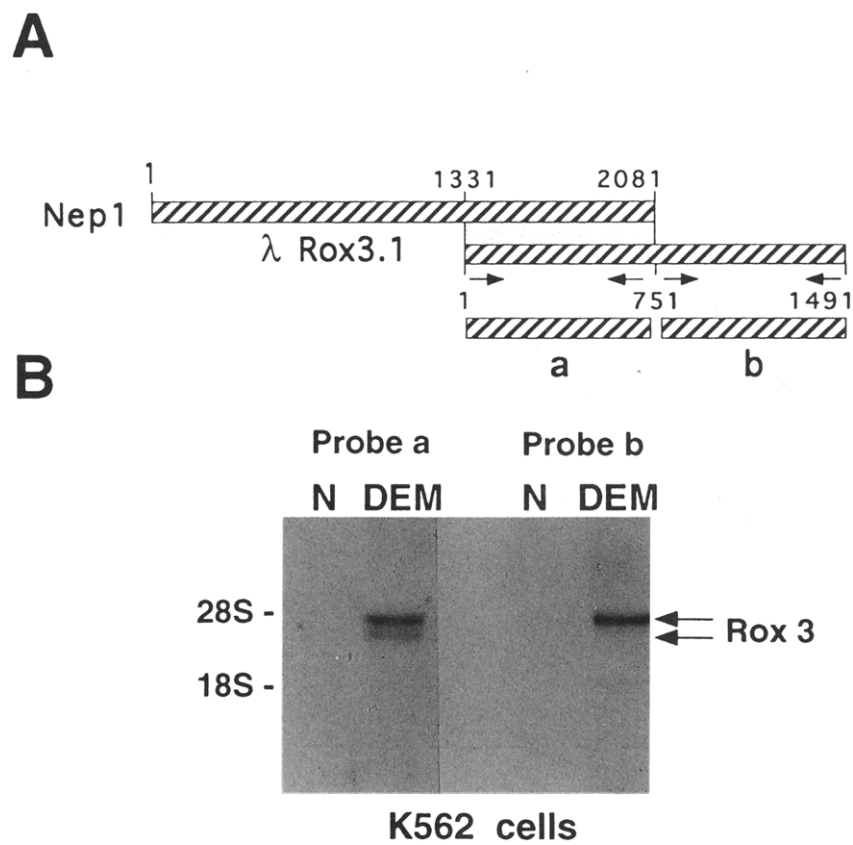

Fig. 3. Rox3 gene gives rise to two different 3′ UTRs. Panel A: overlapping region between λRox3.1 and nep1. The PCR strategy to obtain the two cDNAs fragments from λRox3.1 is also shown. Panel B: Northern blot experiments of RNAs from K562 cells untreated (N) and exposed to 1.2 mM DEM (DEM) hybridized either to the cDNA fragment common to nep1 and to λRox3 1 (a) or to the cDNA fragment corresponding to the 3′ extremity of λRox3.1 cDNA (b).

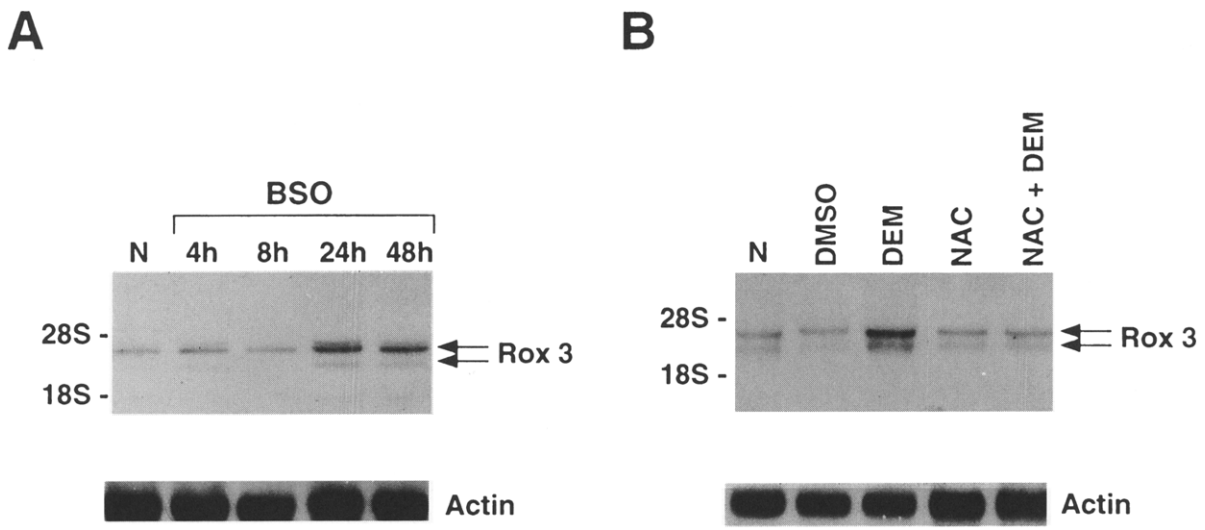

Fig. 4. Rox3 mRNA induction is a consequence of oxidative stress. Panel A: HeLa cells were exposed to 5 mM BSO for the indicated times and RNAs were analysed by Northern blot using as a probe the labeled Rox3 amplicon. Panel B: HeLa cells were exposed to 1.2 mM DEM, to 30 mM NAC or to 30 mM NAC for 2 h and then to 1.2 mM DEM for 3 h; the RNAs from these cells were hybridized to the Rox3 labeled fragment. The filters were rehybridized with the actin cDNA.

and K562 cells. While in HeLa and in Hep3B cells the Rox3 mRNA basal level was increased by DEM treatment, in the K562 cell line Rox3 mRNA was specifically expressed only in the cells exposed to DEM.

3.4. Rox3 is homologous to nepl and gives rise to two 3′ UTRs

We used the Rox3 cDNA fragment to screen a λgt11 cDNA library from K562 cells. We obtained 2 positive clones; one clone (λRox3.1), containing an insert of 1491 bp, was fully sequenced on both strands (AC: DS21576) and analysed by searching homologies with data banks. We found that 751 bp (from nt 1 to nt 751) were 100% homologous with the 3′ end (from nt 1330 to nt 2081) of the human guanine nucleotide regulatory protein (nepl) mRNA (A.M. Chan et al., unpubl. data; AC: U02081) (Fig. 3, Panel A). This gene was isolated as a transforming sequence using an expression cDNA cloning approach [20]. The other 740 bp of our clone did not overlap with nepl gene (Fig. 3, Panel A). We amplified by PCR the 751-bp fragment of λRox3.1 homologous with nepl and the not overlapping 740-bp fragment (Fig. 3, Panel A) and used them as probes for Northern blot experiments with total RNAs from untreated and DEM-treated K562 cells. Fig. 3, Panel B shows the result of this experiment. Two transcripts of ~3400 and 3600 bp selectively expressed in K562 cells exposed to DEM were observed when the 751-bp fragment (a) or the Rox3 amplicon were used as probes. On the other hand, the labeled 740-bp fragment (b) detected only the 3600 bp long transcript specifically expressed in DEM-treated K562 cells (Fig. 3, Panel B), corresponding to the upper band observed when Rox3 or the 751-bp fragment of λRox3.1 were used as probes. This result suggests the hypothesis of an alternative termination of the Rox3/nepl transcript that gives rise to two differently sized 3′ UTRs.

3.5. Induction of Rox3/nepl mRNA by DEM is an oxidation-dependent phenomenon

Induction of Rox3/nepl mRNA induction was also evaluated using buthionine sulfoximine (BSO), a compound that induces GSH depletion by a mechanism different from that of DEM, i.e., by inhibiting γ-glutamylcysteine synthetase that catalyzes the biosynthesis of a GSH precursor [21]. Fig. 4 (Panel A) shows the Northern blot experiment performed with RNAs from HeLa cells exposed to 5 mM BSO for 4, 8, 24 and 48 h and hybridized to the labeled Rox3 cDNA fragment. The treatment with 5 mM BSO for 24 and 48 h induced a significant increase of Rox3/nepl transcripts.

N-Acetylcysteine (NAC) is known to protect the cells from oxidizing radicals by increasing intracellular levels of GSH [22]. Incubation of HeLa cells with 30 mM NAC for 2 h before exposure to DEM completely prevented the induction of Rox3/nepl transcripts (Fig. 4, Panel B). Furthermore, to rule out the possibility that the induction observed was due to DMSO contained in the DEM solution, we exposed HeLa cells to DMSO and found that it did not induce any increase of the Rox3/nepl transcript (Fig. 4, Panel B).

4. Discussion

We used the differential display technique to identify genes that are differentially expressed as a consequence of an oxidative stress, caused by DEM. The expressed tag Rox3 was used as a probe to screen a K562 cDNA library. The sequence of one positive clone (λRox3) showed 100% homology with nepl mRNA. This cDNA, which encodes a human guanine nucleotide regulatory protein, was isolated as a transforming sequence using an expression cDNA cloning approach for detection of novel oncogenes by focus formation assay in NIH3T3 cells [20]. It is well established that GTP-binding proteins play important roles in conveying extracellular signals to downstream effector molecules. Their functional characteristic is the ability to bind and hydrolyze guanine nucleotides as exemplified by the cyclical shuttling between a GTP-bound active state and a GDP-bound inactive state [23]. This specific molecular switch is finely regulated by modulators. Rox3/nepl belongs to this class of

regulatory proteins. Many of the responses to enviromental stimuli are regulated by guanine nucleotide-binding regulatory proteins that transduce signals from cell-surface receptors to intracellular effectors and the versatility of G protein-mediated signal transduction is reflected in the diversity of genes encoding the G protein subunits [24].

ROIs are generated by every cell type as side-products of electron transfer reactions. They are also produced by ubiquitous enzyme systems and continuosly inactivated by a complex scavenging machinery [25]. In addition, their concentration is increased by numerous extracellular stimuli, including TNFα, which causes a modification of mitochondrial electron flow [26], TPA, which activates the membrane-bound NADPH oxidase [27] and many other stimuli, such as cytokines, LPS, UV and γ rays [28], whose mechanisms of action have yet to be clarified. Our finding that a guanine regulatory protein is induced upon oxidative stress could be taken as further indication of the possible involvement of ROIs as modulators of membrane signal transduction machinery [28].

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