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Cloning of a novel human RNA polymerase II subunit downregulated by doxorubicin: new potential mechanisms of drug related toxicity

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Abstract Using the differential display PCR method, we have isolated an mRNA downregulated in doxorubicin resistant human cell lines. The full length cDNA clone was identified as the human homologue of yeast RPB11 subunit of RNA polymerase II. Northern blot analysis of normal tissues detected a particularly high expression of RPB11 mRNA in heart and skeletal muscle. Reduction of this mRNA expression was observed in all the cell lines tested after drug treatment and was paralleled by a similar decrease of the protein levels. These findings suggest that doxorubicin may exert in vivo specific inhibitory effects on a major component of the transcription machinery.

Key words: Differential display PCR; Doxorubicin; Drug toxicity; RNA polymerase II

1. Introduction

The use of chemotherapeutic agents in the treatment of neoplastic diseases is limited by several factors including neoplastic cell-specific drug resistance [1] and systemic toxicity. Among the chemotherapeutic agents, doxorubicin is one of the most effective and widely used drugs [2] and alone or in combination with other drugs constitutes the therapeutic choice for the treatment of a large array of neoplastic diseases. In addition to causing neoplastic cell-specific drug resistance, doxorubicin administration is frequently associated with cardiotoxic effects responsible for a high incidence of congestive heart failure (15–30% of patients), especially if cumulative drug doses exceed 500 mg/m^2 [3].

The differential display PCR is a powerful method with which is possible to identify altered gene expression at the mRNA level in any eucaryotic cell [4]. This procedure allows for selection of differentially expressed genes at the molecular level, as the fragments of cDNA obtained by PCR amplification are easily cloned and sequenced; moreover, the same fragments can be used as probes useful for Northern blot assays and for library screening.

We sought to apply the differential display PCR technique to investigate the pattern of mRNAs expressed in human colon carcinoma cell lines differing for their doxorubicin sensitivity in order to identify possible markers related to drug-induced toxicity or resistance.

The present study describes the isolation and cloning of an mRNA differentially expressed in doxorubicin sensitive and resistant human cell lines. This mRNA was previously hypothesised to have a fundamental role in eucaryotic RNA polymerase subunits assembly similar to the one exerted in prokaryotic cells by the a subunit [5,6]. Our findings show that this message is highly expressed in human cardiac and skeletal muscle tissues, where the highest levels of doxorubicin toxicity are generally observed. Moreover, in vitro experiments on several drug-sensitive cell lines show that the presence of doxorubicin in the culture medium is necessary to downmodulate the expression of this RNA polymerase II subunit, at both the mRNA and protein levels.

2. Materials and methods

2.1. Cell lines and drug treatment

LoVo (human colon carcinoma) and MCF-7 (human breast carcinoma) parental and doxorubicin resistant cell lines were kindly provided by Dr. M.P. Colombo (Istituto Nazionale Tumori, Milan, Italy) and by Dr. K.H. Cowan (NCI, NIH, Bethesda, MD, USA). Details regarding the establishment and characteristics of these cell lines have been previously described [7,8]. The resistant cell lines were maintained by continuous exposure to 0.2 mM (LoVo) or 10 mM (MCF-7) of doxorubicin (Adriblastina, Farmitalia Carlo Erba, Milan, Italy). The erythroleukemia cell line K562 was obtained from the American Type Culture Collection (ATCC, CCL 243) and the bladder carcinoma cell line EJ was kindly provided by Dr. J. Masters (Institute of Urology, London, UK)

2.2. Differential display PCR cDNA cloning and Northern blot analysis

Differential display PCR was performed as previously described [4]. The oligonucleotides used for the isolation of the cDNA described in this paper are 5′ (T)13CT, used in the first strand cDNA synthesis as well as in the subsequent PCR reactions, and 5′ TGAGTAGGGG used as a random decamer in the PCR reactions. The PCR fragments were cloned using the TA cloning system (Invitrogen, San Diego, CA) according to the manufacturer's instructions. A lambda Uni-Zap XR cDNA library from human colon carcinoma cell line HT29 was purchased from Stratagene (Stratagene, La Jolla, CA). DNA sequencing of both strands of all the cloned fragments was performed using specific oligonucleotides synthesised on an Applied Biosystems DNA synthesiser 392 model (Perkin Elmer, Roche Molecular Systems Inc., Branchburg, NJ).

Nucleotide and amino acid sequence analyses were performed using the Hitachi software Dnasis and Prosis (Hitachi Software Engineering America Ltd., Brisbane, CA). Data bases of reference were updated versions of GenBank and EMBL for DNA analysis and NBRF-PIR and SWIS-PROT for protein analysis.

The cDNA sequence described is registered in GenBank with the accession no. L37127 and in EMBL with the accession no. X82385.

Total cellular RNA was isolated using acid guanidinium thiocyanate-phenol-chloroform extraction [9]. RNA was denatured at 65°C for 5 min in 50% formamide, electrophoresed through a 1.2% agarose gel containing 2.2 M formaldehyde, and transferred to either nitro-

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cellulose or nylon membranes (Amersham, Bucks, UK). Filters containing poly(A)$^+$ RNA from 16 different normal human tissues (MTN human blots I and II) were purchased from Clontech (Palo Alto, CA). Hybridizations were carried out under high stringency conditions using Quikhyb (Stratagene) according to the instructions of the manufacturer with randomly primed [^{32}P]dATP- or dCTP-labeled probes. Filters were exposed using X-omat AR film (Eastman Kodak Co., Rochester, NY) and bands were quantitated using a Phosphorimager with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

2.3. Polyclonal antibody production and Western blot analysis

The coding region of human RPB11 comprised between amino acid 10 and 117 was cloned in the pQE-31 prokaryotic expression vector and the recombinant fusion protein, constructed swapping the first 10 amino acids of huRPB11 with the 10 residues already present in the prokaryotic expression vector, was expressed and purified according to the manufacturer's protocol (Qiagen, Diagen, Hilden, Germany). For antiserum production, New Zealand rabbits were immunised 4 times with 250 mg of the purified fusion protein every 2 weeks using Freund's adjuvants (Difco Lab., Detroit, MI) and the antiserum was collected 3 days after the last injection. The specific polyclonal antiserum was purified using an affinity column made with recombinant fusion protein coupled to activated CH Sepharose according to the manufacturer's instruction. (Pharmacia Biotech Inc., Piscataway, NJ). Equal amounts of total cell lysates were immunoprecipitated with the purified polyclonal antibody and the samples separated on a 12.5% polyacrylamide gel by SDS-PAGE under reducing conditions [10]. Proteins were transferred to nitrocellulose (Amersham) and blots were probed with the same purified polyclonal antibody. Immunoreactivity was detected using an enhanced chemiluminescence kit (Amersham). Bands were analysed using the 2202 Ultroscan Laser Densitometer (LKB, Bromma, Sweden).

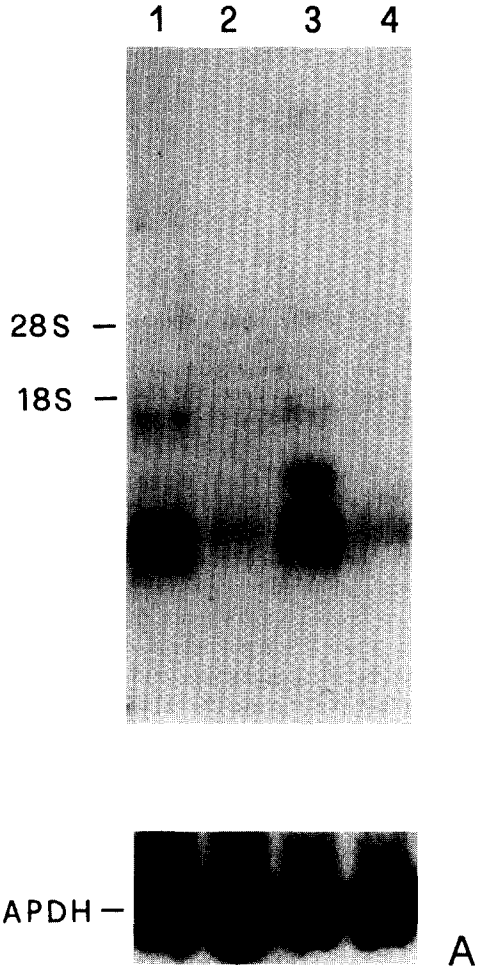
3. Results

3.1. Isolation of differentially expressed mRNAs

Using the differential display PCR method we isolated a cDNA clone hybridising in Northern blot analysis to an mRNA of about 0.6 kb. As detected with the PCR method, this mRNA was found to be highly expressed in both doxorubicin sensitive LoVo and MCF-7 cells, but barely detectable in doxorubicin resistant LoVo and MCF-7 cells (Fig. 1A). Northern blot analysis also detected the presence of two weaker bands with a higher molecular mass, suggesting that this gene might be either alternative spliced or a member of a family of closely related genes. Using the cloned fragment as a probe we then screened a HT29 colon carcinoma cDNA library in order to isolate the full length clone. After three rounds of screening, we obtained several clones that were subjected to insert analysis with restriction enzymes. All of them showed the same pattern; therefore, three clones were randomly picked and sequenced. The three clones were found to be identical one to another with the exception of a few additional bases at the 5'-end of the cDNAs. The longest of these, a 621 bp clone, was subjected to further investigation.

3.2. Nucleotide and amino acid sequence analysis

This latter clone contained a single open reading frame of 351 bp coding for a protein of 117 amino acids with a calculated M_r of 13292.62 and an isoelectric point of 5.53. A search in DNA and protein data banks showed a high homology with yeast RNA polymerase II subunit RPB11 (Fig. 1B) [5]. Indeed, the two proteins shared 52% of identical and 68%

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                10        20        30
huRPB11   MNAPPAFESFLLFEGEKKITINKDTKVPNA
          ::::  :: ::: :::.:..:. :::.:::
yRPB11    MNAPDRFELFLLGEGESKLKIDPDTKAPNA

                40        50        60
huRPB11   CLFTINKEDHTLGNIIKSQLLKDPQVLFAG
          ..:..:::::::::.:...::.:..:::::.
yRPB11    VVITFEKEDHTLGNLIRAELLNDRKVLFAA

                70        80        90
huRPB11   YKVPHFLEHKIIIRVQTTPDYSPQEAFTNA
          ::: ::.  .. .:.::: .:.:..:..::
yRPB11    YKVEHPFFARFKLRIQTTEGYDPKDALKNA

                100       110       120
huRPB11   ITDLISELSLLEERFRVAIKDKQEGIE
          ...:..:. :...:
yRPB11    CNSIINKLGALKTNFETEWNLQTLAADDAF
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B

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Fig. 1. (A) Top: Differential expression of the isolated mRNA in doxorubicin sensitive and resistant cell lines detected by Northern blotting. The blot was probed and washed under stringent conditions and exposed for 6 h at −70°C with an intensifying screen. Each lane contained approx. 20 μg of total RNA from LoVo doxorubicin sensitive (1), LoVo doxorubicin resistant (2), MCF-7 doxorubicin sensitive (3) and MCF-7 doxorubicin resistant (4) cell lines, respectively. The positions of 28S and 18S ribosomal RNAs are indicated. Bottom: results obtained on the same blot with a glyceraldehyde phosphate dehydrogenase (GAPDH) probe. (B) Amino acid sequence alignment between human and yeast RPB11 proteins. Identical residues are indicated by a colon, conserved residues by a period. The amino acid sequence of human RPB11 was deduced from the HT29 derived cDNA clone. The 18 amino acid motif also present in other RNA polymerase subunits is underlined.

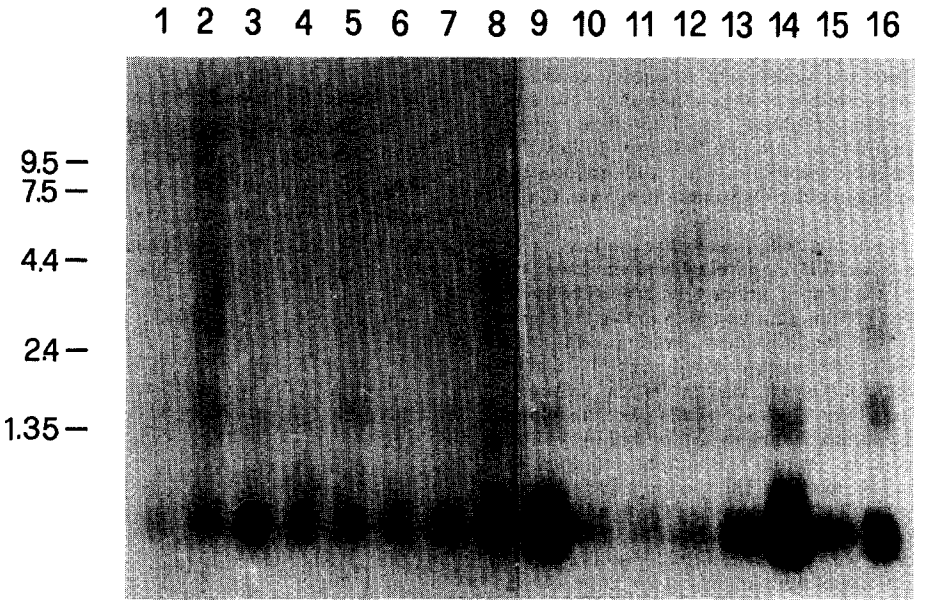

Fig. 2. Expression of RPB11 mRNA detected by Northern blotting in 16 human normal tissues. The blot was probed and washed under stringent conditions and exposed for 3 h at −70°C with an intensifying screen. Each lane contained approx. 2 μg of poly(A)$^+$ RNA from spleen (lane 1), thymus (lane 2), prostate (lane 3), testis (lane 4), ovary (lane 5), small intestine (lane 6), colon (lane 7), peripheral blood lymphocytes (lane 8), heart (lane 9), brain (lane 10), placenta (lane 11), lung (lane 12), liver (lane 13), skeletal muscle (lane 14), kidney (lane 16), and pancreas (lane 16).

of conserved residues, with the major differences localised at the COOH-terminus. This result prompted us to identify our cDNA clone as the human homologue of yeast RPB11. The human sequence contained a previously described highly conserved region of 18 amino acids (underlined in Fig. 1B) that is also present in other RNA polymerase subunits and may be involved in interactions occurring among the different subunits during RNA polymerase assembly [5,6].

3.3. Northern blot analysis on human tissues

The expression of RPB11 mRNA was evaluated in 16 human normal tissues by Northern blotting. Human RPB11 was ubiquitously expressed in all the tissues tested, as expected for an mRNA coding for a subunit of the general transcriptional apparatus. However, in heart and skeletal muscle the expression was 6-fold greater than in the other tissues (Fig. 2). The human Southern blot analysis revealed a pattern of bands compatible with the presence of a single copy gene as previously described for yeast RPB11 [5] (not shown).

3.4. Protein levels of human RPB11 are also reduced in a doxorubicin resistant cell line

In order to investigate whether a decrease in the relative protein expression paralleled the observed human RPB11 mRNA downregulation, cell lysates from doxorubicin sensitive and resistant LoVo cell lines were immunoprecipitated using a specific rabbit polyclonal antibody (see section 2). The immunoprecipitated samples were separated by SDS-PAGE and transferred to nitrocellulose. Filters were subjected to Western blot analysis using the same antibody. As shown in Fig. 3, this antibody identified a protein with a molecular mass of 13 kDa in agreement with the molecular mass predicted from the amino acid sequence and similar in size to the recombinant fusion protein (Fig. 3, lane 1) used to generate the polyclonal antibody. The huRPB11 protein, as observed for the huRPB11 mRNA, was preferentially expressed in the doxorubicin sensitive LoVo cell line (similar results were obtained with MCF-7 cells; not shown).

3.5. Human RPB11 mRNA and protein downmodulation by doxorubicin treatment

In order to investigate the relationship between doxorubicin treatment and human RPB11 expression at the mRNA and protein level, we examined the effect of the drug on three different doxorubicin sensitive human cell lines: LoVo, EJ and K562. Doxorubicin administration caused a rapid and progressive decrease of human RPB11 mRNA in all the three cell lines, with the first effect being readily detectable after 24 h and an almost complete loss of the message after 72 h (Fig. 4A). This downmodulation was observed with a 0.2 mM Dox concentration in the LoVo cell line and with a higher dose (2.0 mM) in EJ and K562. These doses were chosen since they represent equitoxic drug concentrations for the different cell lines (not shown).

When we tested the effects of the drug on human RPB11 protein expression in LoVo, EJ and K562 doxorubicin sensitive cell lines using the same drug concentrations previously found to affect mRNA expression, we observed a progressive decrease of the protein in the cell extracts (Fig. 4B). Therefore, these data indicate that the drug is capable of downregulating human RPB11 both at the mRNA and at the protein level with different kinetics, possibly because of a different half-life of the two products.

4. Discussion

Using the differential display PCR technique, we have identified and cloned an mRNA that is preferentially expressed in doxorubicin sensitive carcinoma cell lines. The corresponding cloned cDNA is the human homologue of yeast RNA poly-

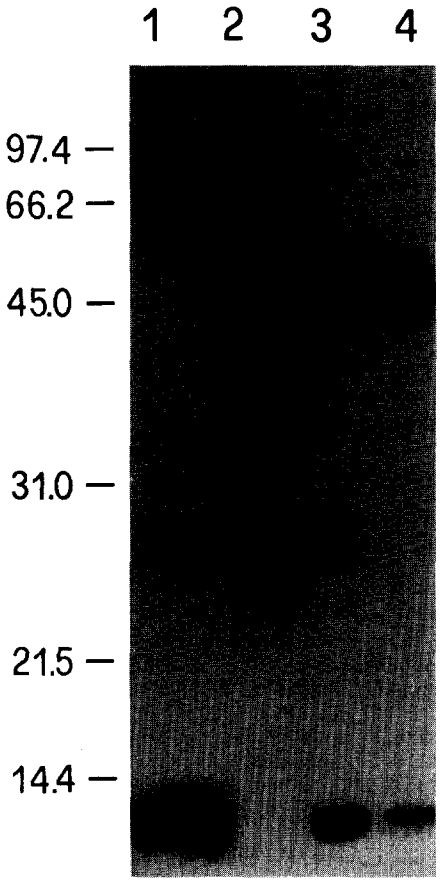

Fig. 3. Expression of RPB11 protein in doxorubicin sensitive and resistant LoVo cell line. The lysates containing equal amounts of proteins were immunoprecipitated with the anti-human RPB11 specific polyclonal antibody. After washing, the immunocomplexes were separated on SDS-PAGE and subjected to immunoblotting with the same antibody. Lanes: 1, recombinant fusion protein used as positive control; 2, pre-immune serum used as negative control on doxorubicin sensitive LoVo colon cancer cells; 3, doxorubicin sensitive LoVo colon cancer cells; 4, doxorubicin resistant LoVo colon cancer cells. Ordinate, molecular weight markers run on the same gel.

merase II subunit RPB11. A screening of several human tissues by Northern blot analysis showed that this mRNA is highly expressed in human heart and skeletal muscle. We also showed that doxorubicin can rapidly modulate with different kinetics human RPB11 both at the mRNA and protein level in different sensitive cell lines. In addition, the complete withdrawal of the drug from the cell culture medium of doxorubicin resistant LoVo cells induced a gradual upmodulation of human RPB11 mRNA levels, which, after 3 weeks, reached the levels observed in doxorubicin sensitive LoVo cells (not shown).

Eucaryotic RNA polymerases are multisubunit enzymatic complexes consisting of 10–12 subunits and RPB11 is a component of the RNA polymerase II multisubunit complex [11,12]. In yeast this protein is associated with 11 other subunits, is not shared among RNA polymerases I and III, and is also essential for cell viability [6,12,13]. The identity between yeast RPB11 and the human homologue discussed in this paper is very high, demonstrating that this subunit is well conserved among eucaryotes as it has been previously shown for other subunits [12–14]. Interestingly, while this manuscript was in progress Pati [6] described the cloning of human RNA polymerase II subunit hRPB14. Our sequence is identical at the DNA and protein level to the one described in that report with the exception of amino acids 14 and 16 that were erroneously translated as glutamine, being instead glutamic acid, and with the insertion of an isoleucine at position 19. These corrections make the homology between the yeast and human gene even more stringent and underscore the fundamental role in transcription of these subunits. It should be noted, however, that the major differences between yeast and human RPB11 subunits are encountered at the carboxy-terminal portion of the protein and that the sequence described by Pati differs completely from ours both at the DNA and protein level in the last 21 amino acids. In addition, while screening the cDNA library, we isolated a single clone that differed from the others in the carboxy-terminal portion of the protein but that showed no homology with the carboxy-terminal portion described by Pati (not shown). These data would suggest

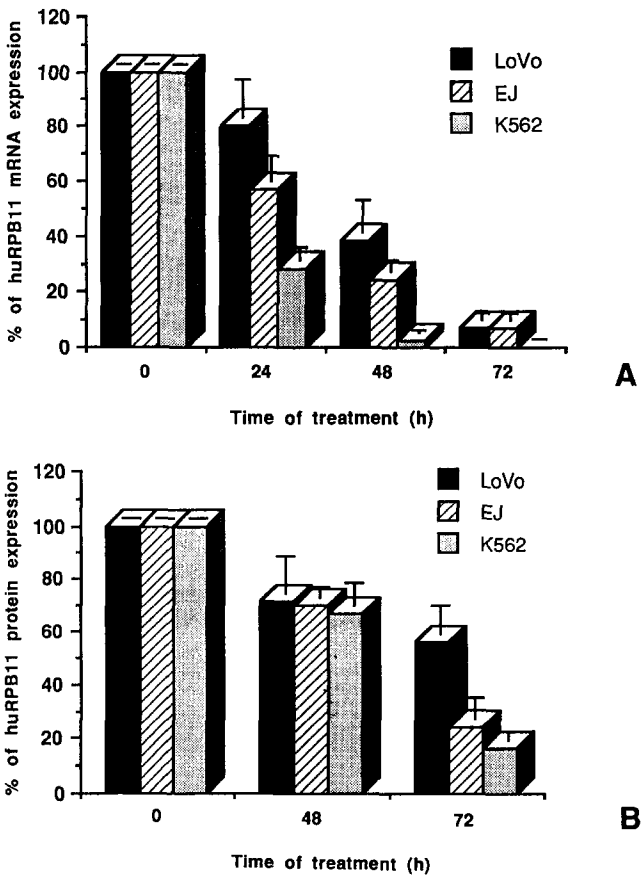

Fig. 4. Human RPB11 mRNA and protein expression is affected by doxorubicin treatment. (A) Modulation by doxorubicin of the levels of human RPB11 mRNA in LoVo, EJ and K562 doxorubicin sensitive cell lines. Cells were treated with 0.2 μM (LoVo) or 2.0 μM (EJ, K562) doxorubicin and mRNA expression was evaluated by Northern blot analysis. The level of mRNAs detected in untreated cells is arbitrarily given as 100%. The percentage of the specific mRNA expression was evaluated in comparison to the β-actin mRNA expression in each sample after quantitation as specified in section 2. (B) Modulation by doxorubicin of the level of human RPB11 protein in doxorubicin sensitive LoVo, EJ and K562 cell lines. Cells were treated with either 0.2 μM (LoVo) or 2.0 μM (EJ, K562) doxorubicin. The level of the protein detected in untreated cells was assumed as 100%. Quantitation of the protein expression was performed as described in section 2.

that possible alternative splicings may occur in this part of the protein generating multiple transcripts. Moreover, since the clone described by Pati was isolated from a different cellular source than the one used in this paper, it is possible that different tissues may utilise peculiar carboxy-terminal sequences, the significance of which has yet to be determined. To clarify this point we are in the process of characterising the genomic structure of the gene.

Doxorubicin has been described to have specific and systemic toxic effects through several mechanisms of action. Recognised among them have been the generation of oxygen free radicals [15], the interaction with cell membrane constituents [16] and the induction of large gene deletions in mammalian cells [17] probably through the stabilisation of the intermediate ternary complex DNA-topoisomerase II [18].

Selective action on several genes has also been reported. In particular, a downmodulation of the mRNA levels for the sarcomeric genes α-actin, troponin I, myosin light chain 2, the muscle specific M isoform of creatine kinase [19], MyoD and myogenin [20] has been associated with the specific cardiotoxic effects of doxorubicin. In addition, other genes have been found to be overexpressed and the presence of doxorubicin response elements in the 5′-region of the human Id2 gene has been hypothesised [21], strongly suggesting that doxorubicin may exert specific functions on the transcription machinery. Interestingly, it has also been reported that a crude preparation of RNA polymerase II was inhibited in its transcriptional activity by doxorubicin and this effect was attributed to a reduction of RNA chain initiation [22]. The data presented in this report further support the latter findings, characterising an essential component of the general transcriptional apparatus [5,6] as a target through which doxorubicin may exert both specific and systemic toxicity. Accordingly, we believe that doxorubicin administration in vivo would result in specific negative effects on the transcriptional activity (this paper and [22]) of those tissues, like the heart, expressing the highest levels of the protein and undergoing a rapid metabolic turnover. In contrast, in doxorubicin resistant cells the selection process may have induced modifications in the half-lives of either human RPB11 mRNA or protein resulting in a proliferative advantage for these cells. Further experimental data are needed to corroborate this pathogenetic hypothesis. In addition, we cannot exclude that doxorubicin may also exert these inhibitory effects on other components of the RNA polymerase II enzymatic complex.

Finally, the cloning and availability of a new subunit of RNA polymerase II provide additional tools to understand the structure and role of this molecule and its subunits in the process of gene transcription and its regulation.

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