FEBS Letters 427 (1998) 236–240 FEBS 20197

The interacting RNA polymerase II subunits, hRPB11 and hRPB3, are coordinately expressed in adult human tissues and down-regulated by doxorubicin

Maurizio Fanciulli[a,*], Tiziana Bruno[a], Monica Di Padova[a], Roberta De Angelis[a], Sarah Lovari[a], Aristide Floridi[a,b], Claudio Passananti[c]

[a] *Cell Metabolism and Pharmacokinetics Laboratory, Regina Elena Cancer Institute, Via delle Messi d'Oro 156, 00158 Rome, Italy*
[b] *Department of Experimental Medicine, Via Vetoio, Coppito 2, University of L'Aquila, 67100 L'Aquila, Italy*
[c] *Istituto di Tecnologie Biomediche, CNR, Viale Marx 43, 00137 Rome, Italy*

Received 11 March 1998

Abstract We previously isolated the human RPB11 cDNA, encoding the 13.3 kDa subunit of RNA polymerase II, and demonstrated that expression of this subunit is modulated by doxorubicin. Using hRPB11 as bait in a yeast two-hybrid system, two cDNA variants encoding a second RNA polymerase II subunit, hRPB3, have now been isolated and characterized. These two hRPB3 mRNA species differed in 3′ UTR region length, the longer transcript containing the AU-rich sequence motif that mediates mRNA degradation. Both hRPB11 and hRPB3 transcripts share a similar pattern of distribution in human adult tissues, with particularly high levels in both heart and skeletal muscle, and the expression of both is down-regulated by doxorubicin as found previously for the hRPB11 subunit. Taken together, these findings suggest that the interaction between hRPB3 and hRPB11 is fundamental for their function and that this heterodimer is involved in doxorubicin toxicity.

Key words: Doxorubicin; Drug toxicity; RNA polymerase II; Alternative splicing; mRNA degradation; Two-hybrid system

1. Introduction

Doxorubicin (dox) is an antineoplastic antibiotic with a broad spectrum of chemotherapeutic activity on several human malignancies [1]. However, its clinical use is often limited because of serious cumulative dose-dependent cardiac toxicity [2] and multidrug resistance. In fact several tumors do not respond to the cytotoxic activity of the drug and/or become resistant to treatment after an initial response [3,4].

In a previous study, we used differential display polymerase chain reaction to identify potential novel molecular mechanisms involved in doxorubicin toxicity and isolated a gene, the expression of which was down-regulated in doxorubicin-resistant human carcinoma cell lines [5]. This gene encoded the human RNA polymerase II (pol II) subunit hRPB11 [5–7]. Treatment of cells with dox down-modulated the expression of this RNA polymerase II subunit at both the mRNA and protein levels [5]. Messenger RNA for hRPB11 was expressed ubiquitously in all normal tissues tested, with highest expression in heart and skeletal muscle, in which the highest toxicity to dox was observed.

Eukaryotic RNA pol II is composed of more than 12 polypeptides ranging from 220 to 10 kDa [8]. The RPB1, RPB2 and RPB3 subunits are structural homologues of the *Escherichia coli* RNA polymerase core subunits β′, β and α, respectively. The minimum and essential subunits required for eukaryotic mRNA synthesis, however, remain to be elucidated. Although the function of the smaller subunits is not yet completely understood, evidence suggests that yeast RNA pol II may influence gene expression by altering its subunit composition in response to stress [9,10]. As hRPB11 is modulated by dox treatment, we used this pol II subunit as bait in the yeast two-hybrid system to identify potential interaction-target protein(s), in an effort to clarify its functions.

We report the characterization of two cDNA variants encoding the hRPB3 subunit of RNA pol II, and confirm their interaction with hRPB11 in the yeast two-hybrid system. Moreover, we demonstrate coordinate hRPB11 and hRPB3 expression in adult human tissues and coordinate repression of expression following dox treatment.

2. Materials and methods

2.1. Yeast two-hybrid selection

The cDNA of hRPB11 modified for sub-cloning in frame with the GAL4 binding domain (BD) was prepared and inserted into the *Pst*I restriction site of vector pGBT9 (Clontech, Palo Alto, CA). The yeast strain HF7c [11], bearing UASg-His3 and UASg-LacZ as reporter genes, was cotransformed with the bait PGBT9-hRPB11 and a library prepared with human skeletal muscle cDNA fused to the GAL4 activation domain (AD), in the vector pGad10 (Clontech). Transformation was performed using the lithium acetate method [12]. Cells were plated directly on minimal synthetic defined medium (SD): 2% glucose, 0.67% Bacto yeast nitrogen base (Difco), supplemented with the required bases and amino acids [13], lacking leucine (Leu), tryptophan (Trp), and histidine (His). Plates were incubated for 7 days at 30°C and His^+ transformants isolated. The His^+ colonies, replica-plated on SD-Leu-Trp-His medium and $LacZ^+$, were identified by a filter-lifting assay for β-galactosidase activity [13]. Plasmid DNA was prepared from candidate clones and electroporated into *E. coli* XLI-blue competent cells (Stratagene, La Jolla, CA). The recovered library-derived plasmids were further analyzed as positive candidates. To investigate the hRPB11 domains involved in the selected interactions, three new baits were constructed in the pGBT9 vector. These three constructs carried different hRPB11 deletions: 11Qui (from aa 24 to aa 117), 11Quo (from aa 54 to aa 117), 11Qua (from aa 87 to aa 117).

2.2. Coimmunoprecipitation

The hRPB11 and hRPB3 cDNAs were modified for sub-cloning in frame in mammalian expression vectors pEGFP-N1 (Clontech) and pcDNA3.1/Myc-His (Invitrogen, Carlsbad, CA), respectively. COS7

*Corresponding author. Fax: (39) (6) 498-52505.
E-mail: fanciulli@crs.ifo.it

Abbreviations: dox, doxorubicin; pol II, polymerase II; hRPB11, human RNA polymerase II subunit 11; hRPB3, human RNA polymerase II subunit 3

PII S0014-5793(98)00431-1

cells (American Type Culture Collection) were cultured to approximately 70% confluence in 10 cm diameter dishes, and transfected using the calcium phosphate procedure [14], with either CMV-myc-tag vector or CMV-myc-tag-hRPB3 and CMV-GFP-hRPB11. After 36 h of incubation at 5% CO_2, cells were rinsed three times with ice-cold PBS, harvested, centrifuged at 4°C, and cell pellets lysed by incubation at 4°C for 1 h in 400 μl of lysis buffer (100 mM NaCl, 1% NP-40, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.02% NaN_3, 1 mM PMSF, 10 mg/ml leupeptin). Lysates were centrifuged in an Eppendorf centrifuge (top speed, 4°C for 5 min). Supernatants recovered were incubated with a monoclonal anti-myc antibody (Invitrogen) for 1 h at 4°C, followed by addition of 20 μl of protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and incubation for an additional 2 h. The resins were then washed three times in the same buffer, the bound proteins were separated by reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [15]. Proteins were transferred to nitrocellulose (Amersham, Bucks., UK) and membranes probed with a monoclonal anti-GFP antibody (Clontech). Immunoreactivity was detected using an enhanced chemiluminescence kit (Amersham) as described by the manufacturer.

2.3. DNA sequencing

All constructs reported were sequenced by Sequenase reaction (Amersham) according to the manufacturer's instructions.

2.4. Cell lines and drug treatment

Human colon carcinoma LoVo H dox-sensitive and LoVo DX dox-resistant cells were kindly provided by Dr. M.P. Colombo (Istituto Nazionale Tumori, Milan, Italy). Establishment and characteristics of these cells have been described elsewhere [16]. LoVo DX cells were maintained in continuous exposure to 0.2 μM dox (Adriblastina, Farmitalia Carlo Erba, Milan, Italy).

2.5. RNA extraction and northern blotting analysis

Total RNA was extracted from dox-resistant and dox-sensitive human LoVo cell lines, as described by Chomczynski and Sacchi [17]. 20 μg of total RNA was separated by 1.2% agarose-formaldehyde gel electrophoresis and transferred to nylon filters (Hybond-N, Amersham) [14]. Pre-made filters containing poly(A)$^+$ RNA from eight normal human tissues (MNT human blot I) were purchased from Clontech. Blots were hybridized using Quikhyb (Stratagene), according to the manufacturer's instructions. ^{32}P-labelled cDNA probes were prepared using a Megaprime probe kit (Amersham). Quantitative loading of RNA was determined by hybridization with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA control probe [18].

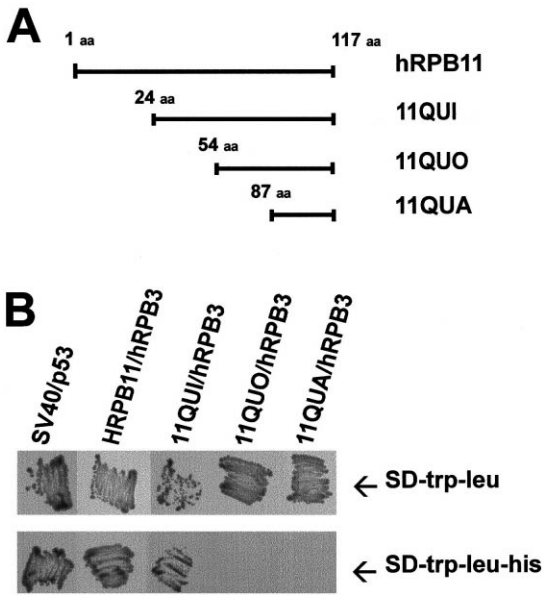

Fig. 1. A: Schematic diagrams of N-terminal deletions of the hRPB11 RNA polymerase II subunit. B: Demonstration of the interaction between hRPB3 and hRPB11 N-terminal deletions by His3 assay. The SV40LT/p53 interaction was been used a positive control.

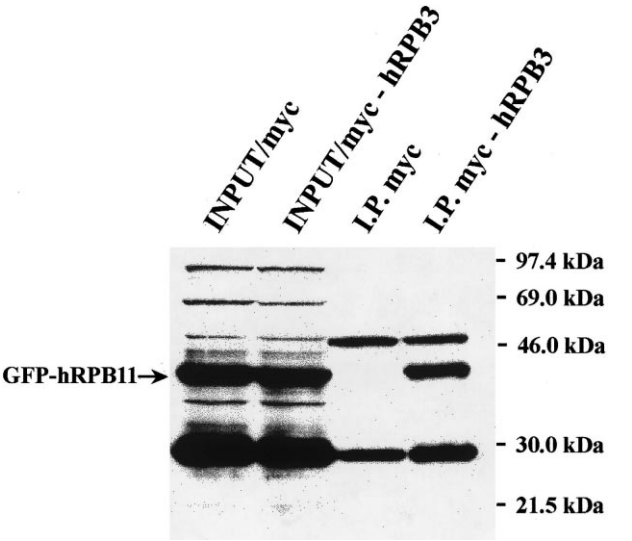

Fig. 2. Western blot analysis of GFP-hRPB11 fusion protein recovered from immunoprecipitates of transfected COS7 whole cell extracts using anti-myc antibody. In each case 20% of input extract (INPUT) shown.

3. Results

3.1. Two-hybrid selection

In order to investigate potential protein/protein interactions driven by hRPB11, screening of a yeast two-hybrid system was performed using the complete cDNA coding sequence for hRPB11 fused to the Gal4 binding domain of the plasmid vector pGBT9. Because of the high level of transcription of the hRPB11 gene in human skeletal muscle, a cDNA library prepared from this tissue and incorporated in the plasmid vector pGAD10 was used. Using the appropriate yeast reporter strain, 1×10^6 clones were screened, and clones exhibiting a positive interaction were further analyzed. Eight clones interacting strongly with the bait were isolated (Fig. 1B), and all encoded a second human RNA polymerase II subunit 'hRPB3' [19], adding to recent reports of a similar interaction in humans, mice and *Arabidopsis* [20–22]. Both hRPB11 and hRPB3 subunits exhibited limited amino acid sequence homology to the α-subunit of *E. coli* RNA polymerase. This region, termed the 'α-like domain', suggests that hRPB11/3 heterodimers may be considered the eukaryotic counterpart of the bacterial α-subunit homodimer [23,24]. Since the interaction between hRPB11 and hRPB3 directly involves the α-like domain of the hRPB3 protein [22], we examined whether the α-like domain of hRPB11 was involved. For this purpose hRPB3 subunits containing three different amino-terminal deletions of hRPB11, 11Qui, 11Quo, 11Qua respectively, were screened in the yeast two-hybrid system (Fig. 1A). Only the deletion 11Qui retained the ability to interact with the hRPB3 subunit, indicating that a minimal region of 30 amino acid containing the α-like domain was essential (Fig. 1B).

3.2. hRPB11 interacts with hRPB3 in vivo

To establish whether hRPB11 interacted with the hRPB3 subunit in mammalian cells, GFP-hRPB11 and myc-tag-

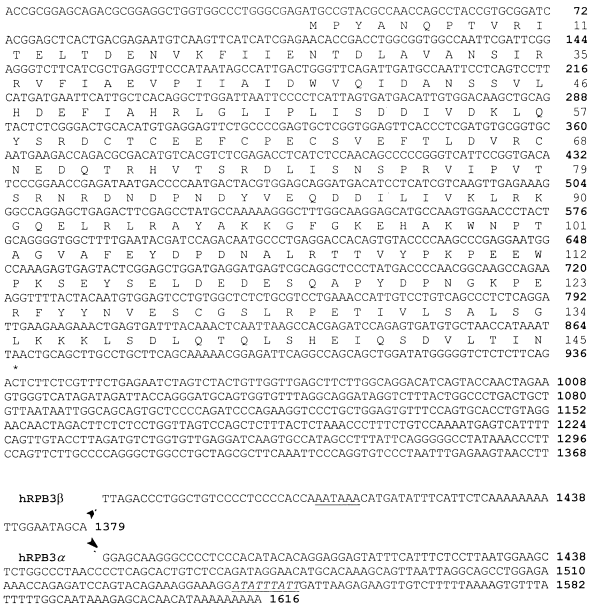

Fig. 3. Nucleotide and amino acid sequences of two hRPB3 cDNA variants (hRPB3α and hRPB3β) encoding a human subunit of RNA polymerase II. Nucleotides are numbered on the right in bold characters, amino acid residues are numbered on the right. Both the hRPB3α and β transcripts encode a complete hRPB3 protein, but differ in the length of the 3′ UTR region. Starting from the divergent nucleotide in position 1379, the two cDNA variants follow independent nucleotide numeration, indicated by two arrows: ↑ for hRPB3β and ↓ for hRPB3α. The polyadenylation consensus signals present in both cDNAs are underlined. The AU-rich sequence motif that mediates mRNA degradation in hRPB3α cDNA is underlined and indicated by italic characters. The hRPB3α and hRPBβ nucleotide sequences have been deposited in GenBank with accession numbers AJ224143 and AJ224144, respectively.

hRPB3 fusion proteins were overexpressed in COS7 cells. Fig. 2 demonstrates Western blot analysis using a monoclonal anti-GFP antibody. In total lysates a protein of 41 kDa, consistent with the size of the GFP-hRPB11 fusion protein, was present. GFP-hRPB11 was also detected in immunoprecipitates of myc-hRPB3, indicating physical interaction between hRPB3 and hRPB11 subunits within mammalian cells.

3.3. Two cDNA variants encoded for the hRPB3 subunit of RNA polymerase II

Fig. 3 shows two cDNA variants encoding the hRPB3 subunit of RNA pol II, previously described by Pati and Weissman [19]. The two variants were independently cloned, six and two times, respectively. DNA sequence analysis of the clones demonstrated that all encode a complete hRPB3 protein and differ only in the length of the 3′ UTR region. The shorter and the longer variants were named hRPB3α and hRPB3β, respectively. Interestingly, both variants differed in the same region from the longer transcript described by Pati and Weissman [19]. The hRPB3β transcript terminated with a canonical polyadenylation signal and contained in the 3′ UTR region an AU-rich sequence motif, which mediates mRNA degradation [25] (Fig. 3). The hRPB3α transcript terminated earlier than the β type with a canonical polyadenylation signal, lacking the AU-rich sequence as a result of alternative splicing.

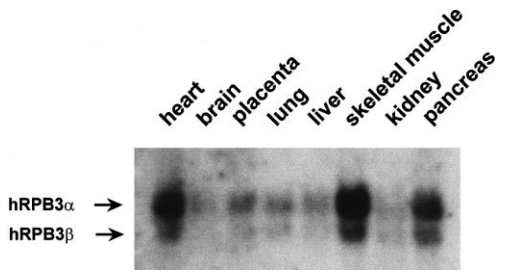

Fig. 4. Expression analysis of hRPB3 by Northern blot analysis of poly(A)$^+$ RNA purified from eight different adult human tissues. The hybridization signals, corresponding to the mRNA variants hRPB3β and hRPB3α, are indicated by arrows.

3.4. hRPB3 and hRPB11 share the same pattern of tissue expression

Since hRPB11 and hRPB3 RNA polymerase II subunits interact, we investigated whether they share the same pattern of tissue distribution. The expression of hRPB3 mRNA was analyzed by Northern blot using poly(A)$^+$ selected RNA prepared from eight normal tissues. As shown in Fig. 4, two mRNA species were detected exhibiting sizes consistent with those of hRPB3α and β variants. The intensity of the hybridization signal for hRPB3α mRNA was greater than hRPB3β. hRPB3 transcripts were found in all tissues analyzed, but they peaked in heart, muscle and pancreas. These results clearly demonstrate that hRPB3 shares the same tissue distribution as described for hRPB11 [5].

3.5. Doxorubicin down-regulates the expression of hRPB3

It has been previously demonstrated that doxorubicin down-regulates the expression of the hRPB11 RNA pol II subunit [5]. We therefore examined the effects of dox on hRPB3 transcription. Fig. 5A shows that both hRPB3α and β variants were highly expressed in the dox-sensitive cell line LoVo H, but barely detectable in dox-resistant LoVo DX cells. This demonstrates the sensitivity of both subunits to dox. To better define the role of dox in the regulation of hRPB3, expression was analyzed in LoVo DX cells after the complete withdrawal of dox from the culture medium. This resulted in the gradual increase in hRPB3 mRNA levels (Fig.

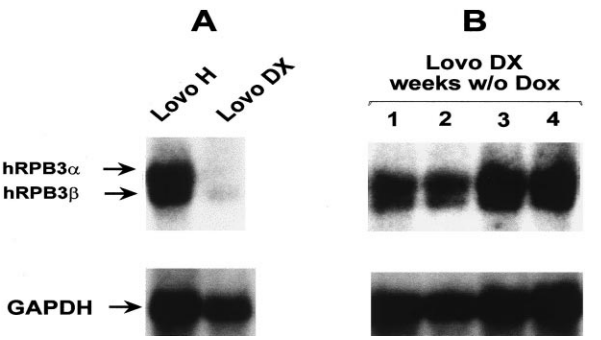

Fig. 5. A (top): Northern blot analysis of hRPB3 subunit expression using total RNA purified from the dox-sensitive human cell line (LoVo H) and the dox-resistant cell line (LoVo DX). B (top): LoVo DX cells cultured in the absence of doxorubicin; numbers indicate weeks of cell growth in culture medium without dox. The absence of the drug induced a gradual up-modulation of hRPB3 mRNA levels, which after 3 weeks reached the levels observed in the LoVo H cell line. The amount of RNA loaded in each lane was normalized by the hybridization signals obtained using as a probe GAPDH (A and B, bottom).

5B), which reached those in the LoVo H cell line after 3 weeks (Fig. 5A). This confirmed a direct correlation between doxorubicin toxicity and the expression of components of the hRPB11/3 complex.

4. Discussion

Doxorubicin exhibits specific and systemic toxic effects through several mechanisms including generation of oxygen free radicals [26], interaction with cell membrane constituents [27], and induction of large gene deletions in mammalian cells [28]. A selective action on several genes has also been reported, suggesting that this drug may exert specific effects on the transcriptional machinery. In particular, a down-modulation of mRNA levels has been described for different muscle-specific genes [29]. Interestingly, the transcriptional activity of RNA polymerase II, in a crude preparation, is inhibited by doxorubicin [30]. Further investigation of the mechanisms involved in dox toxicity has identified a core subassembly subunit of RNA polymerase II, hRPB11, specifically affected by doxorubicin administration [5]. This subunit was used as bait to screen a yeast two-hybrid system to identify potential protein/protein interactions. We report that hRPB11 interacts with a second RNA polymerase II subunit, named hRPB3. hRPB3 was the only RNA polymerase II partner/component isolated, supporting the hypothesis that the hRPB3 subunit is a unique partner for hRPB11 in RNA pol II [20]. The interaction between hRPB11 and hRPB3 subunits has been recently reported in mouse, human and *Arabidopsis thaliana* [20–22], and the α-like domain of hRPB3 was shown to be essential for this interaction [22]. Here, we demonstrate that the α-like domain of hRPB11 is also involved in this interaction, consistent with the prominent role of this domain in RNA polymerase function and its high degree of evolutionary conservation. Indeed, these two subunits have been recently shown to form the core subassembly subunit of RNA pol II [31]. This is reminiscent of the $\alpha_2\beta$ complex of *E. coli* RNA polymerase, which is an intermediate subassembly in the following pathway: $\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta^1$ [32]. All of these observations underscore a fundamental structure role of this heterodimer.

On the other hand, our data suggest a putative regulatory role for these subunits. Analysis of hRPB3 and hRPB11 transcription revealed coordinated tissue expression. Although essential for cellular viability [33], and despite the fact that transcripts were detected in all adult tissues analyzed, both subunits exhibited modulated expression with clear peaks in heart and muscle tissues, which exhibit the highest responses to dox. Furthermore, hRPB3 and hRPB11 [5] expression was strongly down-regulated in dox-resistant LoVo cells and reversed by drug withdrawal.

The necessity for fine regulation of hRPB3 expression was also underlined by the isolation of two mRNA variants, differing in the UTR region for an AU-rich mRNA degradation motif [25]. Interestingly, the longer transcript was expressed several fold higher than the other variant, but was more sensitive to the effects of dox. In fact, in dox-resistant LoVo cells the hRPB3β variant was only slightly detectable.

Further support for a putative regulative role for hRPB3 in the transcription comes from the observation that the cjnC gene product, a homologue of hRPB3 in *Tetrahymena*, is only active during early conjugation, suggesting that it may be involved in the regulation of transcription at that particular time [34]. Moreover, computer analysis of hRPB3 protein has revealed several putative functional domains including the RNA polymerase α-like motif, an EF-hand calcium-binding domain at position aa 136–148 [35], and a cysteine reach region resembling a potential zinc-finger motif in position aa 88–115 [22].

All these considerations support a putative regulative role of hRPB3 subunit, and additional studies will be required to better characterize its functions.

Acknowledgements: We acknowledge Dr. Andrew McKay for critical reading. Miss Roberta Bruno is gratefully acknowledged for artwork. This work was partially supported by Ministero della Sanità, Telethon Project A63, and MURST 60%.

References

[1] Young, R.C., Ozols, R.F. and Myers, C.E. (1981) New Engl. J. Med. 305, 153–158.
[2] Singal, P.K., Deally, C.M. and Weimberg, L.E. (1987) J. Mol. Cell. Cardiol. 19, 817–828.
[3] Ueda, K., Cardarelli, C., Gottesman, M.M. and Pastan, I. (1987) Proc. Natl. Acad. Sci. USA 84, 3004–3008.
[4] Grant, C.E., Valdimarsson, G., Hipfner, D.R., Almquist, K.C., Cole, S.P. and Deely, R.G. (1994) Cancer Res. 54, 357–361.
[5] Fanciulli, M., Bruno, T., Cerboni, C., Bonetto, F., Iacobini, C., Frati, L., Piccoli, M., Floridi, A., Santoni, A. and Punturieri, A. (1996) FEBS Lett. 384, 48–52.
[6] Woychik, N.A., McKune, K., Lane, W.S. and Young, R.A. (1993) Gene Express. 3, 77–82.
[7] Pati, U.K. (1994) Gene 145, 289–292.
[8] Young, R.A. (1991) Annu. Rev. Biochem. 60, 689–715.
[9] Choder, M. and Young, R.A. (1993) Mol. Cell. Biol. 13, 6984–6991.
[10] Woychik, N.A., Lane, W.S. and Young, R.A. (1991) J. Biol. Chem. 266, 19053–19055.
[11] Feilotter, H.E., Hannon, G.J., Ruddel, C.J. and Beach, D. (1994) Nucleic Acids Res. 22, 1502–1503.
[12] Gietz, D., St. Jean, A., Woods, R.A. and Schiestl, R.H. (1992) Nucleic Acids Res. 20, 1425–1426.
[13] Guthrie, C. and Fink, G.R. (1991) Methods Enzymol. 194, 1–932.
[14] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[15] Laemmli, U.K. (1970) Nature 227, 680–685.
[16] Li-Ying, Y. and Trujillo, J.M. (1990) Cancer Res. 50, 3218–3225.
[17] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[18] Piechaczyk, M., Blanchard, J.M., Marty, L., Dani, C., Panabieres, F., El Sabouty, S., Fort, P. and Jeanteur, P. (1984) Nucleic Acids Res. 12, 6951–6963.
[19] Pati, U.K. and Weissman, S.M. (1990) J. Biol. Chem. 265, 8400–8403.
[20] Acker, J., de Graaff, M., Cheynel, I., Khazak, V., Kedinger, C. and Vigneron, M. (1997) J. Biol. Chem. 272, 16815–16821.
[21] Nishi, Y., Yamamoto, K., Yao, Y., Yamamoto, M., Nogi, Y., Matsuo, H. and Muramatsu, M. (1997) Gene 187, 165–170.
[22] Ulmasov, T., Larkin, R.M. and Guilfoyle, T.J. (1996) J. Biol. Chem. 271, 5085–5094.
[23] Woychik, N.A., McKune, K., Lane, W.S. and Young, R.A. (1993) Gene Express. 3, 77–82.
[24] Pati, U.K. (1994) Gene 145, 289–292.
[25] Zubiaga, A.M., Belasco, J.G. and Greenberg, M.E. (1995) Mol. Cell. Biol. 15, 2219–2230.
[26] Doroshow, J.H. (1983) Cancer Res. 43, 460–472.
[27] Siegfried, J.A., Kennedy, K.A., Sartorelli, A.C. and Tritton, T.R. (1983) J. Biol. Chem. 258, 339–345.
[28] Yu, Y., Xu, Z. and Hsie, A.W. (1994) Mutat. Res. 325, 91–98.
[29] Ito, H., Miller, S.C., Billingham, M.E., Akimoto, H., Torti, S.V.,

Wade, R., Gahlmann, R., Lyons, G., Kedes, L. and Torti, F.M. (1990) Proc. Natl. Acad. Sci. USA 87, 4275–4279.
[30] Chuang, R.Y. and Chuang, L.F. (1979) Biochemistry 18, 2069–2073.
[31] Kimura, M., Ishiguro, A. and Ishihama, A. (1997) J. Biol. Chem. 272, 25851–25855.
[32] Ishihama, A. (1981) Adv. Biophys. 14, 1–35.
[33] Kolodziej, P. and Young, R.A. (1989) Mol. Cell Biol. 9, 5387–5394.
[34] Martindale, D.W. (1990) Nucleic Acids Res. 18, 2953–2960.
[35] Kawasaki, H. and Kretsinger, R.H. (1995) Protein Prof. 2, 305–490.