Influence of polyamines on DNA binding of heat shock and activator protein 1 transcription factors induced by heat shock

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Received 6 May 1999; received in revised form 10 June 1999

Abstract Polyamine depletion, obtained in FAO cells with specific inhibitors of biosynthetic enzymes, prevents or decreases the accumulation of hsp 70 mRNA following heat shock [Desiderio et al., Hepatology 24 (1996) 150–156]. The present study shows that under conditions of spermidine depletion caused by α-difluoromethylornithine, the DNA binding capacity of the transcription factor HSF induced by heat shock undergoes a severe and prompt deactivation. Replenishment of the spermidine pool before heat shock re-establishes the DNA binding activity of HSF and the inducibility of hsp 70 mRNA. Similar to HSF, but with a different time-course, the DNA binding of the transcription factor AP-1 activated by heat shock is also impaired in spermidine-depleted cells and reversed by exogenous spermidine. STAT3 provides an example of a transcription factor slightly activated by heat shock but insensitive to polyamine decrease.

Key words: Heat shock; Polyamine; Transcription factor

1. Introduction

The heat shock response occurs when cells are briefly and suddenly exposed to temperatures above their normal growth temperature and is characterised by the synthesis of a unique set of proteins, called heat shock proteins. These proteins are also synthesised in response to a host of noxious agents and unfavourable conditions and are now more appropriately indicated as 'stress proteins'. The final step of stress protein synthesis is preceded by a series of events that include the increase in the steady-state level of the corresponding mRNA, mostly due to increased transcription, and that depend on the activation of the specific heat shock transcription factor (HSF). Such a series of events may easily be influenced by chemical, metabolic or environmental circumstances which are not directly involved in the synthesis of stress proteins, but may behave as permissive conditions [1–3].

Polyamines (putrescine, spermidine and spermine) are ubiquitous polycations that have numerous covalent and non-covalent interactions in eukaryotic cells. Their physico-chemical properties favour the interaction with large anionic molecules such as DNA, RNA and phospholipids [4–6]. Therefore, polyamines exert relevant functions in the processes of mRNA transcription and splicing as well as stabilisation of molecular secondary structures, for instance those of DNA and RNAs [4,5,7,8].

In a previous paper, we have studied the effects of polyamine depletion in FAO cells exposed to heat shock [9]. Polyamine imbalance was realised by inhibition of the key polyamine biosynthetic enzymes, without induction of appreciable growth arrest and impaired expression of growth-related genes. The decrease of spermidine obtained 48 h after treatment with α-difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor of ornithine decarboxylase (ODC), prevented the induction of heat shock protein 70 (hsp 70) mRNA. Depletion of spermidine and spermine, obtained with the combination of two other specific inhibitors of ODC and *S*-adenosylmethionine decarboxylase (AdoMetDC), decreased the intensity and duration of post-heat shock accumulation of hsp 70 mRNA.

We have now tried to investigate the early effects of polyamine imbalance due to DFMO treatment on the expression of hsp 70, and we studied the first step of the transcription of heat shock genes, i.e. the activation of HSF. The inhibitory effect of polyamine depletion on the heat shock-induced expression of c-*jun* and c-*fos* mRNAs [9] was the rationale for the concomitant study of activator protein 1 (AP-1). In some experiments the addition of spermidine to cells treated with DFMO proved effective in re-establishing the inducibility of hsp 70 mRNA. Therefore, the analysis of transcription factor activation was also performed with cells treated with DFMO and then loaded for a short time with exogenous spermidine before exposure to heat.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP, [α-^{32}P]dCTP, and [α-^{32}P]UTP, specific activity 3000 Ci/mmol (Amersham, Bucks, UK). DFMO was generously given by Marion Merrell Dow Research Institute (Strasbourg, France). Polyclonal rabbit anti-STAT1 and anti-STAT3 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All the other chemicals used were of the highest grade available.

2.2. Cell cultures and treatments

The rat hepatocarcinoma cell line FAO (European Collection of Cell Cultures, Salisbury, UK) was cultured as reported before [9]. When the effect of DFMO (5 mM) was studied, the inhibitor was dissolved in the complete culture medium and the pH was adjusted before the medium was dispensed in each flask. Spermidine was added to some flasks 46 h after seeding (final concentration 20 μM) [10] and allowed to act for 2 h at 37°C. All the flasks were heated 48 h after plating by full immersion in a water-bath at 42°C for 30 or 60 min. At the end of this period, some flasks were incubated for 60 min at 37°C to allow the cells to recover from the immediate effects of heating [9].

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Abbreviations: hsp 70, heat shock protein 70; HSF, heat shock factor; AP-1, activator protein 1; STAT, signal transducer and activator of transcription; DFMO, α-difluoromethylornithine; ODC, ornithine decarboxylase; AdoMetDC, *S*-adenosylmethionine decarboxylase; EMSA, electrophoresis mobility shift assay; HSE, heat shock element; TRE, TPA responsive element; SIE, sis inducible elements; oct-1, octamer-1

0014-5793/99/$20.00
PII: S0014-5793(99)00873-X

2.3. Determination of polyamines

Cell extracts in 0.2 N $HClO_4$ and polyamine analysis by HPLC were performed as previously described [9]. Protein content of supernatants used for HPLC analysis was determined by the method of Lowry et al. [11].

2.4. Analysis of the mobility of DNA-protein complexes by gel electrophoresis (EMSA)

Nuclear extracts were prepared [12] and the DNA-protein complexes were analysed by electrophoresis on 5% polyacrylamide gel [13]. The base sequences of the oligonucleotides synthesised by Primm (Milan, Italy) were:

HSE: CTAGAACGTTCT**AGAAGCTTC**GAG [14],
TRE: CTAGTGA**TGAGTCA**GCCGGATC [15],
SIE: GATCCTCCAGCAT**TTCCCGTAA**ATCCTCCAG [16],
oct-1 site: GATCGAATGCAAATCACTAGCT [17].

2.5. Northern blot analysis

Preparation of total RNA and Northern blot analysis were performed as previously described [9]. The following probes were used: cDNA for rat β-actin (S. Gaetani, Rome, Italy); human pURHS70 cDNA for hsp 70 (J.R. Nevins, New York, NY, USA).

2.6. Run-on transcription analysis

Nuclei were isolated from 50×10^6 cells (pool of five flasks) as described by DeLisle et al. [18], resuspended in 150 μl of a solution containing 50 mM Tris-HCl, pH 8.3, 40% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, and frozen in liquid N_2. For the run-on transcription assay, the nuclei were thawed in ice and mixed with 150 μl of reaction buffer (10 mM Tris-HCl, pH 8, 5 mM $MgCl_2$, 300 mM KCl, 0.5 mM each of cold ATP, CTP and GTP, and 200 μCi of [α-^{32}P]UTP), and incubated at 26°C for 30 min [19]. The ^{32}P-labelled RNA was purified as described by Tacchini et al. and hybridised with filter-bound immobilised plasmids [13].

2.7. mRNA half-life determination

hsp 70 mRNA half-life was measured in cells subjected to heat shock, with or without pretreatment with DFMO. Actinomycin D (5 μg/ml) [20] was added at the end of heat shock (60 min at 42°C). Cell samples were harvested from 15 min to 2 h, total RNA was isolated, and quantification of steady-state levels of mRNAs was performed by Northern blotting, followed by densitometric analysis.

2.8. Densitometric analysis

The autoradiograms were quantified by densitometric analysis (OD per mm^2) using a LKB Image Master DTS system (Pharmacia Biotec., Hertfordshire, UK).

3. Results and discussion

The present work was undertaken to study the mechanism(s) by which polyamine imbalance may influence gene reprogramming induced by heat shock. The experiments were performed with FAO cells subjected to heat shock in the presence or absence of DFMO, a specific and irreversible

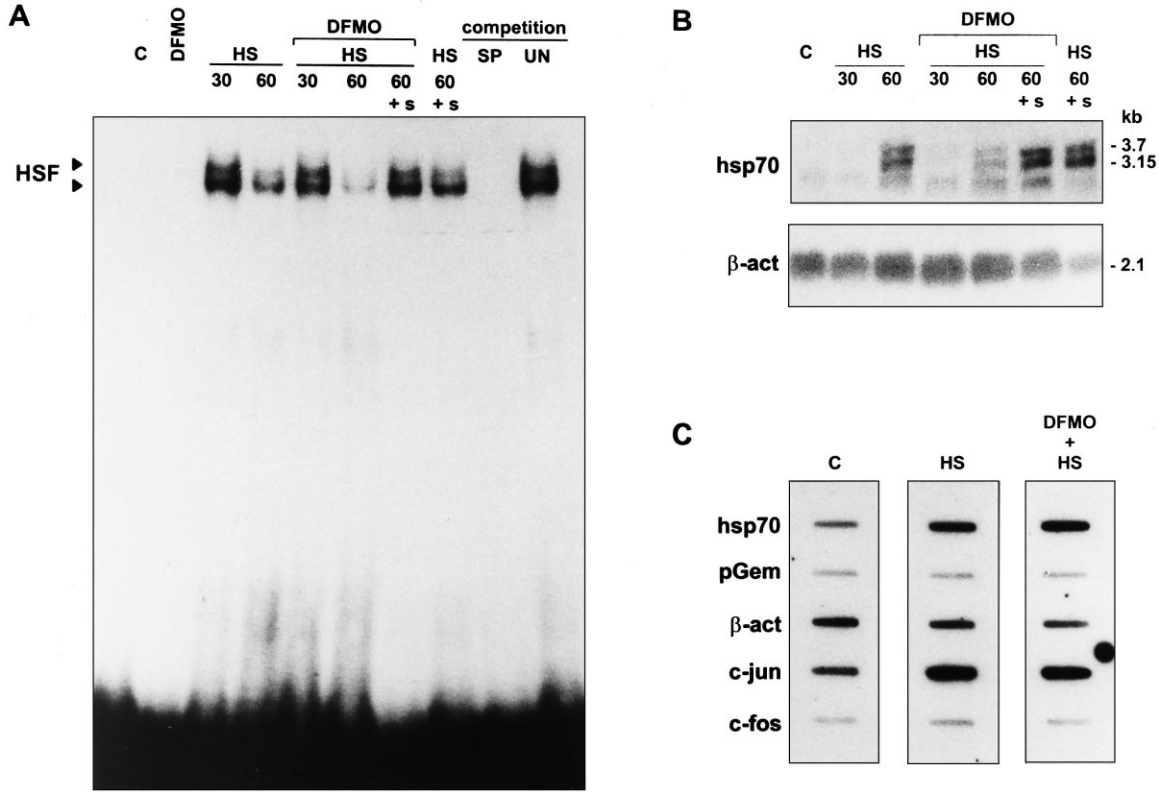

Fig. 1. Effects of DFMO pretreatment of FAO cells subjected to heat shock on HSF DNA binding and hsp 70 transcription. A: EMSA of complexes between HSE and nuclear proteins from FAO cells undergoing the following treatments: C, control; DFMO, 48 h-DFMO pretreatment; HS, heat shock for 30 or 60 min, with addition of spermidine (+s). SP, specific competition; UN, unspecific competition with TRE oligonucleotide. Sample used for competition is HS 30. B: Total RNA was extracted as described in Section 2. 20 μm of each RNA was resolved by electrophoresis on 1% agarose gel, transferred to a nylon membrane, and hybridised with ^{32}P-labelled hsp 70 and β-act (β-actin) cDNA probes. Captions as in A. C: Nuclei were isolated from cells treated as follows: C, control; HS, 60 min heat shock; DFMO+HS, DFMO pretreatment followed by 60 min heat shock. After run-on assay the ^{32}P-labelled transcripts were hybridised with filter-bound immobilised plasmids containing cDNA for hsp 70, β-actin, c-*jun* and c-*fos* as described in Section 2. pGEM is the empty plasmid. This and the following figure show typical results of experiments repeated in triplicate.

inhibitor of ODC, which is the first and rate-limiting enzyme of polyamine biosynthesis [21]. To study DNA binding of HSF we performed EMSA of nuclear extracts obtained from FAO hepatoma cells, subjected to the different treatments, with an oligonucleotide reproducing the base sequence of HSE. In agreement with previous findings [9], 48 h treatment with DFMO causes a 50% decrease of spermidine level (control value at 48 h is 6.2 ± 0.1 nmol/mg protein) without affecting cell viability. The supplementation with 20 μM spermidine for 2 h before heat shock results in a rapid recovery of intracellular polyamine level in 48 h DFMO-pretreated cells (data not shown). As previously reported [9], the treatment with DFMO has the same inhibitory effect on hsp 70 mRNA expression as the combined treatment with inhibitors of ODC and AdoMetDC.

As reported in Fig. 1A, after heat shock we detected the presence of an active HSF competent for DNA binding, while nuclear extracts from control cells or from cells treated with DFMO alone did not bind the oligonucleotide probe. Within 30 min from the beginning of heat shock there was a strong activation of DNA binding which declined, but was still evident, after 60 min of heat shock. Pretreatment of the cells with DFMO reduced only slightly (about 25%) the activation at 30 min, but reinforced the deactivation observed at 60 min (about 60% reduction). Therefore, in DFMO-treated cells the induction of DNA binding seems to start only a little less efficiently, but is clearly reduced in duration indicating an accelerated deactivation of HSF. A possible involvement of polyamines is supported by the rescue effect exerted by exogenous spermidine (DFMO/HS60+s). However, exogenous spermidine added to cells untreated with the inhibitor seemed to maintain HSF in a state of activated DNA binding after 60 min of heat shock (HS60+s). In other words, spermidine may be more related to the stability of HSF DNA binding than to the activation per se. The stronger effect observed with spermidine in DFMO-treated cells is most likely due to the increased uptake of exogenous spermidine by cells under conditions of decreased polyamine intracellular levels [22]. These data are consistent with the results obtained by Northern blot analysis probing the RNA with a specific hsp 70 cDNA (Fig. 1B). hsp 70 mRNA was not detectable after 30 min of heat shock, which is understandable because of the required time interval between activation of the transcription factor and mRNA accumulation. However, remarkable steady-state levels of hsp 70 mRNA were observed after 60 min of heat shock. At this time DFMO-treated cells showed a reduced mRNA level (about 40% of the control), which was completely restored in the cells replenished with spermidine before heat shock. Next, we investigated two steps that are interposed between the DNA binding activity of HSF and the increase in the levels of hsp 70 mRNA, and we performed experiments of run-on transcription and mRNA stability. Run-on rates of both hsp 70 and c-*jun* were enhanced in nuclei isolated from 60 min heat-shocked cells (Fig. 1C), thus demonstrating the role of transcription in the increase in the steady-state levels of the mRNAs of these genes after exposure to heat. In the presence of a short-lived HSF DNA binding and of a decrease of hsp 70 mRNA level we expected a decrease of the transcription rate of hsp 70 in DFMO-treated cells, but this was not the case. The data may be explained by a series of considerations. Transcription rate in vivo depends on the flow of the processes of initiation and

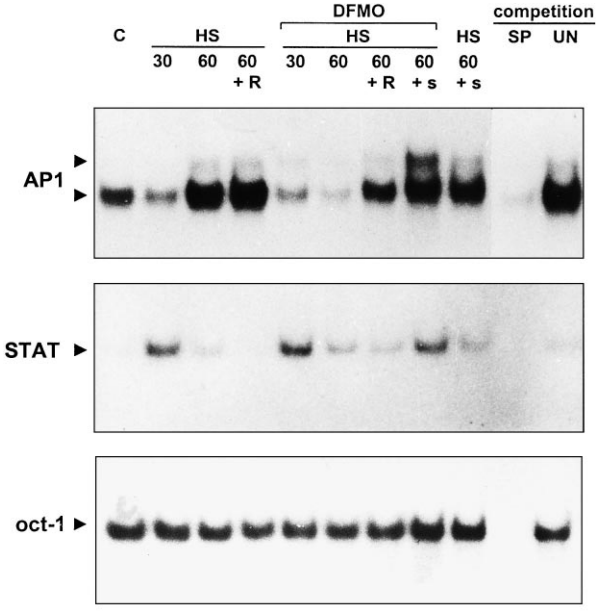

Fig. 2. Effect of DFMO pretreatment of FAO cells subjected to heat shock on AP-1, STAT and oct-1 DNA binding reaction, EMSA of complexes between TRE, SIE, oct-1 and nuclear proteins from FAO cells undergoing the following treatments: C, control; DFMO, DFMO pretreatment; HS, heat shock for 30 or 60 min with addition of spermidine (+s); 60+R, heat shock applied for 60 min followed by 60 min of recovery at 37°C. SP, specific competition; UN, unspecific competition with HSE oligonucleotide. Sample used for competition is HS 60.

elongation of nascent RNA chains: the nuclear run-on assay used to determine transcriptional rates may not accurately reflect all the aspects of the changes in transcriptional activity in vivo, since it only measures the initiated RNA polymerase II transcripts during a single run of elongation [23]. In the context of these data it is worth noting that during the first 30 min of exposure to heat the activation of HSF is essentially the same in untreated and DFMO-treated cells, which would then contain the same number of initiated mRNA chains. What we observed in our run-on experiments is likely to be the elongation of these initiated mRNA chains. Moreover, possible pitfalls in the interpretation of the data obtained by nuclear run-on experiments may arise from the artificial activation of paused RNA polymerase II during the transcription reaction, leading to false results with regard to the level and mode of gene regulation in vivo [24]. Indeed, the presence of paused RNA polymerase in the promoter is a characteristic of the hsp 70 gene [25]. It may be suggested that nuclear polyamines may affect RNA polymerase II activity also by maintaining nucleosome structure and regulating histone displacement [26]. It is known that the measure of polyamine levels in nuclei can give uncertain and sometimes misleading results because the manipulations necessary to isolate nuclei cause loss of their compartmentalisation [27]. Quite recently, with the use of potentiated chromatin templates, which correspond to the in vivo situation best, it has been reported that HSF activates only reinitiation [28].

The measure of mRNA stability implies the use of actinomycin D, an inhibitor of transcription that was added to the cells immediately after heat shock. The results of densitometric analysis were used to plot the regression line. The regres-

sion coefficients, representing the slope of the decay, were 0.046 in untreated heat-shocked and 0.021 in DFMO-pretreated heat-shocked cells: the calculated mRNA half-lives amounted to 100 min and 65 min, respectively. These clear differences show that the imbalance of polyamine pool is associated with decreased stability of the hsp 70 mRNA induced by heat shock. This change may contribute to the lowering of the mRNA steady-state level, but as shown in Fig. 1B and confirmed in these experiments, the mRNA levels at the start of the decay measurement were already 60% lower in DFMO-pretreated than in heat-shocked cells. Obviously, the decreased stability is not directly related to the defect of HSF activity that represents the earliest event in the induction of heat shock genes, but may contribute to the reduction of hsp 70 mRNA in DFMO-treated cells. The existence of multiple defects points to a pleiotropic effect of the depletion of spermidine in the response to heat shock.

Similar to HSF but with a different time-course AP-1 transcription factor is also activated by heat shock (Fig. 2). Soon after exposure to heat the DNA binding capacity of AP-1 seemed to decrease, but then promptly rebounded over the normal level and this stimulated activity was maintained also during the period of recovery from heat shock (3.5-fold increase of the faster band). In cells pretreated with DFMO and then subjected to heat shock (DFMO/HS) an initial (30 min) decrease of binding occurred as in untreated heat-shocked cells. Then, a further reduction occurred at 60 min (90% decrease relative to 60 min heat shock). This decay was reversible and binding activity was re-established, albeit incompletely, during the recovery at 37°C (DFMO/HS60+R). Loading DFMO-treated cells with spermidine before heat shock (DFMO/HS60+s) prevented the effect of the inhibitor on the AP-1 binding activity. Under this condition, the slower band was superinduced.

In Fig. 2, we report the behaviour of another transcription factor, a member of the signal transducer and activator of transcription (STAT) family reacting with the SIE sequence that in experiments of supergel shift, not reported here, has been identified as STAT3. The DNA binding of STAT was activated by heat shock: this activation was small, transient and not inhibited by pretreatment with DFMO. However, exogenous spermidine seemed to stabilise STAT3 DNA binding. DNA binding of oct-1, a constitutively active and heat-shock-insensitive transcription factor, did not change in DFMO-treated cells (Fig. 2). The data obtained with these latter transcription factors indicate that polyamine imbalance has a differential action and interferes only with selected transcription factors activated by heat shock, in particular with HSF which is at the top of the cascade of events leading to increased expression of hsp 70 mRNA.

The significance and the specificity of the effects of polyamine depletion are difficult to assess because these molecules influence many processes, making it impossible to ensure whether or not they have a direct effect. The possibility of restoring the heat shock-induced DNA binding activity of HSF and AP-1, compromised by polyamine imbalance, with exogenous spermidine seems to be in favour of a specific, if not necessarily direct, role of polyamine depletion. A likely target of the imbalance of the intracellular polyamine pool is chromatin [29–31], with some preference for sequences such as TATA elements [8,32]. The intracellular polyamine level is known to influence the degradation rates of both short- and long-lived proteins. Interestingly, recovery of polyamine levels by addition of spermidine to polyamine-depleted cells resulted in the restoration of the degradation rates in both pools of proteins [33]. We cannot exclude that phosphorylations necessary for HSF and AP-1 activities may be affected by polyamines, which are known to influence the activity of protein kinases [34] and the phosphorylation of specific proteins [35].

If we consider the physiological aspects of our results, we should note that the heat shock response is ubiquitous and can be elicited by a wide range of noxious stimuli: indeed the term 'stress response' was introduced to recognise the more general nature and the broad biological importance of the event. The basic machinery of the response needs permissive conditions. Polyamine-depleted cells are defective in the response and may be more vulnerable to injurious agencies or physiological stress.

Acknowledgements: We thank Mr A. Rossi for skilled help in tissue culture work and Mr V. Bianchi for technical assistance. This work was supported by grants from the Consiglio Nazionale delle Ricerche (CNR) and Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST) Italy.

References

[1] Morimoto, R.I., Tissieres, A. and Georgopoulos, C. (Eds.) (1994) The Biology of Heat Shock Proteins and Molecular Chaperons, Academic Press, New York.
[2] Feige, U., Morimoto, R.I., Yahara, I. and Polla, B.S. (Eds.) (1996) Stress Inducible Cellular Responses, Birkhäuser Verlag, Basel.
[3] Downes, C.P., Wolf, C.R. and Lane, D.P. (Eds.) (1998) Cellular Responses to Stress, Portland Press, Colchester.
[4] Tabor, C.W. and Tabor, H. (1984) Annu. Rev. Biochem. 53, 749–790.
[5] Marton, L.J. and Morris, D.R. (1987) in: Inhibition of Polyamine Metabolism. Biological Significance and Basis for New Therapies (McCann, P.P., Pegg, A.E. and Sjoerdsma, A., Eds.), pp. 79–105, Academic Press, Orlando, FL.
[6] Schuber, F. (1989) Biochem. J. 260, 1–10.
[7] Basu, H.S., Schwietert, H.C.A., Feuerstein, B.G. and Marton, L.J. (1990) Biochem. J. 269, 329–334.
[8] Brooks, W.H. (1995) Med. Hypotheses 44, 331–338.
[9] Desiderio, M.A., Tacchini, L., Anzon, E., Pogliaghi, G., Radice, L. and Bernelli-Zazzera, A. (1996) Hepatology 24, 150–156.
[10] Desiderio, M.A., Mattei, S., Biondi, G. and Colombo, M.P. (1993) Biochem. J. 293, 475–479.
[11] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[12] Muroi, M. and Suzuki, T. (1993) Cell. Signal. 5, 289–298.
[13] Tacchini, L., Pogliaghi, G., Radice, L., Anzon, A. and Bernelli-Zazzera, A. (1995) Biochem. J. 309, 453–459.
[14] Mezger, B., Bensaude, O. and Morange, M. (1989) Mol. Cell. Biol. 9, 3888–3896.
[15] Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P. and Karin, M. (1987) Cell 49, 729–739.
[16] Cressman, D.E., Diamond, R.H. and Taub, R. (1995) Hepatology 21, 1443–1449.
[17] Latchman, D.S. (1995) Eukaryotic Transcription Factors, Academic Press, London.
[18] DeLisle, A.J., Graves, R.A., Marzluff, W.F. and Johnson, L.F. (1983) Mol. Cell. Biol. 3, 1920–1929.
[19] Muegge, K., Vila, M., Gusella, G.L., Musso, T., Herrlich, P., Stein, B. and Durum, S.K. (1993) Proc. Natl. Acad. Sci. USA 90, 7054–7058.
[20] Stopeck, A.T., Nicholson, A.C., Mancini, F.P. and Hajjar, D.P. (1993) J. Biol. Chem. 268, 17489–17494.
[21] Marton, L.J. and Pegg, A.E. (1995) Annu. Rev. Pharmacol. Toxicol. 35, 55–91.
[22] McCann, P.P., Pegg, A.E. and Sjoerdsma, A. (Eds.) (1987) In-

hibition of Polyamine Metabolism. Biological Significance and Basis for New Therapies, Academic Press, Orlando, FL.
[23] Kren, B.T. and Steer, C.J. (1996) FASEB J. 10, 559–573.
[24] Eick, D., Kohlhuber, F., Wolf, D.A. and Strobl, L.J. (1994) Anal. Biochem. 218, 347–351.
[25] Lis, J. and Wu, C. (1993) Cell 74, 1–4.
[26] Morgan, J.E., Calkins, C.C. and Matthews, H.R. (1989) Biochemistry 28, 5095–5106.
[27] Hougaard, D.M. (1992) Int. Rev. Cytol. 138, 51–88.
[28] Sandaltzopoulos, R. and Becker, P.B. (1998) Mol. Cell. Biol. 18, 361–367.
[29] Snyder, R.D. (1989) Biochem. J. 260, 697–704.
[30] Celano, P., Baylin, S.B. and Casero, R.A.jr. (1989) J. Biol. Chem. 264, 8922–8927.
[31] Laitinen, J., Stenius, K., Eloranta, T.O. and Hölttä, E. (1998) J. Cell. Biochem. 68, 200–212.
[32] Xiao, L., Swank, R.A. and Matthews, H.R. (1991) Nucleic Acids Res. 19, 3701–3708.
[33] Corella, D., Guillén, M., Hernández, J.M. and Hernández-Yago, J. (1998) Biochem. J. 334, 367–375.
[34] Shore, L.J., Peralta Soler, A. and Gillmour, S.K. (1997) J. Biol. Chem. 272, 12536–12543.
[35] Leroy, D., Jerike, J.K., Filhol, O., Chambaz, E.M. and Cochet, C. (1997) J. Biol. Chem. 272, 20820–20827.