Protein kinase A phosphorylates serine 267 in the homeodomain of engrailed-2 leading to decreased DNA binding

Majbrit Hjerrild[a,*], Allan Stensballe[b], Ole N. Jensen[b], Steen Gammeltoft[a], Thomas E. Rasmussen[a]

[a] *Department of Clinical Biochemistry, Glostrup Hospital, Nordre Ringvej, DK-2600 Glostrup, Denmark*
[b] *Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark*

Received 23 March 2004; revised 10 May 2004; accepted 10 May 2004

Available online 18 May 2004

Edited by Ned Mantei

Abstract Engrailed-2 (En-2) belongs to an evolutionarily conserved family of DNA binding homeodomain-containing proteins that are expressed in mammalian brain during development. Here, we demonstrate that serine 267 in the homeodomain of En-2 is phosphorylated by protein kinase A (PKA) in forskolin-treated COS-7 cells. Furthermore, we analyze the physiological function of En-2 phosphorylation by PKA. The nuclear localization of En-2 is not influenced by the phosphorylation of serine 267. However, substitution of serine 267 with alanine resulted in increased binding of En-2 to DNA, while replacing serine 267 with glutamic acid resulted in decreased En-2 DNA binding. These results suggest that the transcriptional activity of En-2 is regulated by PKA.

Keywords: Engrailed-2; Homeodomain; Protein kinase A; Protein phosphorylation; DNA binding

1. Introduction

Homeodomain proteins are a large evolutionarily conserved family of transcription factors that share a common 60 amino acid DNA binding domain referred to as the homeodomain. These proteins regulate gene expression of specific target genes involved in regulation of major developmental patterning events [1]. The protein encoded by the *engrailed* gene contains a homeodomain, has sequence specific DNA binding activity, and functions as a transcriptional repressor [2–4]. Vertebrates have two *engrailed* genes that encode two homologous proteins referred to as engrailed-1 (En-1) and engrailed-2 (En-2) [5]. During brain development En-1 and En-2 are expressed in the midbrain and cerebellum in a spatially and temporally restricted manner [6,7].

Homeodomain proteins bind short A/T-rich consensus sequences often with an ATTA core motif [8]. Because these motifs are abundant in eukaryotic genomes, the DNA sequence specificity of the homeodomain is relatively low [9]. Hence, the specificity of the homeodomain proteins is regulated through interaction with other transcription factors or by post-translational modifications [10]. Protein phosphorylation and dephosphorylation is a widely described regulation process affecting structure, subcellular localization and DNA binding of transcription factors [11].

It has previously been demonstrated that engrailed proteins are phosphoproteins [12]. Protein kinase CK2 phosphorylates engrailed protein outside the homeodomain leading to enhanced DNA binding [13]. Furthermore, a serine-rich domain within En-2 (amino acids 146–169) is phosphorylated by protein kinase CK2 resulting in regulation of transfer of En-2 between cells [14]. We have recently identified En-2 as an in vitro substrate of protein kinase A (PKA) and determined serine 267 as an in vitro phosphoacceptor site [31]. In the present study, we have demonstrated that serine 267 is also phosphorylated in vivo by PKA in forskolin treated COS-7 cells transfected with En-2. We analyzed the putative physiological functions of the PKA-mediated En-2 phosphorylation and observed that the subcellular localization of En-2 was not affected by phosphorylation. However, replacement of serine 267 with glutamic acid mimicking phosphoserine leads to reduced DNA binding, while replacement of serine 267 with alanine removing the phosphorylation site leads to increased DNA binding. These results indicate that En-2 interaction with DNA promoters is regulated by PKA phosphorylation.

2. Materials and methods

2.1. Plasmid constructs

Plasmid encoding murine En-2 was generously given by Alexandra Joyner, Department of Cell Biology, New York University School of Medicine, New York, USA [5]. En-2 was sub-cloned into a pMT2-MCS vector using *Sal*I and BsiWI cloning sites to generate an expression construct encoding N-terminally haemagglutinin (HA)-tagged En-2. Serine 267 was substituted with alanine or glutamic acid using the QuickChange™ procedure (Stratagene, www.stratagene.com).

2.2. Cell culture and transfection

COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Plasmid encoding HA-tagged En-2 was transfected into COS-7 cells using Lipofectamine™ Reagent (In Vitrogen, www.invitrogen.com) according to the protocol of the manufacturer and the cells were harvested after 48 h. For in vivo PKA studies, cells were grown 16 h without serum after which cells were either stimulated or not by adding forskolin to a final concentration of 50 μM (Sigma–Aldrich, www.sigma-aldrich.com) for 20 min prior to harvesting cells.

* Corresponding author. Fax: +45-43-23-39-29.
E-mail address: mh@dcb-glostrup.dk (M. Hjerrild).

Abbreviations: DTT, dithiotreitol; En-2, engrailed-2; HA-tag, haemagglutinin-tag; LC-ESI-MS/MS, liquid chromatography electrospray tandem mass spectrometry; PKA, protein kinase A

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.05.009

2.3. Immunoprecipitation

COS-7 cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, 0.5% Triton X-100, 5 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 10 mM Na-β-glycerophosphate, 1 mM PMSF, 10 nM calyculin, 10 μM leupeptin, 5 μM pepstatin and 1000 U/ml aprotinin) at 4 °C for 15 min. The lysate was centrifuged at 4 °C for 15 min at 10 000×*g* and the resulting supernatant was incubated for 2 h at 4 °C with monoclonal 12CA5 HA antibody. Anti-HA-tag antibody was prepared from the 12CA5 mouse hybridoma cell line. Protein G–Sepharose beads were added to the immune complexes and the mixture was incubated for 30 min at 4 °C. The immunoprecipitated complexes were subsequently washed three times with lysis buffer and twice with 1.5× kinase buffer (45 mM Tris–HCl, 15 mM $MgCl_2$ and 1.5 mM dithiotreitol, DTT).

2.4. In vitro PKA kinase assay

Prior to PKA assay En-2 was incubated with alkaline phosphatase (New England Biolabs, www.neb.com) at 37 °C for 30 min to remove phosphorylated residues and washed three times with kinase buffer. En-2 was incubated with or without 10 U of PKA catalytic subunit (Sigma–Aldrich), 20 μM ATP, 0.5 μl [γ-^{32}P]ATP (10 mCi/ml), 30 mM Tris–HCl, 10 mM $MgCl_2$ and 1 mM DTT at 30 °C for 20 min. The resins were washed twice with 1.5× kinase buffer to remove excess [γ-^{32}P]ATP. Reactions were stopped by the addition of 20 μl 1× Laemmli sample buffer prior to separating proteins by SDS–PAGE. Finally, phosphorylated protein bands were visualized by autoradiography.

2.5. In-gel digestion

HA-tagged En-2 was excised from Coomassie stained gels. The protein was reduced with 10 mM DTT at 56 °C for 45 min and subsequently alkylated with 55 mM iodoacetamide at room temperature for 30 min [15]. The proteins were digested overnight at 37 °C with an excess of sequencing grade trypsin (Promega, www.promega.com). Peptides were extracted from the gel by addition of 5% formic acid and acetonitrile. The resulting supernatant was subsequently lyophilized and resuspended in 20 μl of 5% formic acid.

2.6. Peptide sequencing by tandem mass spectrometry

Automated nanoflow liquid chromatography electrospray tandem mass spectrometric (LC-ESI-MS/MS) analysis was performed using a quadrupole time-of-flight Ultima mass spectrometer (Waters/Micromass, www.waters.com). A nano-HPLC system (Ultimate; www.lcpackings.com) delivering a flow of 175 nl/min was used to separate the peptide mixture prior to mass spectrometry analysis. Five μl of peptide sample was loaded on the column (Agilent Zorbax SB-C18 3.5 μm; 50 μm internal diameter) using a high-pressure vessel (Proxeon Biosystems, Odense, Denmark) and separated using a gradient of 5–38% acetonitrile in 30 min. The data were analyzed by MassLynx software and the Mascot search engine (www.matrixsciences.com).

2.7. Immunoblotting

Immunoblotting was performed as previously described [16]. Briefly, proteins were separated by SDS–PAGE and HA-tagged En-2 was detected by immunoblotting using an antibody against the HA-tag (rabbit polyclonal HA probe Y-11, Santa Cruz Biotechnology, www.scbt.com). The purity of isolated nuclei was analyzed by immunoblotting using a cytoplasmic marker β-actin (Sigma–Aldrich) and a nuclear marker Rb-1 (Abcam, www.abcam.com).

2.8. Isolation of nuclei

Nuclei were isolated from COS-7 cell expressing wild type or mutant En-2 using the Nuclei EZ PREP kitTM (Sigma–Aldrich) as described by the manufacturer. Nuclei were resuspended in 100 μl 1× Laemmli sample buffer, the proteins were separated by SDS–PAGE and HA-tagged En-2 was immunoblotted using a polyclonal antibody against HA (Y-11).

2.9. DNA binding assay

COS-7 cell extracts were prepared in 400 μl extraction buffer (20 mM HEPES, pH 7.8, 450 mM NaCl, 0.4 mM EDTA, 0.5 mM DTT, 25% glycerol, and 1 mM PMSF) by freezing and thawing three times followed by centrifugation at 16 000×*g* for 10 min. 30 pmol of each oligo (Fig. 4A) was labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. Oligos were combined and annealed by heating to 100 °C followed by slow cooling to 37 °C. The radioactively labeled double stranded oligos were purified using MicroSpinTM G-25 columns (Amersham Biosciences, www.amersham.com). 3 μl cell extract, 10 μl binding buffer (20 mM HEPES, pH 7.9, 1 mM $MgCl_2$, 4% Ficoll, and 0.5 mM DTT) and 1 μg dI–dC were pre-incubated at room temperature for 15 min. 1 μl probe was added and the reaction was incubated for further 25 min. Complexes were loaded onto a 5% (29:1 (w/v) acrylamide, *N*,*N*′-methylenebisacrylamide) non-denaturing 0.5× TBE (90 mM Tris, 90 mM borate, and 2 mM EDTA) polyacrylamide gel. Supershift analysis was performed using 12CA5 antibody against the HA-tag.

3. Results and discussion

3.1. Serine 267 is the major in vitro PKA phosphorylation site in En-2

We have previously identified serine 267 in mouse En-2 as a PKA phosphorylation site in vitro [31]. To confirm that PKA phosphorylates serine 267, we made mutant En-2 S267A. Wild type En-2 and mutant En-2 S267A were transfected into COS-7 cells and the proteins were immunoprecipitated using an antibody against the HA-tag. Immunoprecipitated proteins were incubated with purified catalytic subunit of PKA in the presence of [γ-^{32}P]ATP. The phosphorylated proteins were resolved by SDS–PAGE, stained with Coomassie blue (Fig. 1A) and phosphorylation was detected by autoradiography (Fig. 1B). The amount of incorporated phosphate was quantified by scintillation counting (Fig. 1C). The amount of incorporated phosphate into En-2 by PKA decreased to 20% when serine 267 was substituted with alanine. This confirms that the major in vitro PKA phosphorylation site in En-2 is serine 267.

3.2. Identification of in vivo phosphorylation sites in En-2

In the present study, we analyzed whether serine 267 phosphorylated by PKA in vitro is also modified by PKA in vivo. PKA is activated by increased cAMP concentration, generated through adenylate cyclase as a response to the activation of G protein-coupled receptors [17]. Forskolin is known to stimulate adenylate cyclase. COS-7 cells were transfected with a construct encoding HA-tagged En-2 and cells were serum starved prior to treatment with forskolin. HA-tagged En-2 was immunopre-

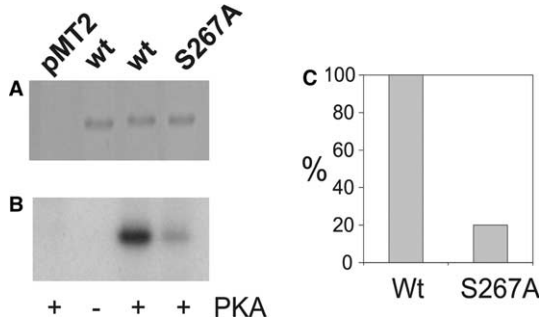

Fig. 1. In vitro phosphorylation of En-2 by the catalytic subunit of PKA. COS-7 cells were transfected with plasmids encoding HA-tagged wild type En-2 and mutant En-2 S267A. After 48 h the cells were lysed and En-2 was immunoprecipitated from the cell lysates with antibody against the HA-tag. The immunoprecipitated proteins were phosphorylated by the catalytic subunit of PKA with [γ-^{32}P]ATP. A: Coomassie blue staining of wild type En-2 and mutant En-2 S267A after in vitro phosphorylation and SDS–PAGE. B: The phosphorylated proteins were detected by autoradiography. C: Quantification of the ^{32}P-labeled phosphate incorporated into wild type En-2 and mutant En-2 S267A.

cipitated, separated by SDS–PAGE and digested by trypsin. Automated nanoflow LC-ESI-MS/MS was performed to locate specific serine or threonine phosphorylation site(s) in En-2. Peptide fragment ions of the b- and y-type [18] allowed exact localization of phosphorylation sites. β-Elimination of phosphoric acid and conversion of phosphoserine to dehydroalanine acid confirmed the phosphoamino acid assignments. This analysis revealed that En-2 was phosphorylated at serine 32 in serum-starved cells and that serine 32 and serine 267 were phosphorylated in forskolin-treated cells. Hence, we conclude that En-2 is phosphorylated by PKA at serine 267 in cultured COS-7 cells. This confirms our previous observation that serine 267, but not serine 32 is phosphorylated by PKA in vitro. Serine 32 is followed by a proline and this site may likely be phosphorylated by a proline-directed protein kinase under basal conditions (see Fig. 2).

3.3. PKA-mediated En-2 phosphorylation does not affect the nuclear localization of En-2

In accordance with the role of En-2 as a transcriptional regulator, it is predominantly localized in the cell nucleus. However, it has been reported that a 11 amino acids nuclear export sequence (250–261) between α helix 2 and 3 of the homeodomain is sufficient and necessary for nuclear export of En-2 [19] (Fig. 3A). Since serine 267 is localized close to the nuclear export sequence, we analyzed whether PKA-mediated

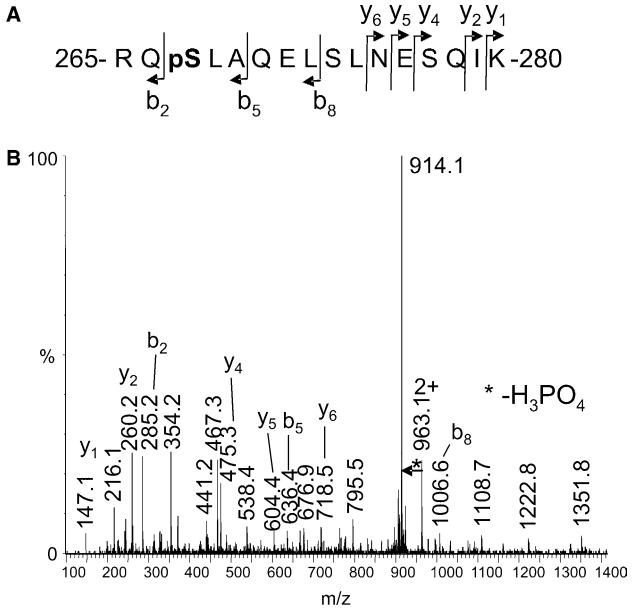

Fig. 2. Identification of in vivo PKA phosphorylation site in En-2. Serum starved COS-7 cells expressing HA-tagged wild type En-2 were treated with 50 μM forskolin for 20 min. After lysis of cells En-2 was immunoprecipitated from the cell lysates with antibody against the HA-tag and isolated by SDS–PAGE. The band containing En-2 was excised from the gel and digested by trypsin. Tryptic peptides were extracted from the gel and analyzed by LC-ESI-MS/MS. A: A doubly charged phosphopeptide was detected carrying one phosphate group (m/z 963.1). B: The fragment ions generated by LC-ESI-MS/MS analysis showed that Ser-267 was phosphorylated in forskolin treated cells. The fragmentation pattern of the peptide is indicated (only *b* and *y* ions are indicated for simplicity). Ions labeled with an asterisk were generated from the peptide in which the phosphoserine had been converted to dehydroalanine by β-elimination (loss of phosphoric acid = 98 Da).

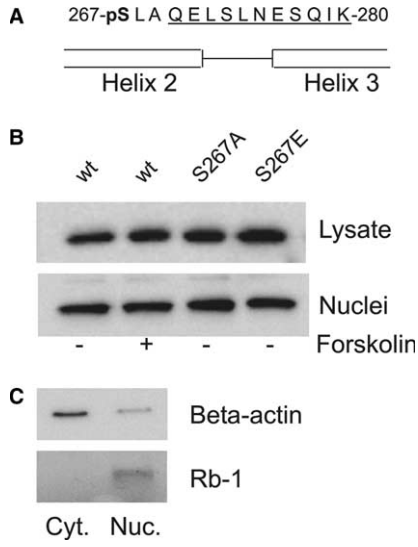

Fig. 3. Subcellular distribution of En-2. A: Amino acid sequence 267–280 between α-helix 2 and 3 in En-2. The nuclear export sequence is underlined. B: COS-7 cells were transfected with HA-tagged wild type En-2 or mutant En-2 S267A or S267E followed by incubation in the absence or presence of 50 μM forskolin for 20 min. After lysis of cells, the nuclei were isolated and the proteins in lysate and nuclei separated by SDS–PAGE. En-2 in lysate and nuclear fraction was determined by immunoblotting using an antibody against the HA-tag. C: Immunoblotting using antibodies against a cytoplasmic marker (β-actin) and a nuclear marker (Rb-1) was used to demonstrate the effectiveness of the nuclear isolation. Cyt. is the cytoplasmic fraction and Nuc. is the nuclear fraction.

phosphorylation could have an effect on the subcellular localization of En-2. COS-7 cells were transiently transfected with constructs encoding wild type En-2, S267A or S267E mutants. Forty-eight hours after transfection, the subcellular localization of En-2 was analyzed directly by isolation of nuclei followed by immunoblotting using an antibody against the HA-tag (Fig. 3B). The total cellular expression of En-2 and the S267A and S267E mutants was identical. The nuclear localization of En-2 was not affected by forskolin-treatment. Furthermore, substituting serine 267 with alanine or glutamic acid did not have any impact on nuclear localization. We conclude that PKA-mediated En-2 phosphorylation does not influence the nuclear localization of En-2. In addition, we analyzed the purification of isolated nuclei by immunoblotting using antibodies against β-actin (cytoplasmic marker) and Rb-1 (nuclear marker). Fig. 3C shows that the nuclear fraction is contaminated by cytoplasmic β-actin to a low extent.

3.4. PKA-mediated En-2 phosphorylation affects the DNA binding

There are several examples of PKA-mediated protein phosphorylation modifying the DNA binding activity of transcription factors either positively or negatively [20–22]. To determine whether phosphorylation of En-2 protein could affect the function of En-2 as a transcription factor, we tested the DNA binding affinity of wild type En-2 and S267A and S267E mutants by gel retardation assay using oligonucleotides containing two binding sites for En-2 (Fig. 4A). As shown in Fig. 4B, the intensity of the complexes formed with the S267A mutant was increased when compared to the intensity of corresponding complexes formed with wild type En-2. This result suggests that phosphorylation of

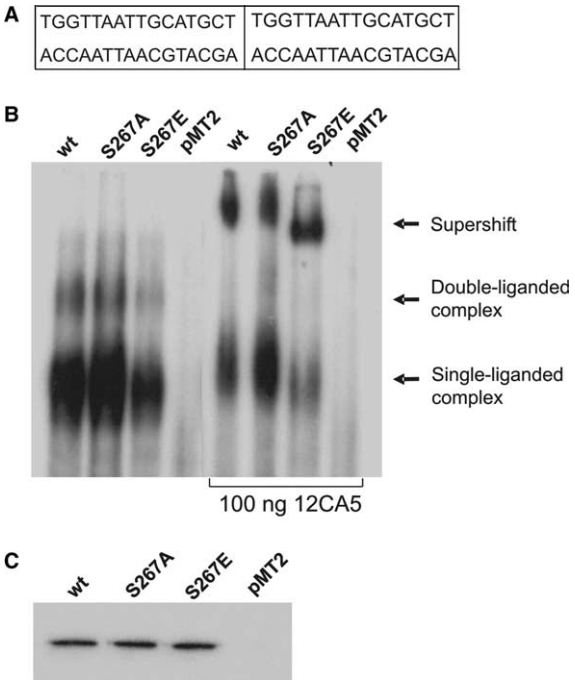

Fig. 4. DNA binding of En-2. A: Nucleotide sequence of the tandem repeat double-stranded oligonucleotide used for gel mobility shift assay. B: COS-7 cells were transfected with empty pMT2 vector, wild type En-2 or mutant En-2 S267A or S267E and lysed by freezing and thawing three times. DNA binding assays were performed by incubation of cell lysates with ^{32}P-labeled oligonucleotide for 25 min at room temperature. For gel supershift analysis 12CA5 antibody was added to the samples. The protein–DNA complexes were analyzed by non-denaturing PAGE. Positions of single-liganded and double-liganded complexes are indicated as well as the supershift with 12CA5 antibody. C: Immunoblot showing the expression level of wild type and mutant En-2 in COS-7 cell extract.

serine 267 in wild type En-2 from COS-7 cells grown in serum-containing medium leads to decreased DNA binding of En-2 compared to the S267A mutant. Serine 267 was also substituted with a phosphoserine mimicking glutamic acid (S267E) [23]. The S267E mutant showed less DNA binding activity than wild type En-2. The expression of wild type and mutant En-2 was determined by Western blotting in Fig. 4C. Taken together these results indicate that En-2 phosphorylated at serine 267 has lower DNA binding affinity than the non-phosphorylated protein. Furthermore, we analyzed if forskolin-treatment of COS-7, HEK and SH-SY5Y cells transfected with wild type En-2 affects the DNA binding of En-2. However, we were not able to detect reduced DNA binding of En-2 after forskolin-treatment of the cells (data not shown). In addition, SDS–PAGE analysis of En-2 immunoprecipitated from forskolin-treated cells did not show any migration shift of En-2 compared with untreated cells. This suggests that only a small fraction of En-2 is phosphorylated after forskolin-treatment. However, changes in the activity of a fraction of transcription factors could also be important for cellular regulation.

4. Conclusion

Differential gene expression regulated through specific functions of transcription factors is important for cellular differentiation. The function of transcription factors is dependent on nuclear localization, DNA binding and transactivation. The cAMP-dependent signaling pathway has long been known to be involved in cell growth, differentiation and cell-specific gene expression [24]. In addition, it has previously been demonstrated that other homeodomain proteins are phosphorylated within their homeodomain region [25–27]. Here, we show that serine 267 is the major PKA phosphorylation site in vivo after forskolin-treatment. This is in agreement with our previous finding that En-2 is phosphorylated in vitro by PKA [31]. Furthermore, we show that serine 267 is important for the En-2 DNA binding. Replacing serine 267 with an alanine increases En-2 DNA binding in an electrophoretic mobility shift assay, while replacing serine 267 with a phosphoserine mimicking glutamic acid results in decreased DNA binding affinity. However, understanding the role of reduced DNA binding of phosphorylated En-2 in regulation of cellular functions requires further study. The most conserved function of En-2 seems to be its role in neuronal development. Differentiation of neural progenitor cells is regulated by a coordinate change in the expression of specific target genes involved in different intracellular signaling pathways. PKA signaling has also been shown to be involved in neuronal differentiation during brain development [28]. PKA phosphorylates CRE binding protein leading to regulation of neuronal differentiation, neurite outgrowth and neuronal plasticity [29,30]. We suggest that PKA-mediated phosphorylation of serine 267 in En-2 could be part of the complex regulation of neuronal development.

Acknowledgements: This study was supported by grants from Danish Medical Research Council and Danish Cancer Society.

References

[1] Gibert, J.M. (2002) Dev. Genes Evol. 212, 307–318.
[2] Desplan, C., Theis, J. and O'Farrell, P.H. (1985) Nature 318, 630–635.
[3] Fjose, A., McGinnis, W.J. and Gehring, W.J. (1985) Nature 313, 284–289.
[4] Tolkunova, E.N., Fujioka, M., Kobayashi, M., Deka, D. and Jaynes, J.B. (1998) Mol. Cell. Biol. 18, 2804–2814.
[5] Joyner, A.L. and Martin, G.R. (1987) Genes Dev. 1, 29–38.
[6] Davis, C.A. and Joyner, A.L. (1988) Genes Dev. 2, 1736–1744.
[7] Davis, C.A., Noble-Topham, S.E., Rossant, J. and Joyner, A.L. (1988) Genes Dev. 2, 361–371.
[8] Gehring, W.J. et al. (1994) Cell 78, 211–223.
[9] Ekker, S.C., Young, K.E., von Kessler, D.P. and Beachy, P.A. (1991) EMBO J. 10, 1179–1186.
[10] Hunter, T. (2000) Cell 100, 113–127.
[11] Hunter, T. and Karin, M. (1992) Cell 70, 375–387.
[12] Gay, N.J., Poole, S.J. and Kornberg, T.B. (1988) Nucleic Acids Res. 16, 6637–6647.
[13] Bourbon, H.M., Martin-Blanco, E., Rosen, D. and Kornberg, T.B. (1995) J. Biol. Chem. 270, 11130–11139.
[14] Maizel, A., Tassetto, M., Filhol, O., Cochet, C., Prochiantz, A. and Joliot, A. (2002) Development 129, 3545–3553.
[15] Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996) Anal. Chem. 68, 850–858.
[16] Jensen, C.J., Buch, M.B., Krag, T.O., Hemmings, B.A., Gammeltoft, S. and Frodin, M. (1999) J. Biol. Chem. 274, 27168–27176.
[17] Robinson-White, A. and Stratakis, C.A. (2002) Ann. NY Acad. Sci. 968, 256–270.
[18] Roepstorff, P. and Fohlman, J. (1984) Biomed. Mass Spectrom. 11, 601.
[19] Maizel, A., Bensaude, O., Prochiantz, A. and Joliot, A. (1999) Development 126, 3183–3190.

[20] Ray, A., Ray, P., Guthrie, N., Shakya, A., Kumar, D. and Ray, B.K. (2003) J. Biol. Chem. 278, 22586–22595.
[21] Viollet, B., Kahn, A. and Raymondjean, M. (1997) Mol. Cell Biol. 17, 4208–4219.
[22] Gupta, M.P., Kogut, P. and Gupta, M. (2000) Nucleic Acids Res. 28, 3168–3177.
[23] Frodin, M., Jensen, C.J., Merienne, K. and Gammeltoft, S. (2000) EMBO J. 19, 2924–2934.
[24] Roesler, W.J., Vandenbark, G.R. and Hanson, R.W. (1988) J. Biol. Chem. 263, 9063–9066.
[25] Coqueret, O., Berube, G. and Nepveu, A. (1996) J. Biol. Chem. 271, 24862–24868.
[26] Kasahara, H. and Izumo, S. (1999) Mol. Cell. Biol. 19, 526–536.
[27] Park, G.T., Denning, M.F. and Morasso, M.I. (2001) FEBS Lett. 496, 60–65.
[28] Kim, G., Choe, Y., Park, J., Cho, S. and Kim, K. (2002) Brain Res. Mol. Brain Res. 109, 134–145.
[29] Zanassi, P., Paolillo, M., Feliciello, A., Avvedimento, E.V., Gallo, V. and Schinelli, S. (2001) J. Biol. Chem. 276, 11487–11495.
[30] Inglefield, J.R., Mundy, W.R., Meacham, C.A. and Shafer, T.J. (2002) Neuroscience 115, 559–573.
[31] Hjerrild, M., Stensballe, A., Rasmussen, T.E., Kofoed, C.B., Blom, N., Sicheritz-Ponten, T., Larsen, M.R., Brunak, S., Jensen, O.N. and Gammeltoft, S. (2004). J. Proteome Res., in press.