

SPERMINE STIMULATES THE PHOSPHORYLATION OF THE NUCLEAR MATRIX PROTEINS CATALYZED BY NUCLEAR KINASE II

Rati Verma and Kuang Yu Chen

Department of Chemistry and The Cancer Institute of New Jersey, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08855-0939

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and Methods
 - 3.1 Purification of NII nuclear kinase
 - 3.2 Phosphorylation of nuclear fractions using exogenously added NII nuclear kinase
 - 3.3 Other procedures
4. Results
 - 4.1 Purification and characterization of NII nuclear kinase
 - 4.2 NII kinase catalyzed phosphorylation of nuclear proteins
 - 4.3 Phosphorylation of subnuclear fractions by NII nuclear kinase
5. Discussion
6. Acknowledgments
7. References

1. ABSTRACT

Nuclear kinase II (nuclear casein kinase 2) is a multifunctional, second messenger-independent protein serine/threonine kinase that phosphorylates many different nuclear proteins, including high mobility group (HMG) proteins, heterogeneous nuclear ribonucleoprotein (hnRNP) fractions, and nuclear matrix proteins, but not histones. The enzyme appears to be essential in growth regulation. However, it is not clear how the enzyme is regulated *in vivo*. To understand the regulation of this enzyme, we have searched for possible effectors for this enzyme. Spermine, at physiological concentrations, significantly stimulates nuclear protein phosphorylation catalyzed by nuclear kinase II (NII kinase). Using various subnuclear fractions as substrates, we showed that the stimulatory effect of spermine was confined only to nuclear matrix proteins. Thus, spermine at 1 mM stimulated a >5-fold increase in nuclear matrix phosphorylation, but had little or no effect on the phosphorylation of HMG and hnRNP proteins catalyzed by NII kinase. Similarly, the inhibitory effect of heparin on NII kinase reaction was also substrate-dependent and appeared to be limited to nuclear matrix proteins. Previously, we have shown that spermine inhibits the phosphorylation of the 11,000- and 10,000-dalton nuclear protein catalyzed by NI kinase. Both of these low molecular weight proteins exist in nuclear matrix fraction. Taken together, our data suggest that NI and NII kinase may be regulated by spermine *in vivo* and that nuclear matrix proteins appear to be the primary target for such a regulation.

2. INTRODUCTION

NII Nuclear kinase is casein kinase 2 (CK2) present in the nucleus. Casein kinase 2 is a second messenger-independent, multifunctional protein kinase capable of using acidic proteins such as phosphovitin and casein as substrates but unable to phosphorylate basic proteins such as histones and protamines (1). The nuclear

localization and the ubiquitous presence of NII nuclear kinase in eukaryotes, from yeast to mammalian cells, suggest that this enzyme may have important physiological functions. Although little is known about the regulation and function of CK2, studies from many laboratories have suggested that it may have an important role in regulating transcription and mitosis (2).

NII kinase has been shown to phosphorylate many important growth-related proteins such as c-fos (3), p53 (4), and other transcription factors (2). CK2 consists of an alpha-catalytic subunit (Mr 35K-44K), and beta-regulatory subunit (Mr 25K-28K) arranged as an alpha₂beta₂ heterotetramer (5). Since the enzyme appears constitutively expressed, it has been difficult to determine whether and how the enzyme activity is regulated *in vivo*. Nevertheless, it has been shown that the CK2 activity is required for progression of the cell cycle (6, 7), suggesting that the enzyme activity is regulated during cell growth. One mechanism of regulation is through the presence of small positive or negative effectors. A number of inhibitors of NII nuclear kinase have been identified, including heparin, 2,3-bisphosphoglycerate, inositol hexasulfate, and poly(U) (1). The physiological relevance of these inhibitors, however, has yet to be established. Other studies have shown that polyamines, particularly spermine, stimulate the phosphorylation of casein catalyzed by NII kinase (8). Polyamines (putrescine, spermidine, and spermine) are naturally occurring organic cations widely distributed in living organisms (9, 10). Studies from many laboratories have indicated that polyamines are essential for growth and play an important regulatory role in various biological processes (9-11). Thus, it is possible that polyamines may serve as a physiological effector of NII kinase. This notion is particularly tempting in view of the abundance of spermine in the nucleus (12) and possible role of nuclear protein phosphorylation in regulating gene expressions (2).

We have previously reported that spermine at physiological concentrations has a dual effect on the endogenous protein phosphorylation pattern of nuclei isolated from NB-15 mouse neuroblastoma cells. Spermine stimulates general nuclear protein

Received 8/19/97 Accepted 8/25/97

Send correspondence to: Dr. Kuang Yu Chen, Department of Chemistry, P.O. Box 939, Rutgers University, Piscataway, New Jersey 08855-0939 Tel: (908) 445-3739, Fax: (908)445-5312, Email: kyuchen@rutchem.rutgers.edu

Spermine and NII nuclear kinase

TABLE 1. Effects of various polyanions on the activity of nuclear NII protein kinase isolated from NB-15 mouse neuroblastoma cells

POLYANION (microgram/ml)	SPERMINE (1 mM)	% ACTIVITY
Heparin (0.1)	-	35
Heparin (0.5)	-	19
Heparin (0.5)	+	111
Poly (U) (150)	-	7
Poly (U) (150)	+	17
Poly (A) (150)	-	80
Poly (A) (150)	+	143
DNA (150)	-	46
DNA (150)	+	135

The chemicals to be tested were added to the standard phosphorylation assay mixture for NII kinase as described in "Experimental Procedures". Results are the average of duplicate measurements, expressed as per cent of control in the absence of added polyanions.

phosphorylation but strongly inhibits the phosphorylation of two low molecular weight nuclear proteins, the 11,000- and 10,000- dalton proteins (13). We subsequently showed that the 11,000- and 10,000- dalton proteins are exclusive substrates of NI kinase, and spermine specifically inhibits the NI catalyzed phosphorylation (14). In this paper, we show that the stimulatory effect of spermine on the phosphorylation of other nuclear proteins is mediated with NII nuclear kinase. We also provide evidence that the stimulatory effect of spermine in nuclear protein phosphorylation is largely confined to nuclear matrix proteins.

3. MATERIALS AND METHODS

3.1. Purification of NII nuclear kinase

Nucleoplasm was prepared by extraction of isolated nuclei as previously described (13), and dialyzed against buffer B (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 10 mM beta-mercaptoethanol, 10% glycerol and 0.25 mM PMSF) containing 0.3 M NaCl. It was then concentrated by an Amicon stirred cell equipped with a PM-10 membrane. The concentrated nucleoplasm was chromatographed twice through phosphocellulose column (1.5 x 20 cm) as previously described (13, 14). Active fractions were pooled and 400 micrograms of Pentax BSA (Fraction V) was added. They were then concentrated and stored at 4° C in buffer B containing 0.65 M NaCl. Under such conditions the enzyme activity could be preserved up to six months.

3.2. Phosphorylation of nuclear fractions using exogenously added NII nuclear kinase

Nuclei and various subnuclear fractions (hnRNP and the HMG and matrix proteins) were prepared as previously described (14). They were briefly heated (65° C for 5 min) to inactivate the endogenous kinase activities before being used as kinase substrates. The phosphorylation mixture generally contained 20 microliters of substrate, 5 to 10 units of NII kinase, 50 micromolar ATP (2.5 x 10⁶ cpm/nmol), 5 mM MgCl₂, 1 mM DTT, 30 mM NaCl in 50 mM Tris-HCl (pH 7.4). The reaction mixture was incubated at 30° C for 15 min and then processed for SDS-PAGE and autoradiographic analysis.

3.3. Other procedures

Tissue culture, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, autoradiography, and

immunoblotting were carried out as described (14). Protein concentration was determined by the method of Bradford (15).

4. RESULTS

4.1 Purification and characterization of the NII nuclear kinase

Nuclear protein kinase NII from NB-15 cells was purified 706-fold over the total casein kinase activity present in the nucleoplasm after a consecutive phosphocellulose column chromatography. The specific activity of the purified NII kinase was 100 units per microgram protein (1 unit=amount of enzyme catalyzing the transfer of 1 pmol of phosphate from ATP to dephosphorylated casein per min at 30° C, pH 7.4). When the NII kinase isolated from NB-15 cells was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining, three bands of apparent molecular weights, 44,000, 40,000, and 26,000 were observed (data not shown). This subunit composition matches that reported for casein kinase II purified from other sources (16). When NII kinase activity was measured at saturating concentrations of casein (2 mg/ml), a Km value of 13 micromolar was found for ATP. Similarly, a Km value of 0.85 mg/ml and Vmax value of 5.3 pmol/min was found for casein when NII kinase activities were measured at saturating concentration of ATP (50 micromolar). These values are in agreement with that reported for casein kinase II isolated from other sources (1, 16). Among the three commonly used non-physiological substrates for kinase assay, NII kinase phosphorylated both alpha-casein and phosvitin but not histone (data not shown). Spermine at 1- 2 mM only stimulated the phosphorylation of alpha-casein but not phosvitin. Kinetic measurement indicated that upon addition of 2 mM spermine, the Km value of NII kinase for alpha-casein was reduced by 11% to 0.76 mg/ml but Vmax was almost doubled to 10.4 pmols/min (data not shown). Among the polyanions examined, heparin was the most potent inhibitor which completely abolished NII activity at a concentration of 0.5-1 mg/ml (Table 1). The inhibition could be reversed by spermine at 2 mM (Table 1). Poly (A), poly (U), and calf thymus DNA also inhibited NII activity but were less effective than heparin (Table 1). Interestingly the addition of spermine to the assay mixture reversed the inhibitory effects of poly (A) and calf thymus DNA, but not that of poly (U), indicating that factors other than counterion interaction were involved in the action of spermine.

Spermine and NII nuclear kinase

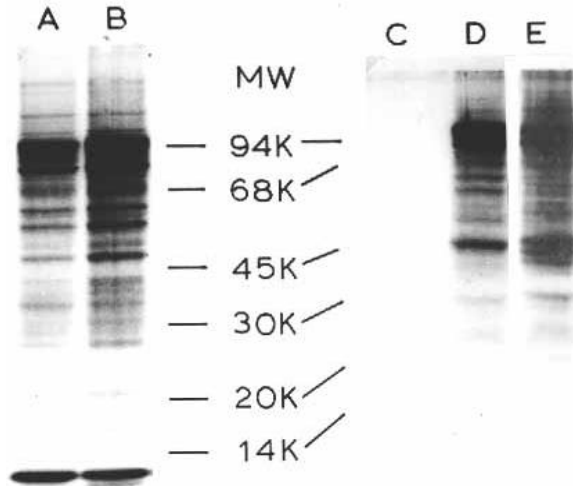


Figure 1. Nuclear protein phosphorylation by endogenous and exogenously added nuclear kinases. Nucleoplasm without (lanes A and B) or with (lanes C-E) prior heat treatment was added to the phosphorylation assay mixture. The final protein concentration was 4 mg per ml. Lane A, no addition; lane B, 6 units NII added; lane C, no addition; lane D, 8 units of NII kinase added; lane E, 8 units of NII kinase and 1 mM spermine added.

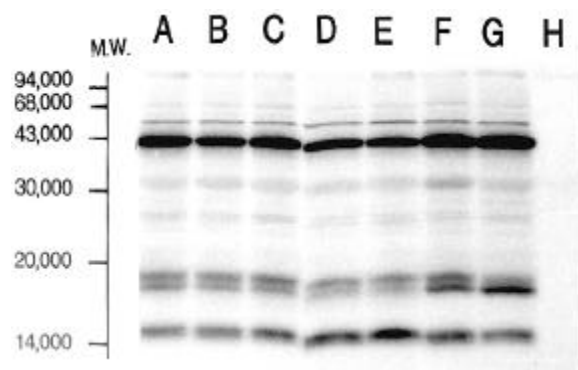


Figure 2. Effect of spermine on phosphorylation of the HMG proteins by NII kinase. The HMG fraction (16 microgram proteins) was added to the standard assay mixture containing 30 μ g/ml bovine serum albumin and 6 units of NII kinase. Activity was assayed in the presence of the following additions: Lane A, no addition; lane B, 1 mM spermine; lane C, 2 mM spermine; lane D, 4 mM spermine; lane E, 6 mM spermine; lane F, 10 mM putrescine; and lane G, 0.5 microgram/ml heparin. In lane H, HMG fraction alone was added to the assay mixture, no NII kinase added.

4.2 NII kinase catalyzed phosphorylation of nuclear proteins

Although casein and phosphovitin have been commonly used as protein substrates for NII kinase (or casein kinase II), neither of which are nuclear proteins. To search for possible physiological protein substrates, we first examined the phosphorylation pattern by adding NII kinase to nuclear extracts. **Figure 1** (lane B vs lane A) shows that addition of NII did not significantly alter the endogenous protein phosphorylation pattern due to high

endogenous protein kinase activity in nucleoplasm. However, brief heat treatment completely abolished all endogenous kinase activities (**Figure 1**, lane C), but did not affect the ability of nuclear proteins to function as substrates for exogenously added NII kinase (**Figure 1**, lane D). The nuclear protein phosphorylation pattern generated by NII kinase closely resembled that of the endogenous phosphorylation except in the low molecular weight region (**Figure 1**, lanes D vs A). Spermine at 1mM significantly stimulated the NII kinase catalyzed phosphorylation of nuclear proteins (**Figure 1**, lanes E vs D).

4.3 Phosphorylation of subnuclear fractions by NII nuclear kinase

To further examine the nature of these phosphorylated nuclear proteins, nuclei were fractionated into three subnuclear fractions: (i) the high mobility group (HMG), (ii) the heterogeneous nuclear ribonucleoprotein (hnRNP) and (iii) the nuclear matrix proteins. HMG represents a family of architectural proteins that may be involved in transcriptional regulation of genes (17). The effects of spermine and heparin on NII catalyzed phosphorylation of HMG proteins are shown in **Figure 2**. The addition of NII kinase to the HMG fraction resulted in the phosphorylation of a number of HMG proteins (**Figure 2**, lane A). The most prominently phosphorylated protein substrates of NII kinase were of apparent Mr 46,000, 20,000, 18,000, and 16,000 (**Figure 2**, lane A). The 20,000, 18,000, and 16,000-dalton bands were identified as HMG 14a, 14b, and 17 protein respectively based on their position on the 15% sodium dodecyl sulfate-polyacrylamide gel (18). Addition of spermine up to 6 mM had neither stimulatory nor inhibitory effect on the phosphorylation of HMG proteins catalyzed by NII kinase (**Figure 2**, lanes B to E). Addition of heparin (0.5 mg/ml) also had no effect on the NII catalyzed phosphorylation of HMG proteins (**Figure 2**, lane G). Interestingly, HMG proteins are very poor substrates for NII kinase (14).

Heterogeneous nuclear ribonucleoproteins or hnRNPs represent a class of about 20 major polypeptides that may be involved in post-transcriptional events such as regulated splicing and mRNA export (19). The hnRNP fraction contained high endogenous kinase activity (**Figure 3**, lane A). Brief heat treatment completely abolished the endogenous kinase activities (**Figure 3**, lane B). The addition of NII kinase added to the heated hnRNP fraction phosphorylated many proteins and yield an almost identical phosphorylation pattern as that of endogenous phosphorylation (**Figure 3**, lanes C vs A). Spermine had a slight stimulatory effect and heparin had a slight inhibitory effect on the action of NII kinase on hnRNP phosphorylation (**Figure 3**, lanes D and E vs C).

The nuclear matrix fraction isolated from NB-15 cells included proteins of apparent Mr of 110,000, 72,000, 68,000 and 62,000 and exhibited similar protein profile as that prepared from other sources (20). Nuclear matrix represents a structural framework of the nucleus and is implicated in DNA replication and RNA processing (21). The endogenous kinase activity associated with the nuclear matrix fraction was insignificant (**Figure 4**, lane A). Addition of NII kinase to the unheated nuclear matrix resulted in the phosphorylation of many proteins, most notably, a 55,000-dalton protein (**Figure 4**, lane B). Spermine at 2 mM stimulated the NII kinase-catalyzed the phosphorylation of all these proteins (**Figure 4**, lane C). The stimulatory effect of spermine was significant and estimated to be more than 5-fold based on densitometric tracing. Addition of 10 mg/ml heparin resulted in

Spermine and NII nuclear kinase

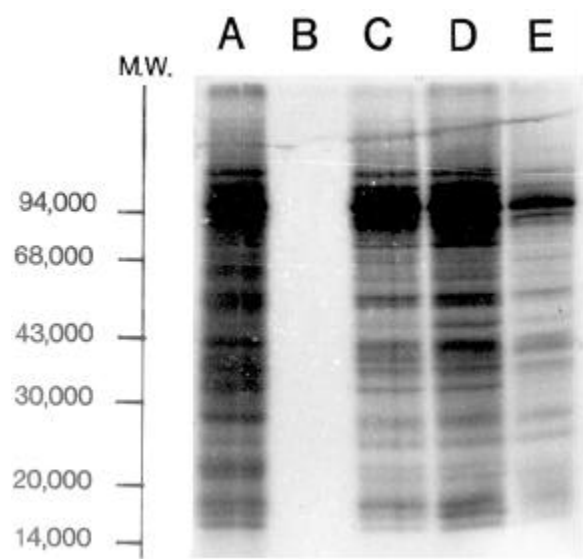


Figure 3. Effects of spermine and heparin on the phosphorylation of the hnRNP fraction by NII Kinase. Lane A, endogenous phosphorylation of hnRNP. Heat-treated hnRNP fraction (2 mg/ml) was used in lanes B-E: lane B, no addition; lane C, 6 units NII kinase added; lane D, 6 units NII kinase and 2 mM spermine added; lane E, 6 units NII kinase and 10 microgram/ml heparin added.

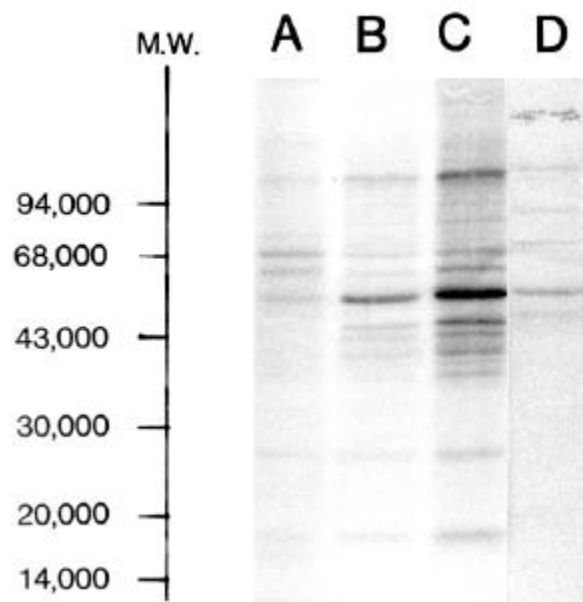


Figure 4. Effects of spermine and heparin on the phosphorylation of nuclear matrix proteins by NII kinase. Nuclear matrix (0.7 mg/ml protein) was added to the standard assay mixture containing 30 microliter/ml bovine serum albumin. Lane A, nuclear matrix fraction alone; lane B, 4 units NII kinase added; lane C, 4 units NII kinase and 2 mM spermine added; lane D, 4 units NII kinase and 10 microliter/ml heparin added.

significant inhibition of the phosphorylation (Figure 4, lane D).

5. DISCUSSION

Nuclear protein kinase NI and NII are both ubiquitously present in eukaryotes and their activities appear to be independent of the regulation by any known second messengers (1, 2). Both enzymes are known to be able to phosphorylate casein, phosphovitin but not histones (1). Although both enzymes appear to be abundantly localized in the nuclei, their precise physiological functions are not clear. The identification of their native substrate proteins and the search for their physiological effector(s) should facilitate our understanding of the physiological roles of these two enzymes.

In the present study, we showed that NII nuclear kinase could phosphorylate proteins in HMG, hnRNP and nuclear matrix (Figures 2-4), and appeared to have a broader substrate spectrum than that of NI kinase (14). The effects of spermine on the phosphorylation of these three subnuclear fractions, however, were very different. Thus, spermine only stimulated the NII kinase-catalyzed phosphorylation of nuclear matrix proteins, and had little or no effect when other subnuclear fractions were used as substrates. We have previously shown that spermine specifically inhibits the phosphorylation of the 11,000- and 10,000-dalton proteins catalyzed by NI kinase. It is of interest to note that these two low molecular weight proteins also exist in nuclear matrix fraction. The nuclear matrix has been shown to be the site of DNA replication (21, 22). Phosphorylation and dephosphorylation of nuclear matrix proteins could affect their binding to double-stranded or single stranded DNA (22). The fact that the specific and opposing effects of spermine on the actions of NI and NII kinase are localized only on nuclear matrix proteins may not be coincidental.

The finding that polyanions such as DNA, poly (U) and poly (A) could affect NII kinase catalyzed reactions, offers an interesting possibility that DNA and/or RNA may affect the phosphorylation status of their own binding proteins through their interaction with NII kinase. The difference in the inhibitory effects between poly (A) and poly (U) and the observation that spermine could reverse the inhibitory action of poly (A), and calf-thymus DNA, but not poly (U) also suggested that the inhibitory actions of these polyanions were not merely due to their negative charges.

In summary, many other protein kinases are regulated by various effectors such as cAMP, cGMP, and Ca^{2+} . In view of the abundance of spermine in nucleus (9, 13), its diverse physiological effects *in vitro* and *in vivo*, and its specific but opposing effects on NI and NII kinase, it is tempting to propose that spermine may serve as the physiological modulator for both NI and NII kinases.

6. ACKNOWLEDGMENTS

We thank Dr. M.E. Dahmus, University of California at Davis for kindly providing us with monospecific antiserum against calf-thymus casein kinase II. This research was supported in part by a grant from National Institutes of Health (CA 24479), a grant from N.J. State Commission on Cancer Research (No. 349695), and a grant from The Charles and Johanna Busch Memorial Fund.

Spermine and NII nuclear kinase

7. REFERENCES

1. G.M. Hathaway & G.A. Traugh: Casein kinases: Multipotential protein kinases. *Curr Topics in Cellular Regul* 21, 101-127 (1982)
2. H. Meisner & M.P. Czech: Phosphorylation of transcriptional factors and cell-cycle-dependent proteins by casein kinase II. *Curr. Opin. Cell Biol* 3, 474-483 (1991)
3. R. Ofir, J. Dwarki, D. Rashid, & I. Verma: Phosphorylation of the C-terminus of Fos protein is required for transcriptional transrepression of the c-Fos promoter. *Nature* 348, 80-82, (1990)
4. D. Eliyahu, A. Raz, P. Gruss, D. Givol & M. Oren: Participation of p53 cellular tumor antigen in transformation of normal embryo cells. *Nature* 312, 646-654 (1984)
5. F. Meggio & L. Pinna: Subunit structure and autophosphorylation mechanism of casein kinase II from rat liver cytosol. *Eur J Biochem* 145, 593-599 (1984)
6. D.E. Hana, A. Rethinaswamy & C.V. Glover: Casein kinase II is required for cell cycle progression during G1 and G2/M in *Saccharomyces cerevisiae*. *J Biol Chem* 270, 25905-25914 (1995)
7. D.R. Marshak & G.L. Russo: Regulation of protein kinase CKII during the cell division cycle. *Cell Mol Biol Res* 40, 513-517 (1994)
8. T. Hara & H. Endo: Polyamines alter the substrate preference of nuclear protein kinase NII. *Biochemistry* 21, 2632-2644 (1982)
9. H. Tabor & C.W. Tabor: Polyamines. *Ann Rev Biochem* 53, 749-790 (1984)
10. S.S. Cohen: Introduction to Polyamines, Prentice Hall, Inc., Englewood Cliffs, NJ. 1-179 (1971)
11. A.E. Pegg: Polyamine metabolism and its importance in neoplastic growth and a target for chemotherapy. *Cancer Res* 48, 759-774 (1988)
12. D. M. Hougaard, L. Bolund, K. Fujiwara & L. I. Larsson: Endogenous polyamines are intimately associated with highly condensed chromatin in vivo. A fluorescence cytochemical and immunocytochemical study of spermine and spermidine during the cell cycle and in reactivated nuclei. *Eur J Cell Biol*, 44, 151-155 (1987)
13. K.Y. Chen & R. Verma: Spermine specifically inhibits the phosphorylation of an 11,000-dalton nuclear protein in various cultured mammalian cell lines. *Biochem Biophys Res Commun* 118, 710-716 (1984)
14. R. Verma & K.Y. Chen: Spermine inhibits the phosphorylation of the 11,000- and 10,000-dalton nuclear proteins catalyzed by nuclear protein kinase NI in NB-15 mouse neuroblastoma cells. *J Biol Chem* 261, 2890-2896 (1986)
15. M.M. Bradford: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248-254 (1976)
16. M.E. Dahmus: Purification and properties of calf thymus casein kinases I and II. *J Biol Chem* 256, 3319-3325 (1981)
17. V. Wunderlich & M.J. Bottger: High-mobility-group proteins and cancer--an emerging link. *Cancer Res Clin Oncol*, 123, 133-140 (1997)
18. M.S. Chapekar, M. Bustin, & R.I. Glazer: Evidence that high mobility group protein 17 is not phosphorylated in human colon carcinoma cells. *Biochim Biophys Acta* 838, 351-354 (1985)
19. F. Weighard, G. Biamonti & S. Riva: The roles of heterogeneous nuclear ribonucleoproteins (hnRNP) in RNA metabolism. *Bioessays* 18, 747-756 (1996)
20. H. Nakayasu & R. Berezney: Nuclear matrices: identification of the major nuclear matrix. *Proc Natl Acad Sci U S A* 88, 10312-10316 1991
21. P. Loidl & A. Eberharter: Nuclear matrix and the cell cycle. *Int Rev Cytol* 162B, 377-403 (1995)
22. D. He, C. Zeng & B.R. Brinkley: Nuclear matrix proteins as structural and functional components of the mitotic apparatus. *Int Rev Cytol* 162B, 1-74 (1995)