Identification of a Substrate for Pkn2, a Protein Ser/Thr Kinase from *Myxococcus xanthus* by a Novel Method for Substrate Identification

**Hiroshi Udo**1,2, Cuong Kim Lam1, Shuji Mori1,3, Masayori Inouye1, and Sumiko Inouye1*

1Department of Biochemistry, Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA
2Present Address: Department of Neurobiology and Behavior, Columbia University, 1051 Riverside Drive, School of Health Sciences, New York, NY 10032, USA
3Present Address: Faculty of Health Science, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700-8558, Japan

**Abstract**

Eukaryotic cells contain a large number of protein Ser/Thr kinases, which play important roles in signal transduction required for cell proliferation, differentiation, and stress response and adaptation. It is also known that some prokaryotes contain a family of protein Ser/Thr kinases. A major challenge in the characterization of these kinases is how to identify their specific substrates. Here we developed such a method using a protein Ser/Thr kinase, Pkn2 from *Myxococcus xanthus*, a Gram-negative soil bacterium. When Pkn2 is inducibly expressed in *E. coli*, cells are unable to form colonies on agar plates. This lethal effect of Pkn2 was eliminated in an inactive Pkn2 mutant in which the highly conserved Lys residue was changed to Asn, indicating that phosphorylation of a cellular protein(s) in *E. coli* resulted in growth arrest. Several clones from an *E. coli* genomic library were found to suppress the lethal effect when co-expressed with pkn2. Four out of seven multi-copy suppressors were identified to encode HUα, (3 for HUα and 1 for HUβ) a histone-like DNA binding protein. Purified HUα was found to be specifically phosphorylated by Pkn2 at Thr-59, and the phosphorylated HUα became unable to bind to DNA, suggesting that the phosphorylation of endogenous HU proteins by Pkn2 contributed at least in part to the lethal effect in *E. coli*. The present method termed the STEK method (Suppressors of Toxic Effects of Kinases) may be widely used for the substrate identification not only for prokaryotic protein Ser/Thr kinases but also for eukaryotic kinases.

**Introduction**

Human cells are assumed to contain more than 1000 protein Ser/Thr and Tyr kinases (Hunter and Plowman, 1997). From the genomic sequence yeast contains 113 protein Ser/Thr kinases, but no protein Tyr kinase (Hunter and Plowman, 1997). These kinases play important roles in various signal transduction pathways involved in cell growth, regulation of the cell cycle, differentiation, cell-cell interaction, tissue-specific gene expression, and stress response and adaptation. Interestingly, some prokaryotes having unique life cycles contain a family of eukaryotic-like protein Ser/Thr kinases such as *Myxococcus xanthus* (Munoz-Dorado et al., 1991; Zhang et al., 1992 and 1996; Udo et al., 1995; and Hanlon et al., 1997). *Streptomyces coelicolor* (Matsumoto et al., 1994 and Ueda et al., 1996), *Anabaena PCC7120* (Zhang, 1993 and Zhang et al., 1998) and *Mycobacterium tuberculosis* (Cole et al., 1998). These kinases are considered to play important roles in their life cycle and not to be replaceable with protein histidine kinases, which are the most prevalent kinase in the prokaryotes.

One of the major tasks in the studies on these protein Ser/Thr kinases is to identify their cellular targets in the signaling pathways as their specific substrates. In the present paper, we developed a novel genetic method to identify unknown substrates of Ser/Thr kinases. This method is based on the cytotoxic effect resulting from inducible expression of a protein Ser/Thr kinase in *E. coli*, which can be suppressed by overproducing its substrates, and thus termed the STEK method for Suppressors for Toxic Effects of Kinases. Here, using this method, we identified HUα and HUβ, histone-like proteins as substrates for Pkn2, one of 13 protein Ser/Thr kinases found in *M. xanthus*. Pkn2 consists of 830 amino acid residues of which the amino-terminal 297-residue region shows high similarity with eukaryotic Ser/Thr kinases (Udo et al., 1995). Pkn2 is autophosphorylated at serine and threonine residues. When Pkn2 is expressed in *E. coli*, it phosphorylates TEM-ß-lactamase to block the secretion of β-lactamase across the cytoplasmic membrane (Udo et al., 1995). We propose that the STEK method may be widely applied for other protein Ser/Thr and Tyr kinases using not only *E. coli*, but also other host cells for identification of their specific substrates. Recently this method has been used to identify a substrate for mouse minibrain kinase from a brain cDNA library (Dr. E. Kandel, personal communication).

**Results**

**Cytotoxicity of Pkn2 Expression in *E. coli***

When the *E. coli* strain SB221 harboring pNIIICm/pkn2 was plated on glucose-M9 agar plates in the presence or
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the absence of 1 mM IPTG, cells were able to form colonies without IPTG, but not in the presence of IPTG as shown in Figure 1A, suggesting that the expression of Pkn2 is toxic in E. coli. PINIIICm is an expression vector, which consists of the E. coli lpp promoter and lac promoter and operator regions with the chloramphenical acetyltransferase gene (Ghrayeb et al., 1984). The cloned gene is thus under the control of these promoters, and its expression is induced by the addition of IPTG. To examine whether the lethal effect of Pkn2 was due to the protein kinase activity of Pkn2, pINIIICm/pkn2(K42N), which expresses an inactive Pkn2 (Udo, et al., 1995), was constructed. In this mutated Pkn2 (Pkn2K24N), the highly conserved Lys residue in the kinase catalytic subdomain II of Pkn2 was changed to an Asn residue, which inactivates the kinase activity of Pkn2. As shown in Figure 1A, E. coli SB221 harboring pINIIICm/pkn2(K42N) was able to form colonies on a plate containing 1 mM IPTG to a similar extent to a plate without IPTG. The same result was obtained when another E. coli strain, JM83 (Vieira and Messing, 1982) was used as a host cell (not shown). These results indicate that a cellular protein(s) was phosphorylated by Pkn2, resulting in a cytotoxic effect.

The inhibition of cell growth was also examined in a liquid culture as shown in Figure 1B. E. coli SB221 (pINIIICm/pkn2) was grown in glucose-M9 medium and pkn2 was induced by adding 1 mM IPTG at a mid-exponential phase as indicated with an arrow. In comparison with the growth curve of SB221 (pINIIICm/pkn2) cells in the absence of IPTG, the growth rate of SB221 (pINIIICm/pkn2) gradually declined during the first 5 hr incubation after the addition of IPTG. When a Klett unit reached 270, the cell growth almost completely stopped. The viability of SB221 (pINIIICm/pkn2) cells steadily dropped during induction in the presence of 1 mM IPTG (Figure 1B).

**Screening of Suppressor Genes in E. coli**

If the cytotoxic effect of Pkn2 is due to the phosphorylation of a specific cellular protein(s) as described above, the cytotoxicity may be suppressed by overproducing the specific protein in E. coli expressing Pkn2, which may act as a Pkn2 substrate, an inhibitor for Pkn2 or a phosphatase for proteins phosphorylated by Pkn2. Therefore, we next attempted to search by screening an E. coli genomic library for multi-copy suppressor genes for the Pkn2 toxicity, whose products may serve as Pkn2 substrates. The E. coli genomic library was constructed by cloning of Sau3AI partial digests (3-4kb) of the E. coli chromosomal DNA into a unique BamHI site of pBRKm which is a pBR322 derivative carrying a Km gene from Tn5 (Beck, et al., 1982) at the DraI site in order to prevent the formation of colonies from drug-sensitive cells after prolonged incubation when the Amp marker is used. E. coli SB221 cells harboring the genomic library were then electroporated with pINIIICm/pkn2. Colonies formed on glucose-M9 agar plates containing 1 mM IPTG with Kanamycin (30 µg/ml) and chloramphenicol (30 µg/ml) were isolated and plasmids were prepared. To confirm the suppression activity, each candidate plasmid was reexamined for the second screening. After the second screening, 7 out of 30 candidates were found to still retain the suppressor function. Seven plasmids designated p3, 4, 7, 9, 10, 27, and 30 were digested with restriction enzymes to classify the type of the insert DNA fragments. Three out of seven candidate plasmids (p3, 4, and 9) contained common DNA fragments (see Figure 3). The protein expression patterns of strains SB221 harboring seven candidate plasmids were also examined by SDS-PAGE. As shown in Figure 2, the strains SB221 harboring p3, 4, and 9 were found to produce a 9-kDa protein at a similar level (lanes 3, 4, and 6 in Figure
expression were almost identical in all strains at least for 6 hr after IPTG (not shown), indicating that the expression of the suppressor gene did not affect the Pkn2 expression.

**Identification of the Suppressor Genes**

In order to map the suppressor gene on the *E. coli* chromosome, p3 DNA was used as a probe to screen the Kohara library (Kohara *et al.*, 1987). Two phages, No. 532 (9B9) and No. 533 (7B7) that were found positive, are located around the 3,550-kb region on the *E. coli* chromosomal DNA (Blattner *et al.*, 1997). Sequencing of the suppressor DNA revealed that p3 and p9 contain a DNA fragment from 65000 to 68714 bp (3,715 bp in length) and p4 contained a DNA fragment from 65000 to 68115 bp (3,116 bp in length), respectively, of U00006 in GeneBank. Thus, plasmids contained common ORFs of *hupA*, *o231*, and *f188* as shown in Figure 3.

The restriction sites *Bst*XI, *Nru*I, *Kpn*I, and *Hinc*I, were used to create deletion mutations of p3 as shown in Figure 3. The resulting plasmids and pINIIICm/pkn2 were cotransformed to SB221 cells and strains were subjected to test for suppression of the Pkn2 toxicity as summarized in Figure 3. The plasmids, which lack *hupA*, p3*ΔNru*I, and p3*ΔKpnI-Hinc*I, failed to suppress the Pkn2 toxicity while a plasmid p3*ΔBstXI*, which contains *hupA*, retained the suppressor function. To further confirm whether the *hupA* gene product is the suppressor, a frame-shift mutation within the *hupA* gene was created by removing 4 bp at the *Kpn*I site. A resulting plasmid, p3*ΔKpnI* lost the suppression activity, clearly indicating that the *hupA* product is responsible for the suppression of the Pkn2 toxicity.

As described previously, SB221 strains harboring p3, 4, and 9 produce a 9-kDa protein (see Figure 2) whose molecular weight is very close to that of the *hupA* gene product, HUα (9.5kDa). Note that the gene products of ORF *o231* and *f188* have calculated molecular weights of 26 and 20 kDa, respectively.

As shown in Figure 2, p27 was also found to produce...
a 9-kDa protein and suppress Pkn2 toxicity at the same level as p3. It is known that E. coli carry two HU genes, hupA for HUα and hupB for HUβ (Kano et al., 1985 and 1987). PCR was performed using two oligonucleotides corresponding to the 5'-end and 3'-end sequences of the hupB as primers, and the p27 plasmid DNA as a template. The resulting DNA fragment was cloned into the NdeI and BamHI sites of pET 11 (Studier et al., 1990), its DNA sequence was determined, and found to be identical to the hupB gene. This plasmid (p27) was thus used for expression and purification of HUβ. Two of the remaining three suppressors (p7 and p30) were found to contain a DNA fragment mapped at 57 min on the E. coli chromosome encoding YfgA and YfgB, hypothetical proteins. The DNA fragment carried by p10 has not yet been identified.

**Phosphorylation of HU by Pkn2**

The results described above suggest that HUα and HUβ, histone-like protein in E. coli, are the targets of Pkn2 and that Pkn2 toxicity caused by phosphorylation of HUα and HUβ by Pkn2, is suppressed by the overproduction of HUα or HUβ. To test this hypothesis, the phosphorylation of HU was examined using purified Pkn2 and HUαβ heterodimers (gift from Dr. Goshima, Hiroshima University) with [γ-32P]ATP. As shown in Figure 4A, 0.5 µg of HUαβ was effectively phosphorylated by 5 ng of Pkn2 (lane 3) and no band was detected at the position of HUαβ without Pkn2 (lane 2).

To further characterize phosphorylation of HU, HUα, and HUβ were purified from Escherichia coli BL21(DE3) harboring pET11hupA and pET11hupB, as described in Experimental procedures. pET11hupA and pET11hupB contained the hupA and hupB gene, respectively, under the control of a T7 promoter (Studier et al., 1990). As shown in Figure 4B, both HUα and HUβ (100 ng) were phosphorylated by Pkn2 (40 ng) as in the case of HUαβ (lane 1 and 2, respectively). The phosphorylation of HUα was dependent upon the concentration of Pkn2, as HUα phosphorylation linearly increased with increasing amounts of Pkn2 (Figure 4C). At the optimal condition, 19% of HUα was phosphorylated by Pkn2 (data not shown).
Phosphorylation Site of HUα

Pkn2 is known to autophosphorylate Ser and Thr residues and to phosphorylate threonine residues of TEM-β-lactamase in vivo (Udo et al., 1995). Since HUα contains 4 Ser and 7 Thr residues, we next examined which residue(s) is phosphorylated by Pkn2. For this purpose, HUα was phosphorylated with [γ-32P]ATP and Pkn2 and then treated with Lys-C (Roche), which cleaved only after a lysine residue. When HUα containing 11 Lys and 3 Arg residues is digested with Lys-C, all 3 Arg residues are resided in a single peptide from Val52 to Lys67. This peptide can be easily identified by reverse phase column chromatography using a C18 column, since the peak corresponding to the peptide disappears by trypsic digestion cleaving at Arg residues. We found that a trypsin-sensitive peptide was phosphorylated by Pkn2 (not shown), and thus concluded that the phosphorylation site(s) resides between V52 and Lys67. Since this peptide contains two Thr residues, Thr59 and Thr65, but no Ser residues, phosphorylation of HUα by Pkn2 occurs at either Thr59 or Thr65 or both.

To elucidate which Thr residue was phosphorylated by Pkn2, Thr59 and Thr65 were mutated to a Ala residue by site-directed PCR mutagenesis, as described in Experimental procedures. Then phosphorylation of HUαT59A and HUαT65A was examined. As shown in Figure 4B, HUαT65A was still phosphorylated as well as the wild-type HUα (lane 4), but HUαT59A was not (lane 3). Note that a faint band detected in lane 3 is considered to be due to phosphorylation of wild-type HUα, which was co-purified with HUαT59A from E. coli BL21(DE3) harboring pET11hupA(T59A), since E. coli BL21(DE3) carries the wild-type hupA gene.

Effect of Phosphorylation of HUα for DNA Binding Activity

Since HU is known to bind to single-stranded and double-stranded DNA, DNA binding activity of phosphorylated HU was examined. A double-stranded DNA of 141 bp in length was prepared from pUC9 digested with BamHI and PvuII and filled in with [α-32P]dGTP and [α-32P]dATP using the Klenow fragment of DNA polymerase I. When different amounts of HUα were added to 0.1 ng of DNA in a binding buffer as previously described (Tanaka et al., 1993), ladders of radioactive bands were observed as shown in Figure 5A. At 20 ng HUα, eight to nine new bands appeared and as more HUα was added, the bands shifted to more slowly migrating positions. The addition of 100 ng HUα resulted in a single band at the most slowly migrating band. Since eleven bands can be detected in the ladder, eleven HUα dimers are considered to be able to bind to the 141-bp DNA. The DNA-binding abilities of HUβ, HUαT59A, and HUαT65A were also examined. HUβ and HUαT59A could bind to the 141 bp DNA as well as HUα, while HUαT65A showed slightly less ability to bind to DNA (Figure 5B).

To examine the effect of phosphorylation of HUα on DNA binding, 100 ng HUα was first phosphorylated with different amounts of Pkn2 and then added to the binding buffer containing the 32P-labeled 141-bp DNA. As shown in Figure 5C, inhibition of DNA binding was observed as the amounts of Pkn2 increased. Since Pkn2 itself could not bind to DNA (Figure 5B, lane 2), the result indicates that Pkn2 inhibits DNA binding activity of HUα by phosphorylation.

To further investigate DNA binding activity of phosphorylated HUα, HUα was first phosphorylated by Pkn2 with [γ-32P]ATP and applied to 5% polyacrylamide gel in Tris-borate buffer. As a result of phosphorylation, 32P-labeled HUα was retained at the well of the gel (Figure 6, lane 3; shown by an arrow b). Under this condition, 8% of HUα was phosphorylated. Next, using the 32P-labeled HUα, the binding assay with 32P-labeled 141-bp DNA was carried out. As shown in lane 4, Figure 6, a new band indicated by arrow a appeared, which migrated at the position identical to the HUα-DNA complex (lane 2). However, if the same binding assay was performed with non-labeled 141-bp DNA, no band was detected at position a (lane 5), indicating that band a did not contain 32P-labeled HUα. On the basis of these results we concluded that only non-phosphorylated HUα can bind DNA, and that the DNA binding activity of HUα is inhibited upon its phosphorylation by Pkn2.

Discussion

In the present paper, we established a novel method to identify the substrate(s) for protein Ser/Thr kinases by

Figure 6. Effect of phosphorylation of HUα by Pkn2 on DNA mobility shift. A. DNA mobility shift assay using the 141-bp DNA and 32P-phosphorylated HUα. 0.1 ng of 32P-labeled DNA (lane 1), with or without 100 ng unphosphorylated HUα (lane 2) were used for the gel shift assay as described in Experimental Procedures. In lane 3, HUα was phosphorylated by 1mM ATP containing 50Ci [γ-32P]ATP and 40 ng Pkn2 was also added. Under the condition used, approximately 8% of HUα was phosphorylated. A HUα mixture (lane 3) was used for the DNA mobility shift assay with 32P-labeled DNA (lane 4) or unlabeled DNA (lane 5). The HUα and DNA complex migrated to position a and phosphorylated HUα was retained in a well, as indicated with an arrow with letter b.
isolating suppressor genes to reduce the toxic effect of kinases in *E. coli*. This method termed the STEK method (Suppressors for Toxic Effects of Kinases) may be applicable to Ser/Thr and probably Tyr kinases from bacteria to animal cells as far as they are toxic when expressed in *E. coli* or other host cells.

The lethal effect of *M. xanthus pkn2* in *E. coli* was found to be suppressed by either *hupA* or *hupB* genes of *E. coli*. The *hupA* and *hupB* genes of *E. coli* are known to encode the histone-like protein HUα and HUβ, respectively and the HU proteins are highly conserved in bacteria (Kano et al., 1986). HUα and HUβ form a heterodimer in vivo and affect gene expression (Lewis, et al., 1999). Strains mutated in both *hupA* and *hupB* genes show reduced viability, perturbed cell division (Wada et al., 1988 and Huisman et al., 1984) and more sensitive to UV (Li and Warters, 1998). Purified HUα heterodimers, HUα and HUβ, were phosphorylated by Pkn2 (Figure 4A and 4B). Peptide-mapping digested with LysC and trypsin, and mutational analysis revealed that Thr-59, which is located in the tip of flexible arms of the three-dimensional structure determined by X-ray crystallography, was phosphorylated. These regions are known to associate with DNA (Drlica and Rouviere-Yanit, 1987). Therefore, the phosphorylation of Thr-59 is likely to affect the binding of HU to DNA. Although Pkn2 can phosphorylate no more than 19% of HU in vitro (the reason for this is unknown at present), HU binding to DNA was inhibited as Pkn2 was continuously added in the reaction mixture (Figure 5C), indicating that the phosphorylated HU is unable to bind to DNA.

At present, it is not unknown how the phosphorylation of HU causes the cytotoxic effect. Since it has been shown that a *hupA ΔhupB* strain is still viable at 37°C (Wada et al., 1988), the cytotoxic effect may not be simply explained by dissociation of HU from DNA. Phosphorylated HU itself may cause a toxic effect to cells by affecting the DNA binding properties of other factors such as H-NS and IHF. Alternatively Pkn2 may have other targets whose phosphorylation by Pkn2 results in a lethal effect to *E. coli*. Indeed, there are two other suppressor genes yet to be identified, which may be the primary target for the *E. coli* lethality caused by Pkn2.

Pkn2 is a receptor type of Ser/Thr kinase which plays an important role in regulation of *M. xanthus* cell cycle presumably by transducing external signals to negatively regulate the transition from vegetative growth to developmental growth (Udo et al., 1996). Since HU proteins are highly conserved in bacteria, and function in the chromosomal structure and gene expression, it is an intriguing question whether HU is also the substrate for Pkn2 in *M. xanthus*. Currently, we are attempting to isolate the suppressor gene(s) from *M. xanthus* library using the same method described in this paper. We are also attempting to isolate *M. xanthus* HU protein(s) to examine whether it can be phosphorylated by Pkn2. It should be noted that the phosphorylation site of *E. coli* HUα is R-A-E-R-T, which is the first sequence identified as the specific phosphorylation sequence for bacterial protein Ser/Thr kinases. The corresponding sequence of *E. coli* HUβ is R-A-A-R-T.

The STEK method described here may be used with other host cells such as *Bacillus subtilis* and yeast. This method was successfully applied to a mouse minibrain kinase to identify substrates from a brain cDNA library (E. Kandel, personal communication), implying a wide application of the STEK method to a large number of protein Ser/Thr kinases from bacteria to kinases, whose substrates have not yet been identified. In addition, the STEK method could be applied for screening for specific inhibitors of a given Ser/Thr kinase as these inhibitors would be expected to function as suppressors for the cytotoxic effect of the kinase.

### Experimental Procedures

#### Materials

Restriction enzymes and Klenow enzyme were purchased from New England Biolabs. T4 DNA ligase was obtained from BRL. Alkaline phosphatase conjugated goat anti-rabbit IgG, 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) and nitro blue tetrazolium (NBT) were purchased from Bio-Rad. [α-32P]dGTP, [α-32P]dATP and [γ-32P]ATP were purchased from Amersham.

#### Bacterial Strains and Growth Conditions

*E. coli* SB 221 (Nakamura et al., 1982) and JM83 (Vieira and Messing, 1982) were used for cloning and suppression assay. Cells were grown at 37°C in LB and glucose-M9 media (Miller, 1972) supplemented with kanamycin (30 μg/ml) and chloramphenicol (30 μg/ml), when necessary.

#### Construction of *E. coli* Genomic Library

The chromosomal DNA was isolated from Escherichia coli SB221 (Nakamura et al., 1982) by the method described previously (Yее and Inouye, 1981). The chromosomal DNA was digested partially with Sau3AI and 3-4 kb fragments were extracted from a 0.7% agarose gel. These fragments were cloned into the BamHI site in pBR327 in which the 615-bp DraI fragment in the α-lactamase gene of pBR322 was replaced with the 1.3-kb HindIII fragment consisting of the kanamycin-resistant gene from Tn5 (Beck et al., 1982). After transformation into *E. coli* SB221, colonies were harvested and used as an *E. coli* genomic library of which more than 90% of plasmids were carrying *E. coli* DNA.

#### Screening of the *E. coli* Genomic Library

*E. coli* SB221 harboring the *E. coli* genomic library consisting of approximately 5×10⁶ independent transformants was grown in LB medium containing kanamycin until the Klett unit reached 100 and electroporated with pNIIICm/pkn2 using a Bio-Rad Gene Pulser (2.5 kV, 25 μF and 200 Ω). After incubation in LB medium for 1 hr, 100 μl of cells containing 20 μl of 1 M isopropyl thio-β-D-galactopyranoside (IPTG) were plated on glucose-M9 agar plates containing chloramphenicol (30 μg/ml) and kanamycin (30 μg/ml). Plates were incubated for 36 hr at 37°C, and 30 colonies were picked and plasmids were prepared. Since these plasmid DNA samples contain both pNIIICm and the candidate plasmids, the plasmids isolated were transformed to *E. coli* SB221 using LB agar plates containing only kanamycin. After overnight incubation, the plasmids containing suppressor genes were isolated from the colonies. Cells expressing candidate suppressor genes were reexamined for their resistance to pNIIICm in pkn2 in the presence of IPTG. pNIIICm/pkn2 was constructed by placing the pkn2 gene under the control of the *E. coli* lpp promoter and lac promoter and operator using a pNIIICm vector (Ghrayeb et al., 1984).

#### Western Blot Analysis of Pkn2

*E. coli* SB221 harboring pNIIICm/pkn2 and the suppressor plasmids were cultured in glucose-M9 medium containing both kanamycin and chloramphenicol. The pkn2 gene was induced with 1 mM IPTG at Klett unit of 0.0 and incubated for 0, 2, 4, and 6 hr. Cells were harvested and washed with 50 mM Tris-HCl (pH 8.0). The cell pellet was suspended in sonication buffer [50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 2 mM 2-mercaptoethanol] and sonicated for 2 min at 4°C. The supernatant after centrifugation at 5,000 × g for 1 min at 4°C was used as the cellular extract. The protein amount was determined by the method (Bradford, 1976) and 100 μg of protein samples were applied to 15% SDS-PAGE. The protein gel was blotted onto PVDF membrane. After the membrane was blocked with 5% skim milk for 4 hr, anti-Pkn2 antiserum was added and secondary antibodies were then applied. Pkn2 bands were developed with alkaline phosphatase substrate, NBT and BCIP.

#### Construction of HUα (T59A) and HUβ (T65A)

HUα (T59A) was constructed by two-step PCR using pUC/HUα as template and the following oligonucleotides as primers: For the first step, T7 primer; TGCAGCACTCCTCCCTT with GCGGCCAGCACGCTCAG, and CTGGAGGTAGCTGCGCGC with T7 3′-end primer; TGCTTTGCGTCT
GTATGT. The PCR products were gel-purified and used as template for the second PCR. HUα(T65A) was constructed by the same procedure described above using oligonucleotides TTATCCGGCCTGGGTG and ACCGGCAGGCCGTGAA as primers. After confirmed nucleotide sequences, HUα(T65A) and HUα(T65A) were placed under the control of a T7 promoter.

Purification of HUα, HUα(T59A), HUα(T59A), and HUB

In order to purify HU protein, expression plasmids of HUα, HUα(T59A), HUα(T59A), and HUB were constructed by placing these genes under the control of T7 promoter (Studier et al., 1990). The production of HUα and its derivatives and HUB were induced by the addition of IPTG at the final concentration of 1 mM in glucose-M9 medium. Purification of HU was performed by the method described by Tanaka et al. (1993) with some modification including, hydroxyapatite (HA) column chromatography to remove DNA fragments.

In vitro Phosphorylation of HU

Purified HU was phosphorylated with purified 40 ng Pkn2 (Udo et al., 1997) and 1 mM ATP containing 5 μCi [γ-32P]ATP (3000Ci/m mol) in the kinase buffer (0.1 M Tris-Cl [pH 7.5]/5 mM MnCl2/1 mM dithiothreitol (DTT)). The reaction mixture was incubated for 30 min at 30°C and terminated by the addition of SDS-sample buffer (80 mM TrisCl [pH 6.8]/2% SDS/10% glycerol/100 mM 2-ME). Proteins were resolved by SDS-PAGE and autoradiographed.

Gel Retardation Assay

The DNA fragment for binding assay was prepared by digestion of pUC9 (Vieira and Messing, 1982) with BamHI and PvuII and the 141-bp fragment was purified by 5% acrylamide gel electrophoresis. The 141-kb fragment was labeled with [α-32P]dGTP and [α-32P]dATP using the Klenow fragment of DNA polymerase I. Binding reaction was performed using 0.1 ng DNA probe by the method described by Tanaka et al. (1993).

The reaction mixtures were separated on 5% polyacrylamide gel electrophoresis in TBE buffer (45 mM Tris-HCl/45 mM boric acid/2 mM EDTA, pH 8.3). The gels were dried and autoradiographed.

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References


