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Synthetic octapeptide pyroGLU-ASP-ASP-SER-ASP-GLU-GLU-ASN controls DNA transcription in vitro by RNA polymerase II

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Abstract. The effect of the synthetic octapeptide pyroGLU-ASP-ASP-SER-ASP-GLU-GLU-ASN (phosphorylated by casein kinase II, CKII) on DNA transcription by RNA polymerase II has been studied. The peptide contains the acidic carboxy-terminus heptapeptide of the largest subunit of RNA polymerase II, which has been demonstrated to be a phosphorylation site for CKII. The aim of this work is to obtain some insights about the possible role of this domain in RNA polymerase II activity and DNA binding. Results demonstrated that the phosphorylated octapeptide causes strong inhibition of transcription of calf thymus DNA or pSVL SV40 plasmid DNA by RNA polymerase II, when used at concentrations between 0.4–4 μg/ml.
Key words. RNA polymerase II; transcription; peptide; protein kinase II; phosphorylation.

Small acidic phosphorylated peptides are present in the chromatin of several tissues[1]. They have been shown to have regulatory activity in the control of transcription in in vitro reconstituted systems, and in cell cultures[2,3]. On the basis of all the available biochemical and mass spectrometrical data on the structure of these peptides, some peptide models have been designed and synthesized[4]. They can be efficiently phosphorylated in vitro by protein kinase CKII[5]. It is of interest that one of the synthesized peptides, the octapeptide pyroGLU-ASP-ASP-SER-ASP-GLU-GLU-ASN, contains the heptapeptide which is found in the carboxy-terminus section of the largest RNA polymerase II subunit (aa 1926–1932). Payne et al.[6] reported that this sequence of the RNA polymerase II represents a phosphorylation site for CKII (serine 1928) since it has the sequence required for optimal phosphorylation by CKII: [Ser or Thr]-x(2)-[Asp or Glu]. However, the possible role of this domain in enzyme activity and DNA-binding has not been studied. In contrast, the phosphorylation of the consensus repeat sequence TYR-SER-PRO-THR-SER-PRO-SER (which is found immediately before the aa 1926–1932) has been studied intensively, and its possible function in the transition from the initiation to the elongation reaction of transcription has been proposed[6]. In this paper we report the phosphorylation of the synthetic octapeptide by CKII, and its effect on in vitro DNA transcription by RNA polymerase II purified from calf thymus.

Materials and methods

Purification of nuclear CKII. CKII was purified from calf thymus chromatin. Nuclear proteins were extracted from purified chromatin using 0.35 M NaCl, and fractionated with ammonium sulfate. The CKII was purified from the 30–50% saturated fraction by DEAE, phosvitin-Sepharose and phosphocellulose chromatography, as previously reported[7]. From 150 g of calf thymus tissue, 7.5 ml of purified protein kinase CKII were obtained. The fraction contained 0.3 mg of protein with an enzymatic specific activity of 580 U/mg of protein[7].

Purification of RNA polymerase II. The RNA polymerase II was isolated from calf thymus by polyethylenimine precipitation and DEAE cellulose, phosphocellulose and BIOGEL A 1.5 m (BIO-RAD) chromatography, according to Hodo and Blatti's method[8].

Preparation of HL-60 protein extract. The 10–60% saturated ammonium sulfate protein extract was obtained from HL-60 cells following the procedure described by Manley et al. for HeLa cell extract[9].

Synthetic octapeptide phosphorylation. The octapeptide (synthesized by Novabiochem, Switzerland) was incubated at concentrations varying from 10^{-5} to 2×10^{-3} M for 30 min at 30 °C with purified CKII (10 μl), 50 mM Tris-HCl (pH 8), 50 mM NaCl, 10 mM $MgCl_2$, 20 μCi γ-^{32}P-ATP (3000 Ci/mmol, Amersham) and 2 μg unlabeled ATP in a final volume of 0.1 ml. The phosphorylation mixture was then added to an equal volume of 0.1 M HCl and heated at 100 °C for 30 min to hydrolyse residual ATP. After lyophilization (twice) the material was resuspended in 100 μl of water and aliquots of the mixture were directly applied to thin layer cellulose plates (20 × 20 cm). Electrophoresis was carried out for 2 h at 600 V in acetic acid/formic acid/water/acetone (8/2/75/15; pH 1.9). The spot corresponding to the phosphorylated octapeptide was visualized by autoradiography. To estimate the value of Km, ^{32}P labeled peptide was eluted from TLC plate by 50 mM ammonium bicarbonate and the radioactivity was measured by Cerenkov counting. To check whether the phosphorylation by CKII does indeed occur on the

serine, an aliquot of the phosphorylated octapeptide was partially hydrolyzed in 6N HCl for 3 h at 110 °C. The hydrolyzate was mixed with a phosphoserine standard, applied onto a cellulose sheet and subjected to ascending thin layer chromatography in n-butanol/pyridine/acetic acid/water (6/2/3/3, by volume). Unlabeled phosphoserine was stained with ninhydrin and ^{32}P-radioactivity was detected by autoradiography.

To obtain enough phosphorylated peptide for the biological assays of in vitro DNA transcription, 200 µg of peptide was phosphorylated in 250 µl of the phosphorylation mixture described above for 30 min at 37 °C. After hydrolysis of the residual ATP in 0.05 M HCl (final concentration) for 30 min at 100 °C the mixture was lyophilized twice and the phosphopeptide was reisolated by anion exchange chromatography on a DE52 column (1 × 0.5 cm), and equilibrated with 50 mM ammonium acetate pH 8. Elution was performed with subsequent steps of 50 mM (1 ml), 100 mM (3 ml), 250 mM (2 ml) and 500 mM (2 ml) ammonium acetate adjusted at pH 8 with ammonia. To follow the phosphopeptide elution, 1 ml fractions were collected and aliquots applied to TLC plate electrophoresis as described above. The peptide-specific radioactivity was 500,000 c.p.m./µg of peptide.

Transcription assay. Calf thymus DNA and pSVL SV40 plasmid DNA (Pharmacia) were transcribed by purified RNA polymerase II at 37 °C in 30 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 0.1 mM creatine phosphate, 0.2 mM ATP, CTP, GTP, 8 µM ^{3}H-UTP (12 Ci/mmol). At various times 60 µl aliquots were taken to determine the radioactivity incorporated into the acid-insoluble fraction of RNA, as previously described[1].

Results and discussion

The electrophoretic analysis of the RNA polymerase II purified from calf thymus confirms the characteristic pattern reported by Dahmus[10]. The major 215 kDa subunit is phosphorylatable in vitro by CKII, confirming the presence of a phosphorylation site specific for this enzyme at the carboxy-terminus section of RNA polymerase II (data not shown). Accordingly, the synthetic octapeptide pyroGLU-ASP-ASP-SER-ASP-GLU-GLU-ASN (comprising the carboxy-terminus heptapeptide of the largest subunit of RNA polymerase II) is highly phosphorylated by CKII ($K_m = 10^{-4}$ M) in vitro (fig. 1A). The amino-terminus of the synthetic octapeptide is a pyroglutamyl residue with no free amino group: the absence of a positive charge should facilitate the phosphorylation by CKII. On the other hand, the charge equilibrium of peptides with a blocked amino-terminus is quite similar to that of protein carboxy-terminus domains with the same amino acid sequence.

Following phosphorylation in vitro by CKII the phosphorylated octapeptide was repurified by anion exchange chromatography; the elution was monitored by high voltage electrophoresis (fig. 1B). Figure 1B shows that after this chromatography, it is possible to obtain the phosphorylated peptide completely purified from hydrolyzed ATP. Thin-layer chromatography data confirm the presence of radioactive phosphoserine in the acidic hydrolyzate of the phosphorylated octapeptide (see Materials and methods; data not shown).

The time-course of calf thymus DNA and pSVL SV40 plasmid DNA transcription by purified calf thymus RNA polymerase II is reported in figure 2; in spite of some kinetic differences, the two molecular systems

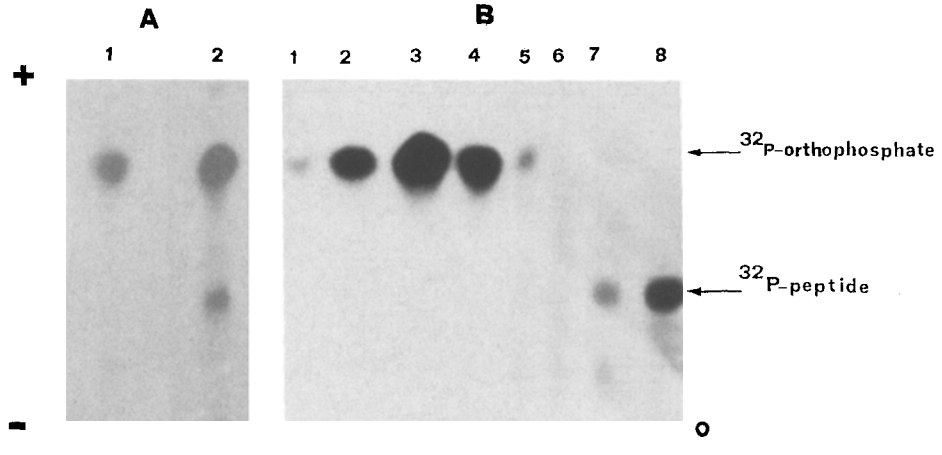

Figure 1. Autoradiograph showing phosphorylation of the synthetic octapeptide and purification of the phosphorylated form. High-voltage electrophoresis was performed as described in Materials and methods. O, origin

A: lane 2, phosphorylation of the synthetic octapeptide by CKII lane 1, phosphorylation mixture as in lane 2, but no peptide

B: TLC plate electrophoresis of the fractions from DE52 chromatography after elution with 50 mM (line 1), 100 mM (2, 3, 4), 250 mM (5, 6) and 500 mM (7, 8) ammonium acetate (see Materials and methods). It is possible to see the phosphorylated peptide in the same position in lane 1, panel A.

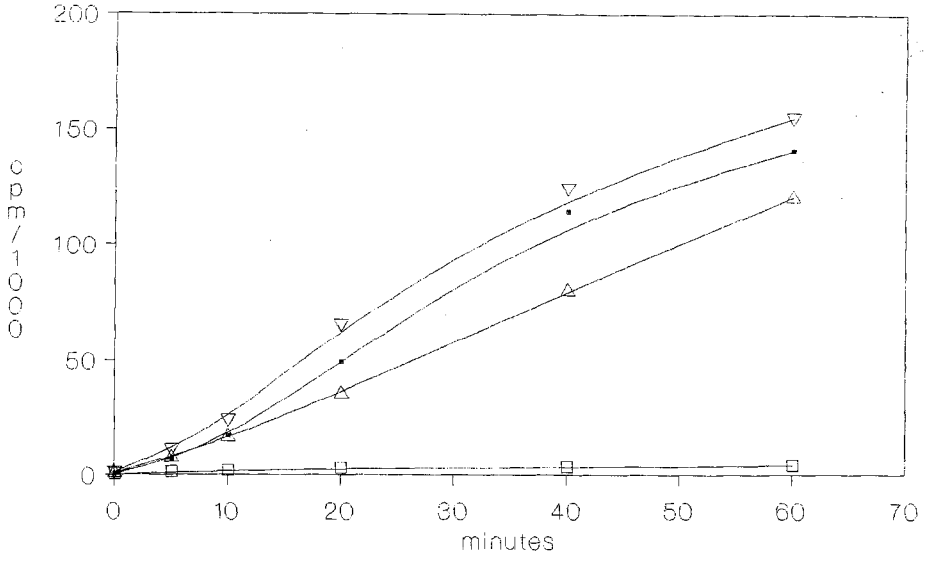

Figure 2. Time-course of calf thymus DNA and pSVL SV40 plasmid DNA transcription by calf thymus RNA polymerase II. Aliquots containing 60 µl of the mixture were taken at various times to determine the incorporation of ^{3}H-UMP as described in Materials and methods.
(■): 1 µg calf thymus DNA
(▽): 1 µg pSVL SV40 plasmid DNA
(△): 1 µg pSVL SV40 plasmid DNA in presence of protein extract from HL60 cells (6 µg)
(□): 1 µg calf thymus DNA plus 20 µg/ml (final concentration) α-amanitin.
Incubation mixture and other conditions are described in Materials and methods.

show essentially comparable transcription rates. As expected, the presence of α-amanitin completely inhibits the enzymatic reaction. The phosphopeptide affects the transcription rate of calf thymus DNA by RNA polymerase II; there is a slight stimulating activity at low concentration, but higher concentrations strongly inhibit transcription until an almost complete suppression of the transcription reaction is achieved (fig. 3). In contrast, the unphosphorylated peptide does not modify the transcription rate.

Very similar effects were observed with phosphorylated octapeptide on pSVL SV40 plasmid DNA transcription performed in the presence (fig. 4A) or absence (fig. 4B) of regulatory protein transcription factors contained in the HL60 cell extract. The data obtained demonstrate that the phosphopeptide strongly affects the transcrip-

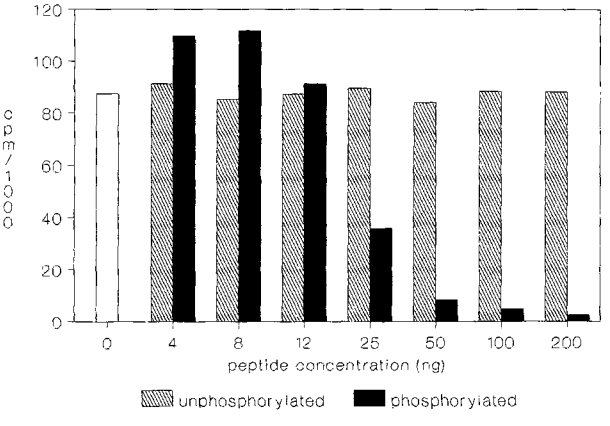

Figure 3. Calf thymus DNA (1 µg) transcription by RNA polymerase II purified from calf thymus. Effect on transcription caused by adding several concentrations of phosphorylated or unphosphorylated octapeptide. Incorporation of ^{3}H-UTP was determined after 30 min incubation as described in Materials and methods.

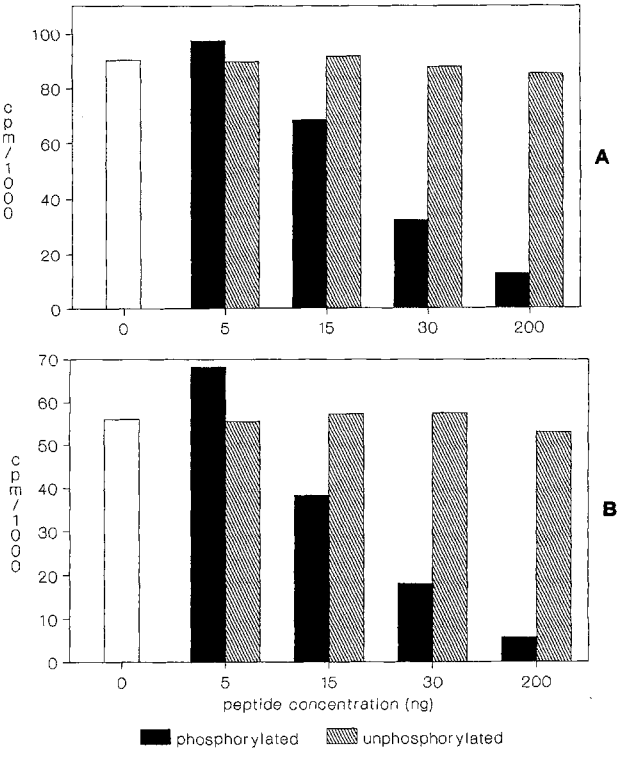

Figure 4. pSVL SV40 plasmid DNA (1 µg) transcription by calf thymus RNA polymerase II in the absence (*A*) or presence (*B*) of HL60 protein extract. Effect on transcription caused by adding several concentrations of phosphorylated or unphosphorylated octapeptide. The incorporation of ^{3}H-UTP was measured after 30 min incubation as described in Materials and methods.

tion reaction directed by both genomic (calf thymus) and plasmidic (pSV SV40) DNA, and the effects can be observed both in the presence and in the absence of regulatory protein factors. This observation seems to indicate that the phosphopeptide activity is exerted directly on the DNA-polymerase complex, and is not mediated by the protein transcription factors involved in the accurate recognition of initiation sites. Therefore experiments are in progress to show whether the phosphopeptide is able to bind to RNA polymerase II or to DNA, or if it impairs enzyme-binding to DNA.

These results also suggest that the carboxy-terminus domain of the largest subunit of RNA polymerase II is probably involved in the enzyme activity and/or DNA binding: the phosphorylation by CKII may be an activating regulatory mechanism of this function. This conclusion is supported by Kim and Dahmus[12], who have shown that transcription performed with the phosphorylated form of RNA polymerase II is slower than that obtained with the unphosphorylated enzyme.

1 Gianfranceschi, G. L., Barra, D., Bossa, F., Coderoni, S., Paparelli, M., Venanzi, F., Cicconi, F., and Amici, D., Biochim. biophys. Acta *699* (1982) 138.
2 Mancinelli, L., Castigli, E., Qualadrucci, P., Gianfranceschi, G. L., Bramucci, M., Miano, A., and Amici, D., Physiol. Chem. phys. Med. NMR. *24* (1992) 97.
3 Castigli, E., Mancinelli, L., Franceschini, M., Gianfranceschi, G. L., Bramucci, M., Miano, A., and Amici, D., Physiol. Chem. phys. Med. NMR. *24* (1992) 109.
4 Gianfranceschi, G. L., Cardellini, E., Piccinini, G., Bramucci, M., Miano, A., Maccari, E., Amici, D., and Durban, E., Physiol. Chem. phys. Med. NMR. *21* (1989) 289.
5 Chillemi, F., Lugaro, G., Boari, D., Cardellini, E., Bramucci, M., Miano, A., Amici, D., Gianfranceschi, G. L., and Durban, E., FEBS Lett. *291* (1991) 67.
6 Payne, J. M., Laybourn, P. J., and Dahmus, E., J. biol. Chem. *264* (1989) 19621.
7 Angiolillo, A., Panara, F., Desgro, A., Petrelli, C., and Gianfranceschi, G. L., Int. J. Biochem. *24* (1992) 1785.
8 Hodo, H. G., and Blatti, S. P., Biochemistry *16* (1977) 2334.
9 Manley, J. L., Fire, A., Cano, A., Sharp, P. A., and Gefter, M. L., Proc. natl. Acad. Sci. USA *77* (1980) 3855.
10 Dahmus, M. E., J. biol. Chem. *256* (1981) 3332.
11 Zandomeni, R., Carrera Zandomeni, M., Shugar, D., and Weinmann, R., J. biol. Chem. *261* (1986) 3414.
12 Kim, W. Y., and Dahmus, M. E., J. biol. Chem. *264* (1989) 2169.