Characterization of the Residues Phosphorylated in Vitro by Different C-terminal Domain Kinases*

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The C-terminal part of the largest subunit of eukaryotic RNA polymerase II is composed solely of the highly repeated consensus sequence Tyr¹-Ser²-Pro³-Thr⁴-Ser⁵-Pro⁶-Ser⁷. This domain, called the C-terminal domain (CTD), is phosphorylated mostly at serine residues during transcription initiation, but the precise role of this phosphorylation remains controversial. Several protein kinases are able to phosphorylate this sequence in vitro. The aim of this work was to define the positions of the amino acids phosphorylated by four of these CTD kinases (transcription factor (TF) IIH-kinase, DNA-dependent protein kinase, and the mitogen-activated protein kinases ERK1 and ERK2) and to compare the specificity of these different protein kinases. We show that TFIIH kinase and the mitogen-activated protein kinases phosphorylate only serine 5 of the CTD sequence, whereas DNA-dependent protein kinase phosphorlates serines 2 and 7. Among the different CTD kinases, only TFIIH kinase is appreciably more active on two repeats of the consensus sequence than on one motif. These in vitro results can provide some clues to the nature of the protein kinases responsible for the in vivo phosphorylation of the RNA polymerase CTD. In particular, the ratio of phosphorylated serine to threonine observed in vivo cannot be explained if TFIIH kinase is the only protein kinase involved in the phosphorylation of the CTD.

A characteristic feature of eukaryotic RNA polymerase II is the carboxyl-terminal domain (CTD)† of its largest subunit, composed of multiple repeats of the sequence Tyr¹-Ser²-Pro³-Thr⁴-Ser⁵-Pro⁶-Ser⁷. The number of repeats differs according to the species. The RNA polymerase II CTD contains 26 repeats in yeast (1), 45 in Drosophila (2, 3), and 52 in mammals (4). The consensus sequence is highly conserved, and this domain is essential for cell viability (2, 3, 5). Partial deletions of the CTD alter the regulatory properties of distinct promoters in different ways. The CTD has been shown to interact with a multisubunit complex containing the TATA-binding protein, which is an integral part of the transcription initiation complex (6).

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1 The abbreviations used are: CTD, C-terminal domain; TF, transcription factor; DNA-PK, DNA-dependent protein kinase; MAP, mitogen-activated protein.

The CTD motif is mainly composed of phosphorylatable amino acid residues, and the RNA polymerase II CTD is actually highly phosphorylated in vivo, mostly at serine and to a lesser extent at threonine and tyrosine (7–10). This phosphorylation appears to play a role in transcription initiation (11–13), but its precise function remains to be established. The in vivo phosphorylation sites are not known, but this issue has been approached indirectly by comparing the ratio of phosphorylated serine and threonine. The predominance of serine phosphorylation in vivo (with a serine/threonine ratio of ~10:1) has been explained by phosphorylation at positions 2 and 5: the serine at position 2 is replaced by a threonine in one-fifth of the repeats, and position 5 has only one threonine out of 51 (9). Moreover, yeast strains in which CTDs have been modified by the substitution of these two serines are nonviable (14).

In the past 5 years, many protein kinases from various organisms have been described as being able to phosphorylate the CTD in vitro (15–22). Some of them are now well identified. δ-Kinase from rat (20) (also called factor b in yeast (16) and TFIIH or BTF2 in humans (22)) is a multisubunit transcription factor that contains DNA-dependent helicase activity, DNA repair activity, and CTD kinase activity (see Ref. 23 for review). A subcomplex called TFIIK is responsible for the CTD kinase activity and is composed of the Cdk-related protein kinase MO15/Cdk7 in mammals (KIN28 in yeast), associated with cyclin H (CCL1) and MAT-1 (24–27, 67, 68). The kinase-cyclin pair MO15-cyclin H (KIN28-CCL1) is already known as a cyclin-dependent kinase (Cdk/Cdc2-activating kinase) in vitro (28, 29). Other protein kinases have also been described as able to phosphorylate the CTD. DNA-dependent protein kinase (DNA-PK) is composed of a catalytic subunit (DNA-PKcs) and a regulatory component corresponding to the Ku autoimmune antigen (30, 31). It acts as a CTD kinase when stimulated by linear double-stranded DNA and by several transcriptional activators (30, 32). Recently, DNA-PK has also been shown to phosphorylate the RNA polymerase I transcriptional apparatus and to inhibit RNA polymerase I transcription. Moreover, this protein kinase plays a major role in DNA repair processes and recombination of immunoglobulin gene loci in the cells of the immune system (33). MAP kinases are induced by mitogenic stimuli and by heat shock. They are able to phosphorylate the CTD among their many known in vitro substrates (34).

The aim of this study was to determine the sites phosphorylated in vitro in the CTD by TFIIH kinase, DNA-PK, and MAP kinases. Comparison of the results obtained in vitro with what is known about CTD phosphorylation in vivo gives some clues to the nature of the protein kinases involved in the in vivo phosphorylation of the RNA polymerase CTD. To determine which of the three serines is phosphorylated by the different CTD kinases, we synthesized a set of peptides containing one or
two CTD motifs in which each serine was successively replaced by an alanine. We devised new electrophoretic conditions to be able to separate the phosphorylated peptides. Similar experiments were performed with another set of peptides to determine the influence of amino acids surrounding the phosphorylated site and to compare the specificity of the different CTD kinases. Using a similar method, we were recently able to distinguish the different CTD kinase activities that are induced by stress and heat-shock treatment (35).

MATERIALS AND METHODS

Kinase Assays

Reaction mixtures (24 μl) contained (final concentrations) 40 mM β-glycerophosphate, pH 7.4, 7.5 mM MgCl2, 7.5 mM EGTA, 5% glycerol, [γ-32P]ATP (0.2 mM, 1 μCi), 50 mM NaF, 1 mM orthovanadate, 0.1% (v/v) β-mercaptoethanol, and the following peptides: hepta-3 (SPTSPSY), 0.4 mg/ml, hepta-2 (SPTSPSY), or modified hepta-2 (0.4 mg/ml), or hepta-1 (SPTSPSY) or modified hepta-1 (1.6 mg/ml) (unless otherwise mentioned in the figure legends).

Phosphorylation reactions were performed with 4 μl of human ERK1 (agarse-conjugated ERK1; Upstate Biotechnology, Inc.) for 90 min at 30 °C, with 0.4 units/ml murine recombinant ERK2 (from Prof. P. Cohen, University of Dundee, Dundee, United Kingdom) for 30 min at 30 °C, with ~40 ng of TFIIH kinase (TSK SP-5-PW fraction (36)) for 2.5 h at 30 °C in the presence of 1.6 mg/ml polyvinyl alcohol and 5 mg/ml bovine serum albumin, or with 2 μl of DNA-PK (from Dr. G. Smith, Wellcome/Cancer Research Campaign Institute, Cambridge, United Kingdom) in the presence of 200 μg/ml salmon sperm DNA for 30 min at 30 °C. The times used for in vitro reactions were chosen to be in initial rate conditions. Peptides were synthesized by Dr. O. Sifort (Organic Chemistry Laboratory, Institut Pasteur). Reactions were stopped by adding 1 volume of Laemmli sample buffer (37) containing 5% β-mercaptoethanol.

Gel Electrophoretic Conditions and Quantification

Hepta-3, Hepta-2, and Modified Hepta-2 Peptide Analysis—Reaction samples were analyzed by 22% SDS-polyacrylamide gel electrophoresis performed according to Laemmli (37).

Hepta-1 Peptide Analysis and Modified Peptides—Hepta-1 peptides were too small to be separated from the radiolabeled ATP front by the usual electrophoretic conditions. We acidified the buffer by replacing the Tris/glycine buffer with a phosphate buffer (38) as we previously described (39). Reaction samples were subjected to electrophoresis on 10% denaturing phosphate buffer gels at pH 6.0.

Gels were fixed in ethanol/acetic acid/trichloroacetic acid/water (3:1:5:1, v/v), dried, and submitted to autoradiography with an intensifying screen at 4 °C. Signals were quantified with a Fuji BAS reader, and measurements made with PC BAS.

Phosphoamino Acid Analysis

The SPTTPSY peptide was phosphorylated by TFIIH kinase and analyzed as described above. The radiolabeled spot was cut out of the gel and washed rapidly in 500 μl of water. The phosphorylated peptide was eluted in 0.5 M ammonium acetate at 37 °C with gentle agitation overnight. Peptide hydrolysis and phosphoamino acid analysis were performed as described previously (39).

RESULTS

TFIIH Kinase and the MAP Kinases ERK1 and ERK2 Phosphorylate Serine 5 of the CTD Motif—Serizawa et al. (40) previously showed that TFIIH kinase phosphorylates the RNA polymerase II CTD at serine residues. To determine which of the three serines was phosphorylated by TFIIH kinase, we assayed a set of peptides in which serines were replaced by alanines. Fig. 1A shows that phosphorylation was completely lost when serine 5 was replaced by alanine, whereas the two other serine substitutions had no effect on phosphorylation. Thus, only serine 5 of the CTD motif appears to be phosphorylated by TFIIH kinase. These results are in agreement with those reported by Roy et al. (25). Identical results were obtained with ERK1 (Fig. 1B) and ERK2 (data not shown).

Phosphorylation by TFIIH Kinase and by ERK1 and ERK2 Reveals Differences in the Recognition of the Hepta-2 Peptide—Within the entire CTD, all the repeats are contiguous, and all (but one) serines at positions 2 and 7 are close to the extremity of another repeat. Therefore, the absence of phosphorylation of these serines by TFIIH kinase (or MAP kinases) might be due to the absence of the amino acids surrounding these residues in the CTD. To rule out this possibility, we performed experiments with similar alanine replacements in hepta-2 peptides (Ser2-Pro3-Thr4-Ser5-Pro6-Ser7-Tyr8-Ser9-Pro10-Thr11-Ser12-Pro13-Ser14-Tyr1) (Fig. 2). These peptides were electrophoresed on a 22% denaturing polyacrylamide gel. Phosphorylation of the hepta-2 peptide by ERK1 or ERK2 revealed two separated bands with similar intensities. Phosphorylation efficiency was very low, and the peptide concentration that we used did not allow the characterization of multiple phosphorylations. The two bands correspond to phosphorylation in the left or right part of the peptide. Indeed, when serine 5 of the right part was replaced by an alanine, the upper band disappeared, and when the same substitution was made in the left side, there was no longer a lower band. Replacement of serine 9 had no effect on the phosphorylation. Therefore, as for hepta-1 peptides, only serine 5 of the CTD motif is phosphorylated in hepta-2 peptides by ERK1 and ERK2.

As far as TFIIH kinase is concerned, the two bands were also observed, and we obtained qualitatively the same effect for the substitutions (Fig. 2), although the relative intensities were different from those obtained by MAP kinase phosphorylation. The intensity of the upper band, which corresponds to phosphorylation of the serine present in the central position of the left motif, was very low compared with the other. This differ-
naturing polyacrylamide gels. Phosphorylated peptides were subjected to electrophoresis on 22% de-
tide A9), and 2 (peptide A2) were successively substituted by alanine
modified hepta-2 peptides (0.4 mg/ml). Serines 5 (peptide A5), 9 (pep-
TFIIH and MAP kinases were assayed for phosphorylation of hepta-2 or
and MAP kinases within the CTD motif using hepta-2 peptides.
rence was still evident with substituted peptides. The two sites
are not equivalent, suggesting that TFIIH kinase recognizes
sequence or conformational information in the right side of the
phosphorylated site. Fig. 3 shows the result of a comparison of
the efficiency of the different protein kinases to use one, two, or
three repeats of the CTD motif. To be able to compare the
phosphorylation of the three different peptides in a single ex-
periment, we used the electrophoretic conditions normally used
for the hepta-1 peptides. Under these conditions, only one spot
is obtained for the hepta-2 peptide. For this experiment, the
same peptide concentration was used for all the peptides.
ERK1 and ERK2 phosphorylated the hepta-3 and hepta-2 pep-
tides 3- and 2-fold better than the hepta-1 peptide, respectively,
whereas TFIIH kinase phosphorylated the hepta-2 and hepta-3
peptides 13- and 20-fold better than the hepta-1 peptide, re-
respectively. The hepta-3/hepta-2 ratio was therefore identical
for the three kinases. This indicates that two repeats increase
the phosphorylation efficiency of TFIIH kinase.

**FIG. 2.** Determination of the serine phosphorylated by TFIIH
and MAP kinases within the CTD motif using hepta-2 peptides.
TFIIH and MAP kinases were assayed for phosphorylation of hepta-2 or
modified hepta-2 peptides (0.4 mg/ml). Serines 5 (peptide A5), 9 (pep-
tide A9), and 2 (peptide A2) were successively substituted by alanine
residues. In peptide A5A2, both serines 5 and 2 were substituted.
Phosphorylated peptides were subjected to electrophoresis on 22% de-
naturing polyacrylamide gels.

**FIG. 3.** Phosphorylation efficiency of TFIIH kinase, MAP ki-

nase, and DNA-PK to use one, two, or three repeats of the CTD
motif. TFIIH kinase, MAP kinase (MAPK), and DNA-PK were assayed
for phosphorylation of hepta-3, hepta-2, or hepta-1 peptides (1.6 mg/ml).
Phosphorylated peptides were separated on phosphate buffer-polyac-
rylamide gels at pH 6.0 and were quantified with a Fuji BAS reader.
The amount of radioactivity incorporated in the hepta-1 peptide was
arbitrarily set to 100 to compare the relative activities of the different
kinases. au, arbitrary units.

**Phosphorylation of Modified Hepta-2 Peptides by DNA-PK—**
The experiment was submitted to autoradiography. Ninhydrin after chromatography on a cellulose plate. Standards were added to the amino acid hydrolysate and revealed by the peptide.

The hepta-1 motif to determine their importance in the recognition of kinases.

A third set of peptides was synthesized. Amino acids surrounding the phosphorylated residue were substituted or displaced in the hepta-1 motif to determine their importance in the recognition of the peptide. The different peptides were assayed with TFIIH (upper panel) or MAP kinase (MAP K, lower panel) at 1.6 mg/ml. Phosphorylated peptides were analyzed as described for Fig. 1. Phosphoamino acid analysis was performed on SPTTPSY phosphorylated by TFIIH kinase. Panel 1, cold phosphoserine (Pser) and phosphothreonine (Pthr) standards were added to the amino acid hydrolysate and revealed by ninhydrin after chromatography on a cellulose plate. Panel 2, the same experiment was submitted to autoradiography.

Since the sites phosphorylated by DNA-PK were different from those phosphorylated by TFIIH and MAP kinases, two other modified hepta-2 peptides were synthesized. Four bands were observed by phosphorylating the hepta-2 peptide (Ser5-Pro6-Ser7-Tyr8-Ser9-Pro10-Thr11-Ser12-Pro13) with DNA-PK and Methods. Taking into account the serines phosphorylated by DNA-PK in hepta-1 peptides (Fig. 1), serines 9 and 14 of the hepta-2 peptide were successively or simultaneously substituted by alanines. Phosphorylated peptides were analyzed as described for Fig. 2.

Despite all the studies on the CTD and its phosphorylation, the role of this domain of eukaryotic RNA polymerase II is still unclear. A model frequently evoked for RNA polymerase II transcription initiation is the recruitment of a hypophosphorylated RNA polymerase II to the preinitiation complex and phosphorylation during the transition from initiation to elongation (11–13). Possible pausing at an early phase of transcription, dependent on proximal activating sequences (42), might necessitate another phosphorylation for the release of RNA polymerase. Therefore, a first phosphorylation might be necessary for initiation and a second for relief of pausing. These two phosphorylations might be carried out by different Ser/Thr or Tyr protein kinases. By using immunofluorescence microscopy, Weeks et al. (43) showed on polytene chromosomes that some specific genes are transcribed by hyperphosphorylated polymerases, whereas hsp70 mRNAs are elongated by a mixture of hypo- and hyperphosphorylated forms. Similar results were obtained by in vivo protein-DNA cross-linking assays (44). The CTD phosphorylation state during transcription elongation may thus be different for the different types of gene. Experiments in yeast suggest the involvement of several protein kinases in CTD phosphorylation in vivo. Lee et al. (45) have
characterized the CTK1 gene encoding a yeast nuclear CTD kinase, which presents some homologies to cdc2/CDC28. CTK1 gene disruption decreases in vivo phosphorylation of RNA polymerase II without abolishing it (45). The different sensitivity of CTD phosphorylation to protein kinase inhibitors suggests that it is the same in higher eukaryotes: CTD phosphorylation in quiescent cells is decreased by the protein kinase inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, whereas it is unaffected in serum-treated cells or heat-shocked cells (34, 46).

The number of protein kinases able to phosphorylate the CTD in vitro is still growing. A CTD kinase has been located in the yeast holoenzyme. This kinase is encoded by a new gene called SRB10, regulated by the cyclin SRB11 (47). Recently, another CTD kinase has been identified as a component of the positive transcription elongation factor of Drosophila. It has been suggested that this protein kinase could phosphorylate the CTD to release the RNA polymerase II from an abortive elongation state (48). In addition, McCracken et al. (69) showed recently that the CTD is required for efficient RNA processing. CTD phosphorylation might also be implicated in mRNA splicing. All these observations suggest that depending on the signal that induces specific gene transcription, the step in the transcription cycle or mRNA splicing, different enzymes might be involved in CTD phosphorylation. These protein kinases may have different site specificities in the CTD motif.

In Vivo Phosphorylation of the RNA Polymerase II CTD

Little is known about the exact phosphorylation state of RNA polymerase II in vivo. A shift on SDS-polyacrylamide gels revealed multiple CTD phosphorylation, and two-dimensional paper electrophoresis of in vivo labeled CTD showed a predominant phosphorylation of serine compared with threonine (ratio of ~10:1). This low level of threonine phosphorylation cannot be explained by threonine 4 phosphorylation because this position is highly conserved, and its phosphorylation would not lead to a correct ratio. However, it can be explained by phosphorylation at positions 2 and 5, which present some non-consensus threonines instead of serines (9).

Specificity of the Different Protein Kinases That Phosphorylate the CTD Motif

TFIIH and MAP Kinases Phosphorylate the Same Site in the CTD Motif—TFIIH kinase, ERK1, and ERK2 phosphorylate the same residue in the repeated motif of the CTD. Surprisingly, the influence of each amino acid of the motif seems very similar for TFIIH and MAP kinases. They are proline-directed protein kinases. The consensus sequence deduced from CTD peptides (PX/S/T/P) is identical to the sequence determined for MAP kinases in the myelin basic protein and epidermal growth factor receptor (41, 49, 50). For both kinases, peptide phosphorylation is strongly enhanced by the replacement of threonine 4 by a leucine. Moreover, the N-terminal proline, which is essential for CTD sequence phosphorylation, is no longer necessary in the presence of a leucine at position 4 (SALSPSY). When the site phosphorylated by MAP kinases in whole proteins does not present a proline at position −2, a hydrophobic amino acid at position −1 is very often observed, confirming a strong influence of this residue on peptide phosphorylation (51). One of the differences we found between MAP and TFIIH kinase specificities is the more stringent requirement of the latter for a serine at the phosphorylated position. The second concerns the difference between hepta-1 and hepta-2 peptides phosphorylation efficiencies. We observed a small and progressive increase in phosphorylation of peptides containing one, two, and three CTD motifs by ERK1 and ERK2. This is in agreement with the data showing that the entire RNA polymerase II CTD is better phosphorylated than a small peptide by most of the CTD protein kinases. However, we showed that TFIIH kinase phosphorylates the hepta-2 peptide 13-fold better than the hepta-1 peptide, whereas the difference between the hepta-3 and hepta-2 peptides is low and identical to that observed for the other CTD kinases. Despite an identical phosphorylation site, these experiments show that MAP and TFIIH kinase recognition sites are not equivalent.

The Cdk2 Sequence Recognized by TFIIH Kinase Is Different from the Consensus Sequence Determined with CTD Peptides—TFIIH kinase is a multisubunit complex containing several enzymatic activities. The CTD kinase catalytic subunit of TFIIH kinase corresponds to the protein kinase MO15/Cdk7 complexed with cyclin H (24–27, 67, 68). MO15 is considered to be a Cdc2-like protein (52, 53). We showed in this study that TFIIH kinase is also a proline-directed protein kinase like Cdc2. However, definition of a general consensus sequence for TFIIH is problematic. It was previously shown that this MO15-cyclin H complex, also called CAK, was implicated in p34cdc2 and p33cdc2 activation by phosphorylation of threonine 161 for p34cdc2 and threonine 160 for p33cdc2 whose surrounding sequences are almost identical (RVYTHHEVTLWYR) (54).
There are no prolines, either at position −2 or at position +1. One could imagine that a subunit supplementary to MO15 and cyclin H would allow a change in specificity. However, this hypothesis seems unlikely because Serizawa et al. (67) showed that TFIIF kinase (identical to the factor we used) phosphorylates both the RNA polymerase II CTD and Cdk2. One similarity between CTD kinase and Cdc2 kinase is their relative efficiency in phosphorylating serine and threonine. We showed that a serine-containing peptide is a better substrate for TFIIF kinase than a threonine-containing peptide. An identical conclusion was reached with Cdk2 when Thr160 was replaced by a serine (54).

**DNA-PK Is Able to Phosphorylate Two Serines in the CTD Motif**—Several proteins such as HSP90, Sp-1, p53, c-Jun, and SV40 large T antigens are in vitro substrates for DNA-PK. Studies of the sites phosphorylated by DNA-PK in these proteins defined a common minimal consensus sequence, which is Q(S/T) or (S/T)(Q)55–60, but these sequences are not present in the CTD, even in the non-consensus motifs. However, it has been previously noted that certain proteins such as c-Fos are also efficient substrates, but are apparently not phosphorylated on Q(S/T) or (S/T)Q motifs (61).

We showed in this study that in contrast to TFIIF and MAP kinases, DNA-PK does not phosphorylate serine 5 of the CTD motif, but rather the serines at positions 2 and 7. The substitution of each amino acid surrounding the phosphorylated serines did not lead to a complete loss of phosphorylation, suggesting that none of these amino acids is absolutely necessary. All the substitutions showed that prolines are not important for CTD phosphorylation by DNA-PK.

For DNA-PK, the replacement leading to the most striking increase is the substitution of threonine 4 by a glutamic acid. The positive influence of glutamic acid was previously shown for p53 and c-Jun. One of the sites phosphorylated in vitro by DNA-PK is ES\(^7\)Q for p53 and ES\(^5\)\(^9\)\(^4\)QE for c-Jun (59, 60). The successive replacement of each of these glutamic acid residues in the c-Jun sequence decreased phosphorylation. The influence of glutamic acid 251 on serine 249 phosphorylation may be similar to the influence of glutamic acid 4 on serine 2 phosphorylation in the CTD sequence. This observation is consistent with the idea that acidic residues contribute to the recognition by DNA-PK, but such sequences are never found in the RNA polymerase II C-terminal domain, even in the non-consensus motifs.

**Tyrosine May Play a Role in DNA-Substrate Binding**—Tyrosine 1 replacement by an alanine significantly decreases peptide phosphorylation. Tyrosine may be important for enzyme-substrate binding or for DNA-substrate binding necessary for good recognition by DNA-PK. Most of the potential substrates phosphorylated by DNA-PK bind DNA, and the colocalization of the enzyme and the substrate on the same DNA fragment appears important. A unique CTD motif with a tyrosine at each end is able to bind DNA, but the case of a simple hepta-1 peptide has not been tested (62). More recently, West and Corden (14) showed that tyrosine replacement by a phenylalanine in yeast CTD is lethal for the cell. However, interpretation of the latter data remains difficult.

**Physiological Significance of the in Vitro Phosphorylation Studies**

The approach that we have chosen is open to criticism since it relies on the assumption that the peptides are recognized with the same efficiency when free in solution or inserted into a polypeptide chain. However, recent studies have determined the optimal consensus sequence phosphorylated by protein kinases by comparing their efficiency in degenerate peptide libraries (63). In the case of the CTD, this approach appears more valid as its highly repeated nature suggests that this domain has a repetitive three-dimensional structure. Indeed, this suggestion is supported by recent NMR studies (64). Therefore, it is likely that the conformation of 1- or 2-fold repeats of the motif is probably similar to the conformation of the same motif in the whole RNA polymerase II subunit.

The CTD kinases that we chose for this study are good candidates to participate in CTD phosphorylation in vivo. Cytoplasmic MAP kinases are activated by a broad range of agents and migrate into the nucleus only in the presence of inducers. Previous studies have shown that DNA-PK is present in preinitiation complexes and phosphorylates the CTD of endogenous RNA polymerase II (65). The sites phosphorylated by DNA-PK defined in this study are not in full agreement with the serine/threonine ratios already published for this protein kinase (66): the CTD phosphorylated by DNA-PK contains approximately equal amounts of phosphoserines and phosphothreonines. However, positions 2 and 7 indeed contain the highest number of substitutions of serines by threonines (64 Ser/14 Thr). Moreover, this ratio can decrease if DNA-PK preferentially phosphorylates the C-terminal end of the CTD, where the number of substitutions is higher, or if DNA-PK is more specific for threonines (a possibility that has not yet been tested). TFIIF kinase is a good candidate for in vivo CTD phosphorylation because of its presence in the holoenzyme. Moreover, we show in this study that its recognition site corresponds to two adjacent motifs, indicating a good specificity for CTD repeats.

All these protein kinases are located very close to the transcription apparatus, but we cannot conclude about their roles in CTD phosphorylation in vivo. If the CTD is phosphorylated by a single enzyme, neither TFIIF kinase nor DNA-PK and the MAP kinases can explain the in vivo ratio of serine to threonine (1:10) (9). In contrast to the former model, several protein kinases might be involved, inducing different CTD modifications. In both cases, a systematic determination of the specificity of each CTD kinase for the CTD motif remains useful.

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