CDK7, CDK8, and CDK9 are cyclin-dependent kinases (CDKs) that phosphorylate the C-terminal domain (CTD) of RNA polymerase II. They have distinct functions in transcription. Because the three CDKs target only serine 5 in the heptad repeat of model CTD substrates containing various numbers of repeats, we tested the hypothesis that the kinases differ in their ability to phosphorylate CTD heptad arrays. Our data show that the kinases display different preferences for phosphorylating individual heptads in a synthetic CTD substrate containing three heptamer repeats and specific regions of the CTD in glutathione S-transferase fusion proteins. They also exhibit differences in their ability to phosphorylate a synthetic CTD peptide that contains Ser-2-P. This phosphorylated peptide is a poor substrate for CDK9 complexes. CDK8 and CDK9 complexes, bound to viral activators E1A and Tat, respectively, target only serine 5 for phosphorylation in the CTD peptides, and binding to the viral activators does not change the substrate preference of these kinases. These results imply that the display of different CTD heptads during transcription, as well as their phosphorylation state, can affect their phosphorylation by the different transcription-associated CDKs.

The three cyclin-dependent kinases, CDK7, CDK8, and CDK9, have an established connection with transcription machinery and are regulated by constitutively expressed cyclins. These CDKs can hyperphosphorylate the CTD of the large subunit of RNA polymerase II (pol II) and therefore are CTD kinases (1). The CTD of mammalian pol II consists of 52 heptad repeats with the consensus sequence 1YSPTSPS7, which is well-conserved in the first half (N-terminal) of the CTD. Of the phosphorylatable amino acid residues, serine 5 is the most conserved, with only one threonine replacement, indicating that it is likely to have a crucial role in the function of the CTD. Pol II CTD is hyperphosphorylated in vivo, mostly at serine (positions 2 and 5) but also at threonine and tyrosine (2). Hyperphosphorylated CTD is correlated with transcription initiation and elongation, whereas the holoenzyme and preinitiation complexes contain pol II with hypophosphorylated CTD (3). In yeast cells, replacing either serine 2 or serine 5 with alanine in each of this organism’s 26 CTD heptad repeats causes lethality (4).

The functions of these kinases correlate with different stages of transcription. CDK7 is a subunit of the general transcription factor TFIIH and is the kinase that phosphorylates pol II CTD after the preinitiation complex is formed (5). CDK7 is the catalytic member of a three-subunit complex called CAF (CDK-activating kinase) composed of CDK7/cyclin H and MAT-1 (6, 7). CAF is also a subassembly of TFIIH (8). Comparison between the kinase activity of CAF and TFIIH revealed differences in substrate specificity: CAF shows a strong preference for CDK2, consistent with its function in the cell cycle, but cannot hyperphosphorylate pol II; TFIIH hyperphosphorylates pol II, but CDK9 is a very poor substrate for this form of CDK7 (9, 10). TFIIH also phosphorylates transcription factors TBP, TFIIIE, and TFIIF, whereas free CAF phosphorylates only TBP (5, 9, 10). Hence, the composition of the complexes that contain CDK7 modulates its substrate preferences.

Human CDK8/cyclin C is found in a variety of pol II holoenzyme complexes (11, 12), the largest of which is slightly smaller than 70 S ribosomes (13), and also in complexes devoid of pol II such as the mammalian SRB/Mediator complex, which contains homologues of the yeast SRB/Mediator (14). The functions of the various CDK8-containing complexes are not fully understood. E1A and VP16 target the human SRB/Mediator and stimulate transcription in vitro (14, 15). CDK8 complexes containing E1A and VP16 activation domains can phosphorylate the CTD (16), implying that they may activate transcription by increasing the processivity of pol II. On the contrary, NAT (negative regulator of activated transcription) is probably a subcomplex of pol II holoenzyme and can interact with pol II to repress activated transcription by phosphorylating pol II (17).

CDK9 was cloned as cdcl2-related kinase called PITALRE (18), and together with its cyclin partners, cyclins T1, T2a, and T2b, was identified as part of human P-TEFb (positive transcription elongation factor b) (19–22). P-TEFb is crucial for activation of transcription by HIV-1 Tat (trans-activator of transcription) in vitro and is essential for HIV-1 replication (22, 23). The Tat-bound P-TEFb complex that contains only cyclin T1 is also referred to as TAK (Tat-associated kinase). It is well established that P-TEFb is necessary for the transition of pol II into productive elongation in both human and Drosophila sys-
tems (24). Tat enhances the function of P-TEFb by further increasing pol II CTD phosphorylation (25, 26). Preinitiation complexes formed on the HIV-1 promoter contain CDK9 (as well as CDK7), which stays with pol II throughout the elongation process (25–27). The role of CDK9 in the preinitiation complex is not yet understood, however. Tat does not increase phosphorylation of the CTD by CDK7 in the initiation complex (25, 26). Moreover, CDK7 is dispensable for Tat transactivation of the HIV long terminal repeat (28).

Despite these differences in function, we and others have shown that CDK7, -8, and -9 all recognize the same amino acid target in various model CTD heptad repeat substrates, namely serine 5 (5, 29–33). Biochemical differences among the three kinases have been reported (25, 31), but the basis for their distinct roles in transcription remains unclear. To elucidate the specificity of the CTD kinases, we compared the activity of natural complexes of each kinase with different CTD substrates. Although they phosphorylate purified pol II similarly, we observed differences in the phosphorylation pattern of various GST-CTD substrates. Most striking is their display of heptad preference when phosphorylating a model peptide, CTD3, containing three heptads. CDK8 is the most indiscriminate whereas CDK7 and CDK9 show preferences toward different heptads. An important distinction was revealed using a phosphorylated CTD peptide substrate. Serine 2-phosphorylated CTD4 is a poor substrate for CTD9 whereas CDK7 and CDK8 are equally active with CTD4 and CTD4–2P. The natural CTD complexes, as well as CDK8 and CDK9 complexes bound to viral activators E1A and Tat, respectively, target only serine 5 for phosphorylation. Binding to the viral activators did not change the substrate preference of these kinases. These results suggest that the differential functions of the three CTD kinase complexes reflects distinct modes of recognition of pol II as a substrate.

EXPERIMENTAL PROCEDURES

Preparation of Cell Extracts—Nuclear extracts and cytoplasmic S-100 were prepared from 293 and HeLa cells essentially as described (34) except that the dialysis step was omitted for the cytoplasmic extract and glycerol was added to a final concentration of 10%. The nuclear extracts were dialyzed against buffer D (20 mM Hepes (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 20% glycerol) and centrifuged for 30 min at 15,000 × g.

CTD Substrates—CTD3 peptides were synthesized in the Molecular Resource Facility of the University of Medicine and Dentistry of New Jersey-New Jersey Medical School with an ABI automated peptide synthesizer. The peptides were purified on an analytical scale by reverse phase high-performance liquid chromatography on a Vydac C18 column (25 × 0.46 cm) developed with a linear acetonitrile gradient of 0–60% in 0.1% trifluoroacetic acid. The peak UV-absorbing fractions were lyophilized, and the lyophilized peptides were dissolved in distilled water and stored in −80 °C until use. The molar concentration of each peptide was determined from the absorbance at 274 nm using the extinction coefficient for tyrosine (1.4 × 10² M⁻¹ cm⁻¹). Biotinylated CTD4 and CTD4 phosphopeptides were described previously (35). RNA pol IIa was a gift from D. Reinberg (36). GST-CTD fusion proteins and CTD4 WT and substituted CTD4 peptides were described previously (31).

Kinase Assays—Immunoprecipitations were done essentially as described previously (32) except that radiolabelled precipitation buffer (150 mM NaCl, 10 mM Tris (pH 8.0), 1% Nonidet P-40, 0.1% Triton, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride) was used to wash the immunoprecipitated complexes. Protein A beads were used for binding antibodies (Santa Cruz Biotechnology, Inc.) to CDK7 (0.2 μg/10-μl beads) or CDK9 (0.4 μg/10-μl beads) and protein G beads to CDK8 (1 μg/10-μl beads). Except where noted, we used the amount of 293 NE (0.5–8 mg of protein/ reaction) that gave similar levels of phosphorylation of pol IIa by the three CTD kinases. Kinase assays were performed essentially as described in a previous study (19). In reactions containing GST-CTD proteins as substrate, an additional step of substrate pull-down was performed. At the end of the kinase reaction 100 μl of EBCD (50 mM NaCl, 0.5% Nonidet P-40, 5 mM dithiothreitol) was added to each tube, tubes were incubated for additional 10 min at 25 °C, and the supernatant was added to 10-μl glutathione-Sepharose beads. After incubation for 30 min at 4 °C, the beads were washed twice with EBCD (0.05% SDS) buffer and resuspended in 50 μl of the same buffer. All beads were prepared by boiling in Laemmli sample buffer and resolved by electrophoresis in 12%, 15%, or 18% polyacrylamide/bis-acrylamide (30%:0.15%) denaturing gels. The gels were dried, quantified on Packard Instant Imager, and exposed to x-ray film. TAK activity assay is described in a previous study (19). GST-E1A plasmid, containing WT E1A-conserved regions 2 and 3, and the mutant GST-E1A (C174S) (37) were a gift from C. Herrmann and A. Rice. GST-E1A proteins and their binding to glutathione-Sepharose beads were performed as described for GST-Tat proteins (19). After the binding of HeLa nuclear extract to GST-E1A bound beads, the complexes were washed with radioimmune precipitation buffer, and kinase assays were performed as described above. Phosphoamino acid analysis was performed as described in a previous study (32). Western blot analysis—Western blot analysis was done as described previously (32). Because goat IgG and CDK8 exhibit similar electrophoretic mobility, in our gels we used different antibodies to CDK5 for immunoprecipitations and Western blots (WB). CDK8 was immunoprecipitated with anti-CDK8 C-terminal goat IgG (Santa Cruz Biotechnology, Inc.), and rabbit anti-CDK8 N-terminal antibodies (Calbiochem) were used for WB. Secondary antibodies against rabbit IgG–furyl antibodies against rabbit IgG do not recognize denatured goat IgG (see Fig. 1A). For detection of pol II, anti-pol II antibodies from three different sources were used. pol II antibodies from Santa Cruz Biotechnology, Inc., which recognize only the IIA form, human autoantibodies against pol II (from patients diagnosed with systemic lupus erythematosus), which recognize both IIA and IIB (a gift from W. Reeves and M. Satoh) (38), and monoclonal antibodies against different phosphorylated forms of pol IIB (BAbCO-Berkeley Antibody Company H5 and H14). Secondary antibodies were from Roche Molecular Biochemicals for human anti-pol II and Kirkegaard and Perry Laboratories for the monoclonals.

RESULTS

Phosphorylation of pol II and GST-CTD Substrates—CDK7, -8, and -9 are present in nuclear extracts (NE) prepared from several human cell types (32, 39–41). Previous studies of these CDKs were performed with kinases isolated from various sources and by different methods, namely with complexes isolated from cellular extracts by chromatography and/or immunoprecipitation, or with recombinant kinase complexes. To allow systematic comparison of the kinases in natural complexes, we used 293 cell NE as the source of in vivo formed complexes of each CDK and assayed their activities on different substrates. Each kinase complex was immunoprecipitated with antibody directed toward its C-terminal peptide and washed in buffer-containing detergent. Only tightly bound proteins are expected to withstand the washing conditions. Immunoblotting assays verified that the complexes contained only the expected kinase (Fig. 1A). When the CDK7, -8, and -9 immunocomplexes were probed for the presence of CDK7 (lanes 1–4), CDK8 (lanes 5–8), or CDK9 (lanes 9–12), only the cognate kinase was detected in each case. Thus, the three CDKs are not found in the same complex or in tightly associated complexes. We also confirmed by immunoblotting that each CDK immunocomplex contained its associated cyclin partner, H, C, and T1 or T2, respectively (data not shown). No pol II was detected by probing the complexes with three different antibodies (data not shown). CDKs 7, 8, and 9 can phosphorylate the CTD of the large subunit of pol II (19, 32, 41, 42). Our CDK complexes all phosphorylated purified human RNA pol IIa (devoid of the IIB subunit), giving similar patterns of phosphorylation (Fig. 1B). In kinetic reactions with added HeLa IIA, we detected weakly phosphorylated bands, presumably due to the presence of traces of pol II that were undetectable by immunoblotting. The slower-migrating hyperphosphorylated form of pol II CTD, IIB, was detected only after phosphorylation as expected (Fig. 1B, lanes 14–16) (1). Because the three CDKs were active with regard to their natural substrate and showed no obvious dif-
CDK7, -8, and -9 Differ in Phosphorylation of Phospho-CTD Substrate—The phosphorylation site specificity of the three CDKs has been tested previously for the mammalian CDKs as well as yeast CDKs, mostly by using serine → alanine substituted CTD peptides or GST-CTD. This approach revealed that the exclusive target of phosphorylation for CDK7, -8, and -9 kinases is serine 5 in the CTD heptad (30–33, 45).

We examined the ability of the three CDK complexes to phosphorylate mutated CTD3 peptides, depicted in Fig. 2A, in which all of the serines at position 2, 5, or 7 were changed to alanine. The substitution of serine 5 in each heptad to alanine (SSA) abolished phosphorylation of the substrate by CDK7 and CDK8 (Fig. 2A). Similar results were obtained with CDK9 complexes (data not shown) and TAK complexes (32). Thus, within a heptad, the amino acid site specificity for the three complexes is indistinguishable. Furthermore, the substitution of all serine 2 and serine 7 residues with alanine did not affect the ability of the kinases to phosphorylate CTD3 (data not shown). Phosphoamino acid analysis confirmed that only serine residues were phosphorylated by the three CDKs (data not shown).

The CTD of pol II is a phosphorylation target for many CTD kinases, some of which phosphorylate serine 2 in the CTD heptad (CDK1 and DNA-PK) (1, 29, 30). It is plausible that, in the cell, each of the CDKs interacts with a CTD that is already phosphorylated at serine 2. Therefore we tested the ability of the three CDK complexes (data not shown) and TAK complexes (32). Thus, within a heptad, the amino acid site specificity for the three complexes is indistinguishable. Furthermore, the substitution of all serine 2 and serine 7 residues with alanine did not affect the ability of the kinases to phosphorylate CTD3 (data not shown). Phosphoamino acid analysis confirmed that only serine residues were phosphorylated by the three CDKs (data not shown).

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CDK7 displayed a slight preference for unphosphorylated CTD4, and CDK8 displayed a slight preference for the phosphorylated form, but in both cases the differences were less than 2-fold. Thus, CDK9 complexes discriminate strongly against the serine 2-phosphorylated CTD peptide, whereas CDK7 and 8 do not. As expected from the results obtained with the CTD3 S5A peptide (see Fig. 2A), CTD4–5P was not phosphorylated by any of the CDKs (Fig. 2B).

The viral activators Tat-1 and Tat-2 target complexes containing CDK9, whereas E1A and VP16 target complexes containing CDK8. E1A and VP16 activation domains recruit CDK8 complexes that can phosphorylate GST-CTD (16). Recently, these complexes were identified as the SrB/Mediator complex that contains, in addition to CDK8, SUR2 (renamed TRAP 150) (14, 46), which binds E1A directly, and TRAP 80, which binds to VP16 activation domain. (15). We wanted to determine whether the binding of the viral activators Tat-1 and E1A to CDK9 and CDK8, respectively, would change their substrate specificity. Fig. 2B shows that TAK complexes, like CDK9 complexes, recognized CTD4–2P as a poor substrate and phosphorylated it 8-fold less efficiently than CTD4 WT (lanes 7 with 8). TAK complexes did not phosphorylate the serine 5-phosphorylated CTD4 (data not shown). These results indicate that the interaction of CDK9 with the CTD peptide does not change when it is complexed with cyclin T1 only (in TAK complexes), instead of with both cyclins T1 and T2 (in CDK immunocomplexes), and the binding of the complex to Tat does not influence its substrate preference.

Similarly, we tested CDK8 complexes with the E1A trans-activator. Fig. 2C shows that CDK8 was pulled down from HeLa NE by a GST-E1A fusion protein carrying the intact E1A activation domain but not by a mutated activation domain. The binding of CDK8 to GST-E1A did not change its preference for CTD4 or its phosphorylated forms; it recognized CTD4 WT and CTD4–2P to about the same extents (Fig. 2D, lanes 3 and 5) and did not phosphorylate CTD4–5P (Fig. 2D, lane 7). CDK8-containing complexes bound to E1A show the same target specificity as CDK8 immunocomplexes (Fig. 2A); they phosphorylated serine 5 only when S2A and S7A were used as substrates (data not shown).

Each CDK Exhibits a Different Pattern of CTD3 Phosphorylation—Phosphorylation of the WT CTD3 peptide by CDK7 and CDK9 gives rise to two bands in SDS-gel electrophoresis (32), and the same is true for CDK8. However, as seen in Fig. 3A, the pattern of phosphorylation differs for the three kinases. CDK7 and CDK9 phosphorylated serine 5 only when S2A and S7A were used as substrates (data not shown).

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** Site specificity of CDK complexes. A, CDK7 and CDK8 complexes phosphorylate serine 5. Kinase assays contained 100 μM of CTD3 mutants S2A, S5A, or S7A and [γ-32P]ATP. The top portion depicts the sequences of WT and mutant CTD3 peptides. Incorporation of label from [γ-32P]ATP was detected by gel electrophoresis and autoradiography. B, phosphorylation of CTD4-WT and phospho-CTD4 peptides by CDK7, -8, and -9 immunocomplexes and TAK. Peptide structures are depicted in the top portion of the panel. Kinase assays contained 1 μg of CTD4-WT (lanes 1, 4, 7, and 10), CTD4–2P (lanes 2, 5, 8, and 11) or CTD4–5P (lanes 3, 6, and 9). Phosphorylated products were resolved in an 18% SDS-polyacrylamide gel and quantitated. C, WB analysis of complexes pulled down from HeLa NE by GST-E1A activation domain (WT) or GST-E1A activation domain mutant (M). The blot was probed with rabbit anti-CDK8 N terminus peptide antibody. HeLa NE was used as marker. D, E1A-bound CDK8 complexes, analyzed in panel C, phosphorylate CTD4 peptides with the same specificity as CDK8 immunocomplexes. Kinase assays contained WT or phospho-CTD4 peptides as in Fig. 2B. The phosphorylated products were separated in a 4–20% SDS-polyacrylamide gel.
CDK8 both showed a preference for the slow band, the ratios were reproducibly different. The intensity of the fast band produced by CDK7 is two-thirds that of the slow band, whereas for CDK9 it is half of the slow band.

Because the three CTD kinases recognize the same target (serine 5) in the CTD consensus sequence, FXSP (47), we focused on the differences observed in the pattern of CTD3 phosphorylation between the kinases. One explanation for the two phosphorylation bands is that each represents phosphorylation of only one heptad out of the three. Another possible interpretation is that one band is multiply phosphorylated, whereas the other band is singly (mono-) phosphorylated. We first tested these possibilities by conducting time course and concentration dependence experiments (Fig. 3, B and C). CTD3 phosphorylation by CDK7 revealed the typical pattern of bands at all time points (Fig. 3B) arguing against a shift from single phosphorylation to multiple phosphorylation. In contrast such a shift was observed in time course phosphorylation experiments with pol II (48), indicating multiple phosphorylation events. Time course experiments with CDK8 and CDK9 complexes gave the same results (data not shown). Titration experiments with CDK9 (Fig. 3C) as well as with CDK7 and -8 (not shown) indicated that even when the peptide concentration was lowered to 6.25 μM or increased to 1 mM (data not shown), neither the pattern of phosphorylation nor the ratio of phosphorylation between the two bands changed. These observations suggest that each CTD3 molecule is phosphorylated only once by each kinase. A similar conclusion was drawn by other investigators who used a di-hepta peptide in kinase assays with other CTD kinases (30, 47).

We also examined the GST-E1A-bound CDK8 complexes described above (Fig. 2C) in kinase assays with WT CTD3 as substrate. These complexes demonstrated the same pattern of CTD3 phosphorylation (Fig. 3D, lane 1) as the CDK8 immunocomplexes (Fig. 3A). Hence, binding to E1A does not change the substrate specificity of CDK8 complexes when tested with all the different substrates described above.

CDK7, -8, and -9 Differ in Their CTD Heptad Preferences—To determine whether the three kinases display preferences for phosphorylating individual heptads, we synthesized three mutated CTD3 peptides (a–c), each containing two different mutations of serine 5 to alanine. Peptides a, b, and c retain serine 5 residues only in heptads 2, 1, and 3, respectively (Fig. 4A). All three kinases recognized the single serine target in each peptide and phosphorylated it, albeit to different extents; CDK7 preferred to phosphorylate heptads 1 and 2 over heptad 3 by at least 3-fold (Fig. 4A, lanes 1–3), CDK8 displayed no heptad preference (Fig. 4A, lanes 4–6), and CDK9 preferred to phosphorylate heptad 1 over heptads 2 and 3 by at least 2-fold (Fig. 4A, lanes 7–9). Thus, this set of model peptides revealed differences between the three CDKs in their CTD heptad preference. The electrophoretic mobility of CTD3 phosphorylated on heptad 2 or 3 is identical, whereas heptad 1 phosphorylation gives rise to a band with faster mobility. The differences in mobility are consistent with the pattern observed with CTD3 WT (Fig. 3A) and support the conclusion that only one heptad is phosphorylated in each CTD3 WT molecule under these conditions.

To confirm this interpretation we tested another set of three mutated peptides (d–f), in which only one serine 5 (in heptad 1, 2, or 3, respectively) was substituted with alanine, leaving two phosphorylation targets (Fig. 4B). All three kinases phosphorylated the substituted peptides, but with notable differences. CDK8 phosphorylated these peptides to approximately equal extents, resulting in one phosphorylated band with peptide d and two bands with peptides e and f (Fig. 4B, lanes 4–6). This is consistent with the finding that CDK8 has no preference for one CTD heptad over another (Fig. 4A, lanes 4–6). However, CDK7 and CDK9 showed marked heptad preferences and phosphorylated the serine in heptad 3 very weakly, even more weakly than when this site was the only target available (Fig. 4A, lanes 3 and 9). These findings confirm that the heptad preferences differ among the kinases: for CDK7, heptad 1 = 2 > 3; for CDK9, 1 > 2 = 3; and for CDK8, all are approximately equal.

TAK complexes show the same heptad preference as the CDK9 immunocomplexes (Fig. 4B, lanes 10–12). TAK complexes also gave the same pattern of phosphorylation with the di-substituted peptides as the immunocomplexes in Fig. 3A (data not shown). Thus, the heptad preference for CDK9 is not influenced by its association with Tat.

**DISCUSSION**

We compared the substrate specificity of natural complexes of the cyclin dependent kinases 7, 8, and 9 from human cells.
Using substrates containing different numbers of CTD heptad repeats, we uncovered differences in substrate preference among these kinases. Specifically, by using CTD3 peptides we showed that CDK7 prefers to phosphorylate serine 5 in the first and second heptad over the third heptad, CDK9 prefers to phosphorylate Serine 5 in the first heptad over the other two, and CDK8 has no preference for either heptad. Moreover, although CDKs 8 and 9 prefer to phosphorylate the highly conserved N-terminal region of the CTD, CDK7 phosphorylates it uniformly. Finally, none of the kinases efficiently phosphorylates serine 5-phosphorylated CTD peptide, and CDK9 (but not CDKs 7 and 8) strongly discriminates against serine 2-phosphorylated CTD peptide. We postulate that the three CDKs exhibit heptad preference when they phosphorylate Pol II CTD. Selective modification of Pol II CTD, before and during transcription, may serve as a regulatory device allowing the specific interaction of various proteins with the CTD to promote or inhibit mRNA transcription.

Differences in Substrate Specificity among the Three Kinases—Taking advantage of differences in gel mobility between the singly phosphorylated specific heptads of CTD3, we demonstrated that the three kinases exhibit different heptad preferences within the CTD. The heptad preference displayed by CDK7 (in purified TFIIH complexes) was noted previously (30). It is possible that these differences in heptad preference are related to differences in the activation segments of the three kinases. The crystal structure of CDK2-cyclin A3 shows that the activation segment is involved in binding the substrate after phosphorylation of the conserved threonine in the T loop (49). Although both CDK7 and CDK9 retain this threonine residue, it is replaced by aspartic acid in CDK8, which is the most promiscuous of these three CDKs with respect to heptad selectivity.

Kinetic experiments indicated that the initial phosphorylation event(s) of Pol II CTD by P-TEFb are slow but additional phosphates are incorporated quickly to reach the I0 position (48). The pattern of phosphorylation of GST-CTD by recombinant CDK9 bound to GST-cyclin T1 (50) and by CDK9 immunocomplexes (Fig. 1C) is consistent with this observation. Hence it seems that CDK9 prefers to phosphorylate Pol II CTD that is partially phosphorylated, consistent with its function at the elongation stage after initial phosphorylation of Pol II CTD by TFIIH. By contrast, the pattern of GST-CTD phosphorylation by CDK7 complexes, leading to an array of phosphorylated molecules, suggests no preference for unphosphorylated or partially phosphorylated CTD. A similar pattern of GST-CTD phosphorylation was observed with CDK7 immunoprecipitated holoenzyme (51). Phosphorylation of full-length GST-CTD by CDK8 did not give rise to the hyperphosphorylated form, suggesting that the rest of the Pol II large subunit has an effect on the enzyme activity. This idea is strengthened by the observation that CDK8 is tightly associated with Pol II in the holoenzyme (41).

Comparing the efficiency of the three CTD kinases for phosphorylating the N- and C-terminal portion of the Pol II CTD revealed further differences between the enzymes in substrate specificity. The first half of the CTD, which is essential for transcriptional activity from many promoters (52), is a good substrate for all three kinases. On the other hand, the C-terminal portion of the CTD, which is required for cell survival (53), although not in transient expression assays (52), provides discrimination among the kinases. This region is strongly phos-
phorylated only by CDK7 and contains many heptads in which serine 7 is substituted with lysine, threonine, or asparagine. Studies with CDT4 mutants containing serine 7 substitutions indicated that the lysine residues comprise the principal determinant of the preference of recombinant CDK7-cyclin H-MAT-1 complexes for GST-CDT-(30–53) (31). In our hands, the efficiency of phosphorylation by CDK5 and -9 was not greatly influenced by any of the CDT4 peptide substitutions (data not shown), indicating that these position 7 differences are not sufficient to explain the kinases' ability to distinguish between the two halves of the CTD.

**Target Specificity of CDK7, -8, and -9 Complexes**—Our CTD peptide assays reinforce the view that serine 5 in the CTD heptad is the only target of phosphorylation by immunocomplexes of CDK7, -8, and -9 as well E1A-bound CDK8 complexes and TAK complexes (32). Corroborative data have been obtained by others using mutant CTD peptides and GST-CTD fusion proteins (5, 29–31, 33). On the other hand, based on an indirect assay, it has been reported that CDKs 8 and 9, but not CDK7, can phosphorylate the CTD on serine 2 and 5, but only the latter targeted recognition of heptad arrays by the CTD kinases will be regulated on the level of substrate specificity. Hence, the differential recognition of heptad arrays by the CTD kinases will affect the structure and exposure of regions of the CTD. At any given time in the dynamic process of mRNA transcription and processing, different protein complexes are bound to the CTD, and these will also affect its heptads' exposure and structure. Moreover, the action of CTD phosphatases could affect heptad recognition by the CDKs. For example, remodeling of the CTD by removing the phosphate from serine 2 may be one way in which cells control the capping of mRNA and productive elongation.

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