THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 8, Issue of February 20, pp. 4689–4694, 1998
Printed in U.S.A.

Growth-related Changes in Phosphorylation of Yeast RNA Polymerase II*

(Received for publication, October 17, 1997, and in revised form, November 21, 1997)

Meera Patturajan‡, Roberta J. Schulte§, Bartholomew M. Sefton§, Ronald Berezney¶, Michel Vincent‖, Olivier Bensaude, Stephen L. Warren‡‡, and Jeffry L. Corden‡§§**

*From the ‡Department of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, The §Salk Institute for Biological Studies, San Diego, California 92186, ‡‡Nexstar Pharmaceuticals, Boulder, Colorado 80301, the ¶Department of Biological Sciences, State University of New York, Buffalo, New York 14260, the ‖Recherche en Sciences de la Vie et de la Sante and Department de Medicine, Universite Laval, Ste-Foy, Quebec, Canada G1K 7P4; **Genetique Moleculaire, Ecole Normale Superieure, 75230 Paris Cedex 05, France*

The largest subunit of RNA polymerase II contains a unique C-terminal domain (CTD) consisting of tandem repeats of the consensus heptapeptide sequence Tyr[1]-Ser[2]-Pro[3]-Thr[4]-Ser[5]-Pro[6]-Ser[7]. Two forms of the largest subunit can be separated by SDS-polyacrylamide gel electrophoresis. The faster migrating form termed IIA contains little or no phosphate on the CTD, whereas the slower migrating II0 form is multiply phosphorylated. CTD kinases with different phosphoryl acceptor specificities are able to convert IIA to II0 *in vitro*, and different phosphoisomers have been identified *in vivo*. In this paper we report the binding specificities of a set of monoclonal antibodies that recognize different phosphoepitopes on the CTD. Monoclonal antibodies like H5 recognize phosphoserine in position 2, whereas monoclonal antibodies like H14 recognize phosphoserine in position 5. The relative abundance of these phosphoepitopes changes when growing yeast enter stationary phase or are heat-shocked. These results indicate that phosphorylation of different CTD phosphoacceptor sites are independently regulated in response to environmental signals.

The largest subunit of RNA polymerase II (pol II)[1] contains a repetitive C-terminal domain (CTD) consisting of tandem repeats of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (1, 2). The CTD plays an essential (3–6) but as yet poorly understood role in mRNA synthesis with evidence indicating potential roles in initiation or promoter clearance (7–9), elongation (10–15), and pre-mRNA processing (16–20).

Phosphorylation of the CTD is a key feature of CTD function. SDS gel electrophoresis separates the largest subunit into two species as follows: IIA contains a hypophosphorylated CTD and pol II0 is hyperphosphorylated on the CTD (21). Serine is the predominant *in vivo* phosphoacceptor with minor amounts of phosphothreonine and phosphotyrosine detected (22, 23). Although *in vivo* phosphorylation sites have not been mapped, *in vitro* studies have identified serines in both positions 2 and 5 (22, 24, 25) and tyrosine in position 1 (23) as potential phosphoryl acceptors. Mutation of these sites to unphosphorylatable alanine or phenylalanine residues in each yeast CTD repeat is lethal, suggesting a requirement for CTD phosphorylation *in vivo* (26).

The preferential inclusion of pol IIA into preinitiation complexes (27–30) together with the observation that elongating pol II is phosphorylated on the CTD (31) led to the hypothesis that the CTD is reversibly phosphorylated with each transcription cycle (8). The unphosphorylated CTD has been shown to contact basal transcription factors TATA binding protein (32), TFIIE, and TFIIF (33), and these contacts, together with as yet undefined interactions with *SRB*s (34–37), suggest that the CTD acts as a structural framework for the preinitiation complex (38). The pol II preinitiation complex also contains several protein kinases that are capable of phosphorylating the CTD (39–45) suggesting that one role of this complex is to effect the conversion of pol IIA to pol II0 thereby releasing pol II from the initiation complex. Finally, CTD phosphatase is required to dephosphorylate pol II0 thus completing the CTD phosphorylation cycle (46, 47).

Several observations complicate this simple two-state CTD phosphorylation cycle. Transcription of some promoters *in vitro* and *in vivo* does not require the CTD (30, 48–50). In addition, CTD phosphorylation can be inhibited without blocking activated transcription *in vitro* (49, 51, 52). Bentley and colleagues (53) have shown that deleting the CTD or blocking CTD phosphorylation by Kin28p does not alter the synthesis of promoter proximal transcripts. Thus, at least for some promoters, transcription initiation can occur in the absence of a CTD or a CTD phosphorylation cycle. Finally, hyperphosphorylation of the CTD does not correlate with pol II's transcriptional activity *in vivo* (54).

Another complication in understanding the role of CTD phosphorylation is the multiplicity of CTD kinases and the diversity of possible phosphate acceptors in the CTD (8). Although *in vivo* CTD phosphorylation sites have not been mapped, the *in vitro* targets of several serine/threonine-specific CTD kinases have been determined. Cdc2 kinase phosphorylates both serine 2 and serine 5 (22), whereas the TFIIH-associated CTD kinase (Cdk7/cyclin H) phosphorylates serine 5 (25). CTD kinases induced by heat shock or arsenite also preferentially phosphorylate serine 5 (55). Genetic evidence indicates that the roles of serines in positions 2 and 5 are different. First, partial substitutions of serines in either position 2 or 5 have different effects on viability (26). Second, mutations in *SRB* genes suppress

* This work was supported by Grant 82371 from the American Cancer Society (to J. L. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§§ To whom reprint requests should be addressed: Dept. of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205-2185. Tel.: 410-955-4719; Fax: 410-502-6718; E-mail: jeff_corden@qmail.bs.jhu.edu.

[1] The abbreviations used are: pol, polymerase; CTD, C-terminal domain; TF, transcription factor; mAb, monoclonal antibody; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; WT, wild type.

position 2 substitutions but not position 5 substitutions (56). Taken together with the differences in CTD kinase specificity, these results suggest that pol II0 may be a collection of different phosphoisomers.

In this paper we describe the results of experiments testing the interaction specificity of different anti-pol II mAbs for a set of phosphorylated CTD binding sites. These mAbs were isolated in screens for antibodies directed against proteins involved in a range of different cell functions (17, 57–61). We show here that each of these antibodies is capable of recognizing wild-type heptapeptide repeats phosphorylated by Cdc2 kinase. The specificities of the different antibodies for mutant CTDs are different, however, indicating that they recognize different phosphoepitopes. Nutritional stress and heat shock result in higher levels of serine 2 phosphorylation suggesting that the serine 2 and serine 5 phosphoepitopes are functionally different.

MATERIALS AND METHODS

Plasmids and Strains—In an earlier study we described the construction and characterization of a set of CTD phosphorylation site substitution mutations (26). These mutations focus on serines in positions 2 and 5 of the consensus heptapeptide YSPTSPS which were shown to be phosphorylated by Cdc2 kinase (22). Each reconstructed CTD consists of multiple wild-type or mutant heptapeptide repeats and a 9-amino acid epitope derived from the influenza virus hemagglutinin gene that is recognized by mAb 12CA5 (62). The mutant yeast CTD sequences were excised and cloned into the pGEX2 vector (Pharmacia Biotech Inc.) allowing the expression of glutathione *S*-transferase-CTD fusions in *Escherichia coli* (Fig. 1*A*). We have also expressed the mammalian CTD as a GST fusion protein. The plasmid expressing this protein was a gift of Dr. David Bentley (Amgen Institute, Toronto).

Purification and Phosphorylation of CTD Fusion Proteins—E. coli (DH5α) strains expressing GST-CTD fusion proteins were grown overnight to saturation. Cultures were diluted 10-fold in fresh L broth containing 100 μg/ml ampicillin and grown for 1 h before addition of isopropyl-1-thio-β-D-galactopyranoside to 0.1 mM. After 4 h of induced expression cells were collected by centrifugation and sonicated, and the fusion protein was purified by glutathione-agarose (Pharmacia) affinity chromatography as described (64). Purified GST fusions containing 16 copies of the wild-type heptapeptide YSPTSPS, 18 copies of YSPTEPS, 15 copies of YESPTSP, 15 copies of YSPTAPS, or 12 copies of YAPTSPS were separated by SDS-PAGE (10%) and stained with Coomassie Blue dye (Fig. 1*B*).

CTD fusion proteins were phosphorylated *in vitro* with baculovirus-expressed epitope-tagged Cdc2 kinase (65) as described previously for RNA pol II (25) but with minor modifications. For each phosphorylation reaction, 10 μl of Cdc2 kinase-bound 12CA5 Affi-Gel beads (~5 μg of Cdc2 kinase) were incubated with 2.5 μg of fusion protein in 60 mM KCl, 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM ATP for 20 min at 30 °C. For labeling, the reaction was pulsed with 20 μCi of [γ-^{32}P-]ATP for 5 min and then chased with 1 mM ATP for 20 min at 30 °C. Phosphorylated GST-CTD fusion protein was removed from the beads by spinning the supernatant through a bovine serum albumin-treated filter (UFC3 OHV 00; Millipore). The level of phosphorylation was assessed by electrophoresis in a 5% polyacrylamide-SDS gel followed by direct autoradiography. Phosphorylation of the mouse CTD fusion protein with c-*ABL* kinase was essentially as described (23).

Anti-CTD Monoclonal Antibodies—mAb 8WG16 is an anti-CTD IgG described by Thompson *et al.* (66). mAbs H5 and H14 are IgMs directed against phosphoepitopes on the CTD (57–59). mAb MARA 3 is an anti-pol II IgM isolated in a screen for mAbs against tyrosine-phosphorylated B cell proteins.[2] mAb CC-3 is an IgG isolated in a screen for chicken proteins with developmentally regulated expression (60). mAb B3 is an IgM directed against nuclear matrix components (17).

Growth Conditions for Yeast—An overnight culture of *Saccharomyces cerevisiae* YPH499 (ATCC 76625) was used to inoculate YEPD (2% bactopeptone, 2% glucose, 1% yeast extract) to an A_{600} of 0.2. Aliquots (50 ml) were withdrawn at intervals of 2 h and cells harvested by centrifugation at 4000 × *g* for 5 min. The yeast cell pellet was washed in ice-cold water and stored at −80 °C. Cells were suspended in 200 μl of buffer A (200 mM Tris-HCl, pH 8, 320 mM ammonium sulfate, 5 mM $MgCl_2$, 10 mM EGTA, pH 8, 20 mM EDTA, pH 8, 1 mM dithiothreitol, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 mM pepstatin, 0.6 mM leupeptin, 2 mM benzamidine HCl). Acid-washed glass beads (425–600 μm; Sigma) were added to the meniscus and vortexed 10 times for 10 s with 30-s intervals on ice. The lysate was cleared by centrifugation at 10,000 × *g* for 10 min, and supernatant was used for SDS-PAGE analysis. The high ionic strength of the grinding buffer allows extraction of almost all of the pol II in the cell. We see no difference when cells are extracted in denaturing buffer (not shown).

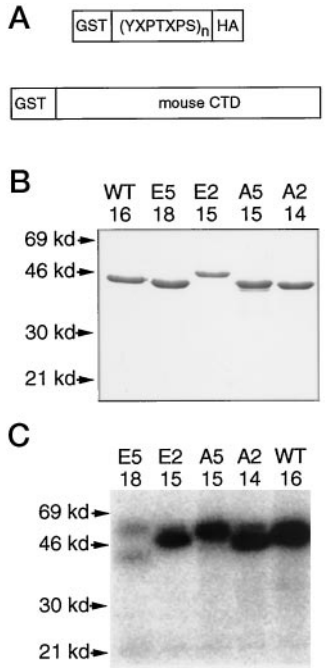

FIG. 1. **GST-CTD fusion proteins.** *A*, maps of GST-CTD fusion protein coding sequences. The construction of vectors is described under "Materials and Methods." The *top line* shows the arrangement of the synthetic CTD constructs. Serine residues that are mutated are indicated by *X*s. The subscript (*n*) refers to the number of heptapeptide repeats. *B*, SDS-polyacrylamide gel electrophoresis of purified GST-CTD fusion proteins. Equal amounts of purified unphosphorylated proteins were separated on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue. *Markers* are indicated to the *left*. Proteins are designated by the nature of the mutation and the number of repeats. *C*, SDS-polyacrylamide gel electrophoresis of [γ-^{32}P]ATP-labeled GST-CTD fusion proteins. Equal amounts (1 μg) of phosphorylated proteins were separated on a 10% SDS-polyacrylamide gel and autoradiographed. *Markers* are indicated to the *left*. Proteins are designated by the nature of the mutation and the number of repeats.

Heat Shock—Yeast cells (YPH499) were grown at 30 °C for 7 h after inoculation (starting A_{600} = 0.2). The culture was diluted 1:1 with fresh medium pre-warmed to 55 °C and then maintained at 39 °C for the indicated times. Control cells were diluted 1:1 using fresh medium at room temperature and then incubated at 30 °C for the indicated times. The heat shock response was terminated by further diluting the culture (1:1) with ice-cold water. The cells were harvested and the extract prepared as described above.

Immunoblotting—Phosphorylated and unphosphorylated CTD fusion proteins (250 ng) were subjected to SDS-PAGE (10%) followed by electrophoretic transfer to nitrocellulose paper (Protran, Schleicher & Schuell). The blots were probed with mAb tissue culture supernatants at a dilution of 1:10 (12CA5, 8WG16, H5, and H14) or ascites fluid at a dilution of 1:1000 (CC3 and B3). Immunoreactive proteins were detected using either anti-mouse IgG (Amersham Corp.) or anti-mouse IgM (Kirkegaard & Perry Laboratories) at a dilution of 1:4000. Supersignal substrate (Pierce) was used to illuminate the reactive bands. To ensure that all proteins transferred efficiently, blots probed with anti-phospho-CTD antibodies were stripped and reprobed with mAb 12CA5 which recognizes an epitope present in each fusion protein. In each case all lanes contained 12CA5 immunoreactive bands.

RESULTS

Expression and Phosphorylation of CTD Fusion Proteins—Fig. 1 shows the purified GST-CTD fusion proteins used in this

[2] R. J. Schulte and B. M. Sefton, submitted for publication.

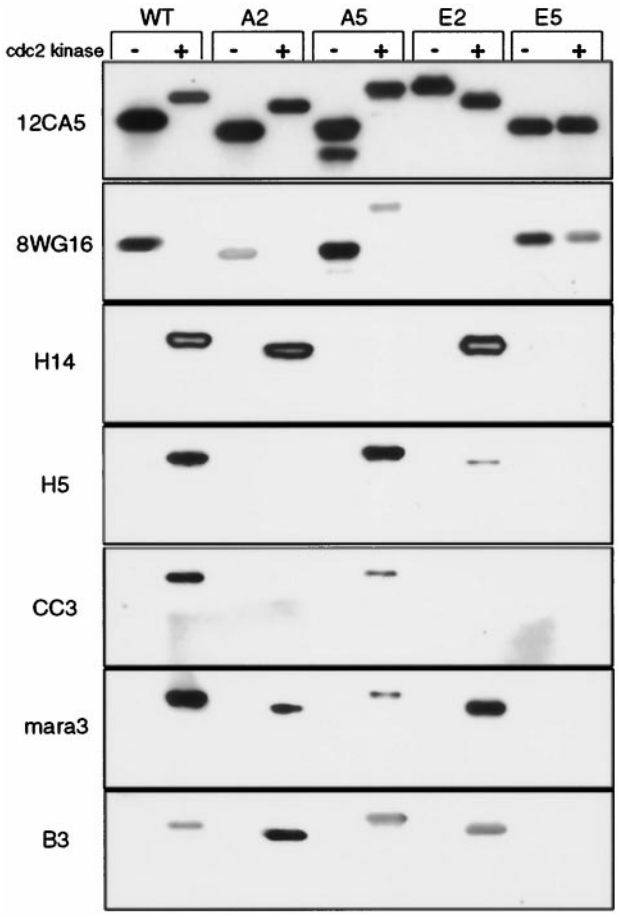

FIG. 2. **Immunodetection of GST-CTD fusion proteins.** Each panel is a Western blot on a set of phosphorylated and unphosphorylated GST-CTD fusion proteins. Equal amounts (1 μg) of phosphorylated and unphosphorylated protein were loaded in each lane. The monoclonal antibody probe is indicated to the *left*.

study. From examination of the relative mobility of these proteins (Fig. 1*B*), it is obvious that the sequence of the unphosphorylated heptapeptide repeat confers aberrant mobility. In particular, glutamate substitution at position 5 seems to increase mobility, whereas glutamate at position 2 results in a marked retardation. The physical basis of this difference is likely due to differences in the ability of SDS to bind to these mutant proteins (22). Phosphorylation of these fusion proteins with Cdc2 kinase results in similar levels of phosphorylation of all proteins with the exception of the E5 mutant (Fig. 1*C*).

The *top panel* of Fig. 2 shows that phosphorylation causes additional shifts in electrophoretic mobility of these proteins in SDS-PAGE. mAb 12CA5 was used to detect the fusion proteins (see "Materials and Methods"). The lower intensity of some phosphorylated species indicates that phosphorylation may alter the binding of mAb 12CA5. For the wild-type and alanine-substituted proteins phosphorylation causes a marked retardation in mobility, much as seen with pol II0 *in vivo*. Phosphorylation of the CTD with glutamate in position 2 causes an increase in mobility. This result suggests that little if any SDS is bound to the unphosphorylated mutant CTD, and phosphorylation thus causes an increase in the charge to mass ratio of the fusion protein in SDS. Phosphorylation of the E5 mutant causes no change in mobility, a result which may be due to the fact that E5 is a poor substrate for Cdc2 phosphorylation as seen in experiments using [γ-^{32}P]ATP (Fig. 1*C*). From the >10-fold molar excess of kinase to substrate used in the phosphorylation reaction and the resulting mobility shift on substrates other than E5, we conclude that the CTD is maximally phosphorylated on serines 2 and 5, the known Cdc2 kinase target sites (22).

Recognition of GST-CTD Substrates by Monoclonal Antibody 8WG16—The mAb 8WG16 was raised against wheat germ RNA polymerase II and found to recognize epitopes conserved among the CTDs of pol IIs derived from a variety of different eucaryotes (66). The *2nd panel* in Fig. 2 shows that mAb 8WG16 recognizes primarily unphosphorylated CTD. Unphosphorylated WT, A5, and E5 interact more strongly than unphosphorylated E2, A2, and phosphorylated A5 and E5. The failure to bind unphosphorylated E2 and weak interaction with unphosphorylated A2 suggests that serine 2 (S2) is an important feature of the 8WG16 epitope. Weak binding to phosphorylated A5 and E5 could mean that position 5 partially overlaps the 8WG16 recognition site. Alternatively, mutation of S5 could interfere with phosphorylation of S2 yielding more unphosphorylated S2 sites and thus partial reactivity with 8WG16.

Recognition of Phosphorylated GST-CTD Substrates by Anti-CTD Monoclonal Antibodies—The remainder of the mAbs tested bind only to phosphorylated CTD (Fig. 2). With the exception of B3, the strongest binding is to the phosphorylated WT sequence. From previous studies we know that Cdc2 kinase phosphorylates the consensus heptapeptide repeat on both S2 and S5 (22). Binding to phosphorylated mutant CTD targets reveals interesting differences in the specificities of the different mAbs.

mAb H14 recognizes both phosphorylated A2 and E2 but not A5 or E5. This result indicates that H14 recognizes phosphorylated S5. In contrast, mAb H5 recognizes phosphorylated A5 suggesting that phosphoserine in position 2 is a critical feature recognized by mAb H5. Consistent with this interpretation mAb H5 does not recognize phosphorylated A2. Weak interaction between H5 and phosphorylated E2 indicates that substitution of the charged side chain at this position mimics the effect of phosphorylation. Note also that the mobility of unphosphorylated E2 is greatly retarded. Glutamate substitution at position 2 is insufficient for H5 recognition, however, indicating that position 5 phosphorylation may induce a conformational change that enables a weak interaction at position 2.

CC3 shows a weak recognition of phosphorylated A5 in addition to recognizing the phosphorylated WT. Thus, like mAb H5, phosphoserine in position 2 appears to be part of the CC3 epitope. None of the other phosphorylated CTDs are recognized by CC3, although in the case of E5 this may be due to the low level of phosphorylation.

MARA 3 recognizes phosphorylated A2, A5, and E2. This could mean that both phosphoserines S2 and S5 are recognized or, alternatively, that the epitope is distinct from either site but requires at least one of the serines to be phosphorylated to achieve the proper conformation. The strong interaction of MARA 3 with phosphorylated E2 suggests that the charged residue in position 2 mimics phosphoserine. The fact that phosphorylated A2 and E2 interact better than A5 suggests that MARA 3 is related to H14. MARA 3 recognizes phosphorylated A5, however, whereas H14 does not recognize it at all. Furthermore, MARA 3 recognizes WT better than A2, whereas H14 interacts with both phosphorylated substrates to the same extent.

mAb B3 is unique in interacting with phosphorylated A2 more strongly than with WT and the other mutants. This could indicate that the epitope recognized by B3 is phosphorylated at position 5. In this respect B3 is like H14. B3's weak interaction with A5, however, suggests a more complicated situation. For example, B3 may prefer a phosphoepitope in the distal nonconsensus repeats of the CTD which are only approximated by the A2 mutation.

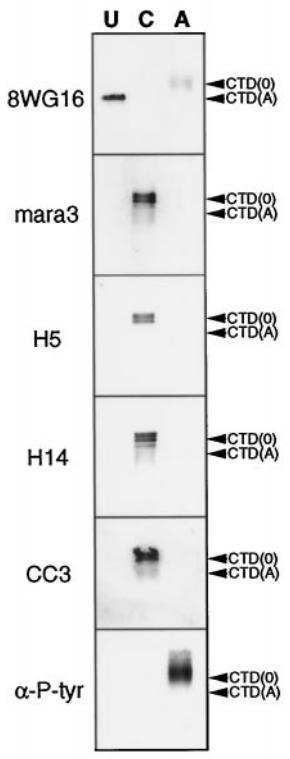

FIG. 3. **Immunodetection of the mouse CTD.** A mouse CTD fusion protein was purified and phosphorylated either with Cdc2 or c-*ABL* kinase. Equal amounts of unphosphorylated (*U*), Cdc2 phosphorylated (*C*), or c-*ABL* phosphorylated (*A*) proteins were separated by 10% SDS-PAGE and immunoblotted with the mAb indicated at the *left*. *Markers* at the *right* indicate the mobilities expected for phosphorylated CTD (*CTD0*) or the unphosphorylated CTD (*CTDA*).

Recognition of Mouse CTD Targets—To verify that the mAbs used in this study also recognize the mammalian CTD, we generated several phosphorylated mouse CTD fusion proteins. Fig. 3 shows the interaction with unphosphorylated, Cdc2, and c-*ABL* phosphorylated mouse CTD fusion proteins. All of the anti-phosphorylated CTD mAbs tested interact solely with the Cdc2 phosphorylated molecules and not with unphosphorylated or tyrosine-phosphorylated CTD. mAb 8WG16 shows a very weak recognition of the CTD phosphorylated by c-*ABL* suggesting that not all repeats have been phosphorylated.

Growth-related Changes in CTD Phosphorylation—Our earlier studies showed that different CTD kinases can recognize different serine phosphoacceptors in the CTD (25). *In vivo*, the plethora of CTD kinases could give rise to a family of pol II phosphoisomers whose populations may change under different growth conditions. To test this hypothesis we examined CTD phosphorylation in cells in different stages of growth. Fig. 4*A* shows a typical yeast growth curve, and Fig. 4*B* shows the results of Western blot analysis using different anti-CTD monoclonal antibodies. As cells reach late log phase pol IIA (detected by mAb 8WG16) and pol II0 (detected by mAb H14 or mAb B3) show maximum expression (Fig. 4; earlier points not shown). Both of these pol II species decrease gradually as cells enter the diauxic phase, in which glucose becomes depleted and cells adapt to respiratory metabolism (67). In contrast, the H5 epitope shows a marked transient increase during the diauxic shift (14 and 16 h of growth) followed by a gradual decline as cells enter stationary phase. A similar pattern of expression is observed with CC3 and MARA 3.

H5 Epitope Is Highly Expressed during Heat Shock—The increase in abundance of the H5 epitope during transition to stationary phase suggests that phosphorylation of serine 2 may

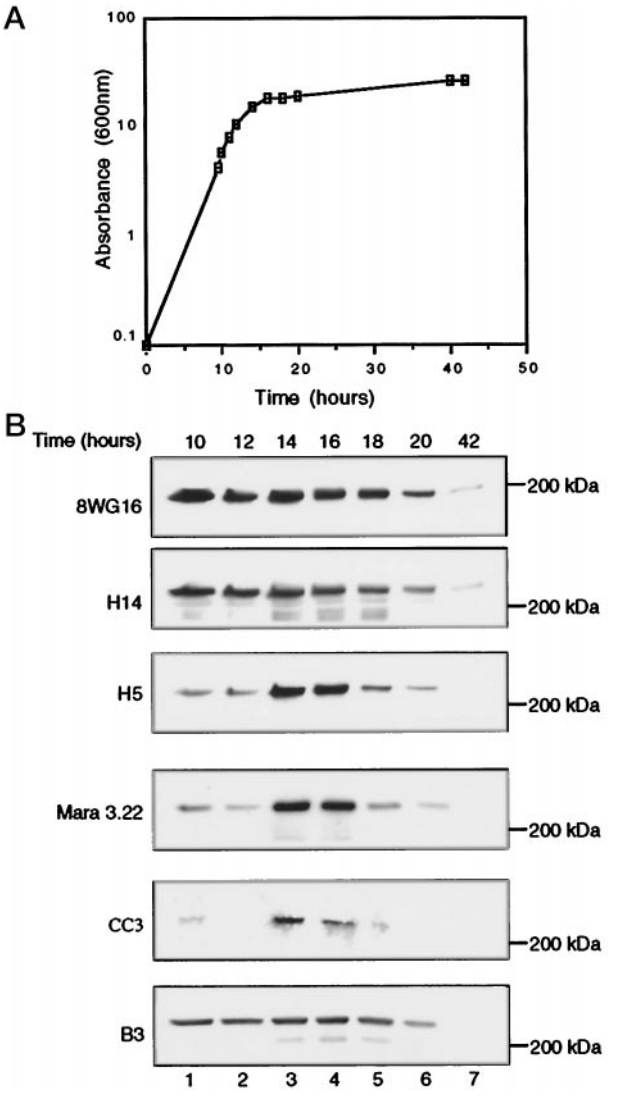

FIG. 4. **Increase in H5 epitope during diauxic shift.** *A*, a typical growth curve of yeast cells grown in YEPD as described under "Materials and Methods." *B*, Western blot analysis of extracts prepared from cells harvested at different times. 100 μg of protein samples were subjected to Western blot and probed with different mAbs shown on *left* as described under "Materials and Methods." *Lanes 1–7*, yeast extract prepared from cells grown for 10, 12, 14, 16, 18, 20, and 42 h, respectively.

be involved in stress response. As both nutrient deprivation and heat shock are generally viewed as stressful (67, 68), it is of obvious interest to determine whether yeast cells respond similarly in both cases. Previous studies have shown that heat shock induces CTD phosphorylation (69). To determine whether both serines 2 and 5 are equally phosphorylated during heat shock, yeast extract prepared from treated and control cells were subjected to Western blot. As shown in Fig. 5 the level of pol IIA decreases in cells subjected to heat shock. Although the level of H14 epitope remains almost constant in both control and heat shock, the level of the H5 epitope dramatically increases in heat shock-treated cells, again suggesting an involvement of serine 2 phosphorylation in stress response.

DISCUSSION

In the present study we have demonstrated that a set of monoclonal antibodies differ in their recognition of phosphoepitopes on the CTD. Fig. 6 summarizes the reactivities of these antibodies. Using these anti-CTD antibodies we show for

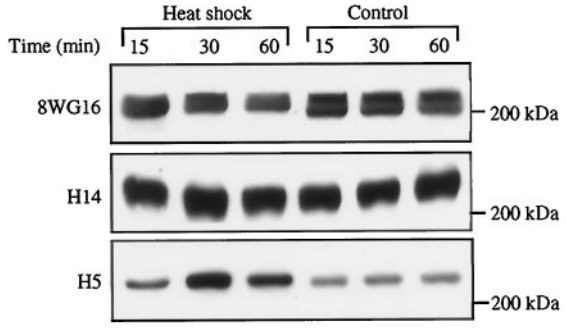

FIG. 5. **H5 epitope is elevated during heat shock.** About 100 μg of protein was subjected to Western blot analysis and probed with different monoclonal antibodies shown to the *left*. *Left three lanes*, yeast extract prepared from cells treated at 39 °C for 15, 30, and 60 min, respectively. *Right three lanes*, extract prepared from cells grown at control temperature for 15, 30, and 60 min, respectively.

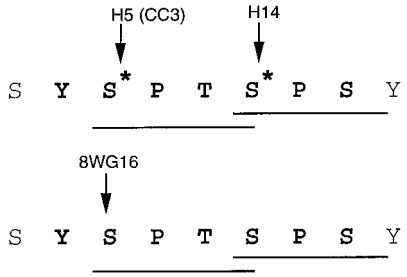

FIG. 6. **Monoclonal antibody recognition sites on the CTD.** This figure summarizes the results of the immunodetection experiments presented here. *Arrows* indicate essential elements of the epitopes recognized by different anti-CTD monoclonal antibodies. *Lines beneath* the sequence indicate residues involved in putative overlapping β-turns (see text).

the first time that the pattern of CTD phosphorylation in yeast varies in response to growth conditions and environmental stress.

mAb 8WG16 is the only antibody that recognizes the unphosphorylated CTD. Indeed, 8WG16 interaction is inhibited by phosphorylation of the wild-type CTD. Strong binding to the unphosphorylated A5 mutant coupled with weak binding to A2 and a complete failure to bind E2 suggests that serine in position 2 is an important element of the 8WG16 epitope. The ability of 8WG16 to recognize *in vivo* phosphorylated pol II suggests that not all CTD repeats are phosphorylated.

The clearest results among the phosphate-specific antibodies were obtained for mAbs H14 and H5 which recognize largely non-overlapping epitopes. H5 recognizes phosphoserine in position 2, whereas H14 recognizes phosphoserine in position 5. This difference in recognition sites is consistent with observed differences in immunolocalization of pol II by these mAbs. H5 stains pol II predominantly in nuclear speckles, whereas H14 staining reveals both speckles and a diffuse nucleoplasmic fraction (59). Western blots with H5 reveal a sharp band at ~240 kDa, and H14 detects a broad band extending from ~240 to 220 kDa (59). Presumably these forms differ in their pattern of phosphorylation, with the H5-reactive form being phosphorylated predominantly on position 2 and the form detected by H14 being phosphorylated predominantly on position 5. Given the overlap in both immunolocalization and Western blotting experiments, it seems likely that some pol II molecules are phosphorylated on both sites. Indeed, immunoprecipitation experiments indicate that some pol II molecules contain both H5 and H14 epitopes on the same CTD (59).

mAb CC3 is similar to H5 but differs in some respects. Although both H5 and CC3 recognize phosphorylated A5 better than phosphorylated A2, CC3 binds phosphorylated WT better than A5. Thus, although phosphorylation of position 2 is important in CC3 recognition, other uncharacterized determinants are also involved. The improved binding to doubly phosphorylated WT CTD suggests that phospho-S5 may be an additional determinant. Dubois *et al.* (70) have also shown that CC3 preferentially binds the non-consensus repeats at the C terminus of the CTD. This interaction is independent of CTD phosphorylation indicating that CTD conformation is also important in recognition by CC3.

mAbs B3 and MARA 3 recognize both phosphorylated A2 and A5. Thus, neither phosphates on serines 2 or 5 are essential elements of the epitope, although at least one site must be phosphorylated. Two explanations are possible. First, the phosphoserines in positions 2 and 5 may be in similar local environments. Structural studies on synthetic heptapeptides (71–73) indicate the presence of overlapping β-turns as indicated in Fig. 6. The Ser-Pro motif in these two turns may comprise part of the epitopes recognized by MARA 3 and B3. Alternatively, B3 and MARA 3 may recognize structural epitopes distinct from serines 2 and 5 that require at least one of these residues to be phosphorylated to achieve the proper conformation.

Differences in immunoblotting and immunolocalization of pol II with antibodies that discriminate between phosphoserines in positions 2 and 5 suggest that phosphorylation of these sites may have different consequences *in vivo*. Genetic and biochemical studies in yeast support this contention. Substitution of glutamate for serine in the most N-terminal CTD repeats is lethal at position 5 but not at position 2, whereas the situation is reversed in C-terminal repeats (26). This result argues that different repeats within the CTD have different functions. Suppressor mutations that allow growth of cells with otherwise lethal serine to alanine or glutamate substitution mutations in position 2 do not suppress similar substitutions in position 5 (56). Finally, while Cdc2 kinase phosphorylates positions 2 and 5 (22), the TFIIH kinase activity and HS-CTD kinases are specific for position 5 (25, 55). Together, these results indicate that serines in positions 2 and 5 are functionally different.

Our present results indicate that the relative phosphorylation level of these two serines changes depending on growth conditions. We show that either nutrient limitation or heat shock induces transient increase in phosphorylated yeast pol II0 expression. In response to stress, phosphorylation of serine 2 in the CTD repeat is markedly increased, whereas serine 5 phosphorylation shows negligible change. Eukaryotic cells respond to environmental changes such as nutritional deprivation or change in temperature by changing the pattern of gene expression (67, 68, 74). The major response to environmental stress is protein modification especially phosphorylation of a number of regulatory proteins by a cascade of stress-related protein kinases. In mammalian systems nutrient limitation or change in temperature induces the kinases belonging to mitogen-activated protein kinase pathway, c-*JUN* kinase pathways, and protein kinase C pathways (55, 69, 75). In *S. cerevisiae* the interplay between growth control and stress is quite intriguing but not well understood. Exposure to increased temperature causes cells to arrest in G_1 (76) while nutritional depletion arrests cells in a G_0-like state (77). In both cases cAMP-dependent protein kinases are thought to play an important role in growth control (67). Which kinase phosphorylates the CTD in yeast in response to stress is not known. Whether serine 2 phosphorylation is required for this growth control or whether it is a consequence of growth-related kinase activation is also not clear.

Different CTD phosphoisomers may play different roles in development. Early germ line nuclei in *Caenorhabditis elegans*

and *Drosophila* react with H14 but not H5, whereas somatic nuclei react with both mAbs (78). Absence of the H5 phosphoepitope in germ line nuclei correlates with failure to express many mRNAs present in somatic cells. This result suggests that phosphorylation in position 2 is required for transcription, a suggestion consistent with the lethal effect of serine to alanine substitution mutations in yeast (26). Furthermore, this result is consistent with a mechanism for transcription repression that involves dephosphorylation (or failure to phosphorylate) of position 2. Clearly, the phosphoepitope-specific antibodies like H5 and H14 will be critical in determining the role of the CTD in developmentally regulated transcription repression.

The multiple potential CTD phosphoryl acceptors, CTD kinases, and possible CTD functions imply that understanding the mechanism of CTD function will not be a simple task. Several possible functions could be imagined for the different phosphoryl acceptors on the CTD. First, results presented in this paper suggest that H5 epitope may be involved in specific gene expression such as stress response gene, whereas H14 epitopes may be required for general overall transcription. Alternatively, phosphorylation on serine 2 may be essential to shut down the general transcription and for directing the inactive pol lI0 to storage sites such as speckles, while appearance of serine 5 phosphorylation may be representing the active pool of polymerase. Finally, modulating the levels of pol II0 phosphoisomers may help the binding of different CTD-associated proteins such as Nrd1p (15, 79) in yeast or mammalian CTD-associated SR-like proteins (16, 20). Other studies in our lab show that these proteins require a specific pattern of CTD phosphorylation for their interaction.[3] Thus, regulation of CTD-binding protein interaction by changes in the CTD phosphorylation pattern may help regulate the assembly of the mRNA processing factors on the growing nascent RNA.

Acknowledgments—We thank David Bentley for the mouse CTD plasmid, R. Baskaran for c-*ABL* kinase, and Dr. Geraldine Seydoux for helpful comments on the manuscript.

REFERENCES

1. Allison, L. A., Moyle, M., Shales, M., and Ingles, C. J. (1985) *Cell* **42,** 599–610
2. Corden, J. L., Cadena, D. L., Ahearn, J., Jr., and Dahmus, M. E. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 7934–7938
3. Nonet, M., Sweetser, D., and Young, R. A. (1987) *Cell* **50,** 909–915
4. Bartolomei, M. S., Halden, N. F., Cullen, C. R., and Corden, J. L. (1988) *Mol. Cell. Biol.* **8,** 330–339
5. Allison, L. A., Wong, J. K., Fitzpatrick, V. D., Moyle, M., and Ingles, C. J. (1988) *Mol. Cell. Biol.* **8,** 321–329
6. Zehring, W. A., Lee, J. M., Weeks, J. R., Jokerst, R. S., and Greenleaf, A. L. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 3698–3702
7. Young, R. A. (1991) *Annu. Rev. Biochem.* **60,** 689–715
8. Dahmus, M. E. (1996) *J. Biol. Chem.* **271,** 19009–19012
9. Zawel, L., and Reinberg, D. (1995) *Annu. Rev. Biochem.* **64,** 533–561
10. O'Brien, T., Hardin, S., Greenleaf, A., and Lis, J. T. (1994) *Nature* **370,** 75–77
11. Yankulov, K., Blau, J., Purton, T., Roberts, S., and Bentley, D. L. (1994) *Cell* **77,** 749–759
12. Yankulov, K., Yamashita, K., Roy, R., Egly, J. M., and Bentley, D. L. (1995) *J. Biol. Chem.* **270,** 23922–23925
13. Bentley, D. L. (1995) *Curr. Opin. Genet. & Dev.* **5,** 210–216
14. Marshall, N. F., Peng, J., Xie, Z., and Price, D. H. (1996) *J. Biol. Chem.* **271,** 27176–27183
15. Steinmetz, E. J. (1997) *Cell* **89,** 491–494
16. Yuryev, A., Patturajan, M., Litingtung, Y., Joshi, R. V., Gentile, C., Gebara, M., and Corden, J. L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 6975–6980
17. Mortillaro, M. J., Blencowe, B. J., Wei, X. Y., Nakayasu, H., Du, L., Warren, S. L., Sharp, P. A., and Berezney, R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 8253–8257
18. Du, L., and Warren, S. L. (1997) *J. Cell Biol.* **136,** 5–18
19. McCracken, S., Fong, N., Yankulov, K., Ballantyne, S., Pan, G., Greenblatt, J., Patterson, S. D., Wickens, M., and Bentley, D. L. (1997) *Nature* **385,** 357–361
20. Corden, J. L., and Patturajan, M. (1997) *Trends Biochem. Sci.* **22,** 413–416
21. Cadena, D. L., and Dahmus, M. E. (1987) *J. Biol. Chem.* **262,** 12468–12474
22. Zhang, J., and Corden, J. L. (1991) *J. Biol. Chem.* **266,** 2290–2296
23. Baskaran, R., Dahmus, M. E., and Wang, J. Y. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11167–11171
24. Stone, N., and Reinberg, D. (1992) *J. Biol. Chem.* **267,** 6353–6360
25. Gebara, M., Sayre, M. H., and Corden, J. L. (1997) *J. Cell. Biochem.* **64,** 390–402
26. West, M. L., and Corden, J. L. (1995) *Genetics* **140,** 1223–1233
27. Laybourn, P. J., and Dahmus, M. E. (1990) *J. Biol. Chem.* **265,** 13165–13173
28. Lu, H., Flores, O., Weinmann, R., and Reinberg, D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 10004–10008
29. Chesnut, J. D., Stephens, J. H., and Dahmus, M. E. (1992) *J. Biol. Chem.* **267,** 10500–10506
30. Kang, M. E., and Dahmus, M. E. (1993) *J. Biol. Chem.* **268,** 25033–25040
31. Bartholomew, B., Dahmus, M. E., and Meares, C. F. (1986) *J. Biol. Chem.* **261,** 14226–14231
32. Usheva, A., Maldonado, E., Goldring, A., Lu, H., Houbavi, C., Reinberg, D., and Aloni, Y. (1992) *Cell* **69,** 871–881
33. Kang, M. E., and Dahmus, M. E. (1995) *J. Biol. Chem.* **270,** 23390–23397
34. Nonet, M. L., and Young, R. A. (1989) *Genetics* **123,** 715–724
35. Koleske, A. J., Buratowski, S., Nonet, M., and Young, R. A. (1992) *Cell* **69,** 883–894
36. Thompson, C. M., Koleske, A. J., Chao, D. M., and Young, R. A. (1993) *Cell* **73,** 1361–1375
37. Koleske, A. J., and Young, R. A. (1994) *Nature* **368,** 466–469
38. Koleske, A. J., and Young, R. A. (1995) *Trends Biochem. Sci.* **20,** 113–116
39. Feaver, W. J., Svejstrup, J. Q., Henry, N. L., and Kornberg, R. D. (1994) *Cell* **79,** 1103–1109
40. Liao, S. M., Zhang, J., Jeffery, D. A., Koleske, A. J., Thompson, C. M., Chao, D. M., Viljoen, M., van Vuuren, H. J., and Young, R. A. (1995) *Nature* **374,** 193–196
41. Shiekhattar, R., Mermelstein, F., Fisher, R. P., Drapkin, R., Dynlacht, B., Wessling, H. C., Morgan, D. O., and Reinberg, D. (1995) *Nature* **374,** 283–287
42. Serizawa, H., Makela, T. P., Conaway, J. W., Conaway, R. C., Weinberg, R. A., and Young, R. A. (1995) *Nature* **374,** 280–282
43. Valay, J. G., Simon, M., Dubois, M. F., Bensaude, O., Facca, C., and Faye, G. (1995) *J. Mol. Biol.* **249,** 535–544
44. Svejstrup, J. Q., Feaver, W. J., and Kornberg, R. D. (1996) *J. Biol. Chem.* **271,** 643–645
45. Rickert, P., Seghezzi, W., Shanahan, F., Cho, H., and Lees, E. (1996) *Oncogene* **12,** 2631–2640
46. Chambers, R. S., and Dahmus, M. E. (1994) *J. Biol. Chem.* **269,** 26243–26248
47. Chambers, R. S., Wang, B. Q., Burton, Z. F., and Dahmus, M. E. (1995) *J. Biol. Chem.* **270,** 14962–14969
48. Buratowski, S., and Sharp, P. A. (1990) *Mol. Cell. Biol.* **10,** 5562–5564
49. Serizawa, H., Conaway, J. W., and Conaway, R. C. (1993) *Nature* **363,** 371–374
50. Gerber, H. P., Hagmann, M., Seipel, K., Georgiev, O., West, M. A., Litingtung, Y., Schaffner, W., and Corden, J. L. (1995) *Nature* **374,** 660–662
51. Jiang, Y., and Gralla, J. D. (1994) *Nucleic Acids Res.* **22,** 4958–4962
52. Makela, T. P., Parvin, J. D., Kim, J., Huber, L. J., Sharp, P. A., and Weinberg, R. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 5174–5178
53. Akhtar, A., Faye, G., and Bentley, D. L. (1996) *EMBO J.* **15,** 4654–4664
54. Kim, E., Du, L., Bregman, D. B., and Warren, S. L. (1997) *J. Cell Biol.* **136,** 19–28
55. Trigon, S., and Morange, M. (1995) *J. Biol. Chem.* **270,** 13091–13098
56. Yuryev, A., and Corden, J. L. (1996) *Genetics* **143,** 661–671
57. Warren, S. L., Landolfi, A. S., Curtis, C., and Morrow, J. S. (1992) *J. Cell Sci.* **103,** 381–388
58. Bregman, D. B., Du, L., Li, Y., Ribisi, S., and Warren, S. L. (1994) *J. Cell Sci.* **107,** 387–396
59. Bregman, D. B., Du, L., Vanderzee, S., and Warren, S. L. (1995) *J. Cell Biol.* **129,** 287–298
60. Thibodeau, A., and Vincent, M. (1991) *Exp. Cell Res.* **195,** 145–153
61. Chabot, B., Bisotto, S., and Vincent, M. (1995) *Nucleic Acids Res.* **23,** 3206–3213
62. Wilson, I. A., Niman, H. L., Houghten, R. A., Cherenson, A. R., Connolly, M. L., and Lerner, R. A. (1984) *Cell* **37,** 767–778
63. Deleted in proof
64. Smith, D. B., and Corcoran, L. M. (1995) in *Current Protocols in Molecular Biology* (Ausubel, F. M. E. A., ed) Vol. 2, pp. 16.7.1–16.7.7, John Wiley & Sons, Inc., New York
65. Desai, D., Gu, Y., and Morgan, D. O. (1992) *Mol. Biol. Cell* **3,** 571–582
66. Thompson, N. E., Steinberg, T. H., Aronson, D. B., and Burgess, R. R. (1989) *J. Biol. Chem.* **264,** 11511–11520
67. Werner-Washburne, M., Braun, E. L., Crawford, M. E., and Peck, V. M. (1996) *Mol. Microbiol.* **19,** 1159–1166
68. Mager, W. H., and De Kruijff, A. J. (1995) *Microbiol. Rev.* **59,** 506–531
69. Bensaude, O., Bellier, S., Dubois, M. F., Giannoni, F., and Nguyen, V. T. (1996) in *Stress-inducible Cellular Responses* (Feige, U., Morimoto, R. I., Yahara, I., and Polla, B., eds) pp. 199–219, Birkhäuser Verlag, Basel
70. Dubois, M. F., Vincent, M., Vigneron, M., Adamczewski, J., Egly, J. M., and Bensaude, O. (1997) *Nucleic Acids Res.* **25,** 694–700
71. Harding, M. M. (1992) *J. Med. Chem.* **35,** 4658–4664
72. Cagas, P. M., and Corden, J. L. (1995) *Proteins* **21,** 149–160
73. Dobbins, J. R., Murali, N., and Long, E. C. (1996) *Int. J. Pept. Protein Res.* **47,** 260–268
74. Voellmy, R. (1996) in *Stress-Inducible Cellular Responses* (Feige, U., Morimoto, R. I., Yahara, I., and Polla, B., eds) pp. 121–137, Birkhäuser Verlag, Basel
75. Venetianer, A., Dubois, M. F., Nguyen, V. T., Bellier, S., Seo, S. J., and Bensaude, O. (1995) *Eur. J. Biochem.* **233,** 83–92
76. Piper, P. (1990) *Antonie Leeuwenhoek* **58,** 195–201
77. Werner-Washburne, M., Braun, E., Johnston, G. C., and Singer, R. A. (1993) *Microbiol. Rev.* **57,** 383–401
78. Seydoux, G., and Dunn, M. A. (1997) *Development* **124,** 2191–2201
79. Steinmetz, E. J., and Brow, D. A. (1996) *Mol. Cell. Biol.* **16,** 6993–7003

[3] M. Patturajan and J. L. Corden, unpublished observations.