HEXIM2, a HEXIM1-related Protein, Regulates Positive Transcription Elongation Factor b through Association with 7SK*§

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The kinase activity of positive transcription elongation factor b (P-TEFb), composed of cyclin-dependent kinase 9 and cyclin T1 or T2, is required for the transition of RNA polymerase II into productive elongation. P-TEFb activity has been shown to be negatively regulated by association with the small nuclear RNA 7SK and the HEXIM1 protein. Here, we characterize HEXIM2, a previously predicted protein with sequence similarity to HEXIM1. HEXIM2 is expressed in HeLa and Jurkat cells, and glycerol gradient analysis and immunoprecipitations indicate that HEXIM2, like HEXIM1, has a regulated association with P-TEFb. As HEXIM1 is knocked down, HEXIM2 functionally compensates for its association with P-TEFb. Electrophoretic mobility shift assays and in vitro kinase assays demonstrate that HEXIM2 forms complexes containing 7SK and P-TEFb and, in conjunction with 7SK, inhibits P-TEFb kinase activity. Our results provide strong evidence that HEXIM2 is a regulator of P-TEFb function. Furthermore, our results support the idea that the utilization of HEXIM1 or HEXIM2 to bind and inhibit P-TEFb can be differentially regulated in vivo.

Transcription by RNA polymerase II is a highly regulated process, influenced by many factors and a cycle of RNA polymerase II phosphorylation (1, 2). The default state of RNA polymerase II transcription is abortive, with RNA polymerase II falling under the influence of negative factors such as DRB1 sensitivity-inducing factor (DSIF), and negative elongation factor (NELF), resulting in the production of short transcripts (3–5). The transition to the productive elongation of full-length transcripts requires positive transcription elongation factor b (P-TEFb) (6, 7), which phosphorylates serine 2 residues in the transcripts requiring positive transcription elongation factor b (3–5). The transition to the productive elongation of full-length transcripts requires positive transcription elongation factor b (P-TEFb) (6, 7), which phosphorylates serine 2 residues in the heptad repeats of the carboxyl-terminal domain of the largest subunit of RNA polymerase II (7, 8). P-TEFb is required for the proper expression of RNA polymerase II genes. The knockdown

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The abbreviations used are: DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; PMSF, phenylmethylsulfonyl fluoride; P-TEFb, positive transcription elongation factor b; Cdk, cyclin-dependent kinase; EMSA, electrophoretic mobility shift assay; HGKEDP, 25 mM HEPES (pH 7.6), 15% glycerol, 100–600 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.1% of a saturated PMSF isopropanol solution; siRNA, small interfering RNA; HGKEDP, 25 mM HEPES (pH 7.6), 15% glycerol, 0.1 mM KCl, 0.1 mM EDTA, and 0.1% of a saturated PMSF isopropanol solution.

of cyclin-dependent kinase 9 or both of the cyclin Ts in Caenorhabditis elegans reduces the expression of early embryonic genes and the level of serine 2 phosphorylation of the carboxyl-terminal domain and leads to the death of the embryo at an early stage (9). Inhibition of P-TEFb kinase activity through treatment with flavopiridol blocks the transcription of RNA polymerase II genes in HeLa cells (10, 11), with complete inhibition causing apoptosis. P-TEFb activity can be targeted to specific genes through association with activators, such as human immunodeficiency virus-1 Tat (12, 13), CIITA (14), nuclear factor-κB (15), and Myc (16, 17).

There are two distinct P-TEFb complexes, which differ in size, composition, and activity (18, 19). The originally identified, smaller P-TEFb complex has kinase activity (7) and is composed of Cdk942 or Cdk955 (20) and a cyclin partner T1, T2, or K (21, 22). A large P-TEFb complex with reduced kinase activity was found to contain the small nuclear RNA 7SK (18, 19) and HEXIM1 (23, 24), in addition to P-TEFb subunits. Independently, HEXIM1 and 7SK have limited inhibitory effects on P-TEFb kinase activity, but together, they strongly inhibit P-TEFb kinase activity (25, 26). 7SK is an abundant and highly conserved small nuclear RNA of 331 nucleotides (27) and associates with up to eight different proteins (28). HEXIM1 was first identified as a protein whose expression was induced in smooth muscle cells in response to hexamethylene bisacetamide treatment (29). HEXIM1 orthologs in chicken and mouse (cardiac lineage protein 1) are expressed during cardiac development (30, 31). HEXIM1 is a highly conserved protein with an RNA binding domain, a nuclear localization signal, and many other areas of high conservation of unknown function (25, 26, 32).

There is a delicate and dynamic balance of the two P-TEFb complexes in cells. The dissociation of P-TEFb from 7SK and HEXIM1 is rapid and reversible. Treatment of cells with UV irradiation, actinomycin D, or DRB results in the dissociation of large P-TEFb complexes, and when cells are allowed to recover from DRB treatment, large P-TEFb complexes reform (18). Physiological signals leading to cardiac hypertrophy converge on the activation of P-TEFb, through dissociation of 7SK, resulting in increased transcription and, ultimately, increased cell size (33–35). Mis-regulation of P-TEFb may lead to cancer because the expression of HEXIM1 (renamed estrogen down-regulated gene 1, EDG1) has been shown to be down-regulated in response to estrogen in breast tumors (36).

A gene encoding a predicted HEXIM1 paralog, HEXIM2, is located less than 10,000 bp downstream of the HEXIM1 gene on chromosome 17 (23, 24, 32). Whereas HEXIM1 has been shown to be involved in controlling P-TEFb, no studies on the role of HEXIM2 in this process have been described. The purpose of this study is to ascertain whether HEXIM2 is expressed and determine whether it plays a functional role in controlling P-TEFb.
MATERIALS AND METHODS

Cloning of HEXIM Proteins—The HEXIM2 coding sequence present in the IMAGE cdNA clone 4559410 (Invitrogen) was amplified with Easy A DNA polymerase (Stratagene) using primers containing a Nhel site (5'-GCTAGCTAGGCGACCACGAAACA-3') and a XhoI site (5'-GCGTCTAGATCGGACCGGACGCC-3') and cloned into pCR2.1 (Invitrogen) in-frame with the His tag, utilizing the Nhel and XhoI stites. Site-directed mutagenesis of pET21a-HEXIM2 of Thr143 to Ala or Asp was carried out with Pfu Ultra HF DNA polymerase (Stratagene) using the primers 5'-GCCCCCTACAAACGGCAGATTTGCTG-3' and 5'- CGGAACGTGGTGTTGAGGGGCTGGG-3' or 5'-GGCGGCCGGCTCAC ACCGATAACCGATTCCCATGATGGA-3' and 5'-CACTGCAGAAGCT GCAGGACGGAGCCGACGCCG-3', respectively. Thr205 in HEXIM1 was also mutated to Ala with 5'-GCCCCCTATAAAGCCGACGCCG-3'.

Expression and Purification of HEXIM Proteins—His-tagged wild type, T205A, and T205D HEXIM1 and wild type, T143A, and T143D HEXIM2 were expressed in Escherichia coli BL21(DE3) cells by induction with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside and growth overnight at 18 °C. The cells lysates were prepared as previously described (25), and the salt was brought up to 750 mM NaCl, followed by incubation with nickel-nitrilotriacetic acid-agarose beads (Qiagen) for 1 h. The unbound material was allowed to flow through the column, and the resin was washed with 10 volumes of 10 mM Tris (pH 7.5), 0.5 mM NaCl, 1% Triton X-100, 0.1% of a saturated PMSF isopropanol solution, 10 mM imidazole, and 10 volumes of Buffer B (10 mM Tris (pH 7.8), 100 mM NaCl, 1% Triton X-100, 0.1% of a saturated PMSF isopropanol solution, 100 mM imidazole). The column was eluted with Buffer C containing 250 mM imidazole. HEXIM1 and HEXIM2 proteins were further purified by loading onto a 1-ml Mono Q column and eluted with a linear gradient from 100 to 600 HGEDP.

Generation and Affinity Purification of HEXIM2 Antibodies—Recombinant histidine-tagged HEXIM2 was injected into a sheep to produce antibodies (Elmira Biologica). 10 ml of the crude antiserum was incubated at 4 °C for 3–5 min, and 5–10 ml of recombinant HEXIM2 covalently attached to 1 ml of Actigel ADL resin (Sterogene). After washing with 30 ml of phosphate-buffered saline, ~3 mg of affinity-purified HEXIM2 antibody was eluted with 2 ml of 100 mM glycine (pH 2.5) and then neutralized with 1 ml of Tris base. The antibody was aliquoted and stored at ~80 °C.

Quantitative Western Blotting—Samples were resolved on 9% SDS-PAGE gels and transferred to 0.45-µm nitrocellulose membranes. The antibodies used in Western blotting were goat anti-cyclin T1 (T-18; Santa Cruz Biotechnology), rabbit anti-Cdk9 (C-20; Santa Cruz Biotechnology), affinity-purified sheep anti-HEXIM2, and affinity-purified sheep antibodies used in Western blotting were goat anti-cyclin T1 (T-18; Santa Cruz Biotechnology), affinity-purified anti-HEXIM2 antibody were generated. Histi-

Immunoprecipitations—HEK 293 cells expressing FLAG-Cdk9 (37) were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1% penicillin and streptomycin, and 0.5 µg/ml puromycin under standard conditions (37 °C in 5% CO₂). The cells were lysed for 15 min on ice in Buffer A (10 mM KCl, 10 mM MgCl₂, 10 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, 0.1% of a saturated PMSF isopropanol solution, and EDTA-free complete protease inhibitor mixture from Roche Applied Science) containing 0.5% Nonidet P-40, followed by cen-

Knockdown of HEXIM1 using siRNA—HEK 293 cells was transfected with 200 nM siRNA using 9000 units of Lipofectamine 2000 per ml of cells. After 8 h, the medium containing the transfection reagent and siRNA was removed, and the cells were cultured in Dulbecco's modified Eagle's medium/F-12 with 10% fetal bovine serum under standard conditions for 36, 48, or 60 h. Cells were lysed for 15 min on ice in Buffer A containing 150 mM NaCl and 0.5% Nonidet P-40, and the lysates were clarified by centrifugation for 20 min at 14,000 rpm prior to fractionation on 5-ml, 5–45% glycerol gradients in Buffer A with 150 mM NaCl at 45,000 rpm for 16 h in a SW-28Ti rotor.

Electrophoretic Mobility Shift Assay—12-µl reactions were carried out in 25 mM HEPES (pH 7.6), 15% glycerol, 80 mM KCl, 0.1 mM EDTA, 5 mM dithiothreitol, 0.01% Nonidet P-40, 1 µM of bovine serum albumin, and 300 ng of poly(I) poly(C) (Amersham Biosciences) and included 500 pg of radiolabeled 7SK RNA and recombinant P-TEFb composed of Cdk9 and cyclin T2a, unless otherwise noted to contain cyclin T1 and recombinant HEXIM1 or HEXIM2 as indicated. 7SK and poly(I) poly(C) were added after 5 min at 75 °C and cooled on ice for another 5 min prior to addition. Reactions were incubated at room temperature for 20 min, and resolved on a 3.5% polyacrylamide (19:1 acrylamide:acrylamide ratio) gel in 0.5x Tris/glycine at 4 °C for 1.5 h at 6 watts. The dried gel was subjected to autoradiography.

Kinase Assay—20-µl kinase reactions containing recombinant purified P-TEFb (Cdk9/cyclin T2a) with Dro sophila RNA polymerase II as the substrate were carried out in 34 mM KCl, 20 mM HEPES (pH 7.8), 7 mM MgCl₂, 30 µM ATP, 1.3 µCi of [γ-32P]ATP (Amersham Biosciences), and 1 µM of bovine serum albumin per reaction and the indicated amounts of wild type or mutant HEXIM1 or HEXIM2 proteins. T7-transcribed 7SK RNA was added last to the pre-incubation after it was heated for 5 min at 75 °C and then cooled on ice for another 5 min. All reactions were incubated for 10 min at 25 °C prior to the addition of ATP. The kinase reactions were incubated at 30 °C for 40 min, and then stopped by the addition of SDS-PAGE loading buffer. Reactions were resolved by 9% SDS-PAGE. The dried gel was subjected to autoradiography and quantified with a Packard Instant Imager.

Purification of P-TEFb—Baculoviruses expressing human Cdk9 and cyclin T1 were generated using the BaculoDirect Baculovirus Expression System (Invitrogen) according to the manufacturer's instructions. Cdk9 and cyclin T1 were tagged with six histidines at its carboxyl terminus, and cyclin T1 was untagged. Sf9 cells were maintained suspended in SF-900 II SFM serum-free medium (Invitrogen) at concentrations between 5 x 10⁹ and 2 x 10⁶ cells/ml. 1 ml of a third amplification stock of each virus was added to 100 ml of cells (at 1 x 10⁶ cells/ml) in a 250-ml Erlenmeyer flask, and the cell lysates were shaken at 140 rpm at 28 °C for 72 h. Cells were spun down at 1000 x g, and the cell pellet was sonicated three 10-s bursts in 1 ml of Buffer L (100 mM Tris (pH 8.0), 100 mM NaCl, 2 mM MgCl₂, 1% Triton, 0.1% of a saturated PMSF isopropanol solution, 1x EDTA-free protease inhibitor mixture (Roche Applied Science), and 10 µg/ml E-64 protease inhibitor (Roche Applied Science)). The cell lysate was spun for 15 min at 192,000 x g in a Beckman table top centrifuge. The supernatant was then incubated with 0.1 µl of nickel-nitrilotriacetic acid resin (Qiagen) for 45 min at 4 °C. The resin was washed once with 1 ml of Buffer L, followed by a wash with 1 ml of 10 mM Tris (pH 8.0), 500 mM NaCl, 1% Triton, 0.1% of a saturated PMSF isopropanol solution, and 40 mM imidazole and with 1 ml of 100 mM HEGK. P-TEFb was eluted with 0.25 ml of 100 mM HEGKPE with 300 mM imidazole and loaded onto a 1-ml Mono S column. P-TEFb was eluted with a 20-ml linear gradient from 0.1 to 0.5 M HGEDP.

RESULTS

Sequence analysis reveals that HEXIM proteins are highly conserved throughout vertebrates. Mammals encode two HEXIM proteins, and in genomes that have been mapped, HEXIM1 and HEXIM2 are located close to each other on the same chromosome. The HEXIM2 gene contains 3 introns, whereas the HEXIM1 gene is unusual in that it does not have any introns. It is possible that HEXIM1 arose in mammals from the insertion of a cdNA copy of HEXIM2 into genomic DNA. Other vertebrates, such as Xenopus laevis, Danio rio, Pugu rubripes, and Gallus gallus, contain only one HEXIM protein, and comprehensive sequence comparison shows similarity with both HEXIM1 and HEXIM2 (supplementary Figure 1S). All HEXIM proteins exhibit conserved domains that include a positively charged RNA binding domain (25, 26), a nuclear localization signal (32), the PYNT domain involved in P-TEFb binding (25), and a potential leucine zipper at the carboxyl terminus that could allow dimerization of the protein through a coiled-coil interaction.

Relative Expression of HEXIM1 and HEXIM2 in Vivo—To examine the function of HEXIM2, recombinant proteins and an affinity-purified anti-HEXIM2 antibody were generated. Histidine-tagged HEXIM2 was expressed in E. coli DE3 cells and
purified using nickel resin and ion exchange chromatography. HEXIM2 has a predicted molecular mass of 32 kDa but has an apparent molecular mass of 45 kDa (Fig. 1A). A similar altered mobility was found for HEXIM1, which has a predicted molecular mass of 41 kDa but an apparent molecular mass of 67 kDa (32) (Fig. 1A). The PYNT domain of HEXIM1 (amino acids 202–205) has been found to be necessary for high affinity binding of P-TEFb to a HEXIM1-7SK complex (25). To determine whether this domain has a similar function in HEXIM2, mutants with Thr143 in the PYNT domain changed to Ala (T143A) or Asp (T143D) were produced (Fig. 1A). They also displayed altered mobilities on SDS-PAGE with apparent molecular masses of 40 and 46 kDa, respectively, compared with their predicted molecular mass of 32 kDa (Fig. 1A).

To examine the relative expression of HEXIM1 and HEXIM2 in vivo, quantitative Western blotting of HeLa and Jurkat whole cell lysates was performed. The signals for HEXIM1 and HEXIM2 obtained with increasing amounts of HeLa and Jurkat whole cell lysates were compared with the signals from a range of HEXIM1 and HEXIM2 recombinant proteins from 0.3 to 10 ng. The signals from equal amounts of recombinant HEXIM1 and HEXIM2 were similar under the conditions used (Fig. 1, B and C). However, the signals from whole cell lysates indicated that HEXIM1 is more highly expressed in HeLa and Jurkat cells than HEXIM2 (Fig. 1, B and C). The amount of HEXIM1 and HEXIM2 at each concentration of cell extract was calculated from standard curves obtained with the recombinant proteins. HEXIM2 was calculated to account for 10% of the HEXIM protein in both cell lines.

**HEXIM2 Is in the Large Form of P-TEFb—** To further characterize HEXIM2, glycerol gradient sedimentation analyses of Jurkat cell lysates were carried out. HEXIM1 has been shown to sediment in two peaks on a glycerol gradient, with a small, free form near the top of the gradient and a larger form associated with P-TEFb (23). Treatment of cells with inhibitors of transcription elongation causes a dissociation of the large P-TEFb complex, shifting all HEXIM1 to the free form (23). Jurkat cells, with or without a 1-h treatment with 50 μM DRB, were lysed to extract all P-TEFb complexes from the cell (18) and subjected to glycerol gradient sedimentation. Fractions were analyzed by Western blotting for cyclin T1, HEXIM1, HEXIM2, and Cdk9. In control cells, both cyclin T1 and Cdk9 remained in fractions 8–11 (Fig. 2A). The sedimentation pattern of HEXIM2 complex was found to sediment with P-TEFb in fractions 4–7 (Fig. 2A). Two peaks of sedimentation were seen for HEXIM1. The free form of HEXIM1 was found in fractions 4–6, and the P-TEFb-associated form was found in fractions 8–11 (Fig. 2A). HEXIM2 exhibited a similar but not identical, sedimentation pattern to that of HEXIM1, with a small amount co-sedimenting with Cdk9 and cyclin T1 and more of the HEXIM2 than HEXIM1 in their respective free forms (Fig. 2A). HEXIM2 that co-sedimented with large P-TEFb exhibited a higher apparent molecular mass during SDS-PAGE compared with free HEXIM2, perhaps due to phosphorylation or another modification. As has been found in studies with HeLa cells (18, 23), in Jurkat cells the sedimentation of P-TEFb subunits and HEXIM1 changes in response to DRB treatment, with P-TEFb and HEXIM1 in their respective free forms (Fig. 2B). The sedimentation pattern of HEXIM2 also changed, such that most of HEXIM2 was found in its free form (fractions 3–7), and little or no HEXIM2 remained in fractions 8–11 (Fig. 2B).

To determine whether HEXIM2 that co-sediments with the large P-TEFb complex is actually associated with P-TEFb, an immunoprecipitation experiment was performed. HEK 293 cells expressing FLAG-tagged Cdk9 (37) were lysed under
conditions that extract the large form of P-TEFb from the nucleus, and the lysate was resolved on a 5–45% 12-ml glycerol gradient. A glycerol gradient fraction containing large, inactive P-TEFb complexes was used as the starting material for M2-FLAG immunoprecipitations. The output contained cyclin T1, HEXIM1, HEXIM2, FLAG-Cdk9, and endogenous Cdk9 (Fig. 3, lane 1). The unbound fraction contained most of the endogenous Cdk9 (Fig. 3, lane 2). About half of the cyclin T1, HEXIM1, and HEXIM2 were not pulled down, presumably because of association to the endogenous Cdk9 (Fig. 3, lane 2). Cyclin T1, HEXIM1, HEXIM2, and a small amount of endogenous Cdk9 were found bound to the M2 FLAG antibody beads in addition to FLAG-Cdk9 (Fig. 3, lane 3), indicating that HEXIM2, like HEXIM1, can be found in a large P-TEFb complex.

HEXIM2 Can Replace HEXIM1 in the Large P-TEFb Complex in Vivo—To examine the function of HEXIM2 in the absence of HEXIM1, siRNA was used to knockdown HEXIM1 in HeLa cells. The effectiveness of HEXIM1 knockdown was examined 36 and 60 h after transfection in cells treated with HEXIM1 siRNA. Western blotting was used to quantitate the amount of HEXIM1 relative to actin (Fig. 4, lane 1). 36 h after the transfection, HEXIM1 was knocked down to ~10% of the level seen in control cells and was further knocked down to 5% of the level seen in control cells after 60 h (Fig. 4A). 48 h after transfection with HEXIM1 siRNA, the cells were lysed to extract all P-TEFb complexes and analyzed by glycerol gradient sedimentation. Gradient fractions were analyzed by Western blotting for cyclin T1, HEXIM1, HEXIM2, and Cdk9. In the control gradient, cyclin T1 and Cdk9 were detected predominately in fractions 8–10, with some signal also present in fractions 4–7 (Fig. 4B). Most HEXIM1 and HEXIM2 proteins were present in their free forms (fractions 2–5) rather than in large complexes with P-TEFb, with more HEXIM2 than HEXIM1 in their respective free forms. In the HEXIM1 siRNA gradient (Fig. 4C) the distribution of Cdk9 and cyclin T1 was unchanged, even though HEXIM1 was knocked down to 2% of its normal level. This value was calculated by comparing the signal from HEXIM1 to the signal from a cross-reacting, non–specific (ns) band in fractions 4–6. The small amount of HEXIM1 remaining co-sedimented with the large form of P-TEFb (Fig. 4C). Most of the HEXIM1 strongly suggests that controlling P-TEFb activity is an important function of both HEXIM1 and HEXIM2. The differential inclusion of HEXIM1 and HEXIM2 in large P-TEFb complexes in Jurkat and HeLa cells also suggests that there may be more than one pathway involved in controlling P-TEFb activity.

HEXIM2 Associates with 7SK and P-TEFb in Vitro—Electrophoretic mobility shift assays (EMSAs) were performed to determine whether HEXIM2 could associate with 7SK and P-TEFb, as previously shown for HEXIM1 (25). The assay utilized radiolabeled 7SK and recombinant proteins and was carried out in the presence of dithiothreitol, which we found had a dramatic effect on the mobility of the complexes (25). The first lane contains 7SK alone (Fig. 5A). Although 7SK was seen to be entirely full length when analyzed on a denaturing gel, it ran as a doublet or a triplet if the native gel was run at low temperature (~4 °C) and as a single band at room temperature (data not shown). This is likely due to stabilization of weak

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2 A. Michels and O. Bensaude, personal communication.

3 S. A. Byers, unpublished data.
secondary or tertiary structures at low temperature that have no effect on HEXIM binding. Inclusion of increasing amounts of HEXIM1 resulted in the sequential formation of two complexes. Whereas we do not know the number of 7SK or HEXIM1 molecules in each complex, the second complex (with lower mobility) began to form only after most of the 7SK had shifted into the first complex (Fig. 5A), suggesting that the second complex resulted in binding of additional HEXIM1, perhaps to a lower affinity site on the 7SK-HEXIM complex. The same pattern of complex formation was seen with HEXIM2, except that the mobility of the complexes was higher (Fig. 5A), presumably due to differences in size or charge of HEXIM2. When equal amounts of HEXIM1 or HEXIM2 were used, the fraction of 7SK shifted was the same, indicating that the two proteins have similar affinity for 7SK.

We next wanted to examine the association of P-TEFb with HEXIM2/7SK complexes. In the absence of either HEXIM protein, P-TEFb containing Cdk9 and cyclin T2a caused a small decrease in the mobility of 7SK at only the highest of two concentrations tested (Fig. 5B, lanes 2 and 9). When P-TEFb was added to reactions containing 3 ng of HEXIM1, a new complex formed (Fig. 5B, lanes 5 and 6) that contains both HEXIM1 and P-TEFb as previously reported (25). The lower mobility HEXIM1/7SK complex, which was the most prevalent complex when 30 ng of HEXIM1 was used, was not shifted upon addition of P-TEFb (Fig. 5B, lanes 8 and 9). When P-TEFb was added to reactions containing 3 or 30 ng of HEXIM2, the mobility of both of the HEXIM2/7SK complexes changed (Fig. 5B, lanes 11, 12, 14, and 15). This suggests that binding of additional HEXIM1 excludes P-TEFb but that binding of additional HEXIM2 does not. However, it is possible that the shift of the low mobility HEXIM1/7SK complex by association of P-TEFb might not be resolvable in the gel system used. At 3 ng there was a small amount of the lower mobility HEXIM1/7SK complex with both proteins, but the response of these complexes to addition of P-TEFb was different. The low mobility HEXIM1/7SK complex did not form in the presence of P-TEFb, but the low mobility HEXIM2/7SK complex was further retarded. Although we do not know the stoichiometry of any of the components in the complexes seen in the EMSA, the high mobility HEXIM1/7SK complex gave rise to two additional shifts as P-TEFb was increased (Fig. 5B, lane 6), suggesting binding of two P-TEFb molecules.

To further characterize the interaction of HEXIM2/7SK complexes with P-TEFb, proteins containing mutations of the PYNT domain (amino acids 202–205 in HEXIM1 and 140–143 in HEXIM2) were examined in the EMSA. The PYNT domain has been shown to be involved in the interaction of HEXIM1 with P-TEFb and plays no role in RNA binding (25). As HEXIM2 T143A or T143D was titrated into the EMSA reactions (Fig. 5C), the set of 7SK complexes that formed was similar to that seen with wild type HEXIM2, indicating that the PYNT region of HEXIM2 is not involved in RNA binding. Addition of P-TEFb did not have much effect on the mobility of the T143A/7SK and T143D/7SK complexes (Fig. 5D), indicating that the PYNT region of HEXIM2 is involved in P-TEFb binding. These results suggest that when bound to 7SK, HEXIM1 and HEXIM2 utilize similar mechanisms for association with P-TEFb.

We next wanted to determine whether HEXIM1 and HEXIM2 differ in their ability to associate with P-TEFb containing either cyclin T2a or T1. Reactions with 10 ng of recombinant HEXIM1 or HEXIM2 and three concentrations of P-TEFb containing cyclin T2a were first analyzed by EMSA. As was found in Fig. 5B, the HEXIM1/7SK complex with the higher mobility was retarded, and the complex with the lower mobility disappeared (Fig. 6, lanes 2–5). Also, both of the HEXIM2/7SK complexes were retarded (Fig. 6, lanes 6–9). When the same analyses were done with P-TEFb containing cyclin T1, the results were identical, except that at the highest concentration of P-TEFb, evidence for a second shift was dramatic (Fig. 6, lanes 10–17). We do not know whether this second shift is due to specific or nonspecific association of P-TEFb. Overall, these results indicate that HEXIM1 and HEXIM2 associate with P-TEFb with similar affinity, regardless of which cyclin is present.

**HEXIM2, in Association with 7SK, Inhibits P-TEFb—Finally, in vitro kinase assays were utilized to compare the ability of HEXIM1 and HEXIM2 to inhibit P-TEFb in the presence of 7SK. The kinase assay examines the ability of P-TEFb containing cyclin T2a to incorporate 32P into the largest subunit of RNA polymerase II. Using this assay, we have previously**
shown that the addition of both HEXIM1 and 7SK inhibits the function of P-TEFb (25). HEXIM1 mutations, Y203D, T205D, and a double mutant (PDND), have been shown to cause a reduction in binding of P-TEFb in EMSAs and a reduction of P-TEFb inhibition in kinase assays in the presence of 7SK (25). The effects of the previously used HEXIM1 mutant, T205D, and a new mutant HEXIM1, T205A, were compared with the effects of two corresponding HEXIM2 mutants, T143A and T143D. Here, the addition of 1 or 5 pmol of 7SK or HEXIM1 individually did not significantly inhibit P-TEFb compared with the control reaction (Fig. 7, A and B). However, when 1 or 5 pmol of 7SK and HEXIM1 were added together, there was a dramatic progression of inhibition to 10% of the activity seen in the absence of 7SK and HEXIM1 (Fig. 7, A and B). When HEXIM1 containing either PYNT mutant was added with 7SK, there was significantly less inhibition of P-TEFb (Fig. 7, A and B). Comparable results (Fig. 7, C and D) were obtained when similar analyses were carried out with HEXIM2 and the corresponding PYNT mutants (T143A and T143D). HEXIM2 inhibited P-TEFb in a 7SK-dependent manner, and the PYNT mutants greatly reduced this inhibition. We do not know why the PYNA mutants are slightly more severe than the PYND mutants. Unexpectedly, we found that both HEXIM1 and HEXIM2 were phosphorylated by P-TEFb and that HEXIM2 was a significantly better substrate.

**DISCUSSION**

We have examined the role of HEXIM2 in the regulation of P-TEFb function both in vivo and in vitro. HEXIM2 was found to be expressed in the three human cell lines examined, HeLa, Jurkat, and HEK 293. Like HEXIM1, it was able to associate with P-TEFb in large complexes in vivo. Furthermore, HEXIM2 replaced HEXIM1 in these complexes when HEXIM1 was knocked down. EMSAs and in vitro kinase assays were used to determine that recombinant HEXIM2 could associate with 7SK and P-TEFb and inhibit P-TEFb kinase activity in a manner similar to HEXIM1. Taken together, these studies strongly implicate HEXIM2 as a P-TEFb-regulatory protein that acts in concert with 7SK RNA to inhibit P-TEFb.

HEXIM proteins appeared late in evolution and may have been selected to accomplish the appropriate degree of P-TEFb regulation needed as the complexity of organisms increased. Analysis of HEXIM proteins across species indicates that the protein arose before chordates and arthropods diverged. Clear examples of HEXIM proteins are found in the arthropods, Drosophila, and mosquito, but not in the nematode *C. elegans* or in yeasts, even though all these organisms have Cdk9 and cyclin T homologs. Perhaps HEXIM proteins evolved to carry out more extensive regulation of P-TEFb. The appearance of a second HEXIM protein in mammals may serve the increased need for intricate regulation of P-TEFb during early development and in highly differentiated tissues. Although the lack of introns in HEXIM1 suggests it might be the duplicated gene, it is not clear which gene is the original. This ambiguity is due to the fact that in both genes all conserved HEXIM sequences are present in one contiguous genomic sequence and because HEXIM proteins from evolutionarily distant species have relatively low similarity to both HEXIM1 and HEXIM2 without containing any characteristic sequence elements specific for HEXIM1 or HEXIM2.

Although it is not clear why mammals have two HEXIM proteins, we favor the hypothesis that the two proteins allow more diverse control of P-TEFb. HEXIM1 and HEXIM2 can be seen to have similar functions because they both inhibit P-TEFb in a 7SK-dependent manner. However, our results suggest that there may be significant differences in the function of HEXIM1 and HEXIM2 in vivo. Although both HEXIM1 and HEXIM2 were found in large P-TEFb complexes in both HeLa and Jurkat cells, we found that HEXIM1 was preferentially included in large complexes. In HeLa cells ~25% of HEXIM1, but only ~10% of HEXIM2, is found associated with P-TEFb. In Jurkat cells the preference for HEXIM1 was even more pronounced, with ~70% of HEXIM1 but only ~10% of HEXIM2 associated with P-TEFb. Evidently, the association of the two HEXIM proteins with P-TEFb is regulated differently in the two cell lines. HEXIM2 appears to be modified in both of these cell lines when it is in large complexes, as suggested by a decreased mobility of the protein seen in Western blots. We also observed preferential phosphorylation of HEXIM2 by P-TEFb during in vitro kinase assays. It has yet to be determined whether the function of HEXIM2 is regulated by phosphorylation in vivo. Although the significance is not clear, a difference between HEXIM1 and HEXIM2 was seen in the EMSA experiments. Both HEXIM1 and HEXIM2 formed a low mobility complex with 7SK, but only HEXIM2 allowed the association of P-TEFb with that complex.

Regulation of P-TEFb by HEXIM1 and HEXIM2 may be controlled in part by differential expression of the two proteins in different tissues. HEXIM2 was found to be 10% of the HEXIM protein in Jurkat and HeLa cells, but HEXIM1 and HEXIM2 were differentially expressed. As a result, both HEXIM1 and HEXIM2 mRNA levels varied widely across the tissues sampled. Importantly, in tissues with relatively high HEXIM1 expression (pancreas, early erythroid, and T and B cells), HEXIM2 expression was relatively low. Also, in tissues with relatively high HEXIM2 expression (liver and testis), HEXIM1 expression was low. Although HEXIM2 is 10% of the HEXIM protein and cyclin T2 is 10% of the total cyclin T in HeLa and Jurkat cells, there is no evidence that HEXIM1 or HEXIM2 preferentially associates with P-TEFb containing cyclin T1 or T2. The EMSAs presented here showed that both HEXIM1 and HEXIM2 form complexes with P-TEFb containing cyclin T1 and T2a, and yeast two-hybrid data have shown association of HEXIM1 with both cyclin T1 and T2 (23). Supporting this, the Gene Sorter data base yielded no correlation in the expression of HEXIM1 or
HEXIM2 mRNAs with those of cyclin T1 or T2. The amino terminus of HEXIM1 and that of HEXIM2 are quite dissimilar, and this region may allow for interaction with different subsets of proteins responsible for differential regulation. Analysis of the regions of HEXIM1 and HEXIM2 important for their inhibitory function in vitro and their regulated association with P-TEFb in vivo will be aided by the EMSA and kinase assays described here, coupled with the knockdown of endogenous HEXIM proteins and replacement with the modified proteins in vivo, as has been done with the termination factor TTF2 (38).

Abnormal expression or altered regulation of HEXIM1 or HEXIM2 may be responsible for inappropriate P-TEFb function and subsequent aberrant RNA polymerase II gene expression during disease progression, especially in cancer. An abnormal increase in the expression of HEXIM1 occurs in HeLa cells treated with the topoisomerase poison camptothecin (39), whereas HEXIM2 is unaffected. Conversely, HEXIM2 is downregulated in response to injection of insulin in skeletal muscle (40), and HEXIM1 is not. The role of P-TEFb and its regulation has not been explored in cancer, although flavopiridol, an antiproliferative compound in clinical trials with cancer patients, has not been explored in cancer, although flavopiridol, an antiproliferative compound in clinical trials with cancer patients.

If the connection is made stronger, it will be useful to search for compounds that target the factors controlling the assembly or dissocation of the large inactive P-TEFb complexes or alter the expression of HEXIM1 or HEXIM2 directly.

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Fig. 7. Inhibition P-TEFb activity by HEXIM proteins. P-TEFb kinase assays with RNA polymerase II as the substrate were performed as described under “Materials and Methods.” HEXIM1 proteins (A) and HEXIM2 proteins (C) and 7SK were added as indicated. Results were quantitated with a Packard Instant Imager, and kinase activity was normalized to control reactions and plotted for HEXIM1 (B) and HEXIM2 (D) proteins.

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