Transcription-dependent Association of Multiple Positive Transcription Elongation Factor Units to a HEXIM Multimer*

Received for publication, March 7, 2005, and in revised form, June 24, 2005 Published, JBC Papers in Press, June 30, 2005, DOI 10.1074/jbc.M502471200

Cyprien Dulac‡‡, Annemieke A. Michels‡‡, Alessandro Fraldi‡‡, François Bonnet‡, Van Trung Nguyen‡, Giuliana Napolitano‡, Luigi Lania‡, and Olivier Benoist‡‡‡

From the ‡‡Unité Mixte de Recherche 8541 CNRS, Ecole Normale Supérieure, Laboratoire de Régulation de l’Expression Génétique, 75230 Paris Cedex 05, France and ‡‡‡Dipartimento di Biologia Strutturale e Funzionale, Università di Napoli Federico II, 80134 Napoli, Italy

The positive transcription elongation factor (P-TEFb) comprises a kinase, CDK9, and a Cyclin T1 or T2. Its activity is inhibited by association with the HEXIM1 or HEXIM2 protein bound to 7SK small nuclear RNA. HEXIM1 and HEXIM2 were found to form stable homo- and hetero-oligomers. Using yeast two-hybrid and transfection assays, we have now shown that the C-terminal domains of HEXIM proteins directly interact with each other. Hydrodynamic parameters measured by gel-permeation chromatography demonstrate that both purified recombinant and cellular HEXIM1 proteins form highly anisotropic particles. Chemical cross-links suggest that HEXIM1 proteins form dimers. The multimeric nature of HEXIM1 is maintained in P-TEFb-HEXIM1-7SK RNA complexes. Multiple P-TEFb modules are found in the inactive P-TEFb-HEXIM1-7SK complexes. It is proposed that 7SK RNA binding to a HEXIM1 multimer promotes the simultaneous recruitment and hence inactivation of multiple P-TEFb units.

The positive transcription elongation factor (P-TEFb) comprises a protein kinase, CDK9, and a Cyclin T1, T2, or K (1, 2). It is required for transcription elongation of most class II genes. P-TEFb phosphorylates numerous substrates, including the C-terminal domain (CTD) of RNA polymerase II and the Sp5 subunit of the DRB sensitivity-inducing factor (DSIF). The DSIF prevents transcription from proceeding efficiently after initiation. The kinase activity of P-TEFb antagonizes this inhibitory effect. Several class II genes such as heat-shock or U2 small nuclear RNA genes do not have a strong requirement for P-TEFb activity to elongate, but rather to terminate, transcription properly (3, 4).

Recent studies have indicated that two major forms of P-TEFb are present in equivalent amounts in HeLa cell lysates (5). The active form consists of “core” P-TEFb, CDK9 and Cyclin T1 or T2. The inactive form consists of CDK9, Cyclin T1 or T2, MAQ1/HEXIM1, and 7SK RNA (5–8). The 7SK RNA is an abundant class III noncoding RNA (9) detected in vertebrates, cephalochordates, and mollusks. Binding of 7SK RNA to HEXIM1 turns this protein into a P-TEFb inhibitor (10). In response to treatments that arrest transcription (5–8) or following cardiac hypertrophic stimuli (11, 12), HEXIM1 and 7SK RNA dissociate from P-TEFb. As a consequence, the P-TEFb activity is up-regulated.

HEXIM1 expression is up-regulated in smooth muscle cells treated with hexamethylene-bisacetamide (13) or down-regulated by estrogen in breast cancer cells and therefore named EDG-1 (14). HEXIM1 has also been reported as a protein accumulating in heart tissues during early embryogenesis and therefore named CLP-1 (cardiac lineage protein) (15). Disruption of the HEXIM1/CLP1 gene results in heart defects leading to death at birth in homozygote CLP-1(−/−) mice (16). Because P-TEFb plays a general role in class II gene expression, such a late developmental defect suggests the existence of a compensatory mechanism at the cellular level. Indeed, a BLAST search within genome and Expressed Sequence Tag data bases suggests the existence of HEXIM2, a mammalian protein strongly related to HEXIM1 but smaller, 286 instead of 359 amino acids, and coded by a distinct gene in the same locus (7).

This work was initiated to investigate HEXIM2 characteristics. In agreement with observations published while it was in progress, it was found that HEXIM2 binding to P-TEFb and 7SK RNA is a transcription-dependent process (17, 18). Furthermore, HEXIM1 and HEXIM2 form stable homo- and hetero-oligomers. These findings were extended, mapping domains involved in HEXIM/HEXIM interactions. Hydrodynamic parameters and chemical cross-links of the HEXIM1 oligomers were used to investigate their stoichiometry. It was demonstrated that multiple P-TEFb units bind to a HEXIM1 multimer.

**MATERIALS AND METHODS**

Yeast Two-hybrid Assays—For two-hybrid assays, L40 yeast cells were co-transformed with LexA DNA binding domain and GAL4 activation domain fusion constructs derived from plasmids pBTM116 and pACTII, respectively (19). Transformants were double-selected on medium lacking both tryptophan and leucine. Quantitative β-galactosidase activities were determined from pools of more than 10 transformed yeast colonies.

* This work was supported by grants from the Association pour la Recherche sur le Cancer, Action Concertee et Incitative Biologie Moléculaire et Structurale, and Agence Nationale de Recherche on AIDS, Associazione Italiana per la Ricerca sul Cancro (to L. L.). 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.

‡ Both authors made equal contributions to this work.

**To whom correspondence should be addressed: Unite Mixte de Recherche 8541 CNRS, Ecole Normale Supérieure, Laboratoire de Régulation de l’Expression Génétique, 46 rue d’Ulm, 75230 Paris Cedex 05, France. Tel.: 33-1-4432-3410; Fax: 33-1-4432-3941; E-mail: bensoude@biologie.ens.fr.

1 The abbreviations used are: P-TEFb, positive transcription elongation factor; CTD, C-terminal domain; HA, hemagglutinin; GFP, green fluorescent protein; DST, diuccinimidyl tartrate; CDK9, cyclin-dependent kinase 9; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole.

2 B. Benecke, personal communication.
Plasmids—HEXIM2 cDNA was generated by reverse transcription PCR from HeLa cell total RNAs. Its sequence was identical to AK056894 (GenBank™). HEXIM2 cDNA was fused to an N-terminal FLAG tag by insertion into pAdRSV-FLAG3 (20). HEXIM2 cDNA was inserted into the EcoRI/BamHI sites of pBTM116 or the EcoRI/XhoI sites of pActII. The HEXIM2 sequence starts at methionine n°2. Deletion mutants were constructed by insertion of PCR-generated fragments of the corresponding HEXIM2 sequences into pAdRSV-FLAG3, pBTM116, and pActII. Full-length HEXIM1 and Cyclin T1 cDNAs were inserted into EcoRI/Sall cloning sites of pEGFP-C2 (Clontech). Full-length CDK9 cDNA was fused to an N-terminal 3XFLAG tag (21). PCR-generated HEXIM1 cDNA was inserted into the Ncol/Xhol sites of pET21d to generate HEX-His6. The HEX-(148–359)His6 and HEX-(162–359)His6 truncated proteins were generated by PCR; amino acids 148 and 162 in the HEXIM1 sequence were placed just after the start codon. The recombinant proteins, expressed in *Escherichia coli* Novablaue DE3, were purified as described (10). Other plasmids used in this work have been described previously (7, 10, 22). Cells, Drugs, Transfections, and Lysis—HeLa (MRL2 strain), G3H, GCM, or 3XFLAG-CDK9 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum. GCM, or 3XFLAG-CDK9 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum. G3H stably express HA-tagged Cyclin T1 (23). GCM cells are HeLa (MRL2) cells stably transfected with pEGFP-HEXIM1, 3XFLAG-CDK9 cells are H1299 cells stably transfected with 3XFLAG-CDK9. Cells were treated or not for 1 h with actinomycin D (1 µg/ml) or DRB (100 µM). For reversibility, after 1 h of DRB treatment, cells were allowed to recover for 1 h in fresh medium. UV irradiation (40 J/m²) was performed at 254 nm. Log-phase cells were used for all experiments. Cells were transfected following the standard calcium phosphate method with coding plasmid supplemented with a noncoding carrier plasmid added up to 10 μg/dish (25 cm²). Cells were transfected in ice-chilled buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM EDTA), supplemented with 1 mM dithiothreitol, 40 units/ml Actinomycin D at 4 °C. Transfections were performed after 5 min at 4 °C in 25 mM, pH 7.9, Hepes buffer supplemented with NaCl. Fractions of 240 µl (checked by weighing) were collected. For each particle, the Stokes radius (r) was determined from the elution parameter obtained with a noncovalent carrier plasmid added up to 10% of the total bed volume of the column provided by the supplier. The void volume, Vₗ, was determined from the elution volume of the total bed volume of the column provided by the supplier. The void volume, Vₗ, corresponded to elution of blue dextran 2000. Protein samples were ultracentrifuged at 38,000 rpm for 21 h at 4 °C on 5–35% glycerol gradients in buffer A supplemented with NaCl using a Beckman SW27 rotor. 240 µl fractions of 300 µl were collected. The sedimentation coefficients (S, Svedberg units) of HEXIM1 complexes were determined by comparison with those of well defined protein standards. By definition, molecules in fraction 1 have a sedimentation coefficient of zero. Proteins from column eluates and glycerol gradients were detected by Coomassie Blue staining or Western blot using low signals to avoid saturation effects. For a given protein, peak fraction determination was highly reproducible from one experiment to the other (within half a fraction). As a calibration control, β-galactosidase was included in all runs and detected by Western blot. The Amersham high molecular weight calibration kit contained aldolase, catalase, ferritin, and thyroglobulin. Bovine serum albumin and E. coli β-galactosidase were from Sigma.

**RESULTS**

Characterization of FLAG-HEXIM2—To investigate HEXIM2 properties, its cDNA was fused to a FLAG epitope and expressed in HeLa cells. The resulting FLAG-HEXIM2 protein was localized in the nucleus and excluded from nucleoli (Fig. 1). Upon deletion of the C-terminal sequences, the (2–286) protein remained nuclear, but the (2–118) and (2–168) proteins were perinuclear. In contrast, deletion of N-terminal sequences in the (84–286) protein did not affect the nuclear localization, whereas the (119–286) protein was spread out through the entire cell. As previously reported (7, 8), FLAG-HEXIM1 coimmunoprecipitated the Cyclin T1 subunit of P-TEFb as well as 7SK RNA in transient transfection assays (Fig. 2A, lane 5). The same observations were made when FLAG-HEXIM2 was used instead (lanes 5 and 6). Actinomycin D triggered the dissociation of the FLAG-HEXIM2/Cyclin T1 interaction (Fig. 2B, lane 5). DRB, another transcription inhibitor, also promoted the disruption of HEXIM2/P-TEFb interaction (lane 3). As previously observed with HEXIM1, DRB action was reversible (lane 4). UV irradiation also resulted in the HEXIM2/P-TEFb disruption (lanes 6 and 7). However, as previously reported for HEXIM1, the UV effect was not immediate. Importantly, equivalent amounts of HEXIM1 co-
immunoprecipitated with FLAG-HEXIM2 independently of transcription and P-TEFb association (lanes 2-7).

Thus, HEXIM1 and HEXIM2 show very similar behavior; both P-TEFb-HEXIM1 complexes are dissociated following transcriptional arrest. HEXIM2 also associates with HEXIM1, but this interaction is not affected by an arrest in transcription.

The HEXIM1 and HEXIM2 C-terminal Domains Interact with Each Other and with Cyclin T1 in a Two-hybrid Assay—To identify which P-TEFb subunit directly interacts with HEXIM2, a yeast two-hybrid study was performed. HEXIM1, HEXIM2, CDK9, and Cyclin T1 were fused to the LexA DNA binding or GAL4 activation domains. Robust growth on medium lacking histidine and elevated β-galactosidase activities were obtained with cells cotransformed with LexA-HEXIM2 and GAL4-Cyclin T1 or with LexA-Cyclin T1 and GAL4-HEXIM2 (Fig. 3A). Slightly stronger responses were obtained with HEXIM1 and Cyclin T1. In contrast, cells co-transformed with HEXIM2 and CDK9 fusions did not show increased β-galactosidase activity compared with negative controls. Thus, the HEXIM2 interaction with P-TEFb occurs through a direct contact with Cyclin T1. In two-hybrids, the HEXIM2/Cyclin T1 interaction did not require the presence of 7SK RNA (Fig. 3A) as previously described for HEXIM1 (7). Strong homotropic HEXIM2/HEXIM2 and HEXIM1/HEXIM1, as well as heterotropic HEXIM1/HEXIM2, interactions were also observed.

To map the HEXIM2 region required for interaction with HEXIM1, HEXIM2, and Cyclin T1, deletion mutants were analyzed. Like the full-length protein, both (84–286) and (119–286) N-terminal deletions interacted with Cyclin T1, HEXIM1, and HEXIM2 (Fig. 3B). In contrast, none of the HEXIM2 mutants with C-terminal domain deletions (mutants (2–118), (2–168), and (2–226)) interacted with HEXIM1, HEXIM2, or Cyclin T1. Thus, sequences in the C-terminal half of HEXIM2 are involved in the interaction with HEXIM1, HEXIM2, and Cyclin T1.

HEXIM1 deletion mutants were probed next for self-association. The HEXIM1/HEXIM1 interaction was maintained for the HEXIM1 N-terminal domain deletions (120–359) and (181–359) (sequence homologous to the (84–286) and (119–286) HEXIM2 mutants, respectively) (Fig. 3C). Furthermore, the HEXIM1 N-terminal domain mutant (181–359) fused to LexA interacted with the same mutant (181–359) fused to the GAL4 activation domain. However, this mutant did not interact with the C-terminal truncations (1–180) and (1–240) (sequence equivalents to the (1–118) and (1–168) HEXIM2 mutants, respectively).

These experiments suggest a direct interaction of the HEXIM2 C-terminal domain with Cyclin T1 as previously reported for HEXIM1 (7). Homo- and hetero-contacts between HEXIM1 and HEXIM2 proteins appear to involve their homologous C-terminal domains.

**Transcription-independent HEXIM/HEXIM Interactions in HeLa cells**—To validate the two-hybrid analysis, we next investigated the coimmunoprecipitation of endogenous HEXIM1 with transfected truncated HEXIM2 mutants in HeLa cells. Truncation of the HEXIM2 83 N-terminal amino acids did not affect the capacity to immunoprecipitate the Cyclin T1 subunit of P-TEFb (Fig. 4A, lane 7). However, the coimmunoprecipitation was suppressed for the truncated mutants (2–118) (lane 5) and (119–286) (lane 9). Anti-FLAG antibodies also retained large amounts of HEXIM1 when the cells were transfected with full-length, N-terminal-deleted (84–286) or (119–286) HEXIM2 (lanes 3, 7, and 9). Although HEXIM2-(119–286) interacted with Cyclin T1 in the two-hybrid test, it did not associate P-TEFb as found for its HEXIM1-(181–359) homolog, possibly because both have lost their basic RNA binding domain (10). No HEXIM1 was retained when the C-terminal domain was deleted in mutants (2–118), (2–168), and (2–226) (lane 5 and data not shown). When actinomycin D was added to the culture medium prior to lysis, no Cyclin T1 was retained by anti-FLAG antibodies. In contrast, the amount of HEXIM1 retained was not affected by actinomycin D treatment (lanes 4, 8, and 10).

The coimmunoprecipitation of endogenous HEXIM1 with transfected truncated HEXIM1 mutants was investigated next in transfected HeLa cells (Fig. 4B). The full-length endogenous protein (H) co-migrated with the transfected one (1–359) and could not therefore be distinguished (lanes 3 and 4). The N-terminal truncated HEXIM1-(120–359) (a) and -(181–359) (b) were equivalent to HEXIM2-(84–286) and -(119–286), respectively. They were detected with the C4 antibody that is directed against the last 15 amino acids of HEXIM1. The C-terminal truncated HEXIM1-(1–240) (c) and -(1–180) (d) were equivalent to HEXIM2-(2–119) and -(2–118), respectively. They were detected with the N2 antibody that is directed against the first 15 amino acids of HEXIM1. The C-terminal truncated HEXIM1-(1–359) (e) and -(1–226) (f) were equivalent to HEXIM2-(2–119) and -(2–118), respectively. They were detected with the N2 antibody that is directed against the first 15 amino acids of HEXIM1. In contrast to the C-terminal truncations (lanes 5–8), both the (120–359) and (181–359) HEXIM1 deletion mutants communoprecipitated endogenous HEXIM1 (lanes 9–12). However, the (120–359) and the (181–359) HEXIM1 differed in that the latter did not bind P-TEFb (7).

Thus HEXIM1 and HEXIM2 show very similar behavior; their interaction with P-TEFb is disrupted by actinomycin D treatment and requires their basic domains. In contrast, the
HEXIM1/HEXIM2 coimmunoprecipitation is not affected by actinomycin D treatment and does not require the presence of the basic domain.

Multiple HEXIM1 Molecules in P-TEFb/7SK RNA Complexes—HEXIM1 cDNA was fused to GFP, and a cell line stably expressing the fusion protein was established from HeLa cells (GCM cells). The fusion protein was nuclear and excluded from nucleoli like the endogenous protein (not shown). Western blot analysis with anti-HEXIM1 antibodies (Fig. 5A) or anti-GFP antibodies (not shown) revealed that lysates from GCM cells express a GFP-HEXIM1 fusion protein that migrates as a 90-kDa polypeptide, whereas endogenous HEXIM1 migrates with an apparent molecular mass of 65 kDa. The capacity of GFP-HEXIM1 to interact with P-TEFb was investigated by immunoprecipitation. Anti-GFP antibodies retained CDK9 and Cyclin T1 from GCM cell extracts (Fig. 5A, lane 6). Coimmunoprecipitation of CDK9 and Cyclin T1 with GFP-HEXIM1 was suppressed when RNase was added to the extracts (lane 7) or when the cells were treated with actinomycin D prior to lysis (lane 8). The anti-GFP antibodies also retained endogenous HEXIM1 from GCM lysates but not from untransfected HeLa cell lysates. Thus, GFP-HEXIM1 and HEXIM1 molecules coexist within the same complexes. This association was affected by neither RNase nor actinomycin D treatments (lanes 7 and 8) in contrast to P-TEFb association to HEXIM1.

HEXIM1 can be fractionated by ultracentrifugation on glycerol gradients into two complexes (7). The large one also comprises CDK9, Cyclin T1 or T2, and 7SK RNA. Because the same HEXIM1 domain interacts with other HEXIM molecules and Cyclin T1, the formation of P-TEFb/7SK RNA complexes might involve the disruption of HEXIM/HEXIM interactions. To address this possibility, lysates from GCM cells were loaded on a glycerol gradient, and both the HEXIM1 and the GFP-HEXIM1 proteins were separated into "small" (frac-
ions 2–4) and “large” (fractions 7 and 8) complexes (Fig. 5B). Buffer A without salt addition was used for cell lysis and gradient to optimize the separation of both complexes. Under these conditions, all soluble CDK9 and Cyclin T1 are bound to HEXIM/7SK. The small GFP-HEXIM1 complexes peaked at a slightly higher molecular mass than small endogenous HEXIM1 complexes. The large GFP-HEXIM1 and HEXIM1 complexes comigrated with Cyclin T1 and CDK9 and thus likely corresponded to GFP-HEXIM1 and HEXIM1 bound to P-TEFb. Indeed, anti-GFP beads retained Cyclin T1 and CDK9 from fraction 7 (Fig. 5C). Furthermore, anti-GFP antibodies retained endogenous HEXIM1 from both small (lane 3) and large (lane 7) GFP-HEXIM1 complex fractions. This observation, combined with the lack of RNase and actinomycin D sensitivity, suggests that HEXIM1 behaves as an oligomeric module independently of its interaction with P-TEFb.

**HEXIM1 Complexes Are Anisotropic**—To establish the stoichiometry of HEXIM1 molecules, the molecular mass of the small HEXIM1 complexes was determined. A HeLa cell extract was loaded on glycerol gradients and on gel-permeation col-

---

*V. T. Nguyen, unpublished results.*

---

**Fig. 4.** HEXIM1 and HEXIM2 oligomerize and associate with P-TEFb through their C-terminal domains in HeLa cells. *A,* HeLa cells transfected with carrier plasmid (control), full-length (2–286), or truncated FLAG-tagged HEXIM2 proteins. Inputs and anti-FLAG beads were probed by Western blot. *B,* HeLa cells transfected with carrier plasmid (control), full-length (1–359), or truncated FLAG-tagged HEXIM1 proteins. FLAG proteins were immunoprecipitated from lysates of transfected cells treated (+) or not (−) with actinomycin D (ActD). Anti-FLAG beads were probed by Western blot. The C4 antibody is directed against an HEXIM1 C-terminal peptide, whereas the N2 antibody is directed against an HEXIM1 N-terminal peptide. H designates the full-length HEXIM1 protein; a, b, c, and d mark truncated HEXIM1 proteins. pSP64 was used as a control empty plasmid.

**Fig. 5.** Endogenous HEXIM1 coimmunoprecipitates with GFP-HEXIM1. *A,* lysates from HeLa (lanes 1 and 5) and GCM (lanes 2–4, 6–8) cells treated (+) or not (−) with actinomycin D (ActD) were immunoprecipitated with anti-GFP beads. Inputs (lanes 1–4) and beads (lanes 5–8) were treated (+) or not (−) with RNase and analyzed by Western blot. B, a GCM cell lysate in buffer A was fractionated on a 5–35% glycerol gradient. C, gradient fractions 3, 6, and 7 were immunoprecipitated with anti-GFP beads and probed by Western blot for CDK9, Cyclin T1, and HEXIM1. Both endogenous HEXIM1 and GFP-HEXIM1 were detected with the C4 anti-HEXIM antibody.
FIG. 6. **Molecular mass and anisotropy of HEXIM1 particles.** Protein standards, purified full-length (HEX-His$_6$) or truncated HEX-(148–359)-His$_6$ recombinant HEXIM1 protein or cellular HEXIM1 (HEX) protein from a HeLa lysate was fractionated on 5–35% glycerol gradients run for 21 or 49 h as indicated (A) or on a Superose 6 gel-permeation column (B). The membranes were stained with Coomassie Blue before Western blot detection with anti-HEXIM1 and anti-β-galactosidase antibodies. Peaks for protein standards as well as for small (S) and large (L) HEXIM1 complexes are indicated. To check the reproducibility and to compare different gradients or columns, β-galactosidase or bovine serum albumin were included in the samples to be fractionated. C, molecular mass (kDa) of protein standards plotted versus $S^*Rs$ product. D, the sedimentation coefficient of protein standards is plotted versus the gradient fraction number in which each standard peak (closed circles/solid line, 21-h gradient; open circles/dashed line, 49-h gradient). The sedimentation coefficients ($S$) of HEXIM1 complexes are determined from the corresponding gradient fraction number. E, the Stokes radii ($Rs$) of protein standards plotted versus the elution parameter, $K_{av}$. The $Rs$ of cellular HEXIM1 and recombinant HEX-His$_6$ complexes were determined from their corresponding $K_{av}$. Parameter determinations for HEXIM1 and P-TEFb-HEXIM1-7SK RNA complexes are underlined by arrows and dotted lines. Bovine serum albumin (1) (molecular mass 67 kDa, $S$ 4.6, $Rs$ 3.55 nm); aldolase (2) (molecular mass 158 kDa, $S$ 7.3, $Rs$ 4.81 nm); catalase (3) (molecular mass 232 kDa, $S$ 11.3, $Rs$ 5.22 nm); ferritin (4) (molecular mass 440 kDa, $Rs$ 6.1 nm); β-galactosidase (5) (molecular mass 454 kDa, $S$ 15.9, $Rs$ 6.9 nm); thyroglobulin (6) (molecular mass 669 kDa, $S$ 19, $Rs$ 8.5 nm); type IgM immunoglobulin (7) (molecular mass 950 kDa, $S$ 19, $Rs$ 12.5 nm); ovalbumin (8) (molecular mass 43 kDa, $S$ 3.5, $Rs$ 3.05 nm); chymotrypsinogen A (9) (molecular mass 25 kDa, $S$ 2.6, $Rs$ 2.09 nm); and ribonuclease A (10) (molecular mass 13.7 kDa, $S$ 2.0, $Rs$ 1.64 nm) were used as protein standards.
amino acid sequences of truncated HEXIM1-(148–359)His6 was evaluated. In an attempt to discriminate between trimers and dimers, proteins were cross-linked.

**Sizing the HEXIM1 Particles**—The parameter obtained by multiplication of the sedimentation coefficient with the Stokes radius \( (S^* R_S) \) is proportional to the molecular mass of the particle and is not affected by its shape (24). Indeed, according to published hydrodynamic parameters for a collection of protein standards, the native molecular mass of proteins verified the relation \( M_w = 4 (S^* R_S) \) over two orders of magnitude (Fig. 6C).

Sedimentation coefficients of HEXIM1 complexes were determined by comparison with protein standards on glycerol gradients (Fig. 6, A and D). Ultracentrifugation during 49 h was optimal to determine sedimentation coefficients of HEXIM1 not bound to P-TEFb-7SK. Under these conditions, ovalbumin (3.5 S) and bovine serum albumin (4.55 S) sedimented in fractions 10.75 ± 0.25 and 13.5 ± 0.25, respectively. Cellular HEXIM1 from HeLa cells was concentrated in only two fractions and showed a sedimentation coefficient of 4.2 ± 0.15 S. Recombinant HEXIM1 peaked in fraction 12.5 ± 0.25 corresponding to a sedimentation coefficient of 4.3 ± 0.15 S. However, a significant amount of this protein was dispersed in upper and lower fractions.

Stokes radii were determined by comparison with elution of protein standards from a gel-permeation column (Fig. 6, B and E). Cellular HEXIM1 eluted like β-galactosidase (6.9 ± 0.5 nm). Hence, its \( S^* R_S \) value corresponded to a protein of 116 ± 15 kDa molecular mass. In contrast, the elution profile of the full-length recombinant HEXIM1 was much more dispersed (6.25 ± 1.5 nm), overlapping β-galactosidase (6.9 nm) and catalase (5.2 nm). Hence, its \( S^* R_S \) value corresponded to a 95 ± 35-kDa particle.

Because sequences N-terminal to the basic domain appeared not to be involved in oligomerization, a purified recombinant truncated protein HEXIM1-(148–359)His6 was investigated. From its Stokes radius (3.55 ± 0.2 nm) and sedimentation coefficient (3.4 ± 0.3 S), a molecular mass of 48 ± 5 kDa was evaluated.

Molecular masses of 26 and 41 kDa are predicted from the amino acid sequences of truncated HEXIM1-(148–359)His6 and full-length HEXIM1, respectively. Hence, the hydrodynamic parameters suggested that truncated HEXIM1-(148–359)His6 forms dimers. The same method suggested that cellular HEXIM1 protein forms trimers or associates with other macromolecular partners, but the full-length recombinant HEXIM1 protein behaves like a mixture of dimers and trimers.

**Covalent HEXIM1 Cross-linking**—In an attempt to discriminate between trimers and dimers, proteins were cross-linked. Addition of DST to truncated HEXIM1-(148–359)His6 and HEXIM1-(162–359)His6 proteins generated two new bands in less than 30 s (Fig. 7B, lanes 2, 3, 5, and 6). Mobilities of the

Fig. 7. Cross-linking of HEXIM1 proteins. A, purified recombinant HEXIM1 (HEX-His6) or HeLa cell extracts (HEXIM1, HeLa) were rapidly mixed with DST at indicated final concentrations from 0.1 to 5 mM. The reaction was allowed to proceed for 5 min at 4 °C. B, truncated (148–359) and (162–359) recombinant HEXIM1 proteins were allowed to react with 0.1 mM DST at indicated final concentrations from 0.1 to 5 mM. The reactions were allowed to proceed for 0.5 or 5 min (min.) and arrested with glycine. Panels A and C were separated on 6% SDS-PAGE; 12% SDS-PAGE was used in panel B. The membranes were stained with Ponceau red before (panel A, lane 9) Western blot detection with N2 anti-HEXIM1 (A and C) or anti-polyhistidine (B). The positions of non-stained molecular mass standards (SDS-6H; Sigma) are indicated.
cross-linked products were, as expected for dimers, two times less than those of uncross-linked monomers. The doublet nature of the DST cross-linked species might correspond to distinct isoforms.

When purified full-length recombinant HEXIM1 was allowed to react with DST, a single new band was generated that migrated like a 160-kDa protein in 6% SDS-polyacrylamide gels (Fig. 7A, lanes 6–8). The mobility of the cross-linked species was therefore intermediate, between two and three times less than the monomer. However, if HEXIM1 forms trimers, one might expect to detect a cross-linked dimer species as well as a cross-linked trimer. A single species was observed in agreement with the dimer hypothesis. Addition of DST to HeLa cell lysates also generated a single cellular HEXIM1 cross-linked product (Fig. 7A, lanes 2–4) that comigrated exactly like the one generated on the full-length recombinant HEXIM1 protein.

When DST was added to GCM cell lysates, three additional bands were generated (Fig. 7C, lanes 5 and 6). GCM cells expressed GFP-HEXIM1 in addition to endogenous HEXIM1. If the HEXIM1 particles were dimers, three cross-linked species should be generated: GFP-HEXIM1 homodimers, HEXIM1 homodimers, and GFP-HEXIM1/HEXIM1 heterodimers. If HEXIM1 particles were trimers, four cross-linked species should be generated. Hence, the cross-link characteristics of both cellular and recombinant HEXIM1 support dimerization.

**Association of Multiple Cyclin T1 Molecules in P-TEFb-HEXIM1/7SK Complexes Involves Sequences N-terminal to the TRM Motif**—Because Cyclin T1 directly interacts with HEXIM1 proteins, the possibility that multiple Cyclin T1 molecules bind to the oligomeric HEXIM module was addressed by testing the immunoprecipitation of endogenous P-TEFb subunits by their tagged counterparts. HeLa cells were transiently transfected with GFP-Cyclin T1, and anti-Cyclin T1 antibodies recognized an additional band migrating slower than endogenous Cyclin T1 with an apparent molecular mass of 110 kDa as expected for GFP-Cyclin T1 (Fig. 8A, compare lanes 1 and 2). In agreement with previous studies using Cyclin T1 fused to other tags, CDK9 and HEXIM1 were retained on anti-GFP beads (compare lanes 5 and 6). Furthermore, in contrast to CDK9, HEXIM1 was not retained when cells were treated with an inhibitor of transcription or when RNase was added to the lysates (lanes 7 and 8). Remarkably, endogenous Cyclin T1 was also retained on anti-GFP beads (lane 6) unless the cells had been treated with actinomycin D or RNase had been added to the lysate (lanes 7 and 8).

To characterize the Cyclin T1 domains required for coimmunoprecipitation of transfected and endogenous Cyclin T1 proteins, HeLa cells were transfected with HA epitope tagged to the N terminus of truncated Cyclin T1. The Cyclin T1 sequence between amino acids 250 and 260 has previously been proposed to be involved in human immunodeficiency virus TAR RNA and Tat protein recognition (TRM motif) (25). In an attempt to test the connection between HEXIM1 binding and endogenous Cyclin T1 binding to truncated Cyclin T1, the TRM was gradually trimmed off in (1–333), (1–254), and (1–248) deletion mutants. All three mutants coimmunoprecipitated HEXIM1 (Fig. 8B, lanes 3, 5, and 7). All three mutants also coimmunoprecipitated the endogenous Cyclin T1. Both coimmunoprecipitations were abolished by RNase treatment. Noteworthy, when the FLAG epitope immediately follows C-terminal of the TRM motif, the Cyclin T1-(1–254) mutant does not appear to bind HEXIM1 (8). Overall, the evolutionary conserved “cyclin T box” is sufficient to associate with HEXIM1 and endogenous Cyclin T1 in transcription- and RNA-dependent complexes.

**Multiple Cyclin T and CDK9 Are Present in P-TEFb-HEXIM1/7SK RNA Complexes**—Because both Cyclin T1 or Cyclin T2 interact with HEXIM1, the existence of complexes containing both cyclins was investigated with HA-tagged Cyclin T2. When a HA-tagged Cyclin T2b expression vector was used, both Cyclin T2a and T2b proteins were expressed due to alternative splicing of the T2b pre-mRNA (26) (Fig. 8C, lanes 3 and 4). Anti-HA beads retained endogenous Cyclin T1 from transfected cells (lane 7), but coimmunoprecipitation was no more observed following actinomycin D treatment (lane 8).

The CDK9 CDNA was fused to a triple FLAG sequence, and a stably transfected cell line expressing 3XFLAG-CDK9 was generated from H1299 cells. The FLAG protein detected with an anti-CDK9 antibody migrated slower than endogenous...
CDK9 (Fig. 9A, compare lanes 1 and 2). Anti-FLAG beads immunoprecipitated CDK9 as well as Cyclin T1 and HEXIM1 (lane 6). However, neither CDK9 nor HEXIM1 was retained when lysates were treated with RNase or when cells were treated with actinomycin D (lanes 7 and 8).

The lysates were next fractionated by ultracentrifugation prior to immunoprecipitation. Anti-FLAG beads retained endogenous CDK9 from gradient fractions that contained P-TEFb-HEXIM1-7SK complexes (Fig. 9B, lane 6), but not from gradient fractions that only contained core P-TEFb (lane 3).

Taken together, these data demonstrate that multiple molecules of both CDK9 and Cyclin T1 are present in the transcription- and RNA-dependent P-TEFb-HEXIM1-7SK complexes.

**Stoichiometry of the P-TEFb-HEXIM1-7SK RNA Complex** — The large P-TEFb-HEXIM1 complex sedimented slower than β-galactosidase (460 kDa) in a glycerol gradient (Fig. 6, A and D). However, it eluted from the Superose 6 column as a particle close to type M immunoglobulins (950 kDa) (Fig. 6, B and E). Thus, P-TEFb-HEXIM1-7SK RNA complex is less anisotropic than “free” HEXIM1 complexes. The $R_g$ product suggested a molecular mass of 685 ± 80 kDa for P-TEFb-HEXIM1-7SK RNA complexes.

To evaluate CDK9, Cyclin T1, and HEXIM1 molar ratios in large P-TEFb-HEXIM1 complexes, we turned back to the autoradiograms of previously published experiments (7). The P-TEFb-HEXIM1-7SK complexes were immunoprecipitated from lysates of cells metabolically labeled with [35S]methionine and [35S]cysteine. To eliminate interference due to core P-TEFb, the P-TEFb-HEXIM1-7SK complex was fractionated on glycerol gradient. The immunoprecipitates were subjected to SDS-PAGE and autoradiography (Fig. 10, lanes 1 and 2). Peaks corresponding to each protein were quantified. The ratio of the counts divided by the total number of sulfur-amino acids in Cyclin T1, HEXIM1, and CDK9 was equal to 80 ± 4 for all three proteins. This indicates a 1/1/1 molar ratio and is supported by the Coomassie Blue staining (lane 3). From amino acid sequences, a 165-kDa molecular mass is predicted for such a unit. A 685-kDa molecular mass would be consistent with three Cyclin T1/CDK9/HEXIM1 units (495 kDa) and a single 7SK RNA (110 kDa) or two Cyclin T1/CDK9/HEXIM1 units (330 kDa) and two 7SK RNA (220 kDa). In conclusion, a multimeric HEXIM1 module inactivates multiple P-TEFb units forming a P-TEFb-HEXIM1-7SK complex.

**DISCUSSION**

In agreement with recently published data (17, 18), the properties of HEXIM2 were found to be very similar to HEXIM1. HEXIM2 associates with P-TEFb in a transcription-dependent manner. Furthermore, HEXIM1 and HEXIM2 form stable homo- and heteromultimers. The multimeric status of HEXIM1 is maintained in P-TEFb-HEXIM1-7SK RNA complexes. We now report that multiple Cyclin T1 or T2 and CDK9 molecules associate with the HEXIM1 multimer in the inactive P-TEFb-HEXIM1-7SK complexes. We therefore propose to describe these complexes as an assembly of P-TEFb modules on a multimeric HEXIM module.

**Functional Similarities between HEXIM1 and HEXIM2 Proteins** — Our observations further underline the functional similarity between HEXIM1 and HEXIM2 proteins. The sequences N-terminal to the basic RNA binding domain (amino acids 150–163 for HEXIM1 or amino acids 89–101 for HEXIM2) are unrelated (7). However, these sequences are dispensable for both proteins to associate P-TEFb and 7SK RNA and can be deleted without visible phenotype. The HEXIM2 amino acids...
84–119 sequence is involved in nuclear localization and contains a sequence (100–116) homologous to the previously identified HEXIM1 amino acids 159–177 bipartite nuclear localization sequence (7, 8, 27).

Sequences C-terminal to the nuclear localization sequence are highly homologous and required for Cyclin T binding. Yeast two-hybrid mapping is less stringent than transfection tests in HeLa cells. In the latter case, the basic RNA binding domain is also required (this work and Ref. 10). HEXIM1 and HEXIM2 self-binding also involves sequences in their C-terminal half but requires neither the basic RNA binding domain nor the nuclear localization sequence.

Data from the laboratories of Price (18) and Zhou (17) show that the ratio of free HEXIM2 to P-TEFb-HEXIM2-7SK is much larger than the ratio of free HEXIM1 to P-TEFb-HEXIM1-7SK. HEXIM2 appears to bind P-TEFb complexes essentially when HEXIM1 is eliminated by small interfering RNA. The two-hybrid data may suggest that HEXIM2/Cyclin T1 interactions are weaker than HEXIM1/Cyclin T1 ones (Fig. 3A).

**Modular Structure of P-TEFb-HEXIM1-7SK RNA Complexes—**In agreement with others (17, 18), we find that multiple HEXIM1 and HEXIM2 molecules are present in the same complexes. Unlike the P-TEFb-HEXIM-7SK complexes, the HEXIM-HEXIM complexes are stable; they are neither affected by an arrest in transcription nor disrupted in vitro by treatments such as RNase or salts. HEXIM1-HEXIM1 complexes are present both as free HEXIM1 and ”complexed” HEXIM1 bound to P-TEFb. It is therefore appropriate to refer to them as a module. Similarly, the CDK9-Cyclin T complex is stable and could be referred as the P-TEFb module. Finally, one has to take into account 7SK RNA. 7SK RNA is not degraded when it dissociates from HEXIM proteins. The properties of the resulting free 7SK RNA remain unclear. Proteins of the free 7SK small nuclear ribonucleoprotein have not been described yet. Nevertheless, one must think of 7SK RNA exchanging with HEXIM to form P-TEFb-HEXIM7SK complexes.

**A Highly Anisotropic HEXIM Module—**Both hydrodynamic parameters and chemical cross-links supported dimerization for truncated recombinant HEXIM1 proteins deleted of their N-terminal domain. Analyzing the shape of isothermal titration calorimetry curves of HEXIM1 C-terminal peptides, the Geyer laboratory recently proposed that HEXIM1 forms dimers (28). A similar conclusion was reached by the Price laboratory (29) as a single hetero-oligomeric species was observed when two HEXIM1 proteins of different length were coexpressed in two-hybrid mapping is less stringent than transfection tests in HeLa cells. In contrast, the multimerization state of the recombinant full-length protein does not appear so well defined. The hydrodynamic parameters suggest a mixture of dimers and trimers, but chemical cross-linking supports dimerization. In the case of cellular HEXIM1, cross-linking also supported dimerization but hydrodynamic parameters suggested that it forms trimers or binds another cellular factor. The discrepancies between stoichiometries obtained using distinct methodologies might possibly be due to a dynamic exchange between dimers and trimers.

In any case, the cellular HEXIM1 particle shows a Stokes radius close to 7 nm, whereas a spherical dimer (82 kDa) or a spherical trimer (125 kDa) is expected to have a radius close to 4 and 4.5 nm, respectively. HEXIM1 thus forms extremely anisotropic particles as illustrated by the fact that it seems smaller than bovine serum albumin (67 kDa) in glycerol gradients and close to β-galactosidase (469 kDa) in Superose columns.

**Multiple P-TEFb Modules in P-TEFb-HEXIM1-7SK RNA Complexes—**The multimeric nature of HEXIM1 is maintained in P-TEFb-HEXIM1-7SK RNA complexes. Yeast two-hybrid analysis and glutathione S-transferase pulldown assays suggest that the evolutionary conserved C-terminal domain of HEXIM1 and HEXIM2 proteins directly interacts with the "cyclin box" in Cyclin T1 or T2 (this work and Refs. 7 and 28). We now show that multiple Cyclin T subunits and multiple CDK9 subunits coexist in P-TEFb-HEXIM-7SK RNA complexes. This finding adds a supplementary level to the originality of the HEXIM1 inhibition of CDK9. To our knowledge, no other Cyclin-dependent kinase has been shown to be inhibited in such a way.

**Acknowledgments—**We thank Fernando Borrega and Mia Edwards for help with experiments, Jean Massoulie and Xavier Darzacq for discussions, and David Price for communicating unpublished data.

**REFERENCES**

1. Price, D. H. (2000) *Cell Biol. Biochim.* 28, 529–544.
2. Garriga, J., and Grana, X. (2004) *Gene* 337, 15–23.
3. Medlin, J. E., Uguen, P., Taylor, A., Bentley, D. L., and Murphy, S. (2003) *EMBO J.* 22, 923–934.
4. Ni, Z., Schwartz, B. E., Werner, J., Suarez, J. R., and Lis, J. T. (2004) *Cell* 13, 55–65.
5. Nguyen, V. T., Kiss, T., Michels, A. A., and Bensaude, O. (2001) *Nature* 414, 322–325.
6. Yang, Z., Zhu, Q., Luo, K., and Zhou, Q. (2001) *Nature* 414, 317–322.
7. Michels, A. A., Nguyen, V. T., Fraldi, A., Labas, V., Edwards, M., Bonnet, F., Lania, L., and Bensaude, O. (2003) *EMBO J.* 22, 4599–4609.
8. Yik, J. H., Chen, R., Nishimura, R., Jennings, J. L., Link, A. J., and Zhou, Q. (2003) *Mol. Cell* 12, 971–982.
9. Zieve, G. and Penman, S. (1976) *Cell* 8, 19–31.
10. Michels, A. A., Fraldi, A., Li, Q., Adamson, T. E., Bonnet, F., Nguyen, V. T., Sedore, S. C., Price, J. P., Price, D. H., Lania, L., and Bensaude, O. (2004) *EMBO J.* 23, 2686–2691.
11. Sano, M., Abdelatif, M., Oh, H., Xie, M., Bagella, L., Giordano, A., Michael, L. H., DeMayo, F. J., and Schneider, M. D. (2002) *Nat. Med.* 8, 1310–1317.
12. Sano, M., Wang, S. C., Shirai, M., Scaglia, F., Xie, M., Sakai, S., Tanaka, T., Kulkarni, P. A., Barger, P. M., Youker, K. A., Tufte, G. E., Hamanori, Y., Michael, L. H., Cragen, W. J., and Schneider, M. D. (2004) *EMBO J.* 23, 3559–3569.
13. Kusuhara, M., Nagasaki, K., Kimura, K., Maase, N., Munabe, T., Ishikawa, S., Aikawa, M., Miyasaka, K., and Yamasugi, K. (1999) *Biochem. Biophys. Acta* *237, 273–279.
14. Wittmann, B. M., Wang, N., and Montano, M. M. (2003) *Cancer Res.* 63, 5151–5158.
15. Huang, F., Wagner, M., and Siddiqui, M. A. (2002) *Gene* 292, 245–259.
16. Huang, F., Wagner, M., and Siddiqui, M. A. (2004) *Mech. Dev.* 121, 559–572.
17. Yik, J. H., Chen, R., Pezda, A. C., and Zhou, Q. (2005) *J. Biol. Chem.* 280, 16360–16367.
18. Byers, S. A., Price, J. P., Cooper, J. J., Li, Q., and Price, D. H. (2005) *J. Biol. Chem.* 280, 16360–16367.
19. Bartel, P. L., and Fields, S. (1995) *Methods Enzymol.* 254, 241–263.
20. Gudzielich, P., Taillebourg, E., Charnay, P., and Gilardi-Hebenstreit, P. (2001) *EMBO J.* 20, 925–934.
21. O’Keeffe, B., Fong, Y., Chen, D., Zhou, S., and Zhou, Q. (2000) *EMBO J.* 19, 31–39.
22. Fraldi, A., Licciardo, P., Majello, B., Giordano, A., and Lania, L. (2001) *Mol. Cell Biol.* 21, 5499–5509.
23. Kusuhara, M., Nagasaki, K., Kimura, K., Maase, N., Munabe, T., Ishikawa, S., Aikawa, M., Miyasaka, K., and Yamasugi, K. (1999) *Biochem. Biophys. Acta* *237, 273–279.
24. Wittmann, B. M., Wang, N., and Montano, M. M. (2003) *Cancer Res.* 63, 5151–5158.
25. Fritz, A., Liciardio, P., Majello, B., Giordano, A., and Lania, L. (2001) *J. Cell. Biol. Biochim.* 36, 247–253.
26. O’Keeffe, B., Fong, Y., Chen, D., Zhou, S., and Zhou, Q. (2000) *J. Biol. Chem.* 275, 273–279.
27. Siegel, I. M., and Monty, K. J. (1966) *Biochem. Biophys. Acta* *112, 346–362.
28. Garber, M. E., Wei, P., KewalRamani, V. N., Mayall, T. P., Herrmann, C. H., Rice, A. P., Littman, D. R., and Jones, K. A. (1998) *Genes Dev.* 12, 3512–3527.
26. Peng, J., Zhu, Y., Milton, J. T., and Price, D. H. (1998) Genes Dev. 12, 755–762
27. Ouchida, R., Kusuhara, M., Shimizu, N., Hisada, T., Makino, Y., Morimoto, C., Handa, H., Ohsuzu, F., and Tanaka, H. (2003) Genes Cells 8, 95–107
28. Schulte, A., Czudnochowski, N., Barboric, M., Schönichen, A., Blazek, D., Peterlin, B. M., and Geyer, M. (2005) J. Biol. Chem. 280, 24968–24977
29. Li, Q., Price, J. P., Byers, S. A., Cheng, D., Peng, J., and Price, D. H. (2005) J. Biol. Chem. 280, 28819–28826
30. Shimizu, N., Ouchida, R., Yoshikawa, N., Hisada, T., Watanabe, H., Okamoto, K., Kusuhara, M., Handa, H., Morimoto, C., and Tanaka, H. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 8555–8560
31. Wittmann, B. M., Fujinaga, K., Deng, H., Ogba, N., and Montano, M. M. (2005) Oncogene 24, in press