

Oligomerization of HEXIM1 via 7SK snRNA and coiled-coil region directs the inhibition of P-TEFb

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ABSTRACT

Transcriptional elongation of most eukaryotic genes by RNA polymerase II requires the kinase activity of the positive transcription elongation factor b (P-TEFb). The catalytically active P-TEFb complex becomes inactive when sequestered into the large complex by the cooperative actions of 7SK snRNA and HEXIM1. In this study, we report that HEXIM1 forms oligomers in cells. This oligomerization is mediated by its predicted coiled-coil region in the C-terminal domain and 7SK snRNA that binds a basic region within the central part of HEXIM1. Alanine-mutagenesis of evolutionary conserved leucines in the coiled-coil region and the digestion of 7SK snRNA by RNase A treatment prevent this oligomerization. Importantly, mutations of the N-terminal part of the coiled-coil region abrogate the ability of HEXIM1 to bind and inhibit P-TEFb. Finally, the formation of HEXIM1 oligomers via the C-terminal part of the coiled-coil or basic regions is critical for the inhibition of transcription. Our results suggest that two independent regions in HEXIM1 form oligomers to incorporate P-TEFb into the large complex and determine the inhibition of transcriptional elongation.

INTRODUCTION

Transcription of eukaryotic protein-coding genes by RNA polymerase II (RNAPII) is tightly regulated at numerous levels, which include the assembly of preinitiation complexes, transcription initiation, promoter clearance, pausing, elongation and termination (1). Studies of the regulation of the

human immunodeficiency virus (HIV) gene expression have uncovered primarily the mechanisms that govern transcription pausing and elongation (2). The transition to productive elongation requires the positive transcription elongation factor b (P-TEFb), which consists of heterodimers between a catalytic subunit, the cyclin-dependent kinase 9 (Cdk9) and one of the four C-type cyclin regulatory subunits, CycT1, CycT2a, CycT2b or CycK (3). Upon its recruitment to the paused RNAPII, P-TEFb phosphorylates serines at position 2 in the C-terminal domain of the Rpb1 subunit of RNAPII, and components of the negative transcription elongation factor (N-TEF), which inactivates N-TEF and facilitates pre-mRNA processing (1–4). P-TEFb is critical not only for productive HIV gene expression, but is a general transcription factor that is recruited to several cellular promoters and is required for proper gene expression of most protein-coding genes in human cells (5,6).

Recently, important aspects of the regulation of P-TEFb have been revealed (2). Notably, P-TEFb exists in two major forms in cells. The small complex (SC) is a heterodimeric complex and is catalytically active. In contrast, the large complex (LC) is inactive and contains 7SK small nuclear RNA (snRNA) and HEXIM1 or HEXIM2 (7–13). The inhibition of P-TEFb is achieved by the cooperative actions of 7SK snRNA and HEXIM1 or HEXIM2 (8,13). The binding between the first 175 nt of 7SK snRNA and the evolutionary conserved basic region (BR) in HEXIM1 via its KHRR motif leads to the interaction between the C- and N-terminal regions of HEXIM1 and CycT1 or CycT2, respectively, resulting in the inactivation of P-TEFb (8,9). The assembly of P-TEFb into the LC can be prevented by disrupting the conserved PYNT motif in HEXIM1 and HEXIM2 (7,8). On the other hand, several stress-inducing agents and cardiac hypertrophic signals disassemble the LC in cells (10,11,14,15). Interestingly, diminution of HEXIM1 in HeLa cells results in the incorporation of an otherwise free form of HEXIM2 into

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the large complex, demonstrating the dynamic and tightly regulated nature of the assembly and disassembly of the large complex (7,12).

Although several studies revealed aspects of the complex assembly of the LC (7–9,12,13), and we presented data on HEXIM1 homodimers and binding between the C-terminus in HEXIM1 and CycT1 *in vitro* (16), no comprehensive analysis of these RNA–protein and protein–protein interactions or their functional correlates *in vivo* has been presented. To these ends, we embarked on an extensive mutagenesis of HEXIM1, defined surfaces that form oligomers in the presence and absence of 7SK snRNA as well as those that bind CycT1 and assemble the LC in cells. In addition, functional studies of these mutant HEXIM1 proteins were performed on a plasmid target that depends uniquely on active P-TEFb complexes (17). A model of the assembly of the LC is presented.

MATERIALS AND METHODS

Cell culture and cell lines

HeLa cells were grown in DMEM containing 10% fetal calf serum at 37°C with 5% CO₂.

Plasmids

The plasmid reporter pG6TAR and plasmid encoding Gal.CycT1 protein were described (17). Plasmids coding for f.Hex1, f.Hex1(1–278), f.Hex1(1–314) were a gift from Dr H. Tanaka (18) and Q. Zhou (13). Plasmid f.Hex1mBR (as well as other Hexim1 mutants carrying mBR) contains the sequence of the SV-40 NLS at its N-terminus and was described previously (19). To construct plasmids encoding the mutant f.Hex1 proteins, the pFLAG-CMV2HEXIM1 plasmids were subjected to site-directed mutagenesis with the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The plasmid encoding f.Hex1mBR(1–315) was prepared according to the procedure described previously (20). The plasmid coding for x.Hex1 was made by cloning the cDNA of HEXIM1 into KpnI and ApaI restriction sites of vector pcDNA3.1HisB (Invitrogen, Carlsbad, CA). Hex1.CFP and Hex1.YFP constructs were created by cloning of the wild-type HEXIM1 into pECFP-N1 and pEYFP-N1 expression vectors (Clontech). To prepare plasmids encoding the mutant Hex1.CFP and Hex1.YFP proteins, the Hex1.CFP and Hex1.YFP constructs were subjected to site-directed mutagenesis with the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene). All plasmids coding for Hex1.CFP and Hex1.YFP and carrying mB2 mutation contain the sequence of the SV-40 NLS at its N-terminus and were prepared as described previously (20).

Immunoprecipitation assay and western blot analysis

HeLa cells were grown on 100 mm plates. A total of 12 µg of plasmids were co-transfected by the FuGENE6 reagent (Roche Applied Science, Indianapolis, IN). After 40 h, HeLa cells were harvested and lysed in 0.75 ml of lysis buffer [1% NP-40, 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% protease inhibitor and either 0.5% RNase inhibitor (Roche, Indianapolis, IN) or RNase A (100 µg/ml final concentration) (Qiagen, Hilden, Germany)] for 1 h at 4°C. The

lysates were immunoprecipitated with anti-FLAG M2 beads for 2–4 h at 4°C and bound proteins were separated by SDS–PAGE electrophoresis and analyzed by immunoblotting with appropriate antibodies.

Transient transfection and CAT assay

HeLa cells were grown on 60 mm plates. Subsequently 0.3 µg of the pG6TAR reporter plasmid, 1 µg of Gal.CycT1 and 2.7 µg of appropriate f.Hex1 plasmid were co-transfected by the FuGENE6 reagent. A CAT assay was performed as described previously (17). In all transfections, the amount of DNA was equilibrated with an empty vector. Fold transactivation represents the ratio between the Gal.CycT1 activated transcription and the activity of the empty reporter plasmid alone, which was given as one.

Immunoreagents

The mouse monoclonal anti-FLAG M2 antibody was obtained from Sigma–Aldrich Corp. (St Louis, MO), the mouse monoclonal anti-Xpress antibody from Invitrogen and the goat polyclonal anti-CycT1 antibody (sc-8127) from Santa Cruz Biotechnology (Santa Cruz, CA).

The rabbit anti-HEXIM1 antibody was generated against HEXIM1 epitope LHRQQERAPLSKFGD and obtained from Antibody Solutions (Mountain View, CA).

Coiled-coil prediction

The prediction of coiled-coil was performed on programs available at the following addresses http://www.ch.embnet.org/software/COILS_form.html and <http://2zip.molgen.mpg.de/cgi-bin/2zip.pl> (21).

Fluorescence resonance energy transfer (FRET) microscopy

HeLa cells were plated onto culture dishes containing a 25 mm coverglass, transfected with the indicated expression plasmid DNAs and subjected to fluorescence microscopy 24 h post transfections as described previously (22). Briefly, the fluorescence images were acquired using an inverted IX-70 Olympus microscope using an Olympus ×40 Plan Apochromat objective lens (Olympus Corp., Lake Success, NY). For each cell, three fluorescence channels were collected. The donor channel consisted of CFP excited with 431–440 nm light and CFP fluorescence collected at 455–485 nm, the acceptor channel consisted of YFP excited with 496–505 nm light and YFP fluorescence collected at 520–550 nm, and the FRET channel consisted of CFP excited with 431–440 nm light and YFP fluorescence collected at 520–550 nm. Image collection and data analyses were conducted with MetaMorph imaging software (Universal Imaging Corp.).

Glycerol gradient centrifugation

Glycerol gradients (10–30%) were established by pipetting 2 ml of each of the glycerol fractions (10, 15, 20, 25 and 30% v/v) in buffer A (20 mM HEPES, pH 7.9, 0.3 M KCl, 0.2 mM EDTA, 0.1% NP-40) into centrifugation tubes (Beckman, Palo Alto, CA), catalog number 331372. Gradients were formed by standing for 6 h at 4°C. HeLa cells either transfected with the corresponding plasmids or not transfected were lysed in 0.5 ml of buffer A containing 0.1% protease inhibitor and either 0.5%

RNase inhibitor or RNase A (100 µg/ml final concentration) for 30 min at 4°C. The lysates were centrifuged at 10000 *g* for 10 min and the supernatants were loaded into tubes with the preformed glycerol gradients. Protein complexes were then fractionated by centrifugation in an SW 41Ti rotor (Beckman) at 38 000 r.p.m. for 21 h. Ten fractions (1 ml) were collected, precipitated with trichloroacetic acid and finally analyzed by immunoblotting with the appropriate antibodies (12).

RESULTS

Prediction of an evolutionary conserved CR in the C-terminal domain of HEXIM1

To obtain further insight into the function of HEXIM1, we first performed multiple sequence alignments between human HEXIM1 and HEXIM1 and HEXIM2 proteins from different species. While the N-termini of HEXIM1 and HEXIM2 proteins differ, the conserved regions include the centrally located basic region (BR; positions 150 to 177 in the human HEXIM1 protein) and the C-terminal domain of the protein (Figure 1A). We further analyzed C-terminal region in greater detail and found that it contains several conserved clusters of amino acids from position 279 to 339 in the human HEXIM1 protein (Figure 1B). Among these, the consensus sequence indicated a high number of conserved leucines. Notably, the leucines are assembled in heptad repeats, which is the hallmark of coiled-coil and oligomer-forming regions (23). Indeed, a prediction program suggested a high probability for a coiled-coil in the C-terminal domain of HEXIM1 from positions 279 to 352 (Figure 1C). Moreover, this analysis further indicated that this CR could be composed of two parts. Its N-terminal part from positions 279 to 315 could form a leucine zipper and its C-terminal part from positions 319 to 352 could constitute a coiled-coil. The highly conserved nature of the C-terminus of HEXIM1 suggests its functional importance. Finally, since it contains a predicted CR, we hypothesized that HEXIM1 could form oligomers.

HEXIM1 forms oligomers in living cells

To test this hypothesis, we first examined whether HEXIM1 proteins can interact with each other in living cells (Figure 2). For these experiments, we used FRET microscopy. The effective energy transfer between cyan (CFP) and yellow fluorescent protein (YFP) fluorophores requires the two proteins to be 1–5 nM apart which typically corresponds to direct interaction of proteins tagged with YFP and CFP. Thus, FRET imaging provides the ability to monitor protein–protein interactions in living cells and has also been recently used to detect protein oligomerization (24–26). We fused the YFP and CFP to the C-termini of different human HEXIM1 proteins, which resulted in the construction of the hybrid Hex1.YFP and Hex1.CFP proteins. Next, we expressed these chimeras individually or in combination in HeLa cells and performed FRET microscopy. When we co-expressed both chimeras, energy transfer resulted in an increased FRET signal at the expense of the donor emission (FRET/donor) relative to the cells expressing the donor alone (Figure 2A, compare image i with c and f). Residual signal in the FRET channel of the images c and f represents

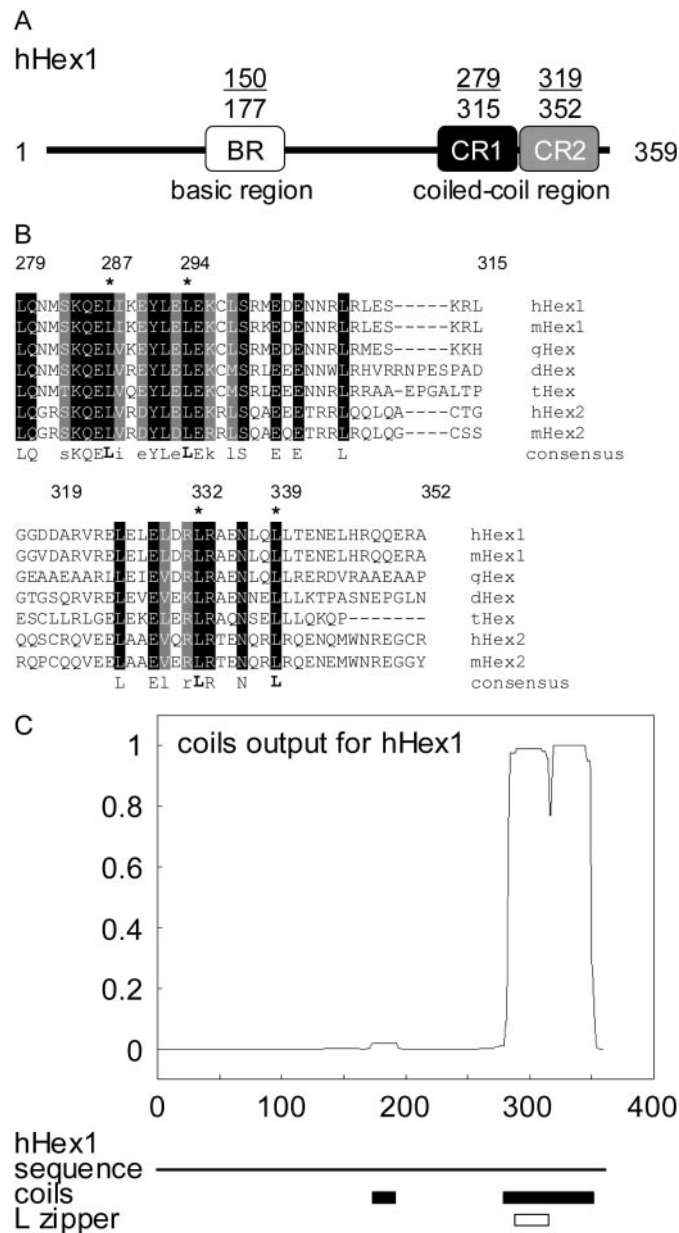


Figure 1. Prediction of CR1 and CR2 in the C-terminal domain of HEXIM1. (A) Schematic depiction of human HEXIM1 protein (hHex1). White box represents the BR. CR1 and CR2 are represented by black and gray boxes, respectively. The numbering above the boxes defines the boundaries of the regions. (B) Alignment of human (hHex1), mouse (mHex1), chicken (gHex1), zebrafish (dHex1), fish (tHex1) HEXIM1 and human (hHex2) and mouse (mHex2) HEXIM2 proteins. Black boxes indicate amino acid identity while shaded boxes the amino acid similarity. The numbering above the alignment corresponds to the boundaries of the predicted CR1 and CR2. Also, the numbered leucines, which are marked by an asterisk, were mutated to alanines. The consensus sequence is indicated below the alignment. (C) The graph indicates the probability of coiled-coil for the human HEXIM1 protein as predicted by the coils prediction program. The line below the graph corresponds to the sequence of hHex1 and the boxes indicate regions with high probability of coiled-coil (black box) and leucine zipper (white box) as predicted by the coils software.

bleedthrough of the donor and acceptor channels to the FRET channel, respectively. Similarly, when we plotted the FRET/donor against the acceptor/donor ratios, we observed an increase in the FRET signal with increasing amounts of

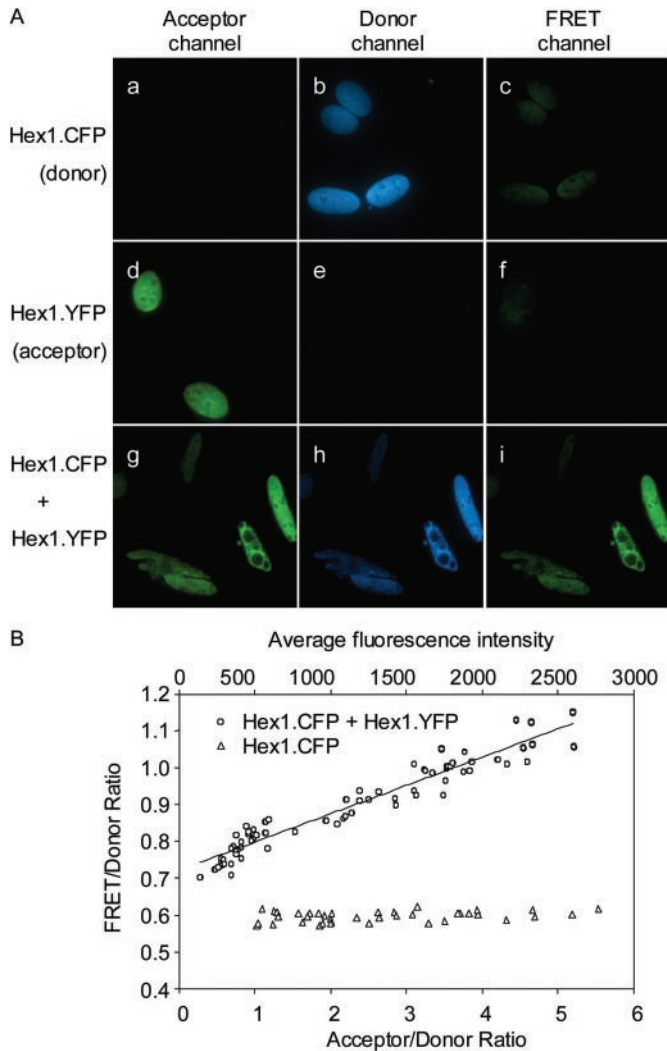


Figure 2. HEXIM1 oligomerizes in the nucleus of cells. (A) Representative images of Hex1.YFP and Hex1.CFP chimeras, which were expressed alone or together in cells. Amounts of nuclear fluorescence were quantified in the yellow, cyan and FRET channels. Energy transfer resulted in an increased FRET at the expense of donor emissions (FRET/donor) in the co-expressing cells relative to the cells containing the donors alone. (B) FRET/donor ratio increased proportionally with the amount of the acceptor relative to the donor in cells co-expressing Hex1.YFP and Hex1.CFP chimeras. The slope of the graph represents the extent of FRET at normalized acceptor/donor levels. There was no FRET between Hex1.CFP proteins expressed alone.

acceptor relative to donor, whereas no FRET signal was observed in cells that expressed the donor alone (Figure 2B). Also, the levels of Hex1.YFP and Hex1.CFP proteins were comparable and these fusion proteins inhibited P-TEFb similarly to the wild-type HEXIM1 protein (data not shown). As a negative control we used an irrelevant protein, the class II transactivator (CIITA), fused to CFP and Hex1.YFP. Co-expression of both chimeras resulted in no FRET signal and no increase in the FRET signal when we plotted the FRET/donor against the acceptor/donor ratios (data not shown). Thus, HEXIM1 proteins interact with each other in living cells and consequently can form oligomers.

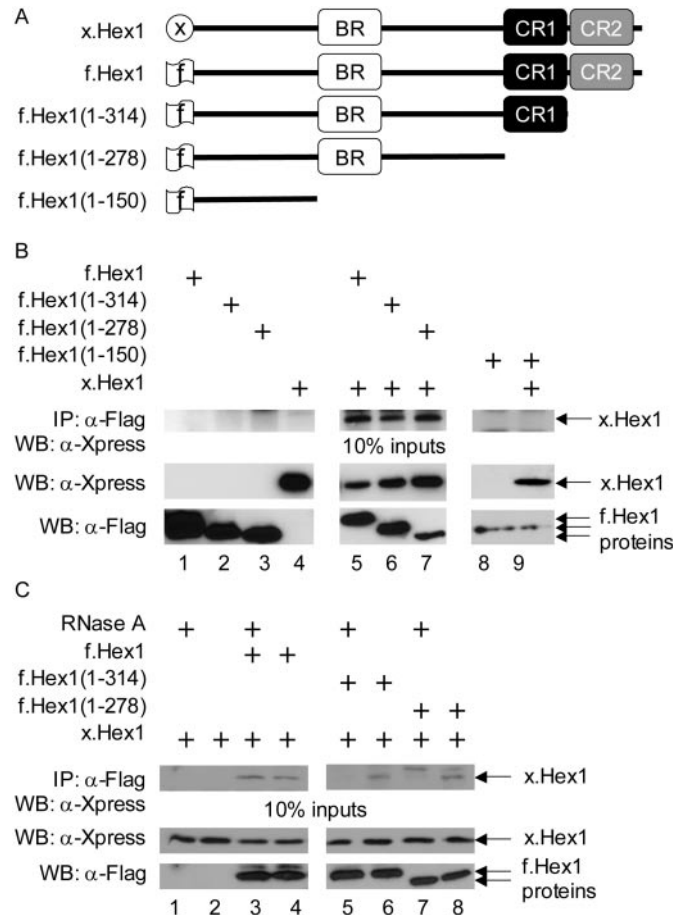


Figure 3. The C-terminal domain and 7SK snRNA mediate the oligomerization of HEXIM1. (A) Schematic diagram of Hex1 proteins used. The signs at their N-termini depict the respective tags. (B) HEXIM1 forms oligomers. The x.Hex1 and f.Hex1 proteins were either expressed alone (lanes 4 and 1, 2, 3, 8, respectively) or f.Hex1 was co-expressed with x.Hex1 in HeLa cells (lanes 5–7 and 9) as indicated. Lysates were co-immunoprecipitated with anti-FLAG agarose beads and immunoprecipitates of x.Hex1 were identified as presented on the upper western blot (WB). The middle and lower WB contain 10% of input proteins for immunoprecipitations (IP). Wild-type and mutant HEXIM1 proteins are identified by arrows. (C) 7SK snRNA and the C-terminal domain of HEXIM1 mediate the oligomerization of HEXIM1. x.Hex1 was expressed alone (lanes 1 and 2) or with the indicated f.Hex1 proteins (lanes 3–8). IP were performed as in (B) and were treated with RNase A where indicated.

The C-terminal domain of HEXIM1 and the 7SK snRNA-bound BR direct HEXIM1 oligomerization

To define the regions that might form oligomers, we performed immunoprecipitation assays (Figure 3). For these studies, we constructed a series of FLAG epitope-tagged HEXIM1 (f.Hex1) proteins, in which their C-termini were shortened progressively (Figure 3A). While the mutant f.Hex1(1–314) protein lacked the CR2, the mutant f.Hex1(1–278) and f.Hex1(1–150) proteins lacked the entire CR. Also, except for the mutant f.Hex1(1–150) protein, the rest of the proteins contained the BR. Finally, we constructed an Xpress epitope-tagged HEXIM1 (x.Hex1) protein. Next, we expressed the f.Hex1 and x.Hex1 proteins in HeLa cells, immunoprecipitated them from the total cell lysates using anti-FLAG agarose beads and followed their binding by western blotting with antibodies directed against the Xpress epitope. Consistent

with the results obtained in Figure 2, the wild-type f.Hex1 protein bound x.Hex1 protein in cells (Figure 3B, lane 5). The same result was obtained with the mutant f.Hex1(1–314) protein (Figure 3B, lane 6). Surprisingly, the mutant f.Hex1(1–278) protein retained the ability to bind x.Hex1 whereas the mutant f.Hex1(1–150) protein did not (Figure 3B, lanes 7 and 9). Since the wild-type x.Hex1 protein was not present in the anti-FLAG immunoprecipitations in the absence of f.Hex1 protein (Figure 3B, lane 4) the binding results were specific. These results suggest that in the absence of the C-terminal domain of HEXIM1, BR is critical for the formation of oligomers in HEXIM1. Furthermore, since 7SK snRNA binds the BR in HEXIM1, we predicted that the BR could mediate the oligomerization via 7SK snRNA.

To address this prediction directly and to assess the requirements of the C-terminal domain for HEXIM1 oligomerization, we repeated the experiments described in Figure 3B and additionally performed parallel experiments in which we digested 7SK snRNA by treatment of the total cell lysates with RNase A (Figure 3C). The wild-type f.Hex1 protein bound x.Hex1 protein in the presence of RNase A (Figure 3C, lanes 3 and 4). This result corresponds with the recent findings that the full-length HEXIM1 protein association does not change significantly upon RNase A treatment (12). In contrast, RNase A treatment disrupted the ability of the mutant f.Hex1(1–314) and f.Hex1(1–278) proteins to bind x.Hex1 (Figure 3C, lanes 5–8). Also, levels of the f.Hex1 and x.Hex1 proteins in cell lysates were comparable (Figure 3C, lower panel). Thus, the oligomerization of HEXIM1 depends on the intact C-terminal domain and the 7SK snRNA-bound BR.

Combined disruptions of the CR and 7SK snRNA abolish HEXIM1 oligomerization

Next, we wanted to elucidate the importance of the predicted CR in mediating HEXIM1 oligomerization (Figure 4). Since the leucines within the heptad coiled-coil repeats are critical for the coiled-coil structure and for the formation of oligomers (21,23), we mutated a series of conserved leucines to alanines. Alanine-mutagenesis was employed since non-polar alanines maintain an α -helical structure of the region but prevent its oligomerization capacity, which is mediated by the hydrophobic side chain methyl groups of the leucines. We mutated the leucine doublets at positions 287 and 294 within the CR1 and at positions 332 and 339 within the CR2 in the C-terminus of HEXIM1 individually or in combination (Figure 4A). In this way, we constructed the mutant proteins f.Hex1mCR1, f.Hex1mCR2 and f.Hex1mCR12, respectively. Next, we tested the ability of the wild-type and these mutant f.Hex1 proteins to oligomerize with the x.Hex1 protein by immunoprecipitation in the presence or absence of RNase A as described above. The wild-type and the mutant f.Hex1 proteins bound x.Hex1 in the absence of RNase A (Figure 4B, lanes 6, 8, 10 and 12). In contrast, the mutant f.Hex1mCR12 protein, in which both parts of the predicted CR were disrupted, failed to bind x.Hex1 in the presence of RNase A (Figure 4B, lane 7). However, the mutant f.Hex1 proteins with individually disrupted coiled-coil parts bound x.Hex1 under the same conditions (Figure 4B, lanes 9 and 11). Also, levels of the f.Hex1 and x.Hex1 proteins in cell lysates

were comparable (Figure 4B, lower panel). Thus, these results indicate that the predicted CR directs HEXIM1 oligomerization. Since the CR1 and CR2 mediate the formation of the HEXIM1 oligomers by themselves in the context of the entire C-terminus of HEXIM1, these two sequences could in principle serve different functions.

To confirm the results observed in immunoprecipitation experiments, we decided to analyze oligomerization properties of HEXIM1 mutants in living cells by FRET microscopy. We constructed mutants of Hex1.YFP and Hex1.CFP with disrupted oligomerization regions in the BR and in the C-terminal domain of the protein. Mutants in the BR, where arginines 154–156 were mutated to alanines, were designated mB2 and represent the situation, where 7SK snRNA does not bind to HEXIM1 (19). We prepared mutant Hex1mB2, Hex1.mB2CR1 and Hex1.mB2CR12 as YFP and CFP fusion proteins, where the 7SK snRNA binding site was disrupted alone or in combination with disrupted CR1 and CR12, respectively. Furthermore, we constructed Hex1.CR12 YFP and CFP fusion proteins with disrupted CR but intact BR. Next, we examined the ability of these mutant Hex1.CFP/YFP proteins to interact with each other in living cells by analyzing the FRET signal. Combined co-expression of YFP and CFP fusion mutants, which carried at least one intact oligomerization region resulted in a FRET signal (Figure 4C, images c, f and i). In contrast, mutant Hex1.mB2CR12YFP/CFP proteins with no functional oligomerization region showed no FRET signal (Figure 4C, image l). Also, when we plotted the FRET/donor against the acceptor/donor ratios we observed an increase in the FRET signal with increasing amounts of acceptor relative to donor with mutant proteins with at least one functional oligomerization region, but not with mutant proteins where all oligomerization regions were disrupted (data not shown). We conclude that combined disruptions of the CR and the 7SK snRNA binding site abolish completely HEXIM1 oligomerization and that at least one functional oligomerization domain is sufficient to mediate the formation of HEXIM1 oligomers in cells.

CR1 in HEXIM1 is required for its binding to P-TEFb and inhibition of transcription

To explore the possibility that the CR1 and CR2 could be functionally separable, we asked whether their individual disruptions could affect the binding to P-TEFb and thus inhibit transcription differently (Figure 5). To accomplish this goal, we first expressed the wild-type f.Hex1 and the mutant f.Hex1mCR1 and f.Hex1mCR2 proteins in HeLa cells and performed immunoprecipitation assays as described above. The presence of P-TEFb in the immunoprecipitations was followed by western blotting with antibodies directed against the endogenous CycT1 subunit of P-TEFb. As expected, the wild-type f.Hex1 protein bound P-TEFb (Figure 5A, lane 1) and the same was true for the mutant f.Hex1mCR2 protein (Figure 5A, lane 3). In contrast, the mutant f.Hex1mCR1 protein decreased considerably its ability to bind P-TEFb (Figure 5A, lane 2). Also, levels of f.Hex1 proteins were comparable (Figure 5A, lower panel). We conclude that CR1 in HEXIM1 is critical for binding to P-TEFb.

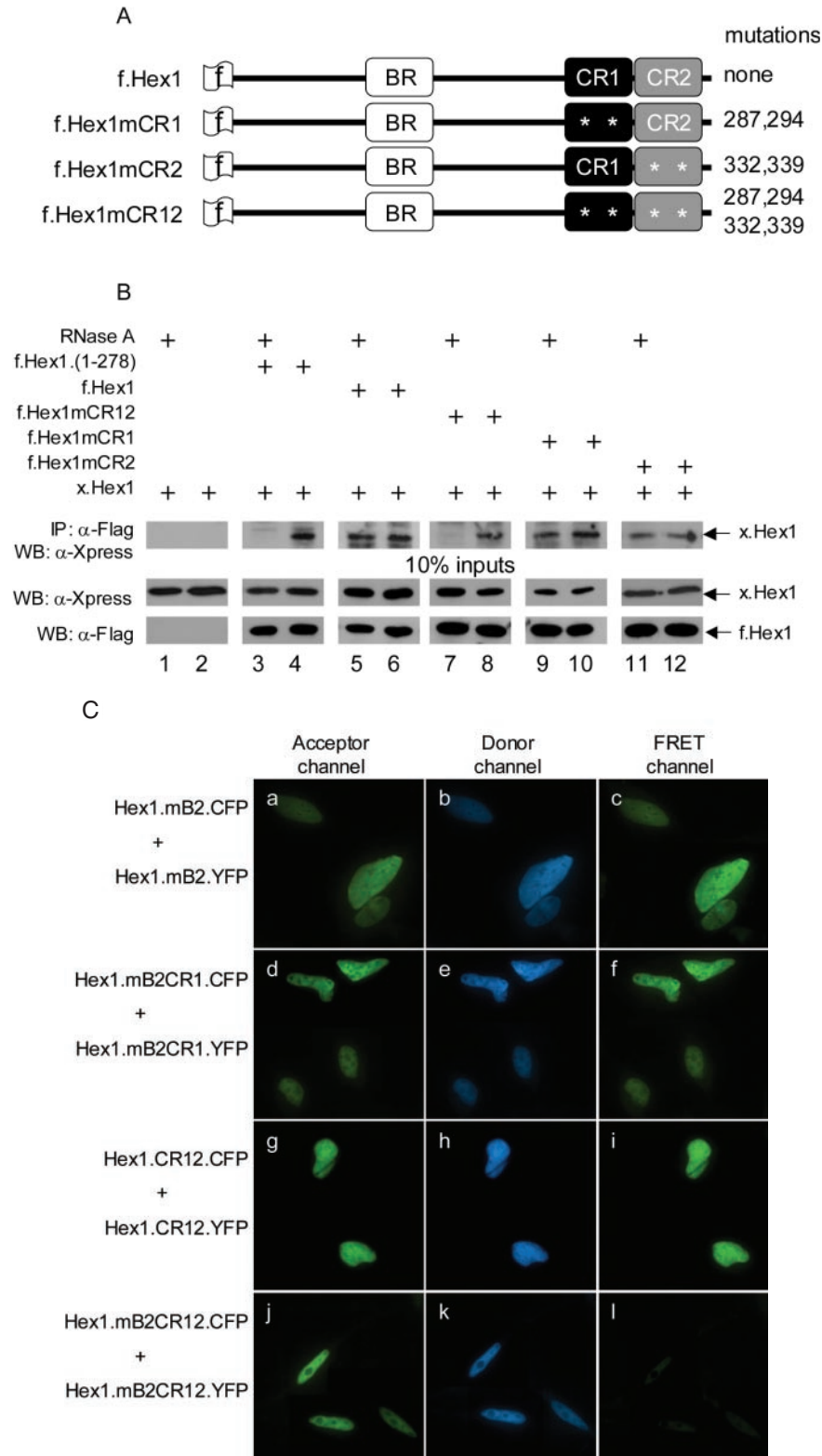


Figure 4. Combined disruptions of the CR and BR abolish HEXIM1 oligomerization. (A) Schematic diagram of Hex1 proteins used. The sign at their N-termini depicts the FLAG tag. The asterisks within the CR1 and CR2 indicate the mutations of leucines to alanines. The numbering indicates the positions of the mutated leucines in f.Hex1 proteins. (B) HEXIM1 with the disrupted CR does not oligomerize in the absence of 7SK snRNA. Proteins with the disrupted CR1 or CR2 (lanes 9–12) or CR (lanes 7 and 8) were co-expressed with x.Hex1 in HeLa cells. The lysates were treated with RNase A where indicated, immunoprecipitated with anti-FLAG agarose beads and immunoprecipitates were subjected to SDS-PAGE and WB (upper panel). Lower panels represent 10% input of proteins. (C) Combined disruptions of the CR and the 7SK snRNA binding site abolish completely HEXIM1 oligomerization in cells. FRET analysis was performed as in Figure 2. Representative images of the nuclei in which Hex1.YFP and Hex1.CFP mutant proteins were co-expressed are presented. The amounts of nuclear fluorescence were quantified in the yellow, cyan and FRET channels.

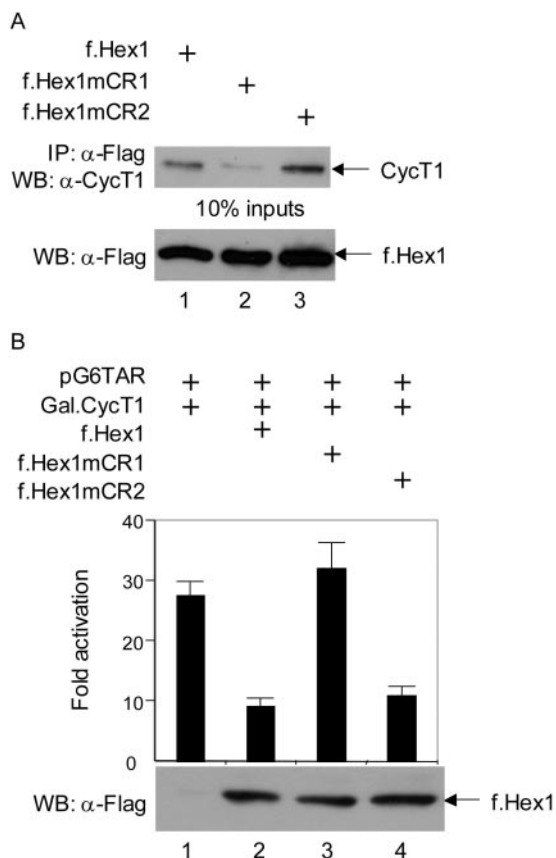


Figure 5. CR1 in HEXIM1 is required for its binding to P-TEFb and inhibition of transcription. (A) Disruption of the CR1 abrogates P-TEFb binding. f.Hex1 proteins were expressed in HeLa cells (6 μ g, lanes 1–3) and immunoprecipitated with anti-FLAG agarose beads. Amounts of bound endogenous CycT1 is presented by WB (upper panel). The amounts of f.Hex1 proteins are indicated in WB below (lower panel). (B) f.Hex1mCR1 does not inhibit transcription. HeLa cells expressed pG6TAR (0.3 μ g), Gal.CycT1 (1 μ g) and f.Hex1 (2.7 μ g) as depicted. Bars correspond to CAT values and the lower panel presents expression (WB) of f.Hex1 as indicated by the arrow.

To extend our binding studies, we assessed the ability of these f.Hex1 proteins to inhibit P-TEFb in a transcriptional assay in HeLa cells (Figure 5B). We employed a system consisting of a plasmid reporter pG6TAR, which contained six Gal4 DNA-binding sites positioned upstream of the HIV long terminal repeat, followed by the CAT reporter gene, and a plasmid effector coding for the chimeric Gal.CycT1 protein. Its recruitment to pG6TAR promoter activates transcription and can be measured by the CAT reporter assay (17). When we co-expressed the Gal.CycT1 chimera with pG6TAR, levels of CAT activity increased 27-fold over basal levels whereas co-expression of the wild-type f.Hex1 protein decreased this activity to 9-fold (Figure 5B, compare bars 1 and 2). Strikingly, when we co-expressed the mutant f.Hex1mCR1 protein, this inhibitory function was lost (Figure 5B, bar 3). In contrast, the mutant f.Hex1mCR2 protein inhibited P-TEFb similarly to the wild-type f.Hex1 protein (Figure 5B, bar 4). Levels of f.Hex1 proteins were comparable (Figure 5B, lower panel). Also, the subcellular localization of both f.Hex1 mutants was the same as the wt f.Hex1 protein (data not shown). Thus, it can be concluded that CR1 and CR2 are functionally separable and only the CR1 in HEXIM1 is

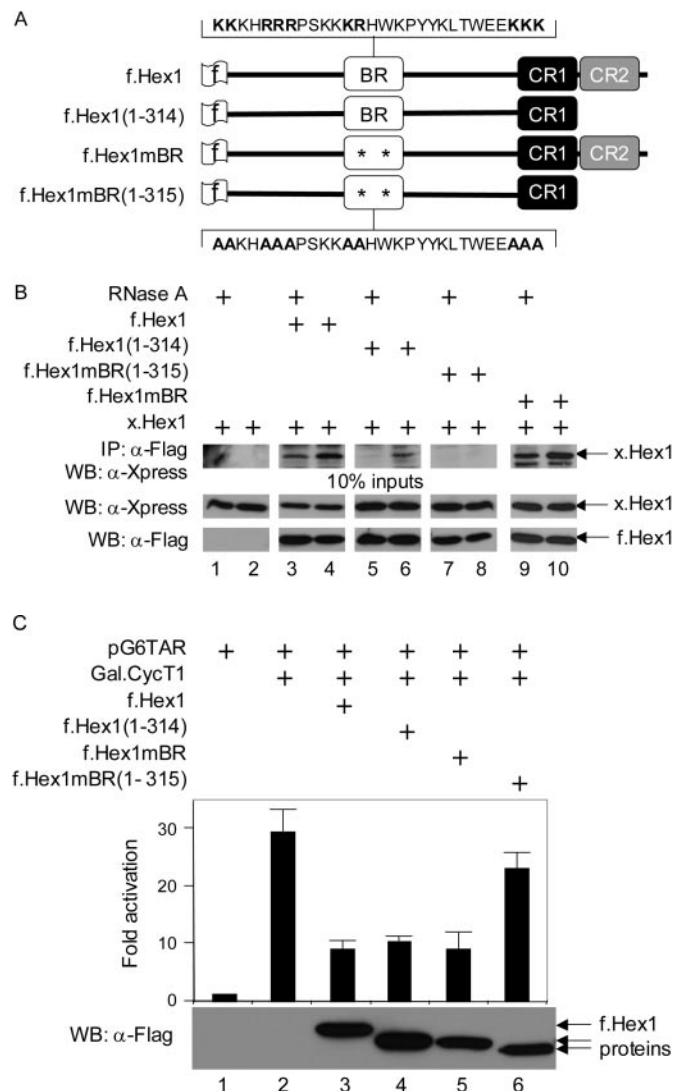


Figure 6. Oligomerization of HEXIM1 via its BR or CR2 is required for the inhibition of transcription. (A) Schematic diagram of Hex1 proteins used. The BR, CR1 and CR2 regions participating in the oligomerization are depicted. The wild-type and the mutated residues of the BR are depicted above and below the diagram, respectively. The mutated BR is indicated by asterisk. The schematic picture represents the f.Hex1 and mutant f.Hex1(1–314), f.Hex1mBR and f.Hex1mBR(1–315) proteins used. (B) HEXIM1 without the BR and the CR2 does not oligomerize. The x.Hex1 and f.Hex1 proteins were co-expressed as depicted. Lysates were treated with RNase A where noted and IP was performed as described. Upper panel represents WB with the immunoprecipitated x.Hex1 proteins, whereas the middle and lower panels show 10% input of proteins used for IP. (C) HEXIM1 without the BR and the CR2 does not inhibit P-TEFb. Bars represent CAT data obtained by co-transfection of HeLa cells with pG6TAR (0.3 μ g), Gal.CycT1 (1 μ g) and indicated f.Hex1 plasmids (2.7 μ g). The lower panel presents the expression of f.Hex1 proteins.

required for the binding to P-TEFb, resulting in the inhibition of its transcriptional activity.

Oligomerization of HEXIM1 via BR or CR2 is required for the inhibition of transcription

To examine the functional importance of HEXIM1 oligomers, we asked whether the ability of HEXIM1 to inhibit P-TEFb depends on its oligomerization (Figure 6). We used the series of f.Hex1 proteins presented in Figure 6A. The mutant

f.Hex1(1–314) and the wild-type f.Hex1 proteins, which possess one or two regions that form oligomers, respectively, were described above. Two additional mutant f.Hex1 proteins were constructed. The mutant f.Hex1mBR protein, in which most of the basic residues in the BR were mutated to alanines, contains a disrupted BR. It does not bind 7SK snRNA *in vitro*, but inhibits P-TEFb in transcriptional assays (19). Finally, the mutant f.Hex1mBR(1–315) has a disrupted BR and CR2. Both of these mutant proteins also contain the SV-40 NLS, which enables their nuclear localization. To examine the oligomerization of these f.Hex1 proteins, we performed immunoprecipitation assays in the presence or absence of RNase A (Figure 6B). As already presented in Figure 3 and in the recent report (12), the RNase A treatment did not change considerably the binding between the wild-type f.Hex1 and x.Hex1 proteins (Figure 6B, lanes 3 and 4) but it prevented the binding with the mutant f.Hex1(1–314) protein (Figure 6B, lanes 5 and 6). Predictably, the mutant f.Hex1mBR protein with the intact CR bound x.Hex1 independently of RNase A treatment (Figure 6B, lanes 9 and 10). In contrast, the mutant f.Hex1mBR(1–315), which lacks the CR2 and the BR, failed to bind x.Hex1 in the presence or absence of RNase A (Figure 6B, lanes 7 and 8). Levels of f.Hex1 and x.Hex1 proteins in cell lysates were comparable (Figure 6B, lower panel). The result in Figure 6B, lane 5, may seem to contradict the result obtained with the immunoprecipitation of mutant f.Hex1mCR2 under RNase A treatment where oligomers were observed (Figure 4B, lane 11). However, it is important to note that in the mutant f.Hex1mCR2 protein just two C-terminally located leucines were mutated in comparison with the deletion of the entire CR2 in the f.Hex1(1–314).

Finally, to determine whether the oligomerization of HEXIM1 correlates with its inhibitory function, we assessed the abilities of these f.Hex1 proteins to inhibit P-TEFb by using transcriptional assays in HeLa cells (Figure 6C). We employed the same system as described in Figure 5. The wild-type f.Hex1 protein decreased the activity of the Gal.-CycT1 chimeric protein from 29- to 9-fold over the basal levels (Figure 6C, compare bars 1–3). Similarly, the mutant f.Hex1(1–314) and f.Hex1mBR proteins inhibited P-TEFb equivalently to the wild-type f.Hex1 protein (Figure 6C, bars 4 and 5). Critically, the mutant f.Hex1mBR(1–314) protein, which could not form oligomers, failed to inhibit P-TEFb (Figure 6C, bar 6). Thus, the separate disruption of two oligomerization domains does not affect the abilities of these mutant HEXIM1 proteins to inhibit P-TEFb. Rather, the combined disruption of the BR and the CR2 prevents not only the formation of oligomers, but also the inhibition of transcriptional activity of P-TEFb. We conclude that the ability of HEXIM1 to inhibit P-TEFb requires its oligomerization.

Oligomerization of HEXIM1 via BR or CR2 is required for the incorporation of HEXIM1 into the LC

To confirm these results, we correlated the inhibitory function of f.Hex1 proteins with their ability to bind P-TEFb and to incorporate into the LC (Figure 7). We hypothesized that mutant f.Hex1 proteins, which do not inhibit P-TEFb, also do not bind it. For this purpose, we expressed our mutant f.Hex1 proteins and performed immunoprecipitation assays as described (Figure 7A). The presence of P-TEFb in the

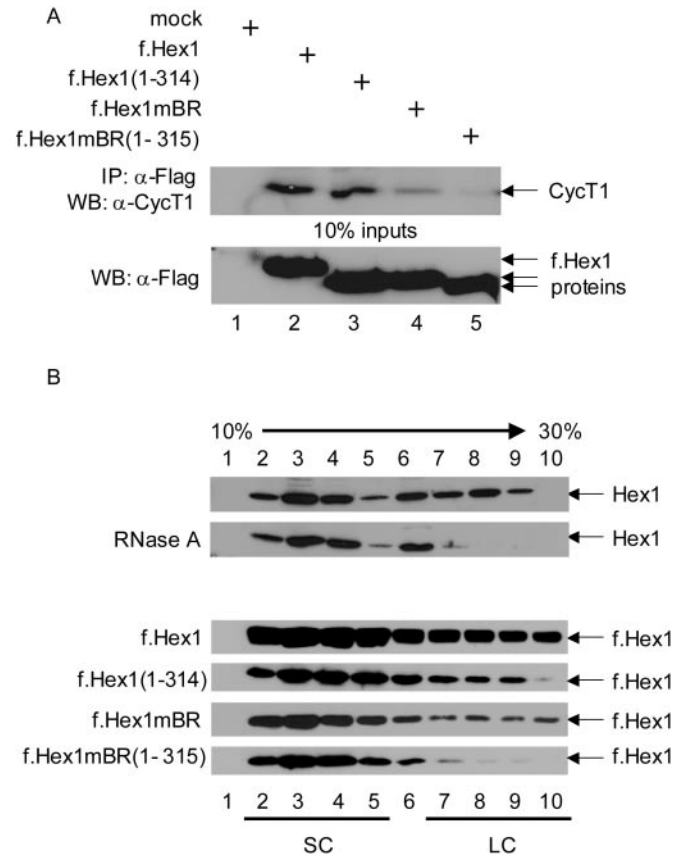


Figure 7. Oligomerization of HEXIM1 via BR or CR2 is required for the incorporation of HEXIM1 into the LC of P-TEFb. (A) HEXIM1 with disrupted BR and CR2 does not bind P-TEFb. HeLa cells were either mock transfected (lane 1) or transfected with f.Hex1 plasmids (6 μ g, lanes 2–5). The f.Hex1 proteins were immunoprecipitated with anti-FLAG agarose beads. Amounts of bound endogenous CycT1 is presented by WB (upper panel). The amounts of f.Hex1 proteins are indicated in WB below (lower panel). (B) HEXIM1 with disrupted BR and CR2 does not incorporate into the LC. Lysates from either mock or f.Hex1 plasmids transfected cells were divided into ten fractions by glycerol gradient centrifugation. Amounts of endogenous HEXIM1 proteins in each fraction were analyzed by immunoblotting as shown on the panels. The numbers below and above panels depict the glycerol fractions and arrow indicates increasing glycerol gradient (from 10 to 30%). SC and LC stand for small complex and large complex of the P-TEFb, respectively.

immunoprecipitations was followed by western blotting with antibodies directed against the endogenous CycT1 subunit of P-TEFb. The wild-type f.Hex1 protein bound P-TEFb (Figure 7A, lane 2) and the lysate from the mock transfected cell did not (Figure 7A, lane 1). As predicted, the mutant f.Hex1(1–314) and f.Hex1mBR proteins with only one disrupted oligomerization region, which inhibited P-TEFb, also bound P-TEFb (Figure 7A, lanes 3 and 4), although f.Hex1mBR shown reduced binding in comparison with f.Hex1. In contrast, the mutant protein f.Hex1mBR(1–315) did not interact with P-TEFb at all, which corresponds well with its abrogated inhibitory function (Figure 7A, lane 5). The levels of f.Hex1 proteins were comparable (Figure 7A, lower panel). Thus, the loss of the inhibitory function of the mutant Hex1 protein with the combined disruption of BR and CR2 is due to its inability to bind P-TEFb.

Part of the P-TEFb in HeLa cells is incorporated into functionally inactive LC. The rest of P-TEFb is catalytically active

and forms SC (8,9,13,27). On the basis that HEXIM1 with the combined disruption of BR and CR2 neither inhibits nor binds P-TEFb, we hypothesized that these two regions are critical for the incorporation of HEXIM1 into the LC. To prove this assumption, we employed glycerol gradient centrifugation of lysates from HeLa cells that expressed different mutant f.Hex1 proteins and analyzed the ability of these mutants to form LC with endogenous P-TEFb (Figure 7B). As a control, we observed endogenous HEXIM1 proteins in SC and LC in lysates of the mock transfected cells (Figure 7B, upper two panels). Upon treatment with RNase A, HEXIM1 was released from the LC and was found only in the free form. Subsequently, we expressed wild-type and mutant f.Hex1 proteins with either separately disrupted BR and CR2 or in combination. As expected, the wild-type f.Hex1 and mutant f.Hex(1–314) and f.Hex1mBR proteins, which oligomerize and inhibit P-TEFb, were also incorporated into the LC. In contrast, the mutant f.Hex1mBR(1–315) protein existed only in the free form and failed to be incorporated into the LC (Figure 7B, compare four lower panels), which is consistent with its inability to inhibit P-TEFb. Thus, we conclude that the BR and CR2 regions are required for oligomerization and incorporation of HEXIM1 into the LC to direct the inhibition of P-TEFb.

DISCUSSION

In this study, we investigated mechanisms dictating the assembly of the 7SK snRNA:HEXIM1:P-TEFb complex that inhibits transcriptional elongation. First, a predicted CR was found in the C-terminus of HEXIM1. Second, HEXIM1 forms oligomers via this CR and BR that binds 7SK snRNA in cells. Third, in the presence of 7SK snRNA, the CR1 binds P-TEFb whereas the CR2 mediates HEXIM1 oligomerization. Finally, we determined that the oligomerization of HEXIM1 is a prerequisite for its incorporation into the LC and thus binding and inhibition of P-TEFb in cells.

This work extends our earlier observations that the C-terminus of HEXIM1 forms homodimers and also binds CycT1 *in vitro* (16). However, in that study, HEXIM1 dimers were not stable in the presence of CycT1, most likely because 7SK snRNA and its binding site on HEXIM1 were not provided. Notably, these findings are consistent with previous reports on the C-terminus of HEXIM1, in which the N-terminal 274 amino acids of HEXIM1 did not interact with P-TEFb in the yeast two-hybrid assay, but the inclusion of additional 26 residues to position 300 partly restored this binding (8,9). However, the precise mapping of the surfaces involved in these protein–protein interactions and the role of 7SK snRNA in this process had not been addressed. For these reasons, we elected to move all our experiments into the physiological setting of the cell, where we could examine our mutations for binding and function in the same context and correlate them to what has been surmised largely from biochemical analyses. Importantly, our study provided further details on surfaces involved in these interactions and presented a kinetic picture of how the LC could be assembled. Namely, it demonstrated the critical contribution of 7SK snRNA to the formation of the stable LC. Combining deletions and clustered point mutations also defined these surfaces precisely, so that we could distinguish between the oligomerization of HEXIM1

via CR2 and its binding to CycT1 and thus P-TEFb via CR1. Since HEXIM1 oligomers do not bind P-TEFb in the absence of 7SK snRNA, it is possible that the entire CR dictates their self-association. In this scenario, the presence of 7SK snRNA then provides the decisive oligomerization function, which exposes CR1 for binding to CycT1, thus leading to the assembly of the LC and P-TEFb inhibition. Importantly, our binding studies were complemented with functional data, where we examined wild-type and mutant HEXIM1 proteins for their ability to block transcription directed by P-TEFb. First, we used a plasmid target that depends uniquely on P-TEFb and then we targeted P-TEFb to the promoter via a heterologous DNA-tethering of CycT1 (17). Previously, we demonstrated that this reporter and target are blocked by dominant negative Cdk9 protein as well DRB or flavopiridol, which are two ATP analogs that inhibit the kinase activity of P-TEFb (17). Thus, the assay depends critically on the catalytically active Cdk9 protein. In this way, we could distinguish directly between HEXIM1 proteins that can and cannot inhibit P-TEFb and correlate the formation of the LC with its functional consequences *in vivo*.

A recent report demonstrated the formation of homo- and hetero-oligomers between HEXIM1 and HEXIM2 proteins, which were thought to be independent of 7SK snRNA (12). Notably, our findings suggest that 7SK snRNA is pivotal in directing the incorporation of HEXIM1 and HEXIM2 proteins into the LC. These LCs with the homo- or hetero-oligomers of HEXIM1/2 could subservise different physiological functions, which would reflect different expression levels of these proteins in various tissues and cells (7,12). This situation would be reminiscent of transcription factors with basic region-leucine zippers (b/ZIP), where homo- and hetero-dimerizations via coiled-coil regions modulate their transcriptional properties (28,29). Although we refer to HEXIM1 oligomers throughout, *in vitro* data (16) and glycerol gradient sedimentation analyses (10) favor strongly the notion of HEXIM1 dimers. In addition, the BR in HEXIM1 that resembles the arginine-rich motif in Tat (27) could bind the top of the first stem loop in 7SK snRNA that resembles the transactivation response (TAR) region in HIV-1. In TAR, Tat and CycT1 bind the 5' bulge and central loop as a heterodimer (30). Analogously, Tat from the equine infectious anemia virus (EIAV) and equine CycT1 bind the central loop in TAR from EIAV as a heterodimer (31). Moreover, the coat protein of bacteriophage MS2/R17 binds the central loop of the operator as a homodimer, which gives it greater affinity and specificity (32,33). From these considerations, we propose that HEXIM1 also forms homodimers on 7SK snRNA. These conclusions are supported by the calculated molecular mass of the LC, which is ~500 kDa (10). This size would encompass one molecule of 7SK snRNA, two HEXIM1, two CycT1 and two Cdk9 proteins, for a final ratio of these subunits of 1:2:2:2.

When this manuscript was in preparation, two papers describing HEXIM1 oligomerization were published (34,35). In agreement with our conclusions, the oligomeric (most probably dimeric) nature of HEXIM1 was reported for free and 7SK snRNA-bound HEXIM1 *in vitro* and *in vivo*. Furthermore, the mutations of leucines at positions 287 and 294 were found to be sufficient for the disruption of HEXIM1 dimerization using native gel electrophoresis in the absence of 7SK snRNA *in vitro* (35). This discrepancy with our data

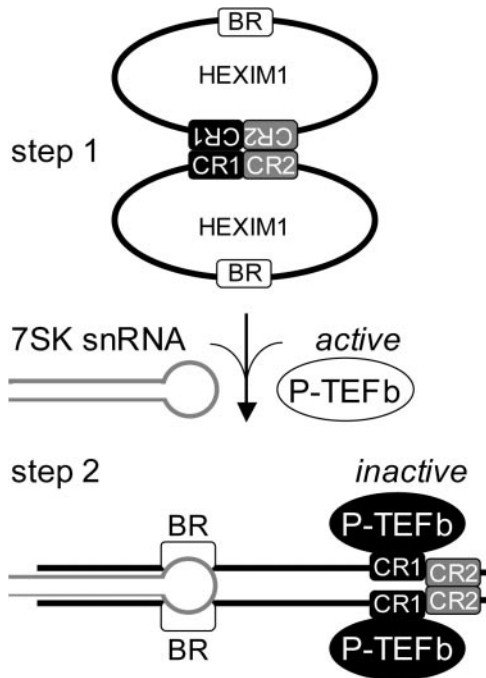


Figure 8. A model how the oligomerization of HEXIM1 via 7SK snRNA and CR directs the incorporation of P-TEFb into the LC. In the absence of 7SK snRNA, free HEXIM1 proteins are oligomers (two ovals, step 1). This oligomerization is mediated solely by the CR1 and CR2 (black and gray rectangles, respectively) in the C-terminal domain of HEXIM1. The BR is depicted as a white rectangle. The small complex of P-TEFb (white oval) is active. In contrast, when 7SK snRNA (hairpin) binds the BRs in HEXIM1, it facilitates the second oligomerization event. Subsequently, P-TEFb is incorporated into the LC, leading to its inactivation (black ovals, step 2). In this complex, the CR1 in HEXIM1 mediates the binding to P-TEFb, whereas the CR2 facilitates HEXIM1 oligomerization.

could be explained by the usage of different leucine mutants in our studies. Their mutations of leucines to positively charged arginines could have more severe effects on dimer stability than our mutations of leucines to non-polar alanines. Interestingly, although their *in vitro* kinase assay revealed only minor decreases in the inhibitory function of the mutant HEXIM1 L287, 294R protein, their analysis of this mutant protein in SC and LC by glycerol gradient centrifugation clearly indicated its absence from the LC *in vivo* (35). This finding corresponds better to the decreased binding of our mutant f.Hex1mCR1 protein to CycT1 and disruption of its inhibitory function in our *in vivo* experiments.

Finally, our study suggests a very dynamic picture for the assembly and disassembly of the LC that dictates the overall transcription in the cell (7–13). Moreover, the balance between the LC and SC is important to the organism as evidenced by the genetic inactivation of HEXIM1 in the mouse that leads to embryonic lethality due to cardiac hypertrophy (36). Additionally, levels of HEXIM1 and P-TEFb are likely to play important roles in cellular activation, proliferation and differentiation. These conclusions are revealed also by the drug hexamethylene-bis-acetamide (HMBA), which is one of the most potent differentiation agents, possibly due to its effects on increased expression of HEXIM1 (12,18,37). Moreover, P-TEFb and HEXIM1 have now been implicated in several cancers and their metastatic potential (38–41).

However, more studies are needed to define the precise roles of these RNA–protein and protein–protein interactions in these diseases. Thus, it is of great importance to determine not only how LC and SC form but their dynamic interplay in cells. To these ends, our study revealed the complex steps in the assembly of the LC, which require 7SK snRNA to coordinate the oligomerization of HEXIM1 proteins and the correct alignment of their C-termini so that they can bind P-TEFb, resulting in its inhibition (Figure 8). Finer details of these structures and the mechanism of P-TEFb inhibition will come from NMR or crystallographic approaches. At the same time, future studies will reveal the post-translational modifications of the respective components that affect the transition between the LC and SC.

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Conflict of interest statement. None declared.

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