The *Drosophila* trithorax group protein Kismet facilitates an early step in transcriptional elongation by RNA Polymerase II

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Summary

The *Drosophila* trithorax group gene *kismet* (*kis*) was identified in a screen for extragenic suppressors of *Polycomb* (*Pc*) and subsequently shown to play important roles in both segmentation and the determination of body segment identities. One of the two major proteins encoded by *kis* (KIS-L) is related to members of the SWI2/SNF2 and CHD families of ATP-dependent chromatin-remodeling factors. To clarify the role of KIS-L in gene expression, we examined its distribution on larval salivary gland polytene chromosomes. KIS-L is associated with virtually all sites of transcriptionally active chromatin in a pattern that largely overlaps that of RNA Polymerase II (Pol II). The levels of elongating Pol II and the elongation factors SPT6 and CHD1 are dramatically reduced on polytene chromosomes from *kis* mutant larvae. By contrast, the loss of KIS-L function does not affect the binding of PC to chromatin or the recruitment of Pol II to promoters. These data suggest that KIS-L facilitates an early step in transcriptional elongation by Pol II.

Key words: Kismet, BRM complex, Polycomb, Trithorax, RNA Polymerase II, Chromatin, Transcription

Introduction

The homeotic (Hox) genes of the Antennapedia and bithorax complexes encode homeodomain transcription factors that specify the identities of body segments by regulating the transcription of downstream target genes (Gellon and McGinnis, 1998). The transcription of Hox genes must be regulated precisely, as their inappropriate expression leads to dramatic alterations in segmental identities. In *Drosophila*, the initial patterns of Hox transcription are established early in embryogenesis by transcription factors encoded by segmentation genes. During subsequent development, these patterns are maintained by two ubiquitously expressed groups of regulatory proteins: the Polycomb group (PcG) of repressors and the trithorax group (trxG) of activators (Kennison, 1995; Simon and Tamkun, 2002). Counterparts of *Drosophila* PcG and trxG genes play conserved roles in other metazoans, including humans (Gould, 1997; Schumacher and Magnuson, 1997).

Although the molecular mechanisms used to maintain heritable states of Hox transcription remain relatively mysterious, a growing body of evidence suggests that they involve changes in chromatin structure (Francis and Kingston, 2001; Simon and Tamkun, 2002). PcG proteins silence the transcription of their target genes via cis-regulatory elements known as Polycomb-response elements (PREs). Two complexes of *Drosophila* PcG proteins have been identified: PRC1 (which contains PC and other PcG proteins) and the ESC/E(Z) complex (Levine et al., 2004). The ESC/E(Z) complex methylates lysine 27 of histone H3; this modification is required for PcG silencing in vivo and may help recruit PRC1 or stabilize the binding of PRC1 to PREs (Cao and Zhang, 2004).

How does PRC1 silence transcription once targeted to a PRE? One popular model is that PRC1 packages chromatin into a configuration that is inaccessible to transcription factors or the general transcription machinery (Francis and Kingston, 2001; Ringrose and Paro, 2004). However, recent studies have indicated that PRC1 may repress transcription via more selective mechanisms. For example, PRC1 may block transcription via direct interactions with components of the basal transcription machinery, as evidenced by the presence of TFIID subunits in PRC1 (Breiling et al., 2001; Saurin et al., 2001). Furthermore, DNA-binding activators TBP and Pol II are present at promoters repressed by PRC1, suggesting that PRC1 selectively interferes with events downstream of Pol II recruitment (Breiling et al., 2001; Dellino et al., 2004).

Other potential targets of PRC1 include the members of the trxG of activators. Mutations in many trxG genes suppress *Pc* mutations and cause homeotic transformations because of the failure to maintain active states of Hox transcription (Simon and Tamkun, 2002). The majority of trxG proteins characterized to date have been implicated in either chromatin remodeling or the covalent modification of nucleosomal histones. For example, the trxG genes *trithorax* (*trx*) and *absent, small or homeotic 1* (*ash1*) encode SET domain proteins with histone methyltransferase activity (Beisel et al.,

2002; Milne et al., 2002; Nakamura et al., 2002; Smith et al., 2004); these histone-modifying enzymes counteract silencing by PcG proteins in vivo (Klymenko and Müller, 2004). Another trxG gene, *brahma* (*brm*), encodes a member of the SWI2/SNF2 family of ATPases (Tamkun et al., 1992). The BRM ATPase, together with the trxG proteins Moira (MOR) and OSA (Collins et al., 1999; Crosby et al., 1999), are subunits of a 2 MDa chromatin-remodeling complex that is highly related to the yeast SWI/SNF and RSC, and the human BAF and PBAF complexes (Mohrmann et al., 2004; Papoulas et al., 1998). By altering the structure or positioning of nucleosomes, these complexes facilitate the binding of transcription factors and other regulatory proteins to chromatin (Flaus and Owen-Hughes, 2004). The BRM complex plays a global role in transcription by Pol II (Armstrong et al., 2002) and is therefore an excellent candidate for a target of PRC1 repression. Consistent with this possibility, PRC1 strongly inhibits chromatin remodeling by human SWI/SNF in vitro (Francis et al., 2001; Levine et al., 2002; Shao et al., 1999).

Like *brm*, *mor* and *osa*, the trxG gene *kismet* (*kis*) was identified in a screen for extragenic suppressors of *Pc*, suggesting that it acts antagonistically to *Pc* to activate homeotic gene expression (Kennison and Tamkun, 1988). Loss of maternal *kis* function causes segmentation defects identical to those caused by mutations in the pair-rule gene *even-skipped* (*eve*) (Daubresse et al., 1999). Loss of zygotic *kis* function causes homeotic transformations, including the transformation of first leg to second leg and the fifth abdominal segment to a more anterior identity (Daubresse et al., 1999). These phenotypes are identical to those resulting from the decreased transcription of the Hox genes *Sex combs reduced* (*Scr*) and *Abdominal-B* (*Abd-B*). Thus, *kis* plays a dual role during development; maternal *kis* activity is required for embryonic segmentation and zygotic *kis* activity is required for the control of cell fate. Mutations in *kis* have also been recovered in screens for genes involved in the Notch and Ras signaling pathways, suggesting that its function is not limited to segmentation and the determination of body segment identities (Go and Artavanis-Tsakonas, 1998; Therrien et al., 2000; Verheyen et al., 1996).

kis encodes two major nuclear proteins with molecular weights of 574 kDa (KIS-L) and 225 kDa (KIS-S) (Fig. 1) (Daubresse et al., 1999; Therrien et al., 2000). KIS-L contains an ATPase domain that is highly related to those found in chromatin-remodeling factors, suggesting that KIS-L, like BRM, catalyzes ATP-dependent alterations in chromatin structure. KIS-L also contains two chromodomains and a BRK domain. Chromodomains mediate protein-protein or protein-RNA interactions, and are found in members of the CHD subfamily of ATPases (including Mi-2 and CHD1) and other proteins that interact with chromatin (Brehm et al., 2004). Some chromodomains are involved in the selective recognition of methylated histone tails (Brehm et al., 2004). The BRK domain is a 41 amino acid segment of unknown function that is conserved in BRM and its human homologs BRG1 and HBRM (Daubresse et al., 1999). The KIS-L protein lacks PHD fingers, a domain characteristic of CHD ATPases (Aasland et al., 1995), and a bromodomain, a domain conserved in SWI2/SNF2 ATPases that mediates interactions with acetylated histone tails (Zeng and Zhou, 2002). Thus, KIS-L is unusual in that it has characteristics of both the CHD and SWI2/SNF2 subfamilies of ATPases, but is a clear member of neither class. Potential orthologs of *kis* are present in nematodes, mice and humans, but not yeast, suggesting that it may play a specialized role in transcription or development in higher eukaryotes (Daubresse et al., 1999; Schuster and Stoger, 2002; Therrien et al., 2000).

Pc mutants are exquisitely sensitive to changes in *kis* levels; *kis* mutations and deficiencies strongly suppress homeotic phenotypes resulting from Hox derepression in *Pc* heterozygotes, while *kis* duplications enhance them (Daubresse et al., 1999; Kennison and Tamkun, 1988). This strong, dose-dependent interaction suggests that the functions of *kis* and *Pc* are intimately related. A functional antagonism between *kis* and *Pc* could occur at several levels. For example, KIS-L may directly counteract the binding of PRC1 or the ESC/E(Z) complex to chromatin by altering the structure or positioning of nucleosomes at PREs. Alternatively, KIS-L might play a more global role in transcription by Pol II as has been observed for the BRM complex (Armstrong et al., 2002). In this case, PRC1 might silence the transcription of Hox genes by blocking chromatin remodeling by KIS-L.

To clarify the mechanism of action of *kis*, we compared the distribution of KIS-L and other proteins involved in chromatin remodeling and transcription on salivary gland polytene chromosomes. Here, we report that KIS-L, like BRM, is associated with virtually all transcriptionally active regions of the *Drosophila* genome. The levels of elongating Pol II and the elongation factors SPT6 and CHD1 are dramatically reduced on polytene chromosomes from *kis* mutant larvae. By contrast, the loss of KIS-L function does not affect the binding of PC to chromatin or the recruitment of Pol II to promoters. These findings suggest that KIS-L facilitates an early step in transcriptional elongation by Pol II.

Materials and methods

Drosophila stocks and genetic crosses

Flies were raised on cornmeal/molasses/yeast/agar medium containing Tegosept and propionic acid. Strains are described in FlyBase (http://www.flybase.org) unless otherwise indicated. Oregon R was used as the wild-type strain for all experiments. To examine phenotypes resulting from loss of *kis* function, salivary glands were dissected from homozygous kis^{k13416} larvae identified among the progeny of $y^1\ w^{67c23}$; *P{w⁺mC lacW}kis*^{k13416}*/ CyO, P{w⁺mW.hs Ubi-GFP.S65T}PAD1* flies by the lack of the GFP marker on the balancer chromosome.

Antibody generation

To generate an antibody that specifically recognizes KIS-L, a DNA fragment was PCR amplified from the *kis* 2 cDNA (Therrien et al., 2000) using the primers 5′-CGCGGATCCGTCACTCAACGATCA-ATTGC-3′ and 5′-CGGAATTCGAGAATGCTGCTCAGGTGATG-3′; digested with *Bam*HI and *Eco*RI; and subcloned in pGEX 3X (Pharmacia). Antibodies against GST fusion proteins were generated in rabbits and rats and purified using columns containing GST-KIS-L coupled to Affigel 15 (BioRad, Richmond, CA) (Harlow and Lane, 1988).

Immunostaining of polytene chromosomes and embryos

Salivary gland chromosomes from third instar larvae were fixed for 5 minutes in 45% acetic acid/1.85% formaldehyde and stained as described previously (Armstrong et al., 2002). Antibodies used in this study include goat antibodies against Pol IIa and Pol IIc (Skantar and

Greenleaf, 1995; Weeks et al., 1993); rabbit antibodies against PC (Strutt et al., 1997), Mi-2 (Brehm et al., 2000), ISWI (Tsukiyama et al., 1995), CHD1 (Stokes et al., 1996) and BRM (Elfring et al., 1998); guinea pig antibodies against SPT6 (Kaplan et al., 2000); and mouse antibodies against PoI IIo^{ser2}, Pol IIo^{ser5} (Covance, Berkeley, CA) and dynein heavy chain (McGrail and Hays, 1997). The specificities of secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were verified as described previously (Corona et al., 2004). Slides were mounted in Vectashield containing DAPI (Vector Laboratories). Images were captured on a Zeiss Axioskop 2 plus microscope using an Axioplan HRm camera and Axiovision 4 software (Carl Zeiss, Germany) and processed using Adobe PhotoShop 7.0 software. Merged and split images were generated as described previously (Corona et al., 2004). To compare the chromosomal levels of proteins in wild-type and mutant larvae, squashes were prepared, stained and photographed at the same time using identical conditions. The images shown are representative of multiple experiments. Embryos (0-12 hour) were fixed and stained with antibodies as described previously (Papoulas et al., 2001).

Protein biochemistry

Native protein extracts were prepared from 0-16 hour embryos as described (Elfring et al., 1998) using an equal volume of ice-cold extraction buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 0.55% Tween-20, 0.125 mM EGTA, 1 mM $MgCl_2$ and 10% glycerol) containing protease inhibitors [1 μg/ml of each aprotinin, leupeptin, chymostatin and Pepstatin A, 1 mM PMSF and 1 mg/ml of complete protease inhibitor cocktail (Roche, Germany)]. For western blotting, proteins were electrophoresed on a 5% SDS-polyacrylamide gel, transferred to nitrocellulose overnight at 4°C, and incubated with primary and HRP-conjugated secondary antibodies (Harlow and Lane, 1988). Secondary antibodies were detected using the Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL) and a BioRad GS-525 Molecular Imager. For gel filtration chromatography, 5 mg of protein extract was fractionated on a Superose 6 HR 10/30 FPLC column (Pharmacia) equilibrated with 50 mM HEPES, pH 7.6, 375 mM NaCl, 0.55% Tween-20, 0.125 mM EGTA, 1 mM $MgCl_2$ and 10% glycerol. Fractions (0.5 ml) were collected and analyzed by western blotting. Co-immunoprecipitations were carried out using affinity-purified rabbit anti-KIS-L or normal rabbit IgG (Santa Cruz Biotech, Santa Cruz, CA) as described previously (Papoulas et al., 1998).

Results

KIS-L is a subunit of a large, ubiquitously expressed protein complex

kis encodes two major protein isoforms – KIS-S and KIS-L – which share a common 2105 amino acid C-terminal segment (Fig. 1). This common segment contains a BRK domain related to those found in *Drosophila* BRM and its human orthologs. The long N-terminal segment unique to KIS-L contains an ATPase domain and two chromodomains. By contrast, KIS-S lacks these domains and is therefore unlikely to have chromatin-remodeling activity. The absence of an ATPase domain in KIS-S suggests that it may function as a naturally occurring dominant-negative form of the KIS protein.

To monitor specifically the expression of KIS-L, we raised antibodies against a region unique to this protein (residues 100-300; Fig. 1). Affinity-purified rabbit polyclonal antibodies against this segment, but not preimmune serum, specifically recognize the 574 kDa KIS-L protein in *Drosophila* embryo extracts by western blotting (Fig. 2A; data not shown). By contrast, antibodies against the C-terminal segment common to KIS-L and KIS-S detect both the 574 kDa KIS-L and 225 kDa KIS-S proteins (Fig. 2A). We next used our antibodies against KIS-L to monitor its temporal and spatial expression. As previously observed for other trxG proteins and KIS-S (Daubresse et al., 1999; Simon, 1995), KIS-L is ubiquitously expressed in nuclei throughout embryogenesis (Fig. 2B).

The majority of *Drosophila* ATP-dependent chromatin-remodeling factors characterized to date, including BRM, Mi-2 (Brehm et al., 2000), ISWI (Corona and Tamkun, 2004) and Domino (Ruhf et al., 2001) function as subunits of large protein complexes. To determine if KIS-L is also a subunit of a protein complex, we determined its native molecular mass in embryo extracts by gel filtration chromatography (Fig. 2C). KIS-L eluted from a Superose 6 gel filtration column with an apparent molecular mass of 1 MDa (Fig. 2C; fractions 18-22); KIS-S fractionates as a distinct complex with an apparent molecular mass of 650 kDa (Fig. 2C; fractions 21-26). The molecular mass of the KIS-L complex is smaller than that of the ~2 MDa BRM complex (Fig. 2C; fractions 16-20). The elution profiles of KIS-L and KIS-S are also distinct from those of the chromatin-remodeling factors CHD1 and Mi-2 (Fig. 2C; fractions 20-22 and 18-24, respectively). These findings suggest that KIS-L functions as the ATPase subunit of a novel chromatin-remodeling complex.

KIS-L is associated with virtually all transcriptionally active regions of chromatin

To directly visualize interactions between KIS-L and chromatin, we examined its distribution on salivary gland polytene chromosomes. Antibodies against KIS-L recognize

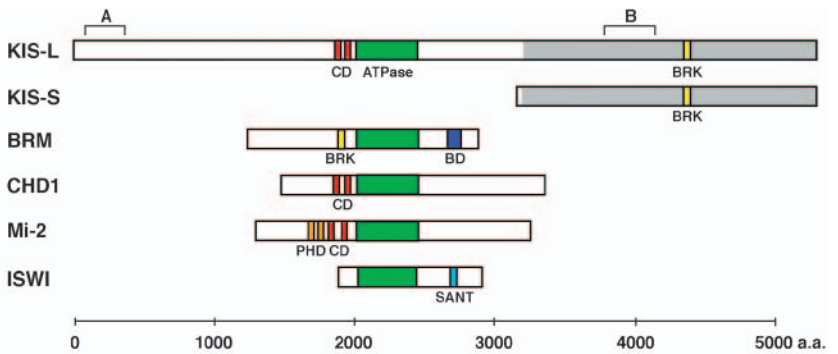

Fig. 1. KIS-L is related to chromatin-remodeling factors. The two major KIS isoforms (KIS-S and KIS-L) are compared with other *Drosophila* ATPases involved in chromatin remodeling, including BRM, Mi-2, CHD1 and ISWI. The ATPase domain of KIS-L is most closely related to that of BRM (44% identical) and CHD family members (50% identical). The C-terminal segment common to KIS-S and KIS-L (shaded light gray) contains a BRK domain, but lacks the bromodomain (BD) found in BRM and other SWI2/SNF2 family members. The N-terminal extension unique to KIS-L contains an ATPase domain and two chromodomains (CD), but lacks the PHD fingers (PHD) and putative DNA-binding domains found in other CHD proteins. KIS-L also lacks a SANT domain, a nucleosome-recognition module found in ISWI and other proteins. A and B indicate regions of KIS proteins against which antibodies were raised (residues 100-300 and 3902-4200, respectively).

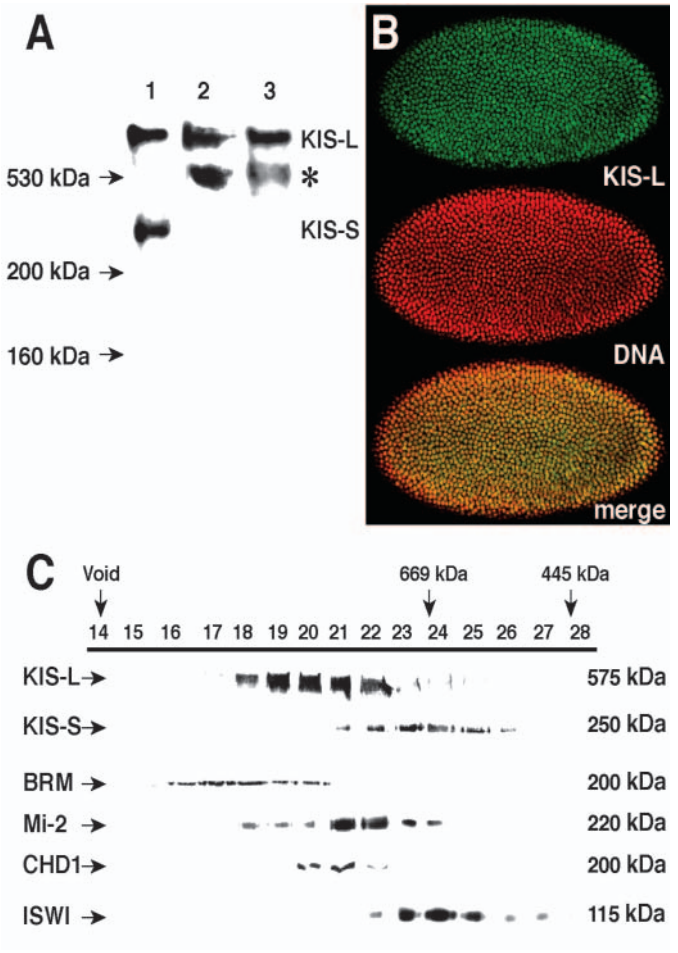

Fig. 2. *kis* encodes multiple large nuclear proteins that are subunits of distinct complexes. (A) Embryo and salivary gland extracts were resolved on a 5% SDS-polyacrylamide gel and analyzed by western blotting. Molecular masses were determined relative to prestained markers and cytoplasmic dynein (detected by western blotting). Antibodies against the common C-terminal segment detect both KIS-L and KIS-S in embryo extracts (lane 1) but only KIS-L in salivary gland extracts (lane 2). Antibodies against the N-terminal segment unique to KIS-L recognize only this protein in embryo extracts (lane 3). The asterisk marks minor bands that are occasionally observed and probably represent degradation products or minor isoforms of KIS. (B) *Drosophila* embryos were stained with antibodies that specifically recognize KIS-L. DNA was visualized using propidium iodide. There is uniform nuclear distribution of KIS-L. (C) Native embryo extracts were fractionated on a Superose 6 gel filtration column and assayed for chromatin-remodeling factors by western blotting. Fraction numbers are indicated at the top. Void and elution volumes of native molecular mass standards are shown by vertical arrows. Denatured molecular masses of the proteins are indicated on the right.

~300 sites in euchromatin (Fig. 3A). The vast majority of these sites reside in interbands: regions of less condensed DNA that stain lightly with DAPI (Fig. 3B). By contrast, KIS-L is not associated with the heterochromatic chromocenter of polytene chromosomes (Fig. 3A). These findings suggest that KIS-L plays a relatively general role in transcription or other processes, perhaps by creating open regions of chromatin. We could not examine the distribution of KIS-S on polytene

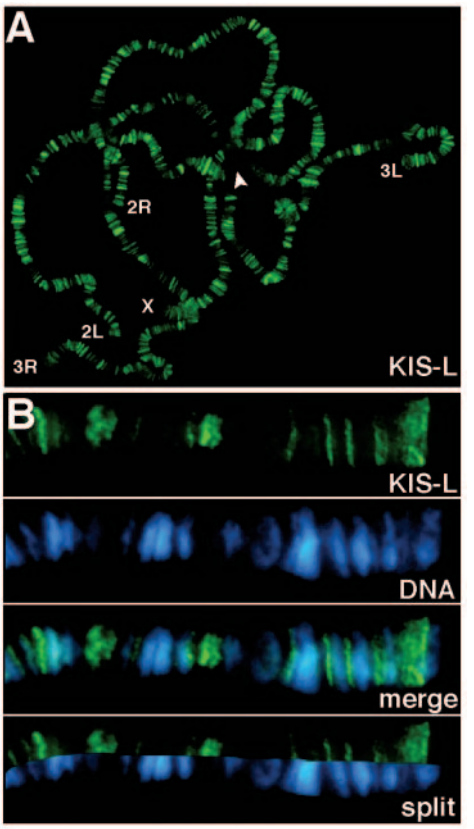

Fig. 3. KIS-L binds a large number of sites on polytene chromosomes. (A) KIS-L (green) binds to ~300 sites on wild-type polytene chromosomes and is not associated with the heterochromatic chromocenter (white arrowhead). (B) Higher magnification of the distal region of chromosome arm 2R. KIS-L (green) is compared with DNA (blue); the last two panels show the merged and split images of the first two panels. KIS-L is primarily associated with interband regions.

chromosomes because it is not expressed in the salivary gland (Fig. 2A).

To gain insight into the potential role of KIS-L in gene expression, we compared its distribution on polytene chromosomes with that of Pol II. For our initial experiments, we detected Pol II using an antibody against its second largest subunit, Pol IIc. This antibody recognizes the initiating, paused and elongating forms of Pol II. The distributions of KIS-L and Pol II overlap extensively (Fig. 4), as previously observed for the BRM complex. Although the relative levels of KIS-L and Pol II vary from site to site, it is clear that KIS-L is associated with the vast majority (~98%) of transcriptionally active regions in this tissue. This striking observation suggests that KIS-L plays a global role in transcription by Pol II.

During the transcriptional cycle, the C-terminal domain (CTD) of the largest subunit of Pol II (which contains multiple repeats of the heptad sequence YSPTSPS) is differentially phosphorylated (Sims et al., 2004). When recruited to promoters, the CTD is unphosphorylated (Pol IIa) (O'Brien et al., 1994; Weeks et al., 1993); after promoter clearance and during early stages of elongation, serine 5 of the CTD is phosphorylated (Pol IIo^{ser5}); at later stages of transcriptional elongation, serine 2 is phosphorylated (Pol IIo^{ser2})

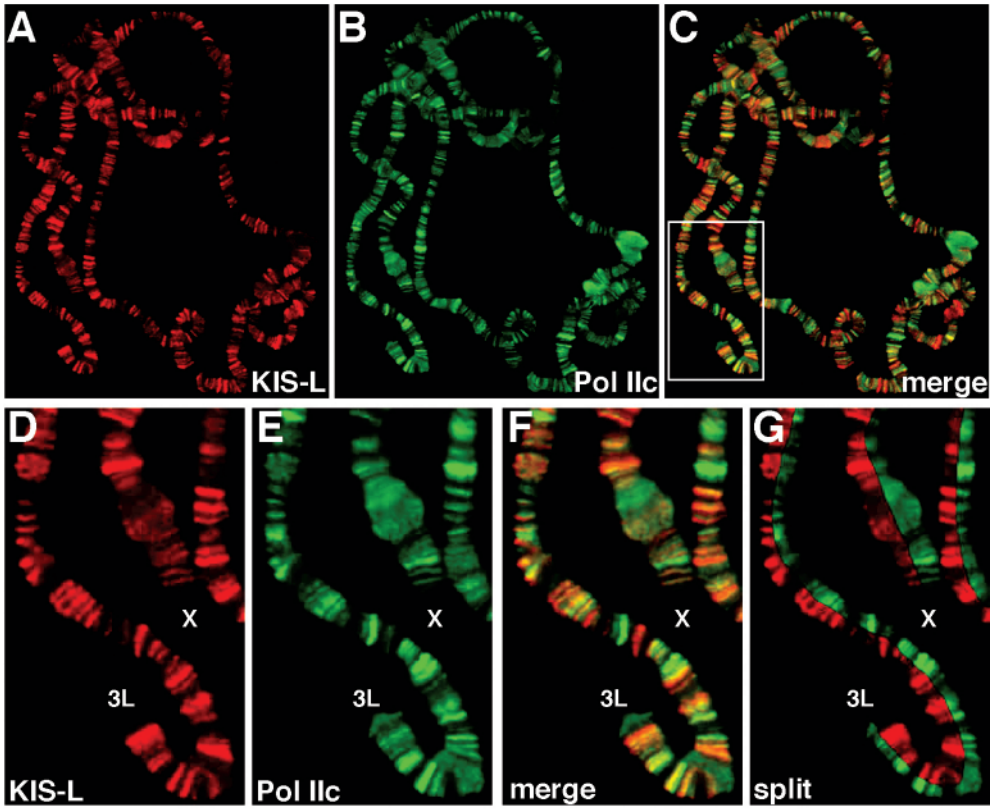

Fig. 4. KIS-L is associated with regions of active transcription. (A-C) KIS-L (red) and Pol II (green) were detected on wild-type polytene chromosomes using antibodies against KIS-L and the 140 kDa subunit of Pol II (Pol IIc); the merged image is shown in C. (D-G) Magnifications of the distal regions of the X chromosome and chromosome arm 3L (white rectangle in C) are shown. The distributions of KIS-L and Pol II are highly overlapping, although the levels vary from site to site, as evident in the split image (G).

(Komarnitsky et al., 2000; Svejstrup, 2004). The distribution of a protein relative to the different forms of Pol II can provide clues to its role in the transcription cycle (Kaplan et al., 2000; Saunders et al., 2003).

CHD1, which is related to KIS-L, has been implicated in transcriptional elongation in yeast and mammals. CHD1 associates with transcriptional elongation factors in yeast (Krogan et al., 2003; Simic et al., 2003) and mammalian cell lines (Kelley et al., 1999). CHD1 is also associated with interband regions of *Drosophila* polytene chromosomes (Stokes et al., 1996) and the body of actively transcribed genes in yeast (Simic et al., 2003). Consistent with a role in transcriptional elongation, CHD1 and Pol IIo^{ser2} have identical patterns on polytene chromosomes (Fig. 5A). By contrast, the distribution of KIS-L is not identical to any one form of Pol II. Instead, staining of KIS-L extensively overlaps with that of Pol IIo^{ser2} and Pol IIa (Fig. 5B,C) and to a lesser extent with Pol IIo^{ser5} (Fig. 5D). These findings suggest that KIS-L is required for an earlier step in transcriptional initiation or elongation than CHD1.

The chromosomal distribution of KIS-L is similar to that of BRM and other chromatin-remodeling factors

To clarify the functional relationship between KIS-L and other chromatin-remodeling factors, we compared their distributions on polytene chromosomes. We first compared the distributions of KIS-L and BRM, as previous studies have suggested that the two proteins have similar functions. For example, *brm* and *kis* were both identified in genetic screens for dominant suppressors of *Pc* (Kennison and Tamkun, 1988) and mutations in the two genes cause similar homeotic transformations (Brizuela et al., 1994; Daubresse et al., 1999). In addition, BRM plays an extremely general role in transcription by Pol II and – like KIS-L – is associated with almost all transcriptionally active regions of polytene chromosomes (Armstrong et al., 2002). Consistent with a close functional relationship between the two proteins, we found that the distributions of BRM and KIS-L on polytene chromosomes are virtually identical (Fig. 6A). In addition, the relative levels of the two proteins do not vary from site to site. The striking similarities between the chromosomal distributions of BRM and KIS-L strongly suggest that the functions of the two trxG proteins are intimately related.

We next compared the chromosomal distribution of KIS-L with members of the CHD family of ATPases, including CHD1 and Mi-2. As discussed above, CHD1 has been implicated in transcriptional elongation and perfectly co-localizes with elongating form of Pol II (Pol IIo^{ser2}) on polytene chromosomes. By contrast, CHD1 and KIS-L have partially overlapping, but not identical, chromosomal distributions (Fig. 6B), suggesting that they play distinct roles in the transcription cycle.

Based on its association with histone deacetylases and transcriptional repressors (Brehm et al., 2000; Kehle et al., 1998; Zhang et al., 1998), Mi-2 is thought to be involved in transcriptional repression. Genetic studies in *Drosophila* also suggest that Mi-2 acts in concert with PcG proteins to repress Hox transcription (Kehle et al., 1998). We therefore anticipated that the chromosomal distributions of KIS-L and Mi-2 would be very different, if not mutually exclusive. Much to our surprise, we found that the patterns of KIS-L and Mi-2 are actually very similar (Fig. 6C). Although the relative levels of KIS-L and Mi-2 vary from site to site, only 1 to 2% of the binding sites of the two proteins fail to overlap. These data suggest that Mi-2 plays an unanticipated and relatively general role in transcription by Pol II.

KIS-L is not physically associated with Pol II and other chromatin-remodeling factors

Physical interactions between KIS-L and BRM, Mi-2 or Pol II could account for their similar chromosomal distributions.

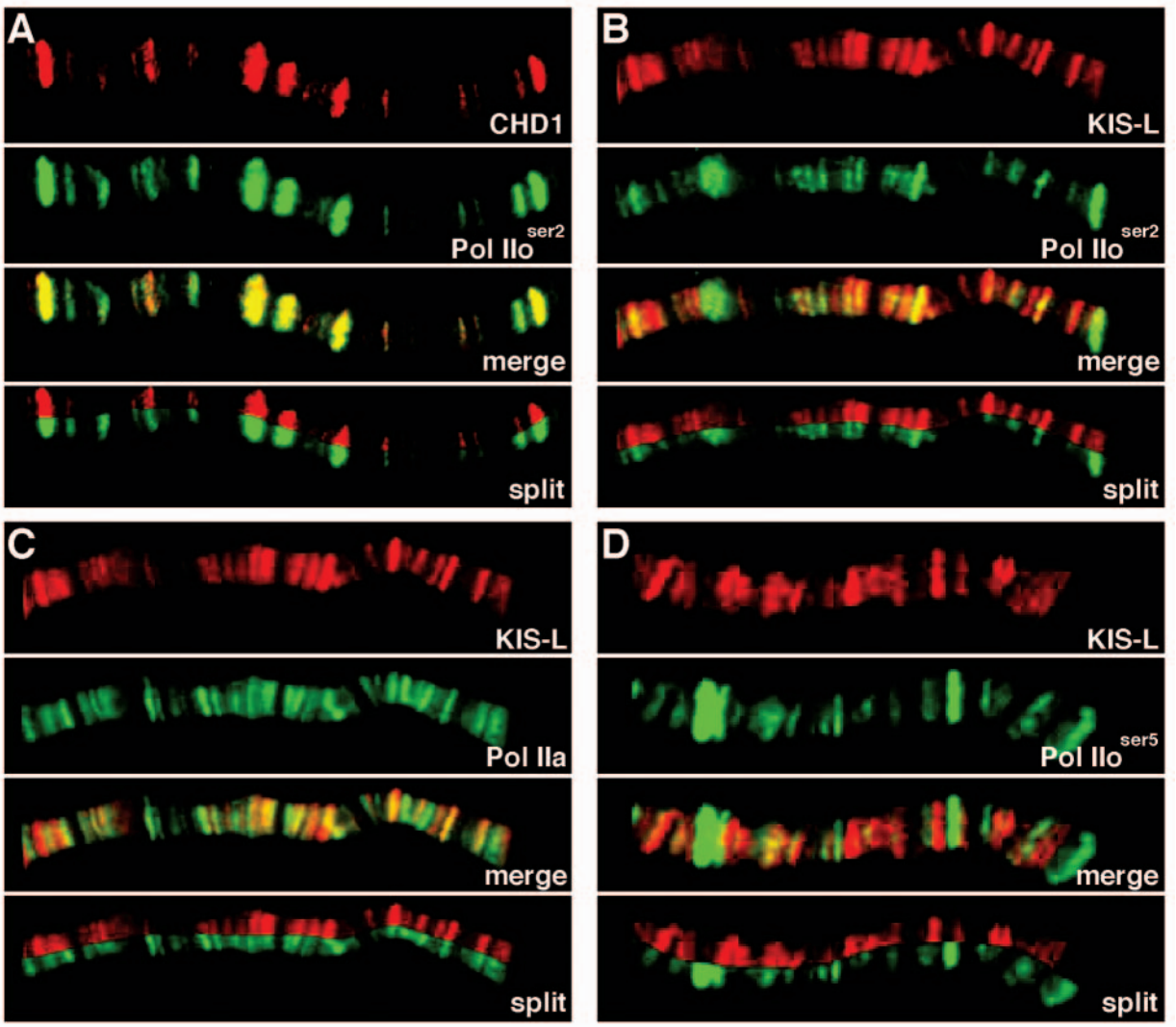

Fig. 5. KIS-L co-localizes with both the paused and elongating forms of Pol II. The distributions of CHD1 and Pol IIo^{ser2} (A); KIS-L and Pol IIo^{ser2} (B); KIS-L and Pol IIa (C); and KIS-L and Pol IIo^{ser5} (D) are shown. Magnifications of the distal chromosome arm 2R are shown in A-C. A magnification of the distal chromosome arm 2L is shown in D. The distribution of KIS-L is highly coincident with all forms of Pol II, whereas CHD1 is associated primarily with the elongating form of Pol II (Pol IIo^{ser2}).

Indeed, physical interactions between yeast SWI/SNF complexes and Pol II holoenzyme have been reported (Neish et al., 1998; Wilson et al., 1996), but these findings remain somewhat controversial. To investigate this possibility, we attempted to co-immunoprecipitate KIS-L, other chromatin-remodeling factors and Pol II from embryo extracts using antibodies against KIS-L. KIS-L, but not KIS-S, could be efficiently immunoprecipitated from embryo extracts using affinity-purified antibodies against the N-terminal segment unique to the KIS-L protein (Fig. 7). In addition to confirming the specificity of this antibody, this result suggests that KIS-L and KIS-S do not stably interact with each other. We were also unable to co-immunoprecipitate KIS-L with other chromatin-remodeling factors (including BRM, Mi-2 and ISWI) or Pol II, even when very mild conditions were used (Fig. 7). These findings are consistent with our gel filtration data, suggesting that KIS-L, BRM and Mi-2 are subunits of distinct protein complexes. Thus, physical interactions between KIS-L, BRM, Mi-2 and Pol II are probably not responsible for their similar chromosomal distributions.

KIS-L is not required for the maintenance of chromosome structure

kis is an essential gene and individuals homozygous for extreme *kis* alleles die prior to the third larval instar (Daubresse et al., 1999). By contrast, individuals homozygous for a hypomorphic, P element-induced *kis* allele (*kis*^{k13416}) (Roch et al., 1998; Spradling et al., 1999) survive until late larval or early pupal development. The recessive lethality of *kis*^{k13416} is rescued by a duplication spanning the *kis* gene [*Dp(2;Y)L124*], but not by a copy of this duplication bearing a *kis* mutation (*Dp(2;Y)L124, kis*^{7}) (Kennison and Tamkun, 1988) (data not shown). Thus, the recessive lethality of the *kis*^{k13416} chromosome is due to the P-element insertion in the *kis* gene, as opposed to another mutation. No KIS-L protein could be detected in extracts of salivary glands from *kis*^{k13416}

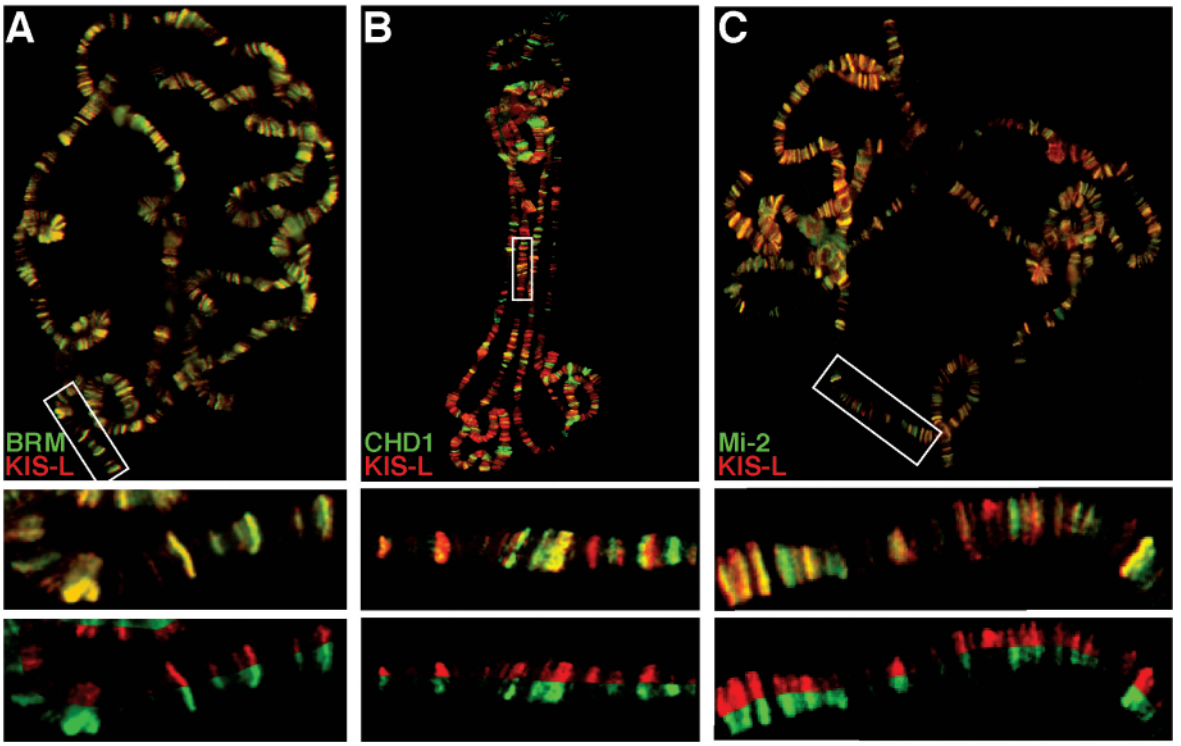

Fig. 6. KIS-L colocalizes with BRM and other chromatin-remodeling factors. Merged images of the distributions of KIS-L (red) and BRM (green; A), CHD1 (green; B) and Mi-2 (green; C) are shown. Extensive overlap between KIS-L and the other proteins are evident in the enlarged merged and split images of the regions bound by white rectangles. The distribution of KIS-L is identical to that of BRM and very similar to that of Mi-2. Although not as striking, there is also considerable overlap between the chromosomal distributions of KIS-L and CHD1.

homozygotes by western blotting (data not shown). Consistent with this observation, KIS-L was absent from the polytene chromosomes of *kis*^{k13416} larvae (Fig. 8). Although the polytene chromosomes of *kis*^{k13416} homozygotes sometimes appear slightly thinner than normal, the loss of KIS-L function does not significantly alter their overall morphology or banding pattern (Fig. 8B).

We next examined whether KIS-L regulates the association of PC with chromatin. PC binds to ~60 sites on polytene chromosomes (Fig. 9B) (Zink and Paro, 1989). Consistent with the functional antagonism between PC and KIS-L, the chromosomal distributions of the two proteins are predominantly non-overlapping (Fig. 9C-E). The number and intensity of PC-binding sites is not altered in *kis*^{k13416} larvae (Fig. 9F-H). Thus, KIS-L does not appear to play a general role in blocking the association of PC with chromatin in vivo. Upon close examination, ~80% of the PC-binding sites lie adjacent to or slightly overlap KIS-L. It remains possible that KIS-L restricts or regulates the activity of PcG proteins in the immediate vicinity of PREs.

KIS-L is required for an early step in transcriptional elongation by Pol II

The association of KIS-L with most sites of active transcription suggests that it might be required for one or more stages of the transcription cycle. To investigate this possibility, we compared the levels of Pol IIa, Pol IIo^{ser5} and Pol IIo^{ser2} on polytene chromosomes from wild-type and *kis*^{k13416} larvae (Fig. 10). Although the total level of Pol II in wild-type and *kis* mutant larvae is similar by western blotting (data not shown), the level of Pol IIo^{ser2} associated with the polytene chromosomes of *kis*^{k13416} larvae is dramatically reduced relative to wild type (Fig. 10I,L). This striking phenotype suggests that KIS-L, like BRM, plays a global role in transcription by Pol II. By contrast, the level and distribution of Pol IIa in *kis* mutant larvae is unchanged relative to wild type (Fig. 10B,E,H,K), indicating that KIS-L is not required for the recruitment of Pol II to promoters. The level and distribution of Pol IIo^{ser5} is also unchanged in *kis*^{k13416} larvae relative to wild type (Fig. 10C,F),

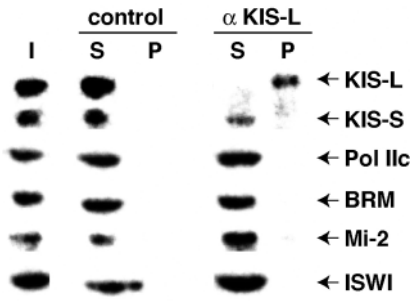

Fig. 7. KIS-L is not physically associated with other chromatin-remodeling factors or Pol II. Proteins were immunoprecipitated from embryo extracts using rabbit IgG (control) or antibodies against the N-terminal segment unique to KIS-L (α-KIS-L). One-fifth of the total input extract (I) and supernatant (S), and four-fifths of the total pellet (P) were separated by SDS-PAGE and analyzed by western blotting.

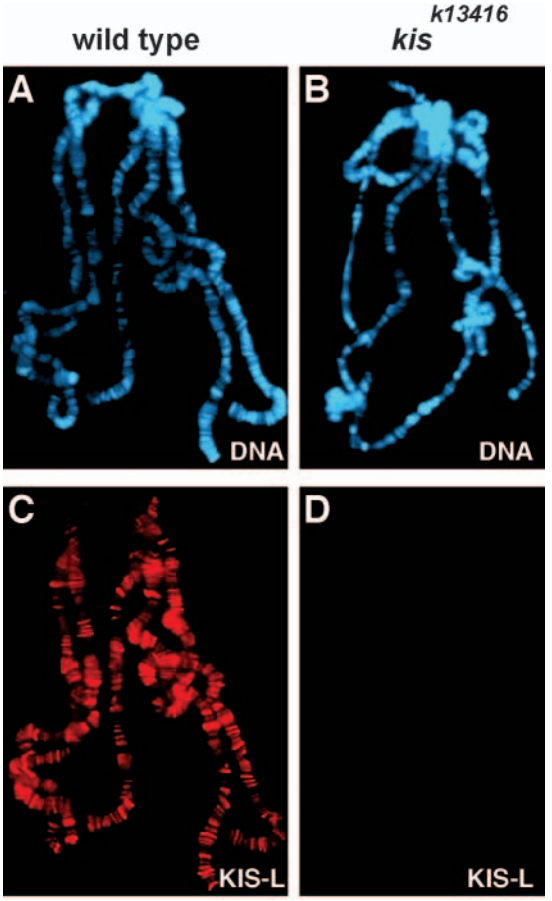

Fig. 8. The levels of KIS-L are dramatically reduced on polytene chromosomes of *kis* mutant larvae. KIS-L (red; C and D) was detected on chromosomes of wild type (A,C) and *kis*^{k13416} (B,D) larvae. DNA (blue; A,B) was visualized by DAPI staining. The level of KIS-L associated with chromatin is drastically reduced in *kis* mutants, but the overall banding pattern and morphology of polytene chromosomes is normal.

suggesting that the initial stages of elongation are not dependent on KIS-L function. Taken together, these data demonstrate that KIS-L facilitates a relatively early step in transcriptional elongation after serine 5 phosphorylation but prior to serine 2 phosphorylation.

The absence of Pol IIo^{ser2} on the polytene chromosomes of *kis* mutant larvae might result from a failure to phosphorylate serine 2, as opposed to a block in an early step of elongation. Inactivation of kinases that phosphorylate serine 2 in yeast (ctk1) (Ahn et al., 2004) and *Drosophila* (the Cdk9 subunit of P-TEFb) (Ni et al., 2004) do not impair transcriptional elongation. However, serine 2 phosphorylation is necessary for the recruitment of factors required for processing the 3′ ends of mRNAs (Ahn et al., 2004; Hirose and Manley, 2000; Proudfoot et al., 2002). Defects in 3′ end processing resulting from the loss of serine 2 phosphorylation lead to the rapid degradation of mRNAs. The failure to detect Pol IIo^{ser2} in *kis* mutants could result from either the failure to recruit the serine 2 kinase P-TEFb to promoters, or a defect in transcriptional elongation. To clarify the mechanism of action of KIS-L, we examined the distribution of the elongation factor SPT6 on the polytene chromosomes of *kis*^{k13416} larvae. We chose SPT6 for these experiments as the association of this elongation factor

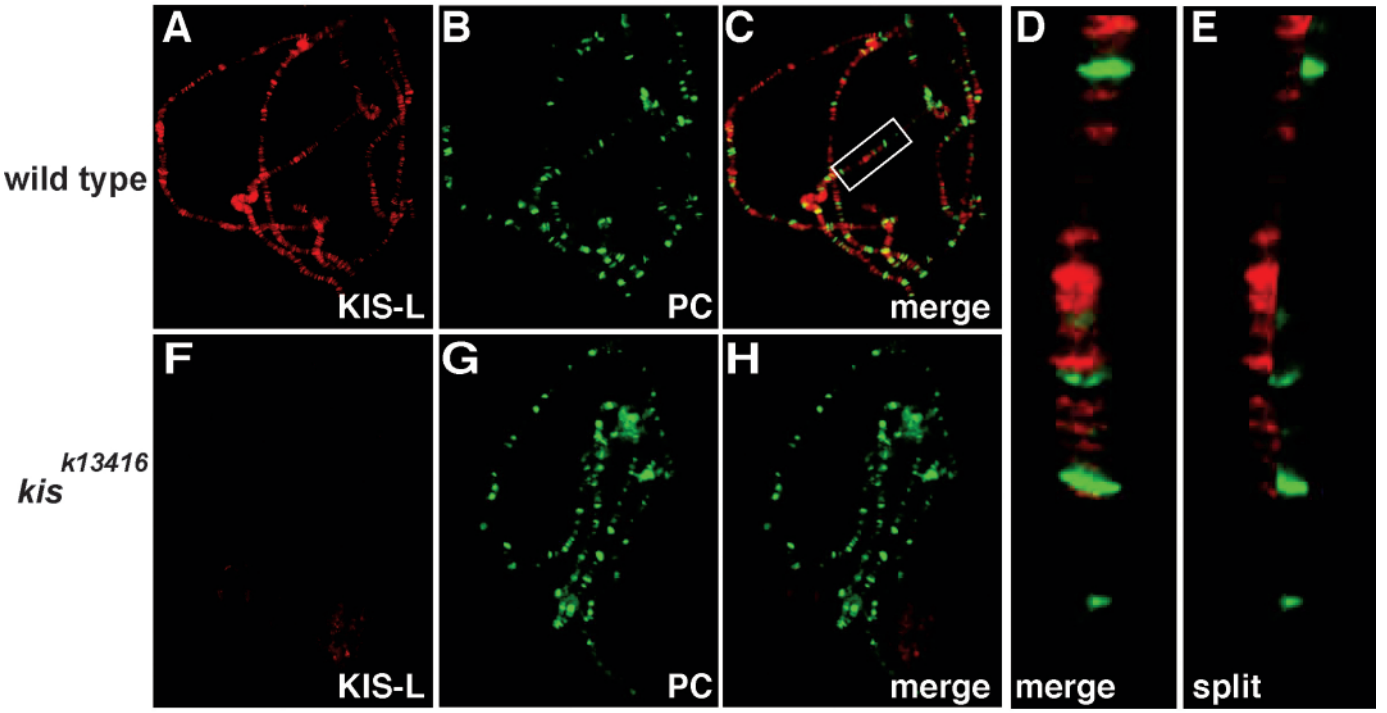

Fig. 9. Loss of KIS-L does not affect the association of PC with chromatin. The distribution of KIS-L (red; A) is predominantly non-overlapping with PC (green; B) on wild-type chromosomes as seen in merged (C) and magnified (D,E) images corresponding to the region bounded by the white rectangle in C. The number and intensity of PC bands are similar on the chromosomes of wild-type (B) and *kis*^{k13416} (G) larvae.

with Pol II is not dependent on serine 2 phosphorylation in yeast (Ahn et al., 2004). The loss of *kis* function dramatically reduces the level of SPT6 associated with polytene chromosomes (Fig. 11A,E) without affecting the overall level of this protein in the salivary gland as assayed by western blotting (data not shown). We therefore conclude that the absence of Pol IIo^{ser2} on the polytene chromosomes of *kis* mutant larvae is probably due to an early defect in elongation.

Chromatin-remodeling factors act sequentially during the transcription cycle

Finally, we examined whether KIS-L is required for the recruitment of other chromatin-remodeling factors to their target genes. The association of BRM with chromosomes is not affected by the loss of KIS-L function (Fig. 11B,F). This observation is consistent with previous studies indicating that BRM is required for relatively early stages of transcription, including the recruitment of Pol II to promoters (Armstrong et al., 2002). By contrast, loss of KIS-L function blocks the association of CHD1 with chromatin (Fig. 11D,H) without affecting the overall level of CHD1 as assayed by western blotting (data not shown). As CHD1 has been implicated in transcriptional elongation and colocalizes with Pol IIo^{ser2}, this result provides additional evidence that an early step in transcriptional elongation is blocked in *kis*^{k13416} larvae. The association of Mi-2 with chromatin is not dependent on KIS-L function (Fig. 11C,G), suggesting that Mi-2 may act prior to or independently of KIS-L in the transcription cycle. These data suggest that chromatin-remodeling factors act sequentially during the transcription cycle, with BRM acting prior to KIS-L and CHD1 acting during the later stages of elongation (Fig. 12).

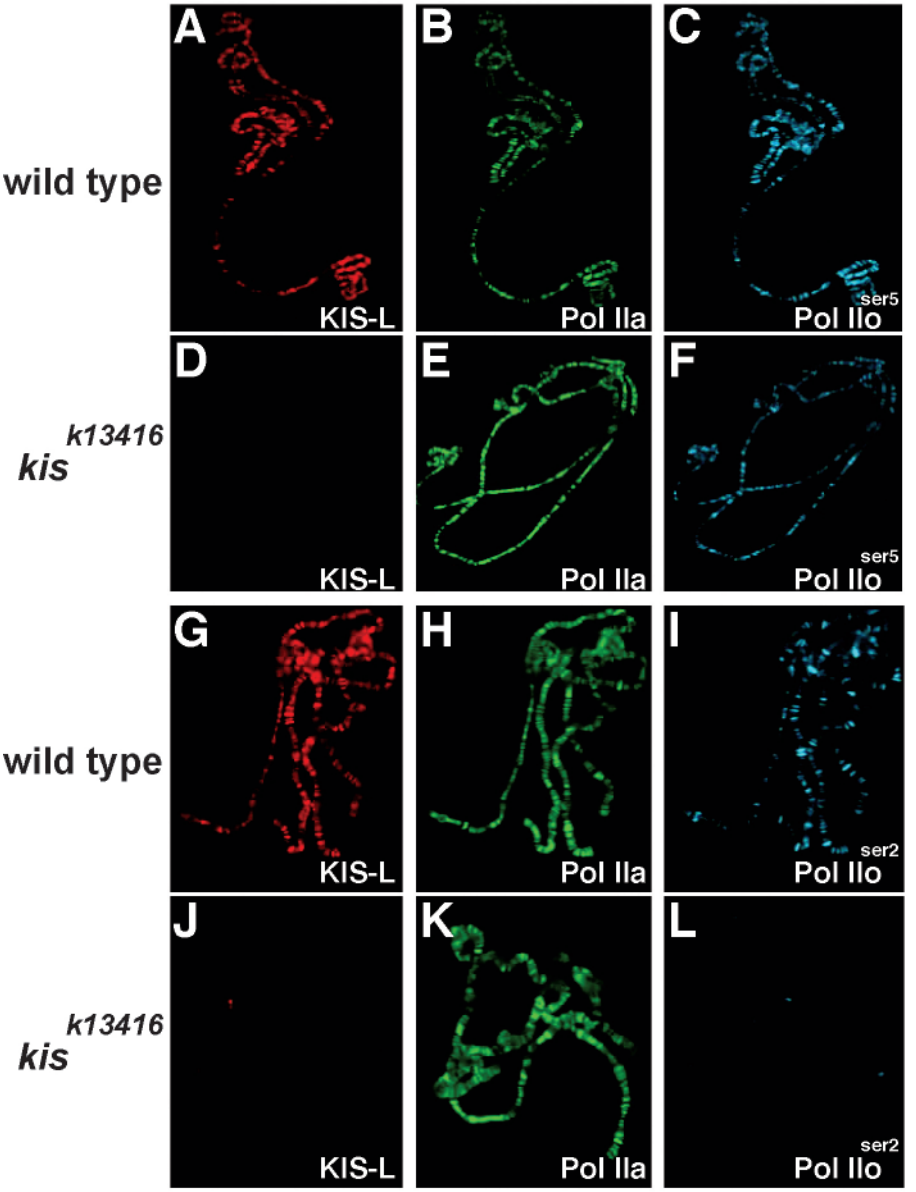

Fig. 10. KIS-L is required for the association of Pol IIo^{ser2} with polytene chromosomes. The distribution of KIS-L (red; A,D,G,J), Pol IIa (green; B,E,H,K), Pol IIo^{ser5} (blue; C,F) and Pol IIo^{ser2} (blue; I.L) on chromosomes isolated from wild type (A-C,G-I) and *kis*^{k13416} mutant larvae (D-F,J-L) is shown. The levels of both Pol IIa and Pol IIo^{ser5} are unaffected by the loss of KIS-L. By contrast, loss of KIS-L results in a dramatic reduction in the level of Pol IIo^{ser2} associated with chromosomes.

Discussion

Eukaryotic transcription involves a highly coordinated cycle of events, including the assembly of the pre-initiation complex, initiation, promoter clearance, elongation and termination. In theory, any step in the transcription cycle could be subject to developmental regulation by PcG and trxG proteins. Basal transcription factors and Pol II are recruited to the promoter during the initial stages of transcription (Buratowski, 2000; Svejstrup, 2004). After Pol II is recruited to a promoter, the phosphorylation of the C-terminal domain (CTD) of its largest subunit coincides with promoter clearance and the transition from initiation to the elongation phase of transcription (Svejstrup, 2004). A number of CTD kinases have been identified, including CDK7 (a subunit of TFIIH) and CDK9 (a component of P-TEFb), which phosphorylate serine 5 and serine 2 residues of the CTD, respectively (Prelich, 2002). The phosphorylation of serine 5 is highest near the promoter, whereas the phosphorylation of serine 2 increases as Pol II proceeds toward the 3′ end of genes (Cho et al., 2001; Komarnitsky et al., 2000). CTD phosphorylation modulates interactions between Pol II and other factors involved in transcription. For example, phosphorylation of the CTD may

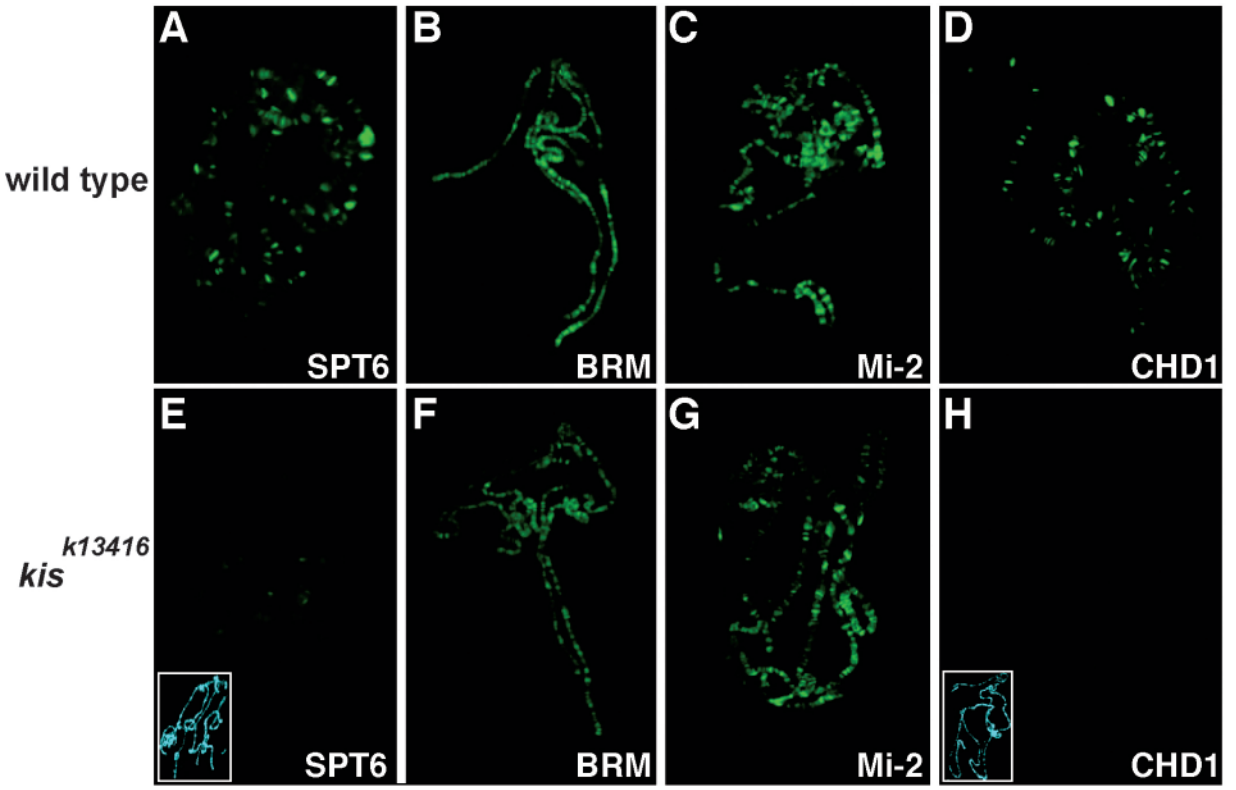

Fig. 11. KIS-L is required for the recruitment of the elongation factors SPT6 and CHD1, but not BRM and Mi-2, to chromatin. The distributions of SPT6 (A,E), BRM (B,F), Mi-2 (C,G) and CHD1 (D,H) on wild type (A-D) and *kis*^{k13416} (E-H) chromosomes are compared. Loss of KIS-L function does not affect binding of BRM and Mi-2 to chromosomes but the levels of both SPT6 and CHD1 are greatly reduced. Insets (E,H) show DAPI staining of chromosomes.

disrupt interactions between Pol II and mediator, thus facilitating promoter clearance (Pokholok et al., 2002; Svejstrup et al., 1997). The phosphorylated CTD can also act as a docking site for factors involved in mRNA processing and termination (Hirose and Manley, 2000; Proudfoot et al., 2002).

Our findings strongly suggest that KIS-L plays a global role in transcription by Pol II. Consistent with this view, KIS-L is associated with virtually all transcriptionally active regions of chromatin in salivary gland nuclei. The initial stages of transcription are normal in *kis* mutant larvae; Pol II is efficiently recruited to promoters and the phosphorylation of serine 5 of the CTD is not affected. However, the absence of Pol IIo^{ser2} and the elongation factors SPT6 and CHD1 on the polytene chromosomes of *kis* mutant larvae strongly suggests that KIS-L is required for an early step in transcriptional elongation.

The discovery that KIS-L plays a global role in transcription by Pol II was unanticipated, as previous genetic studies suggested that *kis* plays relatively specialized roles in development (Daubresse et al., 1999). The limited phenotypes resulting from the loss of zygotic *kis* function may be due to the high maternal contribution of *kis* gene products. It is also

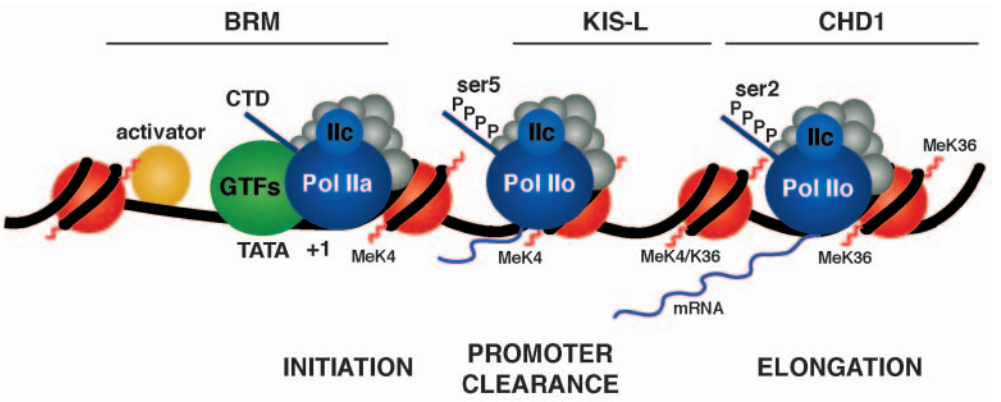

Fig. 12. Chromatin-remodeling factors facilitate distinct stages of transcription. The stage at which each chromatin-remodeling factor is hypothesized to function is marked by an unbroken line. BRM physically interacts with transcriptional activators and facilitates a step prior to the recruitment of Pol II to promoters. CHD1 has been implicated in the later stages of transcriptional elongation. Our findings suggest that KIS-L is required for the transition from the early to late stages of elongation. The activities of KIS-L and CHD1 may be influenced by the methylation of histone H3 lysine 4 and lysine 36 near the 5′ end and in the body of transcribed genes, respectively.

possible that the mutations used in previous genetic studies of *kis* are not null alleles, or that other factors can partially compensate for the loss of *kis* function in tissues other than the salivary gland.

How might KIS-L facilitate an early step in transcriptional elongation by Pol II? Based on its similarity to chromatin-remodeling factors, it is likely that KIS-L promotes transcription by altering chromatin structure. Nucleosomes and other components of chromatin can repress transcription at many different levels (Narlikar et al., 2002). For example, nucleosomes can interfere with the assembly of the preinitiation complex by blocking access of gene-specific and general transcription factors to promoter regions. Nucleosomes also present a physical barrier to Pol II during transcriptional elongation. Histone-modifying enzymes, chromatin-remodeling factors and numerous other factors are therefore crucial for transcriptional initiation and elongation in a chromatin environment (Berger, 2002; Flaus and Owen-Hughes, 2004).

Recent studies of the mammalian *hsp70* gene have suggested that the remodeling of nucleosomes near promoters is important for early stages of transcriptional elongation. Prior to induction, a paused polymerase is located just downstream of the hsp70 promoter (Brown et al., 1996). Following induction, heat shock factor 1 targets mammalian SWI/SNF to the *hsp70* promoter resulting in the disruption of this nucleosome, thus allowing elongation to proceed (Corey et al., 2003). By analogy, KIS-L may promote elongation by remodeling nucleosomes immediately downstream of promoters.

The presence of two chromodomains in KIS-L suggests that the methylation of N-terminal histone tails may also be important for its targeting or function. This possibility is intriguing in light of recent studies suggesting that histone methyltransferases modulate distinct stages of transcriptional elongation by Pol II. The phosphorylation of serine 5 of the CTD promotes interactions between Pol II and the SET1 methyltransferase (Ng et al., 2003), resulting in the methylation of lysine 4 of histone H3 in the vicinity of promoters (Nagy et al., 2002; Roguev et al., 2001; Santos-Rosa et al., 2002). The subsequent phosphorylation of serine 2 of the CTD promotes interactions with the SET2 methyltransferase (Krogan et al., 2003; Xiao et al., 2003), resulting in the methylation of lysine 36 of histone H3 in the body of transcribed genes. The pattern of histone methylation resulting from the dynamic interactions between Pol II and histone methyltransferases may facilitate the transition from early to late stages of elongation by regulating interactions between chromatin-remodeling factors and nucleosomes near promoters.

The yeast SET1 histone methyltransferase is a subunit of a large protein complex known as COMPASS (Miller et al., 2001). Functional counterparts of yeast COMPASS have been identified in humans; these complexes contain subunits related to *Drosophila* TRX (human MLL1 and MLL2) and ASH2 (human ASH2L) (Hughes et al., 2004; Yokoyama et al., 2004). Human MLL1 and MLL2 methylate lysine 4 of histone H3 (Hughes et al., 2004; Milne et al., 2002; Nakamura et al., 2002; Yokoyama et al., 2004), as does *Drosophila* TRX (Smith et al., 2004), suggesting that they are functional counterparts of SET1. Another *Drosophila* trxG protein, ASH1, also methylates lysine 4 of histone H3 both in vitro and in vivo (Beisel et al., 2002; Byrd and Shearn, 2003).

The above findings suggest a plausible model for how KIS-L interacts with other trxG proteins to activate transcription (Fig. 12). Perhaps KIS-L, like SWI/SNF and other chromatin-remodeling factors, is targeted to promoters via interactions with transcriptional activators or components of the general transcription machinery (Hassan et al., 2001; Peterson and Logie, 2000). Once targeted to the vicinity of a promoter, KIS-L may recognize promoter-proximal nucleosomes methylated on lysine 4 of histone H3 (by TRX or ASH1) via its chromodomains, leading to the localized remodeling of nucleosomes that pose a barrier to elongation by Pol II.

Our findings may help explain the functional antagonism between *kis* and PcG proteins. PcG proteins do not merely render chromatin inaccessible to the general transcription machinery, as transcriptional activators, basal transcription factors (including TFIID and TFIIF) and even Pol II are associated with targets of PcG repression (Breiling et al., 2004; Breiling et al., 2001; Dellino et al., 2004). Thus, PcG proteins may act directly on components of the general transcription machinery assembled at promoters. Consistent with this possibility, Pol II is efficiently recruited to an *hsp26* promoter silenced by the *bxd* PRE, but is unable to melt the promoter and initiate transcription (Dellino et al., 2004). A separate study of a promoter silenced by PcG proteins in its natural context (the *Ubx* promoter in wing imaginal discs) revealed that PcG proteins bind to both PREs and a very narrow region just downstream of the start of transcription (Wang et al., 2004). Although their precise mechanism of action remains to be determined, the above studies suggest that PcG proteins exert their influence during the later stages of transcriptional initiation or early stages of elongation. It is therefore tempting to speculate that PcG proteins may repress transcription by blocking KIS-L activity. Further analysis of the role of KIS-L in transcription, together with the development of systems for analyzing its function in vitro, will be necessary to test this hypothesis and clarify the role of KIS-L in gene expression and development.

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