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Molecular and genetic analysis of JIL-1, a chromatin associated protein kinase implicated in transcriptional regulation in Drosophila

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Molecular and genetic analysis of JIL-1, a chromatin associated protein kinase implicated in transcriptional regulation in *Drosophila*

by

Ye Jin

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For the Major Program

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For the Graduate College
To my parents and my husband, for their love and patience.
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GENERAL INTRODUCTION

Dissertation organization

This dissertation begins with the background introduction. Current knowledge on chromatin structure is examined. The importance of chromatin remodeling in chromatin related fundamental cellular processes, especially transcription, is described. Then the two major kinds of protein machines involved in chromatin remodeling are introduced. The mechanisms used by these protein machines are discussed. The connections between signal transduction pathways, cancer and chromatin structure are addressed. Finally, the mechanisms of gene dosage compensation, a process clearly manifested by chromatin remodeling over one entire chromosome, are described.

The dissertation is then organized into two papers. Portion of the first paper is published in Molecular Cell in July of 1999. The second paper is a manuscript being prepared for submission. The first paper describes the cloning and initial functional characterization of JIL-1, a novel tandem protein kinase associated with chromosomes throughout the cell cycle. Ye Jin made the GFP transgenic flies, carried out the indirect immunofluorescent staining of polytene chromosomes using JIL-1 antibodies and was involved in confocal imaging of the in vivo dynamic movement of GFP-JIL-1 fusion protein and indirect immunofluorescence labeled polytene chromosome preparations. The second paper describes the further genetic and molecular analysis of JIL-1. The JIL-1 ribozyme and mutant ribozyme constructs were cooperative work of Hao Dong and Ye Jin. The remainder of the studies were done by Ye Jin.
The last part of this dissertation is the general conclusions. Current knowledge of JIL-1 and its potential functions are generalized and discussed. A model based on current data is presented and the future works are proposed.

References cited in the general introduction and general conclusions are listed in the final section entitled Literature Cited.

Background

Chromatin structure and organization

In the nucleus, the eukaryotic genome is organized into a highly compacted nucleoprotein structure called chromatin. The chemical components of chromatin are DNA, histones and other nonhistone chromosomal proteins, such as topoisomerase II, HMG proteins and transcription factors. The primary structure of chromatin is the 10-nm diameter filament that appears as "beads-on-a-string" observed under the electron microscope (reviewed in Alberts et al., 1994).

The beads comprise the unit particle of chromatin called the nucleosome, which contains 160 bp of DNA wrapped around the core histone octamer with two full turns in a left-handed superhelix. The elliptically shaped histone octamer is composed of 2 molecules of each histone H2A, H2B, H3, and H4 with a mass of approximately 206 kD (Luger et al., 1997) and maximal dimensions of 11.0, 6.0 and 6.5nm (Richmond et al., 1993). The histone octamer is thought to be assembled first by deposition of an H3/H4 tetramer followed by the incorporation of 2 H2A/H2B dimers (Eickbusch and Moudrianakis, 1978). Each histone of the octamer has a long, basic, unstructured N-terminal domain and a globular C-terminal domain which has multiple α helices. The globular domain, also called the fold domain, is
responsible for the 160 bp core DNA binding and histone-histone interaction within the histone octamer. The core DNA is bound to the histone fold domain through electrostatic forces between the negatively charged phosphate groups in the DNA backbone and positively charged amino acid in histones, such as lysine and arginine (Paranjape et al., 1994).

Generally, the binding of DNA to histone octamer is not based on sequence specificity. However, A-T rich sequences are easier to compress than G-C rich sequences and wrapping around the histone octamer causes severe distortion of the DNA double helix. Therefore some sequences, like A-T-rich sequences, are more easily bound by the histone octamer. The specific binding of a histone octamer by nucleosomal DNA based on its primary sequence is called nucleosome positioning. Nucleosome positioning can be classified into “rotational positioning” and “translational positioning”. Rotational positioning refers to the orientation of DNA to the face of the histone octamer (for example, at a specific base pair, whether the minor groove of the DNA helix faces toward or away from the core histone surface). Histone octamers preferentially position the minor groove of A-T rich sequence to the surface of the core histones and the minor groove of G-C rich sequence away from the core histone surface (Paranjape et al., 1994). Translational positioning refers to the specific location of a nucleosome along a gene. For example, in the promoter region of the Adh gene in Drosophila, a nucleosome is passively positioned on a site between the enhancer and the distal adult promoter and this position is entirely dependent on the intrinsic DNA structure features. This specific nucleosome positioning facilitates the interaction of the enhancer and the distal promoter (Jackson and Benyajati, 1993). Sequence specific binding of transcriptional factors also affects the nucleosome positioning. For instance, α2 repressor, a sequence specific DNA-binding factor in yeast, is observed to be required for positioning a nucleosome over the TATA boxes of the a cell specific genes, STE6 and BAR1. Additionally
it is proposed that α2 repressor inhibit the transcription of a cell specific genes through translational positioning (Herschbach and Johnson, 1993).

The extended N-terminal tail domain of core histones are believed to mediate internucleosomal contact and assembly of 30 nm chromatin fiber into higher order chromatin structure. Thus, post-translational modifications of the N terminal tail domain, such as acetylation, phosphorylation and methylation, are proposed to alter the conformation of the chromatin fibers by changing the interactions of histone tail domains with adjacent nucleosomes (Wolffe and Hayes, 1999).

The DNA between two nucleosomes is called linker DNA and has an average length of 60 bp. The length of linker DNA varies among different species and different tissues within one organism. Wolfe (1993) suggested that the linker DNA length tends to be longer in higher eukaryotic animals than that in lower eukaryotic organisms such as yeast. It is also noticed that, in some species, the chromatin repeat length (the length of core DNA wrapped around the histone octamer plus the length of linker DNA) is correlated with the transcriptional activity (Holde, 1989). In other words, the length of chromatin repeats increase with the increment of transcriptional activity.

In living cells, chromatin seldom adopts the "beads on a string" form. Rather, under the electron microscope, chromatin isolated using conditions similar to cellular conditions appears to be organized in 30 nm fibers. The exact structure and assembly of 30 nm chromatin fibers is unknown, although several models have been proposed. The first model is the solenoid model in which the 10 nm fibers are coiled like a spring into a left-handed superhelix with six nucleosomes per turn and histone H1 is in the central, lengthwise axis of the fiber (Thoma et al., 1979). This model was further modified by McGhee et al. (1983) with the histone H1 either in the periphery or center of fiber depending on the function of the linker length. The second model is the cross-linked model in which the 10 nm fibers are
folded into a zig-zag like structure twisting along the length of chromatin into a double-superhelical structure (Williams et al., 1986). The third model is the twisted ribbon model in which the 10 nm fiber is folded into a zig-zag like ribbon and this ribbon is coiled into a superhelical structure with nucleosome peripherally arranged and linker DNA internally located (Bednar et al., 1998). Recent X-ray structure of a nucleosome resolved at 2.8 angstroms seems to support the solenoid model (Luger et al., 1997). In addition to that, the N-terminal tail of core histone can mediate interactions between nucleosomes, packaging nucleosomes into 30 nm fiber is also realized with the help of linker histone H1 (Alberts et al., 1994). Histone H1 has a central globular domain flanked by a short basic unstructured N-terminal tail and a long C-terminal domain. The globular domain has a winged-helix domain and is believed to bind to DNA next to the nucleosomal pseudo-dyad (the surface of histone octamer where the DNA enters and exits (Paranjape et al., 1994)). Its extended N and C termini contact with linker DNA and pull nucleosomes together to form solenoids (Alberts et al., 1994). Because incorporation of histone H1 stabilizes the nucleosomes and facilitates higher order chromatin assembly, it is believed histone H1 plays a role in repression of gene expression. Depletion of histone H1 is found in active or competent genes relative to inactive genes.

Besides DNA and histones, there are also nonhistone chromosomal proteins which exist abundantly on chromatin and play important roles in chromatin structure and function. The high mobility group (HMG) proteins are such a set of proteins. There are three classes of HMG proteins which are not related to each other: the HMG1/2 proteins, the HMG14/17 proteins and the HMG-I/Y proteins. They are commonly termed HMG proteins only because of their similar extraction and solubility properties (Paranjape et al., 1994). HMG1/2 proteins have a C-terminal acidic domain and two HMG boxes upstream, a motif found in many chromatin related proteins. HMG1/2 preferentially binds to cruciform DNA and single
stranded DNA relative to double stranded DNA, which suggest their function may be related to recombination, replication and DNA repair (Zlatanova and Holde, 1998). HGM1/2 also can efficiently bend the DNA and both in vivo and in vitro transcription assays show it can activate gene transcription (Jayaraman et al., 1998; Boonyaratanakornkit et al., 1998). It is estimated for every nucleosome, there are 0.1-0.5 molecules of HMG1/2 (Paranjape et al., 1994). HMG14/17 are small proteins with a basic-rich N-terminal domain and an acidic-rich C-terminal domain. They are relatively enriched in actively transcribed regions relative to inactive regions and are shown to be able to stimulate transcription possibly through unfolding the high-order chromatin structure by the interaction of its C-terminal acidic domain with the N-terminus of histone H3 (Paranjape et al., 1994; Trieschmann et al., 1998). Like HMG1/2, HMG14/17 proteins are also abundant in chromatin with 0.1-0.5 molecules per nucleosome. HMG-I/Y proteins are small proteins containing a motif called the AT hook that binds to the minor groove of AT-tracts in DNA. They have been found both in AT-rich sequences of heterochromatin (Martelli et al., 1998) and AT-rich sequences upstream promoters of genes, such as enhancers, SARs (nuclear scaffold-associated regions) and MARs (nuclear matrix-associated regions). Many reports indicate HMG1/Y is an architectural transcription activator of gene expression by bending DNA and facilitating the assembly of transcriptional machinery (Falvo, et al., 1995; Perrella et al., 1999).

The 30-nm chromatin fiber is further compacted and organized by nonhistone proteins into higher order chromatin structures, the molecular basis of which is still poorly understood. These higher order chromatin structures consist of a series of domains of distinct characters. Correlations exist between these distinct domains of chromatin with their functional states (Holde, 1989; Wolfe, 1993). For example, when chromatin is stained with fluorescent dyes, it is visually divided into heavily stained and weakly stained regions, which correspond to highly condensed chromatin and loosely compacted chromatin regions. The
condensed chromatin regions, referred to as heterochromatin, generally have fewer genes and less transcriptional activity. The loosely compacted chromatin regions, termed euchromatin, have more genes and more active transcription. Although a visual system of chromatin classification such as this does not provide quantitative information about the structural difference between domains of chromatin, it provides some clues about the correlation between the functional state with distinct chromatin domains. There are two types of heterochromatin: constitutive heterochromatin and facultative chromatin (Sterwart, 1997). Constitutive heterochromatin remains condensed and transcriptionally inert throughout the cell cycle and development stages. They generally contain non-coding highly repeated DNA sequences and especially localize in the centromere and telomeres of chromosomes. Facultative heterochromatin is condensed chromatin that unfolds and become transcriptionally active during some portion of the cell cycle or development stages. Like heterochromatin, there are also two kinds of euchromatin: transcriptionally active euchromatin and inactive euchromatin. Inactive euchromatin does not have active gene transcription although it is unfolded and accessible to transcription machinery. This suggests that the chromatin structures of transcriptionally active euchromatin and inactive euchromatin are different (Holde, 1989).

At the nuclear level chromatin organization occurs through compartmentalization (Lamond et al., 1998). Euchromatin tends to be in the interior of the nucleus and the heterochromatin is found to sequester in the periphery of the nucleus (Csink and Henikoff, 1996). It is proposed such compartmentalization may provide a nuclear context to influence gene expression (Cockell and Gasser, 1999). In budding yeast, the compartmentalization of telomeres, which resemble the heterochromatin of higher eukaryotic organisms, is realized through Ku complex, a telomere binding protein complex, tethering telomeres to the nuclear envelope. Deletion of the genes encoding components of Ku complexes results in
mislocalization of telomeres and the disappearance of telomere proximal silencing (Boulton and Jackson, 1998; Gravel et al., 1998).

Gene expression and chromatin remodeling

The packaging of the genome into highly compacted chromatin and chromatin compartmentalization provide the organization for chromatin related fundamental processes such as gene transcription, replication, recombination and mitosis (Paranjape et al., 1994). However, at the same time, packaging the genome into highly compact chromatin also prevents the access of protein factors to the DNA template to initiate gene transcription, replication, recombination and chromatin condensation upon mitosis. The nucleosome is the primary determinant of DNA accessibility due to the distortion of DNA that blocks the binding sites. To initiate transcription or replication, eukaryotic organisms utilize a group of specialized molecular machines to unfold or disrupt the normal highly packaged eukaryotic chromatin locally or globally. Throughout this dissertation, this process will be termed chromatin remodeling and it will include chromatin modification and chromatin disruption induced by ATP-dependent chromatin remodeling complexes.

To help understand the chromatin remodeling involved in gene expression, Paranjape et al. (1994) define the ground state of a gene as the inactive state in which the gene is packaged into a regular array of nucleosomes and is inaccessible to transcription factors. Gene expression is the process in which a gene transitions from a ground state to a derepressed state and further to an activated state by the relief of nucleosome-mediated repression. These transitions are accomplished by remodeling of gene chromatin structure, which results in disruption of the regular nucleosome array, histone depletion, interaction of
enhancer and promoter, and recruitment of the transcription machinery to the opened promoter region.

DNA replication and gene repression also require specialized proteins and chromatin remodeling is required in these two processes. CHRAC, a large protein complex with chromatin remodeling activity was found to be associated with the replication origin of the SV40 genome and to allow efficient replication in vitro (Alexiadis et al., 1998). Some ARS (autonomous replication sequences) are functionally dependent on SWI/SNF, a chromatin remodeling complex (Flanagan and Peterson, 1999). In the case of gene silencing, the chromatin-remodeling complex RSC is required for transcriptional repression of the yeast CHA gene. In the absence of Sthlp/Npslp, the ATP-requiring molecule of the RSC complex, expression of the CHAl gene in non-induced cells increased to a level comparable with that of a fully induced cell (Moreira and Holmberg, 1999).

There are mainly two kinds of protein complexes which work independently or synergistically in chromatin remodeling. One group includes chromatin remodeling complexes which disrupt the nucleosome by using the energy from ATP hydrolysis. According to the structural similarity of the subunit that hydrolyzes ATP within the remodeling complexes, chromatin remodeling complexes can be grouped into three classes: the SWI/SNF-type complexes, the ISWI-type complexes and the complexes containing CHD proteins (Travers, 1999). The engine molecules (the subunit that hydrolyzes ATP) in SWI/SNF-type complexes has a helicase/ATPase domain and a bromodomain. These molecules include the SWI2/SNF2 in the SWI/SNF complex, the Sth1 in the RSC complex, the Brm in the Drosophila SWI/SNF-like complex, the BRG1 and the hBrm in the human SWI/SNF-like complex. In CHARC (chromatin accessibility complex), NURF (nucleosome remodeling factor) and ACF (ATP-utilizing nucleosome assembly and remodeling factor), which were isolated from Drosophila embryo extracts (Pazin and Kadonaga, 1997), ISWI is
the engine molecule and contains a helicase/ATPase domain but not the bromodomain (Tsukiyama et al., 1995). In complexes containing CHD proteins, CHD contains a helicase/ATPase domain, two chromodomains and a PHD domain or another DNA binding domain. All of the classes of chromatin remodeling complex can facilitate the access of transcription factors to nucleosomal DNA by unfolding the nucleosome. However, each class shows different substrate specificity and modeling activity and is involved in different aspects of chromatin related processes, which are determined by the protein context within the complex. For example, the ISWI type complexes not only stimulate transcription factor binding like SWI/SNF complexes do, they also promote nucleosome mobility (the respositioning of the histone octamer on a DNA sequence) (Travers, 1999). Furthermore, even having the same/similar engine molecule, different complexes may have different functional activities, which again are dependent on the protein context within the complexes. For instance, while SWI/SNF complexes are only found in a small subset of genes in yeast, RSC complexes are more widespread. It is suggested that RSC may play a general role in the maintenance of a particular chromatin structure. Another example is that certain CHD containing complexes are associated with histone deacetylase and show repressive chromatin remodeling (Zhang et al., 1998; Tong et al., 1998).

Recent studies of RSC mediated chromatin remodeling (Lorch et al., 1998; 1999) indicate that the molecular mechanisms for chromatin disruption by ATP-driven protein complexes consist of two steps. The first step is formation of an activated intermediate containing RSC and core histones together with nucleosomal DNA. The nucleosomal DNA shows greatly enhanced sensitivity to exo- and endonucleases comparable to that of naked DNA. Upon removal of RSC complex, the nucleosome remains in the altered state with only slightly reduced nuclease sensitivity. The second step is dislocation of this histone octamer to a nearby nucleosome-free DNA sequence. The advantage of this mechanism is that it can
explain both the enhanced DNA accessibility and nucleosome mobility, especially for ISWI-containing complexes (Travers, 1999).

Recent studies also suggest the mechanism of bidirectionality of remodeling complexes (Zhang et al., 1998; Verreault et al., 1996). Bidirectionality means, besides disruption of nucleosome and facilitation of the access of transcription factor to DNA, remodeling complexes are also required for removal of these factors and reformation of nucleosomal array (Travers, 1999).

Another kind of protein complex involved in chromatin remodeling contains enzymes capable of post-translational modification of histones and nonhistone chromosomal proteins. These post-translational modifications include acetylation, phosphorylation, methylation, ubiquitination and ADP-ribosylation. Histone tail domains are usually target sites for these covalent modifications. As mentioned previously, the N-terminal tails of histones contact nucleosomal DNA (Hansen et al., 1998), mediate interactions between nucleosomes (Garcia-Ramirez et al., 1992; Garcia-Ramirez et al., 1995) and are essential for the self-assembly of condensed fibers into higher order structures (Tse and Hansen, 1997; Tse et al., 1998). Thus site-specific modification of residues on N-terminal tail of histones may cause the alteration of functional states of the chromatin (Hansen et al., 1998). Acetylation of core histones, especially H3 and H4, has been directly linked to gene activation (Brownell et al., 1996; Ogryzko et al., 1996; Kuo et al., 1998) by identification of histone acetyltransferase as a subunit of many transcriptional coactivator complexes and histone deacetylase as a subunit of transcriptional corepressor complexes (Taunton et al., 1996; Alland et al., 1997). Acetylation of the lysine residues at N terminal tails of core histones is thought to neutralize their positive charges and presumably reduces the interaction of the core histone tail with DNA so that the DNA is more accessible to transcription factors. Also acetylation reduces the stability of the 30 nm compacted chromatin fiber (Tse et al., 1998) and the interaction
among adjacent nucleosomal arrays (Perry and Chalkley, 1982). The role of histone acetylation in gene activation is probably to potentiate gene transcription, rather than to take part in the transcriptional process, as high enrichment of acetylated histone are found even in an inactive globin gene in chicken erythrocytes (Grunstein, 1997).

Compared with the extensively studied histone acetylation, reports on other post-translational modifications of histones, such as phosphorylation, are fewer, and their roles in regulation of gene expression are still underexplored. Phosphorylation of histones is expected to destabilize nucleosomes and higher order chromatin structure, because the addition of negative charge on N-terminal tail can lead to reduced affinity between N-terminal tail and DNA just like acetylation does. Phosphorylation of histone H1 has been directly shown to weaken the interaction of histone H1 with DNA (Hills et al., 1991) and occurs predominantly at conserved (S/T-P-X-K/R) motifs. Phosphorylated histone H1 proteins are enriched in actively transcribed chromatin (Lu et al., 1995; Sweet et al., 1996) relative to inactive chromatin. Research on the mouse mammary tumor virus promoter (MMTV) indicates the transcription competence of MMTV depends on phosphorylation of histone H1 (Lee and Archer, 1998). Besides histone H1 phosphorylation, phosphorylation of histone H3 at serine 10 is also reported, and this phosphorylation is required for correct chromatin condensation and segregation (Wei et al., 1997; Wei et al., 1998). Intriguingly, phosphorylation of the same site at histone H3 is also correlated with activation of gene transcription. Small subsets of nucleosomes on early response genes (IE genes), such as c-fos and c-jun, are found to be rapidly phosphorylated in response to mitogenic growth signal (Mahadevan et al., 1991). And these fractions are also extremely sensitive to hyperacetylation (Barratt et al., 1994). But acetylation itself does not predispose H3 to phosphorylation, nor does phosphorylation predispose H3 to acetylation, which suggests H3 phosphorylation event is independent of its acetylation. Furthermore, chromatin conformation changes have been observed concomitant
with the phosphorylation state of H3 within the chromatin of induced IE genes (Chen et al., 1987; Chen et al., 1990). Thus, besides histone acetylation, histone phosphorylation also can potentiate gene transcription independently or synergistically with other remodeling ways to cause changes in chromatin structure. In addition, histone H3 phosphorylation is likely to provide an important link between cellular mitogen activated signal transduction pathways and chromatin remodeling.

Signal transduction and chromatin remodeling

In the past three years, great progress has been made in studying retinoid, steroid and thyroid hormones induced transcriptional activation and repression through chromatin remodeling complexes (Xu et al., 1999). These studies give us some insight on how signal transduction pathways can change chromatin structure to potentiate gene transcription. Receptors for retinoid, steroid and thyroid related hormones are nuclear transcription factors. In unliganded states, these receptors interact with corepressor factors, such as NcoR (nuclear receptor corepressor) and its homolog SMRT (silencing mediator of retinoid), and recruit complexes containing Sin3, histone deacetylases and additional proteins to bind the targeted genes and repress their expression (Horlein et al., 1995; Chen et al., 1995). Once bound by ligands, these corepressor complexes disassociated from the receptor and are replaced by coactivator complexes, like SRC-1/ncoA-1 (steroid receptor coactivator-1/nuclear receptor coactivator-1), TIF-2/GRIP-1/NcoA-2 (transcriptional intermediary factor 2/glococorticoid receptor interacting protein 1/nuclear receptor coactivator 2), p/CIP/ACTR/AIB1 (p300/CBP co-integrator associate protein/activator of the thyroid and RA receptor/amplified in breast cancer), CBP/p300 (CREB-binding protein) and p/CAF (p300/CBP-associated factor), then
potentiate targeted gene expression through chromatin remodeling by the intrinsic histone acetylase activities within those complexes (Xu et al., 1995) (Figure 1).

Activities of corepressor and coactivator complexes are regulated by cellular signal transduction pathways. In the case of corepressor, the interaction between antagonist-bound ER and NcoR is significantly weakened by activation of the cAMP pathway (Lavinsky et al., 1998). In the case of coactivator, CBP/p300, the integrator of many signal transduction pathways (Perissi, et al, 1999) and an essential component of many coactivator complexes, is differentially regulated at different steps of coactivation by nuclear calcium/calmodulin-dependent (CaM) protein IV and by cAMP. Cytoplasmic calcium signals that stimulate the RAS mitogen-activated protein kinase pathway provide the CBP recruitment signal by phosphorylation of CREB at serine 133, but the final activation of gene transcription by CBP has to be achieved through nuclear calcium/CaM mediated activation on CBP (Chawla et al., 1998). Another example of CBP mediated coactivation regulated by a signal transduction pathway is pit-1 transcriptional activation. Pit-1 is a tissue specific transcription activator important for the differentiation of three pituitary cell types. Its transcriptional activation response to cAMP and growth factors are mediated through CBP containing complexes. Distinct functional domains of CBP were used by cAMP and growth-factor-induced signaling pathways. Furthermore, the HAT (histone acetyltransferase) activity of p/CAF is required by growth-factor-induced pathway but not by cAMP pathway which use CBP intrinsic HAT activity (Xu et al., 1998).

Signal transduction pathways also regulate the assembly and functional state of chromatin remodeling complexes. For example, phosphorylation of a human SWI/SNF complex upon mitosis may be correlated with its inactivation during mitosis. hSWI3 and Brg1 are phosphorylated and hBrm is missing from mitotic inactivated SWI/SNF complex.
**Fig. 1 Hormone induced gene activation pathway.** Ligands of nuclear receptors induce recruitment of the coactivator complexes, leading to chromatin remodeling and activation of transcription. The double-headed arrow implies a sequential model in which the TRAP or DRIP complexes activate transcription initiation after the chromatin remodeling step catalyzed by CBP/p300/CAF or SWI/SNF complexes. The CBP/p300/p/CAF complexes, as well as the TRAP or the DRIP complexes, may provide link between nuclear receptors and the transcription machinery (as indicated by dashed arrows). The mSin3/HDAC corepressor complexes which have intrinsic histone deacetylase activities, are linked to nuclear receptors via NCoR or SMRT in the absence of ligands (figure adapted from XU, 1999)
In vitro assay further shows active hSWI/SNF can be phosphorylated and inactivated by erl kinase (Sif et al., 1998).

Cancer and chromatin remodeling

Chromatin remodeling is an essential step for regulation of gene transcription. Mutations in genes encoding important components in chromatin modifying and remodeling complexes can cause the deregulation of transcription of some important oncogenes or tumor suppressor genes and lead to cancers.

CBP/p300, a transcriptional integrator for multiple signal transduction pathways, is connected to many forms of human cancers. Patients lacking one functional allele show Rubenstein-Taybi syndrome (RTS), an autosomal dominant condition characterized by cranial and digital malformations, mental retardation and predisposition to cancer (Giles et al., 1998). p300 missense mutations and loss of heterozygosity on chromosome 22q at the p300 loci are correlated with colorectal tumors (Muraoka et al., 1996). These haplo-insufficient phenotypes of CBP/p300, together with studies of transgenic CBP and p300 knockout mouse, suggest that gene dosage of CBP/p300 is important in embryonic development and somatic growth control (Jacobson and Pillus, 1999).

Lesions in human SWI/SNF complexes are also associated with tumors. Somatic mutations truncating the hSNF5 gene, a component of SWI/SNF, are identified in several aggressive pediatric malignant rhabdoid tumors (Verstege et al., 1998). These lesions consist of frameshift, nonsense and deletion mutations mainly affecting the conserved carboxy-terminal 200 amino acids that mediate interaction with hBRM.

Fusions between genes involved in chromatin remodeling caused by chromosomal arrangements have been found in multiple cases of leukemia. For example, a MOZ-CBP
(MOZ, a human homologue of Drosophila histone transferase mof) recurrent translocation of the genotype t(8;6)(p11;p13) is associated with M4/M5 subtype of acute myeloid leukemia (AML) (Borrow et al., 1996). Fusion proteins produced by this arrangement retain the PHD domain, zinc finger and HAT domain of MOZ and a mostly intact CBP. Another gene frequently found in abnormal chromosomal arrangements resulting in leukemia is the MLL gene. MLL is a human homologue of Drosophila trithorax, a chromatin protein required for proper homeotic gene expression and regulation of chromatin structure (Waring and Cleary, 1997). MLL-CBP fusion is found in t(11;16)(q23;p13) genotype of patients having myelodysplastic syndrome, therapy-related AML and chronic myelomonosytic leukemia. The exact mechanisms beneath these types of leukemia induced by fusions between two chromatin remodeling genes are unknown. However, Jacobson and Pillus (1999) proposed three possible ways: mistargeting of fusion proteins may cause aberrant gene expression; misregulated association of the fusion partners may cause constitutive activation of genes; and the fusion may result in partial loss of function of one or both partners due to sequestration and inability to interact with other proteins.

Gene dosage compensation in Drosophila

Gene dosage compensation is a process clearly manifested by chromatin remolding over one entire chromosome. Gene dosage compensation is the process by which the same amount of transcription of X linked genes is reached regardless of the dosage difference in males and females. In mammals, dosage compensation is achieved by inactivating one of the female X chromosomes (Ballabio and Willard, 1992; Penny et al., 1996). In the nematodes C. elegans, the transcription level of each X chromosome in the XX hermaphrodite is
reduced to half of the level of a single male X chromosome (Meyer and Casson, 1986; Kelley and Kuroda, 1995).

In Drosophila, the male X chromosome is hypertranscribed at a two-fold level compared with one female X chromosome (Mukherjee and Beerman, 1965; Baker et al., 1994). This higher transcription from the male X chromosome is correlated with changes in chromatin structure (Gorman and Baker, 1994; Bone et al., 1994; Hilfiker et al., 1997). The Drosophila male X chromosome appears wider and more diffuse relative to the rest of autosomes although its DNA content is half of the paired autosomes. It is also enriched in H4Ac16, an isoform of histone H4 correlated with active gene transcription (Bone et al., 1994).

The molecular mechanism behind Drosophila gene dosage compensation is currently an active research field. Establishing dosage compensation is initiated with sex determination in early Drosophila development. The ratio of X-chromosomes to autosomes determines the expression of the sex determination gene, Sex lethal (Sxl), an RNA binding protein. When the X: A is 1, Sxl is expressed by female-specific transcription activation of an early embryonic promoter; later, a positive autoregulation loop is formed maintaining the expression of Sxl in female from a constitutive promoter (Bell et al., 1991; Keyes et al., 1992). When the X: A ratio is 0.5, Sxl is not expressed, and the male specific gene product, MSL2, is translated (Gorman et al., 1993). MSL2, a ring finger protein having the zinc-binding motif, is the key protein to initiate the assembly of MSL complexes. MSL2 first interacts with MSL1, a novel protein with an acidic domain, through the ring finger. Then the MSL1-MSL2 complex recruits and stabilizes MSL3 protein, also a novel protein with a chromodomain. Later by further recruiting MLE and other components, the MSL complexes are assembled with a molecular weight about 2 MD (Copps et al., 1998). These large protein complexes then associate with the male X chromosome at hundreds of sites and are proposed
to recruit \textit{mof}, a histone acetyltransferase, to the male X chromosome, thereby increasing H4Ac16 so that the chromatin structure is altered and elevated transcription levels result. (Bone et al., 1994).

Besides MSL proteins, other known proteins or components within \textit{MSL} complexes are \textit{MLE, rox1} and \textit{rox2}. \textit{MLE} is an RNA/DNA helicase for which DNA/RNA helicase activity has been shown in vitro (Kuroda et al, 1991; Lee et al., 1997). Abolishment of the ATPase/helicase activities of \textit{MLE} impairs its function in gene dosage compensation. Based on its DNA and RNA helicase activity, \textit{MLE} is proposed to disrupt chromatin structure in a similar way as SWI/SNF and NURF complex and alter the structure of nascent RNA to facilitate the process of reinitiation and elongation. The interactions of \textit{MLE} with other components of \textit{MSL} complex are weak and are dependent upon RNA components. Immunostaining of \textit{MLE} on polytene chromosomes disappears upon treated with RNase A (Lee et al, 1997). \textit{rox1} and \textit{rox2} are two non-coding RNAs shown to specifically bind to the male X chromosomes (Amrein and Axel, 1997; Meller et al., 1997). Their appearance on the male X is dependent on the \textit{MSL} complexes. It is proposed that \textit{rox1} and \textit{rox2} may be RNA components of the dosage compensation pathway and inhibit female specific gene expression on male X chromosomes (Amrein and Axel, 1997; Meller et al., 1997). However the requirement for either one of the rox RNAs in male viability has not been demonstrated. It is possible their role in dosage compensation is redundant (Copps et al., 1998).

\textit{Sxl} inhibits dosage compensation in females by negatively regulator translation from \textit{MSL2} transcripts so that \textit{MSL2} protein only exists in males (Bashaw and Baker, 1995; Kelley et al, 1995; Zhou et al. 1995). Other \textit{MSL} proteins, \textit{MSL1, MSL3} and \textit{MLE} are expressed both in male and female (Gorman et al., 1995). In female flies in which \textit{MSL2} is ectopically expressed, \textit{MSL} complexes are assembled and bind to female X chromosomes. Based on this observation, it is proposed that \textit{MSL2} seeds the assembly of \textit{MSL} complexes and targets \textit{MSL}
complexes to male X chromosomes by specifically distinguishing male X chromosomes from autosomes (Zhou et al., 1995; Kelley et al., 1995; Bashaw and Baker, 1995).

The advantages of Drosophila system to study chromatin structure and gene regulation

Drosophila is an excellent system for studying chromatin-associated proteins and signal transduction pathways. The huge polytene chromosomes from salivary glands provide a very powerful system to view the distribution of chromosomal proteins on chromatin. The interbands and bands of polytene chromosomes reflect the loosely packaged chromatin regions, in which genes are actively transcribed, and highly condensed chromatin regions, in which consist of heterochromatin and primarily inactive genes (Rykowski, et al., 1988; Jamrich et al., 1977; Zhimulev et al., 1981). Thus, proteins involved in active gene transcription are generally found in interbands and puffs, such as RNA polymerase II and splicing factors (Weeks et al., 1993). Drosophila embryonic extract has been widely used as a system for studying chromatin assembly and regulation of gene transcription in vitro. Many chromatin remodeling complexes such as CHARC, NURF and ACF were first identified and isolated from embryo extracts (Travers, 1999). Overall, Drosophila is a system in which genetic, cytological, molecular and biochemical means can be easily combined to study complicated biological processes. Through powerful genetic screens and molecular cloning, proteins involved in a signal transduction pathway can be identified and their interaction can be inferred from determination of epigenetic relationships and other molecular and biochemical methods, such as the case with MSL genes.

Although tremendous progress has been made in understanding gene regulation and chromosome structure, this field still has much to be explored and is currently a very active
area. For example, although histone phosphorylation has been correlated with active transcription, a direct link has yet to be demonstrated. Also, the exact mechanisms of chromatin modifications affecting transcription are still unknown. How do the signal transduction pathways connect extracellular stimuli, the resultant chromatin remodeling and the activation of gene transcription? How is substrate specificity achieved for remodeling complexes, and how can one chromatin remodeling complex be involved in two different, even opposite aspects, of chromatin related processes? To answer these questions and to define the signaling molecules, pathways and how they are interrelated in control of gene expression and chromatin structure will be a major challenge in this field in the near future.
JIL-1, A NOVEL CHROMOSOMAL TANDEM KINASE IMPLICATED IN TRANSCRIPTIONAL REGULATION IN DROSOPHILA

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SUMMARY

We have cloned and characterized JIL-1, a novel tandem kinase in Drosophila that associates with the chromosomes throughout the cell cycle. Antibody staining and live imaging of JIL-1-GFP transgenic flies show that JIL-1 localizes to the gene-rich interband regions of larval polytene chromosomes and is up-regulated almost two-fold on the hypertranscribed male X chromosome compared to autosomes. Phylogenetic analysis suggests JIL-1 together with human MSKs defines a new family of tandem kinases. That JIL-1 is a functional kinase was demonstrated by autophosphorylation and phosphorylation of histone H3 in vitro. Based on these findings we propose that JIL-1 may play a role in transcriptional control potentially by regulating chromatin structure.

INTRODUCTION

DNA tightly packed together with histones and other proteins into nucleosomes is not easily accessible to the enzymes which use it as a template for transcription or replication. Consequently, remodeling of chromatin structure plays an essential role in regulation of fundamental cellular processes such as gene expression (Wolffe and Hayes, 1999; Varga-
Weisz and Becker, 1998) and DNA replication (Alexiadis et al., 1998). This change in accessibility to the DNA is affected by remodeling complexes such as NURF or SWI/SNF that reorganize nucleosomal positioning in an energy-dependent process (Carlson and Laurent, 1994; Varga-Weisz and Becker, 1998) or by modifications of the histone tails that lower their affinity for the DNA (Davie, 1998; Wolffe and Hayes, 1999). The most prevalent histone tail modification is acetylation, which has been convincingly linked with the establishment of transcriptionally active chromatin (Csordas, 1990; Brownell and Allis, 1996). Another post-translational modification of histone tails which has been shown to be functionally important is phosphorylation (Davie, 1998; Wolffe and Hayes, 1999). The phosphorylation occurring on serine 10 in histone H3 is necessary for chromosomal condensation and occurs at high levels in mitotic cells (Wei et al., 1999). However, phosphorylation of histone H3 serine 10 also has been found to occur in a small subset of nucleosomes in mitogenically stimulated cells (Mahadevan et al., 1991; Barratt et al., 1994). These observations imply a role for phosphorylation of histones in regulation of gene expression in addition to its well established role in chromosome condensation and is further supported by evidence that H3 can physically interact with both transcriptional activators (Alevizopoulos et al., 1995) as well as with transcriptional repressors and silencers (Wan et al., 1995; Bortvin and Winston, 1996). In addition, phosphorylation of histone H3 appears to be regulated by mitogen-activated signal transduction cascades, indicating a direct link between signal transduction pathways and chromatin structure (Mahadevan et al., 1991; Barratt et al., 1994).

Drosophila is an excellent model system in which to identify and study signal transduction molecules such as protein kinases that may directly regulate chromatin structure and gene expression. It is well established that changes in chromatin structure are correlated with dosage compensation in the male (Gorman and Baker, 1994; Hilfiker et al., 1997) providing a readily accessible assay for studying the molecular mechanisms mediating this process. In order to achieve equal levels of expression of genes on the X chromosome, a two-
fold hyper-transcription of the male’s single X chromosome occurs relative to the female’s two X chromosomes and the autosomes (Lucchesi and Manning, 1987). This increased transcriptional activity correlates with a more diffuse chromosome structure, such that despite the fact that it contains half the DNA content, the male X chromosome appears to be the same width as the paired female X chromosomes and the autosomes (Gorman and Baker, 1994). In this study we have identified a novel tandem kinase, JIL-1, which localizes to chromosomes throughout the cell cycle and which is present at nearly two-fold higher levels on the male X chromosome as compared to autosomes. Furthermore, JIL-1 is preferentially localized to the gene rich euchromatic interband regions of polytene chromosomes and we show that JIL-1 is a functional kinase that can phosphorylate histone H3 in vitro. Based on these findings we propose that JIL-1 is a protein kinase which plays a functional role in transcriptional regulation potentially by facilitating chromatin remodeling at regulatory regions in the genome in Drosophila.

RESULTS

Molecular Cloning and Characterization of JIL-1

The mAb2A, which was previously shown to exhibit a dynamic nuclear staining pattern (Johansen, 1996; Johansen et al., 1996), was used to screen a λgt11 genomic Drosophila expression library. A partial clone identified in this expression screen was subsequently used to probe embryonic and ovary-specific cDNA libraries resulting in isolation of several overlapping cDNAs from both developmental stages spanning approximately 6.5 kb. Analysis of the cDNA transcripts revealed a single large open reading frame of 3,621 nucleotides which conceptually translates into a 1,207 amino acid protein with a calculated molecular mass of 137 kDa. As diagrammed in Figure 1 the protein contains two tandemly arranged serine/threonine kinase domains (Figure 1, shaded regions) (Hanks and Quinn, 1991). Based on this tandem
kinase domain structure, which is reminiscent of the JAK family of tyrosine kinases, we have named the newly identified protein, JIL-1. The first kinase homology domain (KDI) is between residues 261 and 546 and is separated by an 85 amino acid linker region from the second kinase domain (KDII) which is located between residues 633 and 886. Whereas KDI contains all of the conserved residues found in greater than 95% of all kinases (Hanks and Quinn, 1991) (Figure 2A), KDII is lacking the first of two conserved glycines usually found in subdomain I and the conserved glutamate residue usually found in subdomain III. The tandem kinase domains are flanked by a unique 260 residue NH$_2$-terminus and by a unique 321 residue COOH-terminus. The NH$_2$-terminal domain contains an asparagine-rich stretch (9 out of 10 residues) and an alanine-rich stretch (16 residues). In addition, JIL-1 contains a bipartite nuclear localization signal starting at position 58 (Figure 1, boxed region). Three regions characterized by a low hydrophobicity index and high proline, glutamic acid, aspartic acid, serine, and threonine content were similar to PEST sequences that have been implicated in targeting proteins for rapid turnover (Rodgers et al., 1986). Two of these regions are found in the NH$_2$-terminal domain and one is found in the inter-kinase domain region (Figure 1, underlined sequences).

The two kinase domains, KDI and KDII, of JIL-1 were compared with all sequences in the current databases in order to identify the most related sequences. KDII was not closely related to any other kinase family; however, KDI had the highest sequence identity with the first kinase domain of a novel protein tandem kinase in human reported in two recent studies called mitogen- and stress-activated kinase, MSK1 (Deak et al., 1998) or RSK-like protein kinase, RLPK (New et al., 1999). An alignment of KDI of JIL-1 and MSK1 and its isoform MSK2 (Deak et al., 1998) is shown in Figure 2A. In addition, Figure 2B shows a domain comparison of JIL-1 with MSK1 and Drosophila RSK, which is the most related kinase to JIL-1 next to the human MSKs. Whereas JIL-1 is 63% identical in KDI to MSK1, it is only 47% identical to Drosophila RSK. In KDII JIL-1 is 32% and 28% identical to the second kinase
domain in MSK1 and Drosophila RSK, respectively, a level of shared residues reflecting the general level of conserved features among kinase domains. Compared to these other tandem kinases, JIL-1 shows extended NH2- and COOH-terminal domains. To further determine the evolutionary relationship between JIL-1 and other protein kinases we constructed phylogenetic trees based on maximum parsimony. Figure 2C shows a consensus tree based on KDI sequences from protein kinases that had the highest sequence identity with JIL-1 in database searches. The tree is rooted using sequences from the yeast PKC kinase domain. The phylogenetic analysis indicates that JIL-1 is grouped with 95% bootstrap support with human MSK1 and MSK2 in a monophyletic clade that is distinct from the RSK, S6 and RAC kinase families and their Drosophila homologs. Consequently, these data suggest that JIL-1 is the Drosophila representative of a novel tandem serine/threonine kinase family which it defines together with MSK1 and MSK2. However, the fact that JIL-1 and MSK1 are only 32% identical in KDI suggests that vertebrate kinases which would be overall more closely related to JIL-1 than the MSK1 kinase may exist. Interestingly, this phylogenetic analysis also suggests that the S6 kinases, which are single domain kinases, may have evolved from tandem kinases by a deletion of the second kinase domain (Figure 2C).

Antibody and Northern Analysis of JIL-1

In order to further characterize the JIL-1 protein, new JIL-1 specific polyclonal antibodies were generated against a β-galactosidase JIL-1-fusion protein (Odin antiserum) as well as a GST-JIL-1 fusion protein (Hope antiserum) in rabbits. Figure 3 shows a syncytial blastoderm Drosophila embryo stained with affinity-purified JIL-1 antiserum where all of the synchronously dividing nuclei were labeled confirming the nuclear localization of JIL-1. This staining pattern is similar to the interphase nuclear staining pattern obtained with mAb2A (Johansen, 1996; Johansen et al., 1996). On immunoblots of embryo protein extracts (0–6 hr)
both the Odin and Hope antisera detect JIL-1 protein as a doublet or triplet of bands migrating at approximately 150-160 kDa (Figure 4A). However, post-translational modifications of JIL-1 may be developmentally regulated as JIL-1 protein from later developmental stages and in the S2 cell line was detected as a single band by the antisera (data not shown). The specificity of the JIL-1 antibody was confirmed by preadsorption experiments. As shown in Figure 4A (lane 3 and 4) preadsorption with GST-fusion protein of the Hope antiserum completely abolished staining on immunoblots whereas preadsorption with GST protein was without effect as compared to control lanes. Northern blot analysis using a JIL-1 probe reveals a single transcript migrating at approximately 6.5 kb suggesting that alternative splicing is not involved (Figure 4B).

**JIL-1 is a Functional Kinase which Phosphorylates Histone H3 in vitro**

Many kinases have been shown to be regulated by autophosphorylation. In order to address whether JIL-1 encodes an active kinase and to determine whether JIL-1 could potentially regulate its activity by autophosphorylation, we immunoaffinity purified JIL-1 protein from S2 cell tissue culture extracts and tested the purified JIL-1 protein in an in vitro kinase assay. Immunoprecipitates were incubated in a kinase reaction buffer with 10 μCi of \([\gamma-32P]\)-ATP added. After the incubation the immunoprecipitates were fractionated by SDSPAGE, Coomassie Blue stained, dried, and incorporation of radiolabeled phosphate was visualized by autoradiography. Control immunoprecipitations using pre-immune sera were performed simultaneously. Autoradiographs of gel fractionated kinase assays done with immunoprecipitated JIL-1 revealed a labeled band migrating at the same position as JIL-1, as detected by immunoblot analysis (Figure 5A). Lanes containing the control pre-immune immunoprecipitations showed neither phosphorylation labeling nor JIL-1 protein present (Figure 5B). The immunoprecipitations using both immune and pre-immune sera were found to contain equivalent levels of primary antibody (Figure 5A and 5B, arrows). These results
demonstrate that JIL-1 possesses an inherent kinase activity and is able to autophosphorylate in an in vitro kinase assay.

Histone H3 phosphorylation has been shown to correlate with activation of gene expression (Mahadevan et al., 1991; Barratt et al., 1994) as well as chromatin condensation during mitosis (Wei et al., 1998; 1999). We therefore tested whether the nuclear JIL-1 kinase could phosphorylate bovine histone H3 in vitro. The bovine histone H3 NH2-terminal tail is identical to that of its Drosophila homolog except that alanine 31 is substituted for a proline. Kinase assays with either JIL-1- or pre-immune antisera immunoprecipitation were performed as above but with 15 μg of histone H3 included in the reaction. Samples were fractionated by SDS-PAGE, Coomassie Blue-stained, and autoradiographed (Figure 5C). Whereas no phosphorylation was observed in the control pre-immune immunoprecipitation assay, histone H3 in the JIL-1 immunoprecipitation reaction showed clear labeling after autoradiography. Coomassie Blue staining of the gel showed that equivalent amounts of histone H3 were present in both reactions. Thus, these experiments suggest that histone H3 can serve as a substrate for the JIL-1 kinase in vitro.

**JIL-1 Localizes to Chromosomes Throughout Mitosis in Live Embryos**

In order to examine in living tissue the dynamics of JIL-1 distribution throughout the cell cycle, transgenic fly lines expressing a green fluorescent protein JIL-1 fusion protein (JIL-1-GFP) were generated. JIL-1 containing GFP coding sequence at its NH2-terminus was constructed in the pCaSpeR hs83 P-element vector (Figure 6A) and used to generate 14 independent lines of transgenic flies. Although all lines showed the same localization pattern, the strongest fluorescing line (G59.1) was selected for further study and crossed into a background containing the vinS deficiency which removes one copy of the JIL-1 gene (JIL-1 maps to the 68A region on polytene chromosomes, Y. Jin, unpublished observations) increasing the proportion of detectable JIL-1-GFP product. Immunoblotting of transgenic
embryonic proteins with JIL-1 antibody revealed an additional protein band not present in wild type flies that was consistent with the predicted size of the JIL-1-GFP protein (Figure 6B, arrow). This indicates that the fusion protein could readily be detected on immunoblots and was being stably expressed.

To study the cellular localization of the JIL-1-GFP fusion protein various tissues, including ovaries, imaginal discs, 3rd instar larval bodywall muscles, and salivary glands were dissected and imaged live in physiological saline using confocal microscopy. In all tissue examined JIL-1-GFP was detectable at high levels in the nucleus approximately 4 hours after heat shock induction. An example of this is illustrated in Figure 6C which shows a projection image of 10 confocal sections of two ovarian egg chambers. The JIL-1-GFP fusion protein is clearly localized to the nucleus of all the cells, including the nuclei of the large polyploid nurse cells and the smaller diploid follicle cells. The JIL-1-GFP fluorescence in the nucleus was not uniform but was organized in a discrete pattern as shown at high magnification of a single confocal section in Figure 6D. This pattern overlapped with Hoechst fluorescence in double labeled preparations (data not shown) suggesting that JIL-1 is localized to chromosomes in the nucleus. The JIL-1-GFP distribution in the interphase nucleus closely matched that observed with JIL-1 antibody (Figure 3).

To further analyze JIL-1's possible localization to chromosomes we imaged JIL-1-GFP distribution during the cell cycle in early syncytial blastoderm embryos. Time lapse movies were generated from confocal sections obtained with 20 s intervals from live embryos. Figure 6E1-4 shows four frames selected from such a movie. JIL-1-GFP is widely distributed throughout the nucleus at interphase (Figure 6E1), condenses during prophase (Figure 6E2), aligns at the metaphase plate (Figure 6E3), and moves to the spindle poles at anaphase, where its distribution then decondenses back into the interphase pattern (Figure 6E4). This sequence of events is very similar to chromosomal dynamics during the cell cycle as observed in Drosophila by video time-lapse of fluorescently-tagged histone (Therkauf, 1995) and suggests
that the JIL-1 nuclear kinase is chromosomally localized throughout the cell cycle. We did not observe any indications of dominant-negative effects as a consequence of the overexpression of the JIL-1-GFP construct.

**Expression of JIL-1 Correlates with Enhanced Transcription of the Male X Chromosome**

In order to better characterize JIL-1's apparent chromosomal localization, we analyzed its distribution pattern in confocal images of larval polytene chromosomes. Chromosomal squashes prepared from salivary glands of climbing third instar larvae were stained with JIL-1 antisera and double-labeled with Hoechst to visualize the DNA. It has previously been shown that Hoechst staining is brightest in the condensed banded regions of the chromosome due to the higher concentration of DNA (Beermann, 1972). Conversely, Hoechst signal is weaker or absent in the gene-rich interband regions which are comprised of less tightly packed euchromatin (Rykowski et al, 1988). The results obtained in a chromosomal squash from a female larva (Figure 7A-C) demonstrate that JIL-1 localizes to hundreds of sites along the polytene chromosome (Figure 7A) which correspond to interband regions, and that this staining shows only a very limited overlap with regions of strong Hoechst labeling (Figure 7B and 7C). Since interbands arise from partial unfolding of the 30 nm chromatin fiber and have been proposed to be the sites of actively transcribed genes (Jamrich et al., 1977; Zhimulev et al., 1981; Rykowski et al., 1988), these findings suggest that JIL-1 may be involved in gene activity potentially by regulation of chromatin structure via histone phosphorylation. However, it is clear that JIL-1 is not required at all locations of decondensed chromatin since there are interband regions which do not show JIL-1 labeling (Fig. 7C).

To further test the hypothesis that JIL-1 expression and localization may be correlated with transcriptional regulation we compared the expression of JIL-1 on female and male X chromosomes, respectively, in relation to JIL-1 expression on autosomes. The unpaired male
X contains only half the amount of DNA as the paired female X chromosomes and the autosomes, and to compensate for this the transcriptional level of the male X is upregulated about two-fold (Lucchesi and Manning, 1987; Gorman and Baker, 1994; Kelley and Kuroda, 1995). Figure 7D and 7E shows two examples of male polytene chromosomes labeled with JIL-1 antibody. The X chromosomes are clearly much more intensely labeled than the autosomes although the banding pattern is maintained. In contrast, in Figure 7F which is a Hoechst labeling of the same preparation as in Figure 7E, Hoechst binding to the X chromosome is much less than to the autosomes. Figure 7G shows a composite of the JIL-1 and Hoechst labeling of the X chromosome from this preparation indicating the non-overlapping banding pattern as in the female X and autosomes (Figure 7C). To verify the localization pattern of JIL-1 obtained by antibody labeling we imaged live polytene nuclei from JIL-1-GFP transgenic flies using confocal microscopy. Figure 7H shows a 3D stereo-reconstruction of female polytene chromosomes from such a nucleus. JIL-1-GFP is clearly localized on all chromosomes in a banded pattern similar to that observed by antibody labeling. Furthermore, the level of JIL-1-GFP in live transgenic animals is also upregulated on the male X as compared to autosomes (Figure 7I).

In order to quantify the difference in labeling of the X chromosomes in males and females, we determined the average pixel intensity of JIL-1 immunostaining for X chromosomes in males and females and compared it to the autosomal staining intensity which was normalized to a value of 1.0. In 11 female polytene squashes examined, there was no significant difference between autosomal (1.0 ± 0.0) and X-chromosome staining intensity (1.0 ± 0.1); whereas in 17 males, a highly statistically significant difference (p < 0.0001, student's t-test) between autosomal (1.0 ± 0.1) and X-chromosomal (1.8 ± 0.1) staining intensities was observed. Thus, there is an almost two-fold increased level of JIL-1 on the Drosophila male X chromosome compared to autosomes which correlates well with the roughly two-fold increased transcription level on this chromosome due to dosage compensation.
mechanisms (Lucchesi and Manning, 1987; Kelley and Kuroda, 1995). These results support a model whereby JIL-1 activity is involved in regulating gene expression.

DISCUSSION

In this study we report the cloning and characterization of Drosophila JIL-1, one of the antigens recognized by the mAb2A (Johansen et al., 1996; Johansen, 1996). JIL-1 encodes a novel nuclear protein that shows an unusual organization of two serine/threonine kinase domains in tandem flanked by unique NH2- and COOH-termini. This structural organization is reminiscent of the organization of the RSK family of protein kinases (Jones et al., 1988). However, phylogenetic analysis shows that JIL-1 defines a new kinase family together with the recently described human mitogen- and stress-activated kinases, MSK1 and MSK2 (Deak et al., 1998; New et al., 1999) that is distinct from the RSK and S6 kinase families. We demonstrate that JIL-1 is a functional kinase by its ability to autophosphorylate as well as to phosphorylate histone H3 in in vitro kinase assays. The nuclear localization of JIL-1 was confirmed in all tissues examined, including embryos, imaginal discs, salivary glands, and ovaries, both by labeling of fixed tissues using newly generated JIL-1-specific antibodies as well as by live imaging of a JIL-1-GFP construct in transgenic flies. The nuclear localization may be mediated by a bipartite nuclear localization signal in the NH2-terminal domain. Furthermore, JIL-1 antibody labeling of polytene chromosomes combined with imaging of the JIL-1-GFP in dividing nuclei showed that JIL-1 is a chromosomal kinase and that it remains associated with the chromosomes throughout the cell cycle.

The finding that JIL-1 immunolocalization on polytene chromosomes is complementary to the Hoechst staining pattern indicated that JIL-1 preferentially localizes to the gene-rich interband regions that are comprised of less compact DNA (Rykowski et al, 1988). This correlation was further strengthened by the observation that JIL-1 kinase is enriched almost
two-fold on the male larval polytene X-chromosome which as a consequence of dosage compensation mechanisms has an altered chromatin structure and is transcribed at twice the level of the female X chromosome in order to yield equivalent levels of X-linked gene products (Lucchesi and Manning, 1987; Gorman and Baker, 1994; Kelley and Kuroda, 1995). Thus, JIL-1's ability to phosphorylate histone H3 in conjunction with its localization to the more open chromatin interband regions and its enrichment on the hyperactive male X chromosome suggests a model whereby the JIL-1 kinase may function in transcriptional regulation. However, since JIL-1 is localized at most but not all decondensed regions of the euchromatic interbands it is not obligatory for open chromatin domains raising the possibility that it may be associated with regulating the functional state of discrete regulatory domains on the chromosome.

Evidence for an involvement of kinases in regulation of chromatin structure and gene expression comes from studies which show that phosphorylation of histone H3 appears to be governed by mitogen-activated signal transduction cascades. For example, treatment of cells with growth factors or phorbol esters resulted in rapid phosphorylation of histone H3 (Mahadevan et al., 1991) which was specific to a small subset of hyperacetylation-sensitive nucleosomes implying a role in gene activation in response to signaling events (Barratt et al., 1994). Correlation of the strength and duration of H3 phosphorylation with induction levels of c-fos/c-jun also suggested a possible mechanistic link between gene induction and H3 phosphorylation (Barratt et al., 1994). However, many of the previously described kinases implicated in these processes are not obligatory nuclear kinases but may relocate from the cytoplasm in response to extracellular signaling (Chen et al., 1992; Zhao et al., 1995). In contrast, our studies show that the JIL-1 kinase is localized to chromosomes throughout the cell cycle suggesting that JIL-1 may play a more direct or specific role in regulating chromosomal structure and function.
While this study is the first report of a kinase found to be up-regulated on the male X chromosome, chromatin modifying enzymes such as the histone acetyltransferase MOF (maleless on the first) have also been found to be preferentially localized to the male X chromosome and are necessary to facilitate hypertranscription in the male (Hilfiker et al., 1997; Gu et al., 1998). This is correlated with strong antibody labeling of a specific acetylated isoform of histone H4 (H4Ac16) on the X chromosome as compared to autosomes (Turner et al., 1992; Bone et al., 1994; Hilfiker et al., 1997). Thus, acetylation of nucleosomes due to the targeting of MOF to the male X chromosome is thought to be one of the factors responsible for the more diffuse chromatin structure necessary to achieve higher levels of transcription (Hilfiker et al., 1997), consistent with results from a number of studies where histone acetyltransferases (HATs) serve as transcriptional coactivators (Csordas, 1990; Wolffe and Hayes, 1999). The relaxation of chromatin structure may result from the modification of positive residues in the histone tails with negatively charged acetyl groups. Though less well studied, histone tail phosphorylation may similarly result in negative-charge modifications leading to a more open chromatin configuration which may also facilitate gene expression (Davie, 1998; Wolffe and Hayes, 1999). Thus, histone modifications both by phosphorylation as well as by histone acetylation may be connected to transcriptional regulation. The chromosomal localization of JIL-1 and its ability to phosphorylate histone would be consistent with JIL-1 providing such kinase activity. It should be emphasized, however, that kinases may show promiscuous activity on non-physiological substrates and that we do not know the in vivo targets for JIL-1 phosphorylation. There are a number of potential chromatin-localized targets besides histones which are also implicated in transcriptional regulation. For example, the cAMP response element binding protein CREB must be phosphorylated to activate its associated genes (Yamamoto et al., 1988), and the JIL-1 related kinase MSK1 has been reported to phosphorylate CREB in vitro (Deak et al., 1998).
In summary, the localization of JIL-1 to specific sites of particular chromatin domains argues strongly for a functional role for JIL-1 in site-specific phosphorylation, though it does not indicate the precise nature of this role. Nonetheless, the upregulation of JIL-1 on the hypertranscribed male X-chromosome and its in vitro phosphorylation of histone H3 suggest a model where JIL-1 is involved in transcriptional regulation possibly through modification of chromatin. The future isolation and characterization of mutants defective in JIL-1 in Drosophila, an animal amenable to genetic manipulation, promises to provide further insights into the function of this protein.

EXPERIMENTAL PROCEDURES

Drosophila Stocks

Fly stocks were maintained according to standard protocols (Roberts, 1986). Oregon-R was used for wild-type preparations. The deficiency stock Df(3L)vin5, ru1 h1 gl2 e4 ca1/TM3, Sb1 Ser1 was obtained from the Bloomington Drosophila Stock Center. The w; Δ2-3/TM2Ubx stock was the generous gift of Dr. Linda Ambrosio.

Molecular Cloning and Sequence Analysis

Library screening was performed using standard procedures (Sambrook et al., 1989). The mAb2A was used to screen a λgt11 library containing genomic sequence (Goldstein et al., 1986) and a JIL-1 positive clone was identified. This clone was used to isolate overlapping clones from embryonic cDNA libraries in λgt10 (Poole et al., 1985; Zinn et al., 1988; Clontech) and an ovarian cDNA library in λgt22 (Stroumbakis et al., 1994). DNA sequencing was performed at the Iowa State University DNA Sequencing and Synthesis Facility. JIL-1 sequence was compared with known and predicted sequences using the National Center for
Biotechnology Information BLAST e-mail server. PEST sequences were identified using the ExPASy server's PEST search utility (Rodgers et al., 1986). Phylogenetic analysis was performed by first generating alignments of kinase domains with the computer program Clustalw version 1.7; gaps in the resulting alignments were removed by deleting residues corresponding to the gaps. Trees were constructed by maximum parsimony using the PAUP program version 3.1.1 (Swofford, 1993) on a Power Macintosh G3. All trees were generated by heuristic searches and bootstrap values in percent of 1000 replications are indicated on the bootstrap 50% majority rule consensus tree.

**JIL-1-GFP Transgenic Flies**

An hs83 promoter-driven JIL-1-GFP cDNA fusion construct (P[hs83-GFP-JIL-1, w+f]) was produced in the P element germline transformation vector pCaSpeR-hs83 generating a JIL-1 cDNA sequence with GFP in-frame at its NH2-terminus. The P[hs83-GFP-JIL-1, w+f] plasmid was purified, injected into w; A2-3/TM2Ubx embryos using standard techniques (Roberts, 1986), and 14 transgenic lines were recovered. GFP-tagged JIL-1 driven by the hs83 promoter is expressed at low levels at room temperature; however, for maximal expression levels, transgenic flies were heat-shocked 30 min daily at 37°C for 3-4 days prior to experiments. To further enhance JIL-1-GFP fluorescence levels, a homozygous JIL-1-GFP transgenic stock was constructed in a Df(3L)vin5 heterozygous background which removes one copy of the endogenous JIL-1 gene (Y. Jin, unpublished observations).

**Antibody Generation**

Two regions from the COOH-terminal region of JIL-1 were subcloned using standard techniques (Sambrook et al., 1989) into the β-gal fusion protein expression vector pUR288 to generate the construct 288B1 encoding residues 928 to 1132, and into pGEX4T-3 (Pharmacia) to generate the construct pGEXFI encoding residues 886 to 1013. The correct orientation and
reading frame of the inserts were verified by sequencing. 288B1 β-gal fusion protein was induced and harvested essentially as described in Carroll and Laughon (1987). pGEXFI GST fusion protein was expressed in XL1-Blue cells and purified over a glutathione agarose column (Sigma) according to the pGEX manufacturer's instructions (Pharmacia). The purified fusion proteins were used to generate polyclonal antibodies in rabbits. Rabbits Tor and Odin were injected with 200 μg of 288B1 and rabbits Hope I and Hope II were injected with pGEX1I and then boosted at 21-day intervals, as described in Harlow and Lane (1988). After the second boost, serum samples were collected 7 and 10 days after injection. The sera was analyzed for specificity by comparing the staining obtained with the antisera and the preimmune sera on nitrocellulose filters spotted with fusion protein and with vector-protein only. Affinity purification of antibodies was performed using positive and negative affinity columns made by coupling 1 g of CNBr-activated Sepharose 4B (Pharmacia) to 5 mg of the appropriate protein as per the manufacturer's instructions.

**Northern and Western Blot Analysis**

PolyA+ mRNA was purified from 0-15 hr embryos using the FastTrack kit (Invitrogen), and 20 μg of polyA+ mRNA was fractionated on 1.2% agarose formaldehyde gels, transferred to nitrocellulose and hybridized with the addition of dextran sulfate (10%) according to standard protocols (Sambrook et al., 1989). JIL-1 specific probes were generated by purifying 1.2 kb of JIL-1 cDNA encompassing the COOH-terminal tail domain using GeneClean (Bio 101) and synthesizing random primer [32P]-labeled probe using the Prime-A-Gene kit (Promega) according to manufacturer's instructions. High stringency hybridization and washing conditions were employed (Sambrook et al., 1989).

Protein extracts were prepared from dechorionated embryos homogenized in lysis buffer (0.137 M NaCl, 20 mM Tris pH 8.0, 10% glycerol, 1% NP40). Protease inhibitors
were routinely added to the homogenization buffers. Proteins were separated on SDS-PAGE gels and transferred to nitrocellulose, and incubated with JIL-1 antibody overnight, washed in TBS (0.9% NaCl, 100 mM Tris, pH 7.5), incubated with HRP-conjugated goat anti-rabbit antibody (1:3000) (Bio-Rad) for 2.5 hr, washed in TBS, and the antibody complex visualized by incubation in 0.2 mg/ml DAB/0.03% H2O2/0.0008% NiCl2 in TBS.

**JIL-1 Kinase Assays**

1 x 10^6 Schneider-2 cells were lysed in ip buffer (20 mM Tris 8.0/150 mM NaCl/ 1 mM EDTA/ 1 mM EGTA/ 0.2% NP-40/ 0.2% Triton X-100) containing 2 mM Na3VO4 and protease inhibitors and clarified by centrifugation. The lysate was pre-cleared by incubating with 5 |l| pre-immune rabbit serum at 4°C for 2 hr followed by addition of 25 |l| Protein G-Sepharose (Sigma) for 1 hr. The sample was spun at 3000 rpm and the cleared lysate supernatant collected and incubated for 2 hr at 4°C with either 5 |l| Odin anti-JIL-1 antibody or 5 |l| of pre-immune Odin control sera. Incubation was continued for 1 hr at 4°C after addition of 5 |l| Protein G-Sepharose, samples were then washed 5 times in ip buffer and 3 times in kinase buffer (20 mM Hepes pH 7.4, 10 mM MgCl2, 2 mM Na3VO4). The immunoprecipitates were resuspended in 30 |l| kinase buffer with 10 |m|Ci [γ32P]ATP (6000 Ci/mmol) and incubated at 24°C for 30 min with continuous mixing. For histone H3 kinase assays, 15 |g| bovine histone H3 (Boehringer Mannheim) was added to the kinase reaction to a final volume of 30 |l|. Samples were then boiled for 4 min, separated by SDS-PAGE, Coomassie-blue stained, dried, and visualized by autoradiography.
Immunohistochemistry and GFP Imaging Studies

Antibody labelings of Drosophila 0-3 hr embryos were as previously described (Johansen et al., 1996). The HRP-labeled preparations were photographed on a Zeiss Axioskop using Ectachrome 64T film. The color positives were digitized using Adobe Photoshop and a Nikon Coolscan slide scanner. In Photoshop the images were image processed and converted to black and white before being imported into Freehand (Macromedia) for composition and labeling.

Polytene chromosome squash preparations from late third instar larvae were immunostained by the JIL-1 antibodies Hope and Odin essentially as previously described by Zink and Paro (1989). The polytene chromosome spreads were incubated with JIL-1 antibody diluted in 0.2% Tween-20, 0.2% BSA in PBS (1:25 dilutions for affinity purified Hope; 1:100 for Odin serum) at room temperature for 1 hr, washed three times for 10 min, and blocked with 5% normal goat serum for 30 min. Subsequently the preparations were incubated with FITC-conjugated goat anti-rabbit IgG (1:200 dilution), washed in 0.2% Tween-20, 0.2% BSA in PBS, rinsed in water, and stained with 10 µg/ml Hoechst 33258 (Molecular Probes) for 5 min. After a final brief rinse with distilled water the preparations were mounted in 90% glycerol containing 0.5% n-propyl gallate.

For imaging of JIL-1-GFP in living tissues, imaginal discs and ovaries were dissected and mounted in physiological saline (110 mM NaCl, 4 mM KCl, 2 mM CaCl2, 10 mM glucose, 10 mM HEPES, pH 7.4). For observation of JIL-1-GFP in developing embryos, embryos were collected on apple juice plates at 1 hr intervals, aged for 1 hr at room temperature, and manually dechorionated. Dechorionated embryos were then mounted on slides with a drop of light halocarbon oil (Halocarbon oil series 95, Halocarbon Products Corp.). The embryos were examined immediately by laser scanning confocal microscopy.
Confocal Microscopy

Confocal microscopy was performed with a Leica confocal TCS NT microscope system equipped with separate Argon-UV, Argon, and Krypton lasers and the appropriate filter sets for Hoechst, GFP, FITC, and TRITC imaging. A separate series of confocal images for each fluorophor of double labeled preparations were obtained simultaneously with z-intervals of typically 0.5 μm. An average projection image for each of the image stacks was obtained using the NIH-image software. These were subsequently imported into Photoshop where they were pseudocolored, image processed, and merged. During live imaging of JIL-1-GFP transgenic embryos a timelapse series of images were obtained with 20 s intervals and made into QuickTime movies. Stereo pairs of images of live JIL-1-GFP polytene nuclei at -7.2 and +7.2 degree angles, respectively, were generated using the Leica TCS 3D-reconstruction software.

Quantification of Polytene Chromosome Antibody Labeling

For quantification of the relative levels of antibody staining of X chromosomes and autosomes, confocal images of polytene chromosome squashes were analyzed using the NIH-Image software. In these images the grayscale was adjusted such that only a few pixels were saturated by using the glowover feature of the Leica TCS acquisition software. In NIH-Image the area of each X chromosome and the autosomes were traced using the outline tool and the average pixel value determined. This analysis assumes that the relative area of band and interband regions is roughly the same for the X chromosome as for the autosomes (this assumption was verified for female X chromosomes and autosomes, see Results). The staining intensity of the autosomes for the male and female preparations, respectively, was averaged and normalized to a value of one and compared to the average pixel intensity for the X chromosomes using a student's t-test.
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REFERENCES


FIGURE LEGENDS

Figure 1. The Predicted Protein Sequence for JIL-1 Contains Tandem Kinase Domains

The complete 3,621 nt open reading frame derived from the JIL-1 cDNA sequence translates into a 1,207 amino acid protein with a predicted molecular mass of 137 kDa. The shaded boxes in the sequence indicate the two domains with homology to serine/threonine kinase catalytic domains. The sequence also includes a putative bipartite nuclear localization signal (boxed region) and three underlined regions which show significantly high PEST sequence prediction scores (11.90, 12.98, and 11.58, respectively). The schematic diagram below the sequence indicates that the tandem kinase domains are flanked by unique NH$_2$ and COOH-terminal domains (white boxes). In the NH$_2$-terminal domain there is also an asparagine-rich stretch (9/10 residues) and an alanine-rich stretch (16 residues). This sequence data and the JIL-1 cDNA nucleotide sequence are available from EMBL/GenBank/DDBJ under accession number AF142061.

Figure 2. Alignment and Phylogenetic Relationship of JIL-1 with Related Kinases

(A) The sequence of JIL-1 KDI is aligned with the first kinase domain of the human kinases, MSK1 and MSK2 (Deak et al, 1998). Shared amino acids of these kinases with JIL-1 are in white typeface outlined in black and the overall homology in percent is indicated at the end of the sequence alignment. Catalytic kinase subdomains I through XI are indicated in
Roman numerals above the alignments. Residues nearly invariant in all serine/threonine kinases are marked by asterisks. (B) Schematic diagrams of JIL-1, human MSK1 and Drosophila RSK drawn to scale to compare the domain organization of these tandem kinases. JIL-1 Kinase Domain I (KDI) shares 63% identity with the first kinase domain of MSK1 but only 47% identity with the first kinase domain of Drosophila RSK. The comparisons reveal a much lower level of identity between JIL-1 KDII and the second kinase domains of MSK1 and Drosophila RSK which are only 32% and 28%, respectively. Sequences outside of the kinase domains of JIL-1 show no similarity to sequences of other proteins in the databases. (C) Consensus maximum parsimony tree derived from an alignment with all the gaps removed of the first kinase domains of JIL-1 and members from the most closely related serine/threonine kinase families. The tree is rooted using sequences from yeast PKC as an outgroup. The bootstrap 50% majority rule consensus of 1000 maximum parsimony trees is depicted with associated bootstrap support values. The following kinase database sequences were used (corresponding top to bottom to the kinases depicted in the tree): AF074393.1, AF074715.1, AF142061, P10665, P18652, A57459, AAA50509, AAA42103, P23443, AAC47429, P31751, P47197, CAA58499, Q12236.

Figure 3. JIL-1 Antibody Labeling of Drosophila Embryo Nuclei

(A) Whole-mount preparation of a precellular blastoderm embryo demonstrating the labeling of interphase nuclei with Odin antiserum. (B) Higher magnification Nomarski image of the labeled nuclei in (A).

Figure 4. Immuno- and Northern Blot Analysis of JIL-1

(A) Immunoblots of SDS-PAGE fractionated embryonic Drosophila proteins labeled with Odin and Hope antisera against JIL-1. Both antisera recognize a doublet or triplet of bands with relative molecular masses of 150-160 kDa (lane 1 and 2). The antibody labeling is
abolished after preadsorption with 20 µg of JIL-1-GST fusion protein (lane 3) but not by 20 µg of GST fusion protein only (lane 4). The migration of molecular weight markers in kDa is indicated. (B) On Northern blots JIL-1 transcripts are identified as a single band of approximately 6.5 kb.

Figure 5. In vitro JIL-1 Kinase Assays

(A) JIL-1 was immunoprecipitated from Schneider-2 cells using Odin antiserum (Odin ip). A portion was added to an in vitro kinase assay, fractionated by SDS-PAGE, stained, dried, and autoradiographed, or alternatively was subjected to Western Blot analysis. The radiolabeled band in lane 1 corresponds in size to the band detected by JIL-1-specific antibody on Western blot (lane 2). (B) In contrast, immunoprecipitation with Odin preimmune serum (preimmune ip) results in neither Odin antiserum detectable JIL-1 protein on western blots (lane 2) nor in any incorporation of radiolabeled ATP at the corresponding position (lane 1). (C) The top panel shows autoradiographs of histone H3 protein. The presence of JIL-1 protein immunoprecipitated from S2 cells with Odin antiserum (Odin ip,) leads to radiolabeling of histone H3 in the in vitro kinase assay. In contrast, histone H3 shows no labeling after incubation with Odin preimmune serum immunoprecipitated protein (preimmune ip). The bottom panel shows Coomassie Blue staining of the gels demonstrating that equivalent amounts of histone H3 were present in both reactions. The migration of molecular weight markers in kDa is shown for (A) and (B).

Figure 6. Imaging of Live Nuclei from JIL-1-GFP Transgenic flies

(A) Expression of JIL-1-GFP NH2-terminal fusion protein is under the control of the hs83 promoter. (B) JIL-1-GFP is detectable on immunoblots with Hope antiserum in transgenic flies as an additional band (arrow) compared to wild type flies. (C) Average
projection image of 10 confocal sections of two live ovarian egg chambers showing JIL-1-GFP fluorescence localized to the nuclei. (D) Higher magnification of a single confocal section of JIL-1-GFP fluorescence from one of the nuclei in (C). (E1-E4) Four frames from a time lapse movie of dividing nuclei in live JIL-1-GFP transgenic syncytial embryos. (E1) interphase, (E2) prophase, (E3) metaphase, (E4) second interphase.

Figure 7. JIL-1 Expression in Salivary Gland Polytene Chromosomes

(A-C) Double labeling of female polytene chromosomes with Hope antiserum (A) and Hoechst (B) shows that JIL-1 is expressed in a banded pattern complementary to that of Hoechst. In the composite image (C) there is little overlap between JIL-1 (green) and Hoechst (red) labeling (indicated by minimal regions of yellow). (D-E) JIL-1 antibody labeling of male X chromosomes (indicated by X) is greatly enhanced as compared to autosomes. In contrast, double labeling of the preparation in (E) with Hoechst (F) shows that the male X is less intensely labeled with Hoechst as compared to autosomes. (G) The composite image of the JIL-1 (green) and Hoechst (red) labeling of the preparation in (E) and (F) shows that the complementary banding pattern is maintained on the male X (white lines). (H Stereo pair of JIL-1-GFP fluorescence in a live female transgenic polytene nucleus. The expression of JIL-1-GFP in discrete bands is clearly discernible. (I) Average projection image of 3 confocal sections from a live male JIL-1-GFP transgenic polytene nucleus. The X chromosome exhibits enhanced fluorescence as compared to autosomes.
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GENETIC AND MOLECULAR ANALYSIS OF JIL-1, A CHROMATIN ASSOCIATED PROTEIN KINASE IN DROSOPHILA

A paper prepared for submission to Genetics

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ABSTRACT

In this paper, we report the genetic and molecular characterization of JIL-1, a chromatin associated protein kinase. JIL-1 maps to the 68A4-5 region of polytene chromosomes and is very likely to be a nonessential gene in this region as suggested by a series of deficiency mapping and rescue experiments. However, JIL-1 ribozyme was able to mildly affect the late embryogenesis by reducing the hatch rate compared to the control group. We also further address the possible role of JIL-1 in gene transcription by double-label immunostaining of polytene chromosomes using anti-Pol IIA, anti-Pol II0, anti-phosphorylated histone H3 at serine 10 and anti-JIL-1 antibodies. The JIL-1 banding pattern is distinct yet overlaps with the banding pattern of Pol IIA, Pol II0 and phosphorylated histone H3 at serine 10. Most significantly, JIL-1 does not preferentially localize within the highly transcriptionally active puff; instead it binds more strongly to boundaries of puffs compared to its weak staining of the central region of puffs. Polytene chromosome staining suggests that JIL-1 may play a role in defining competent chromatin domains for transcription possibly by phosphorylation of histone H3 at residues which are different from serine 10.
INTRODUCTION

Eukaryotic genomes are organized into a highly compacted chromatin structure. To overcome the repression mediated by nucleosomes, chromatin structure must be disrupted or remodeled to allow the entrance of protein factors to DNA templates. Chromatin remodeling is an essential step for many chromatin-related fundamental cellular processes, such as DNA replication (ALEXIADIS et al., 1998), gene expression (SUDARSANAM et al., 1999; BIGGAR and CRABTREE, 1999), recombination (POLLARD and PETERSON 1998) and silencing (MOREIRA et al., 1999). There are mainly two groups of proteins involved in chromatin remodeling. One utilizes of large multi-subunit protein complexes, such as RSC, NURF, and SWI/SNF (LOGIE et al., 1999; Varga-Weisz and BAKER, 1998). These protein complexes use the energy of ATP hydrolysis to disrupt or displace nucleosomes. Another is enzymatic modification of N-termini of core histones by acetylation, phosphorylation, methylation, ubiquitination and ADP-ribosylation. (HANSEN et al., 1998; WOLFFE and HAYES, 1999). The most prevalent and well-studied histone modification is acetylation, which has been directly linked to transcriptional activation (BROWNELL et al., 1996; OGRYZKO et al., 1996 and KUO et al., 1998). Besides histone acetylation, the functional importance of histone phosphorylation in chromatin remodeling has also been reported. Phosphorylation of histone H3 at serine 10 is found to be required for proper chromosome condensation and segregation (WEI et al., 1998, 1999). And this is not the only role of phosphorylation of histone H3 at serine 10, there are other reports that indicated this isoform of H3 is rapidly accumulated in a small fraction of nucleosomes in response to external mitogenic signals (MAHADEVAN et al., 1991; BARRATT et al., 1994).

We have previously reported the cloning and initial functional characterization of JIL-1, a novel tandem protein kinase associated with chromosomes during the entire cell cycle (JIN et al., 1999). The observations that JIL-1 localizes to interbands of polytene
chromosomes, localizes to Drosophila male X chromosomes with about two-fold higher level compared to autosomes and JIL-1 can phosphorylate histone H3 in vitro strongly suggest that JIL-1 is implicated in up-regulation of gene transcription by remodeling of chromatin structure.

In this report, we further pursue the role of JIL-1 in gene transcription by genetic analysis of JIL-1 and double-immunostaining of polytene chromosomes using JIL-1, Pol IIA, Pol II0 and anti-phosphorylated histone H3 at serine 10 antibodies. Genetic mapping and rescue experiments indicate JIL-1 may be a non-lethal gene. Partial knockout of JIL-1 gene by ribozyme mildly effects the late embryogenesis. Double-immunostaining of Drosophila polytene chromosomes show JIL-1 banding pattern is distinct yet overlaps with Pol IIA, Pol II0 and phosphorylated histone H3 at serine 10. JIL-1 is not highly enriched in puff regions, but in edges of puffs. These localization data are consistent with a role for JIL-1 in the establishment of chromatin domains competent for gene transcription.

MATERIALS AND METHODS

Fly Stocks: Flies were raised on standard Drosophila media at 25°. Genetic markers used here can be found in LINDSLEY and ZIMM (1992).

A set of 8 deficiency strains uncovering the 68A-C region were used in deficiency mapping to determine the localization of JIL-1 locus. Df(3L)vin2/TM3, Df(3L)vin3 e[1]/TM3 ry[+], Df(3L)vin4 e[1]/TM3 ry[+], Df(3L)vin5 e[1]/TM3 Sb[1] Ser[1] and y[1]; Df(3L)lxd6/TM3 y[+] Sb[1] e[1] Ser[1] were from the Bloomington Stock Center. Df(3L)lxd8 cur /TM3 Sb and Df(3L)H9/TM3 were gifts from Dr. Vicky Finnerty. Df(3L)h76 red/TM3 was kindly provided by Dr. Arthur Hilliker. 5 lethal mutants around 68A4-5 region were used in rescue experiments. 1(3)58 e^ sr ca/TM3 Sb Ser, 1(3)EMS1-1 sr e® ca/TM3 Sb Ser and 1-55 sr e^ ca/TM3 Sb Ser were from Dr, Arthur Hilliker; myc517/TM1 Me was from
Dr. Allan Shearn; 1 P element induced lethal mutant: P{ry^{	ext{r7.2}}=PZ}l(3)01239^{	ext{01239}} ry^{	ext{506}}/TM3, ry^{	ext{RkSb^1 Ser^1}} was from the Bloomington Stock Center. The w; Δ2-3/TM2Ub stock which supplied the transpose for P element transformation was the generous gift of Dr. Linda Ambrosio. For stocks used in polytene chromosome immunostaining, Oregon-R was used for wild-type preparations.

**Polytene chromosome in situ hybridization:** Salivary glands from late third instar larvae were dissected in 0.7% saline solution, fixed in 45% acetic acid and squashed in 20 ul lactic and acetic acid solution (1 lactic acid, 2 H2O, 3 acetic acid). Squashed slides with good chromosome spreads are immersed in liquid nitrogen and coverslips were removed using razor blade. Samples on slides were further subjected to denaturation in 0.2N NaOH for late hybridization and then were hybridized with digoxigenin labeled JIL-1 cDNA prepared using Boehringer Mannheim’s DNA labeling kit. After hybridization overnight at 42°, polytene chromosomes were washed sequentially with 2XSSC, PBS and AP buffer (100 mM Tris, 100mM NaCl, 5mM MgCl2, pH 9.5) at room temperature and incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody in Blotto (5% dry milk in PBS) for 2 hours. The hybridization signal was detected by incubating slides in color reaction buffer (57 ul NBT, 52ul BCIP in 15 ml AP buffer) for 1 hr to 2 hr and observing by phase contrast microscopy. Polytene chromosomes with good morphology and signals were photographed using Kodak film.

**Northern Analysis:** PolyA+ mRNA was purified from 0-15 hr embryos using the FastTrack kit (Invitrogen), and 20 μg of polyA+ mRNA was fractionated on 1.2% agarose formaldehyde gels, transferred to nitrocellulose and hybridized with the addition of dextran sulfate (10%) according to standard protocols (Sambrook et al., 1989). JIL-1 specific probes were generated by purifying 1.2 kb of JIL-1 cDNA encompassing the COOH-terminal tail domain using GeneClean (Bio 101) and synthesizing random primer [32P]-labeled probe
using the Prime-A-Gene kit (Promega) according to manufacturer's instructions. High
stringency hybridization and washing conditions were employed (SAMBROOK et al., 1989).

**Developmental Western Blot and SDS – PAGE:** Embryos from 0-3 hour, 3-6 hour,
6-24 hour's collection, larvae, pupae and adults were homogenized respectively in NP40
lysis buffer (0.137 M NaCl, 20 mM Tris pH 8.0, 10% glycerol, 1% NP40) which contained
protease inhibitors. 2X SDS sample buffer was immediately added after homogenization.
Protein extracts were boiled, spinned and separated on SDS-PAGE gels and transferred to
nitrocellulose, and incubated with JIL-1 antibody overnight, washed in TBS (0.9% NaCl, 100
mM Tris, pH 7.5), incubated with HRP-conjugated goat anti-rabbit antibody (1:3000) (Bio-
Rad) for 2.5 hr, washed in TBS, and the antibody complex visualized by incubation in 0.2
mg/ml DAB/0.03% H2O2/0.0008% NiCl2 in TBS.

**Construction of JIL-1 transgenic rescue lines and ribozyme lines:** Full length JIL-1
cDNA was cloned into the EcoRI site and Not I site of the P element germline
transformation vector – pCaSpeRhs70 for rescue of potential JIL-1 mutants. For the JIL-1
ribozyme construct, a 43 nucleotide long fragment, which consists of a catalytic domain
flanked by antisense JIL-1 sequence, was synthesized and first cloned into pGEMT BamH1
and KpnI sites. Then this fragment was digested with EcoR I and BamH1 restriction enzyme
and cloned into pCaSpeR-hs70 vector. At the same time, we also made a mutant JIL-1
ribozyme construct with three nucleotide changes at the catalytic domain that abolishes the
catalytic activity of ribozyme. These P element transformation plasmids were purified,
injected into w; \( \Delta2-3/TM2Ubx \) embryos using standard techniques (ROBERTS, 1986), and
several transgenic lines for each construct were recovered and homozygous lines were
established.

**Immunostaining of polytene chromosomes:** Polytene chromosomes from late third
instar larva were first fixed in 3.7% paraformaldehyde for about 30 seconds, then re-fixed in
solution of 50% glacial acetic acid and 3.7% paraformaldehyde for 2-5 minutes and squashed. Preparations with good spreads were double immunostained by JIL-1 antibody (1007 chicken antibody) and Pol IIa, Pol IIo and anti-phosphorylated histone H3 at serine 10 antibodies according to the protocol previously described by Zink and Paro (1989). The polytene chromosome spreads were incubated with primary antibodies diluted in 0.2% Tween-20, 0.2% BSA in PBS (1:100 for 1007 chicken serum) at room temperature for 1 hr, washed three times for 10 min, and blocked with 5% normal serum for 30 min. Subsequently the preparations were incubated with FITC-conjugated and TRITC-conjugated secondary antibodies (1:200 dilution), washed in 0.2% Tween-20, 0.2% BSA in PBS, rinsed in water, and stained with 10 µg/ml Hoechst 33258 (Molecular Probes) for 5 min. After a final brief rinse with distilled water the preparations were mounted in 90% glycerol containing 0.5% n-propyl gallate. The chromosomes were examined under epifluorescence optics using a Zeiss Axioskop microscope and the images were captured and digitized using a high resolution Paultek cooled CCD camera and the NIH-Image software. Subsequent image processing was carried out using Adobe Photoshop version 3.0. Images were sharpened, scaled, pseudocolored and overlaid.

RESULTS

JIL-1 locus is mapped to 68A4-5 region: By in situ hybridization with Oregon-R polytene chromosomes using digoxigenin labeled JIL-1 cDNA, the JIL-1 locus was mapped to the 68A region of the third chromosome in Drosophila (Figure 1a). To further define its location, 8 deficiency lines with deletions spanning 68A-C region were mapped using cDNA probes from 5' end, 3' end and middle part of JIL-1 coding sequence. As shown in Figure 1a and Figure 1b, JIL-1 locus was covered by Df(3L)vin3, Df(3L)vin4, Df(3L)lxd8 and Df(3L)h76, but was uncovered by Df (3L)vin2, Df(3L)vin5 and Df(3L)lxd6. By this
information, we localized JIL-1 locus in 68A4-5 of the third chromosomes and JIL-1 falls between the deletion Df(3L)lxd8 and Df(3L)h76. One of the deletions, Df(3L)h9, is found to remove the 3' end of JIL-1 gene but not the 5' end (Figure 1a), which indicates that the proximal breakpoint of this deletion falls within the JIL-1 locus. The JIL-1 mRNA expressed from this deletion chromosome may still include coding sequence as revealed by Northern blot(data not shown).

**JIL-1 does not correspond to any previously reported lethal complementation group and might be a nonlethal gene:** 68A-C region has previously been mutated to saturation by Meyerowitz and Hilliker's groups using different mutagens. (CROSBY et al. 1986; CAMPBELL et al. 1986; STAVELEY et al. 1990). There are 3 lethal complementation groups in 68A4-5 region: l(3)D, l(3)J and l(3)E group. One lethal mutation induced by P element insertion is also reported in this region: l(3)01239. However, these lethal mutations are uncovered either by Df(3L)lxd8 or Df(3L)h76 (Figure 1c). This suggested that JIL-1 does not correspond to any of these lethal complementation groups. However, considering the probes used in deficiency mapping coming from JIL-1 cDNA, we can not exclude the possibility that the most 3' and 5' of JIL-1 genomic sequence can be removed by either Df(3L)lxd8 and Df(3L)h76. These deleted sequences can effect the transcription level of JIL-1 by alteration of the local chromatin conformation so that the transcription level is reduced. Such a case is found with human myotonic dystrophy that is correlated with expanded CTG trinucleotide repeat sequence in the 3' untranslated region of a protein kinase gene. (TAKAHASHI et. al., 1996). To determine whether l(3)J group and other adjacent lethal mutations could be JIL-1 mutants, we made several JIL-1 rescue transgenic lines in which JIL-1 cDNA was either under hs70 promoter or hs83 promoter control and crossed them with potential JIL-1 mutants to rescue them using the genetic scheme illustrated in Figure 2. None of the lethal mutants were
rescued. From the mapping data and rescue experiment, it is likely that JIL-1 is a redundant gene.

**Molecular characterization of JIL-1:** JIL-1 gene encoded a approximately 6.5 Kb long messenger RNA (Figure 3a) as revealed by Northern Blot analysis. We did not find any sign of differential splicing during development, as it appeared to be a single band in a developmental Northern Blot (data not shown). Additionally we observed a single reading open frame in our several cDNA library screenings, which resulted in isolation of several overlapping cDNAs. These cDNA libraries are constructed from embryonic and ovary specific RNA.

This 6.5 kb RNA is translated into a protein of approximately 150-160 kDa as shown on Western Blots. The predicated molecular mass for JIL-1 from the open reading frame is 137 kDa and the difference between the predicated and observed molecular weight may due to posttranslational modifications. Qualitative developmental Western blots shows that JIL-1 protein was expressed as a single or triplet band during different development stages (Figure 3b) as detected by JIL-1 specific antibody — Odin which has been described in previous paper (Jin et al., 1999). Three bands are evident during early embryogenesis. After late embryogenesis, JIL-1 is expressed as a single band which corresponds to the higher detected in earlier embryogenesis. These different bands of JIL-1 during development suggest that JIL-1 may be developmentally regulated by post-translational modifications.

**JIL-1 ribozyme disturbed the late embryogenesis of Drosophila:** To understand the role of JIL-1 in Drosophila development, we made the JIL-1 ribozyme construct to decrease the expression of JIL-1 mRNA. Ribozymes have been used successfully to generate loss-of-function phenotypes of the fushi tarazu (ZHAO and LESLIE 1993) and paired genes (VANARIO-ALONSO et al. 1995) and to reduce expression of the white gene in Drosophila (HEINRICH et al. 1993).
As described in Materials and Methods, JIL-1 ribozyme and defective ribozyme consisted of the hammer-head RNA catalytic domain flanked by two JIL-1 antisense sequences from kinase domain I, which is unique to JIL-1. (Figure 4a). The defective ribozyme has three nucleotide changes within the catalytic domain so that the enzymatic activity is abolished (ZHAO and LESLIE, 1993).

To examine the effect of JIL-l ribozyme on embryogenesis, we collected 0-2 hour embryos from homozygous JIL-1 ribozyme flies, JIL-1 defective ribozyme flies and yw control flies. Embryos (AEL Shr) were subjected to heat shock at 37° for 4 times until hatching. Each heat shock lasted 1 hour. The hatched larvae were counted and the hatch rate of each group was calculated. Figure 4b and Table 1 showed the hatch rate of each group treated by heat shock and non-heat shock. Control groups (yw) have almost no differences in hatching rate between heat-shock treated embryos (93%) and non-heat-shock treated embryos (94%). The hatch rate of homozygous JIL-1 ribozyme embryos treated by heat-shock was 61% and was 16% less than that of non-heatshocked JIL-1 ribozyme embryos (77%). The heat-shocked defective JIL-1 ribozyme embryos hatched out with 50% and were 9% less than the nonheat-shocked embryos. Because defective JIL-1 ribozyme worked only as antisense RNA, it was expected that it had less effect than the ribozyme as the ribozyme can more efficiently remove the endogenous mRNA (ZHAO and LESLIE 1993). The low hatch rate of defective JIL-1 ribozyme (50%) may be the result of position effect of the transgene insertion. Statistic analysis using X square indicated that the differences among JIL-1 ribozyme, mutant ribozyme and control groups are significant at 95% level.

**JIL-1 is associated with interbands of polytene chromosomes that are transcriptional active regions:** We previously reported that JIL-1 binds to numerous sites on Drosophila salivary gland polytene chromosomes. These sites are complementary to Hoechst bands and correspond to interbands, which are gene rich and loosely packaged
chromatin region (JIN et al., 1999). This global banding pattern of JIL-1 is highly reproducible and resistant to RNase treatment. The JIL-1 immunostaining pattern on polytene chromosomes suggests that JIL-1 may be preferentially associated with sites of gene expression.

Here we further pursued the possible role of JIL-1 in gene transcriptional regulation by double-labeling polytene chromosomes with JIL-1 antibody and either RNA polymerase IIA (Pol IIA) antibody or RNA polymerase II0 antibody and JIL-1. Both Pol IIA and Pol II0 have been used as markers to designate actively transcribed loci in polytene chromosomes (BETTLER et al., 1996). Pol IIA antibody recognizes the hypo-phosphorylated or non-phosphorylated carboxyl-terminal repeat domain of RNA polymerase. Pol II0 antibody recognizes the hyperphosphorylated carboxyl-terminal repeat domain of RNA polymerase. Both Pol II0 and Pol IIA antibody stain numerous bands on polytene chromosomes and their banding patterns are overlapping yet distinct from each other. Major developmental puffs (regions of polytene chromosomes with extended diameter in which highly transcribed loci reside) and heat-shock induced puffs contain Pol II0. Heat shock puffs contain both Pol IIA and Pol II0 (WEEKS et al. 1993). Double label immunostaining experiments demonstrate that JIL-1 and Pol IIA are broadly distributed (Figure 5a and Figure 5b) and share many common immunostaining sites as represented by yellow or orange shading in Figure 5c. But there are also a fair number of bands that are different as shown by the presence of green bands (JIL-1 immunostaining bands) or red bands (Pol IIA or Pol II0 immunostaining bands). JIL-1 had a much broader distribution pattern than that of Pol II0. (Figure 5d and 5e). One main difference between JIL-1 and Pol II0 staining is that JIL-1 did not significantly accumulate in developmental puffs as Pol II0 did. Instead, JIL-1 seems to be immediately adjacent to Pol II0 sites at the edge of puff as indicated by arrow at ecdysone induced site 2B5.
We also carried out JIL-1 antibody staining of the polytene chromosomes from heat shock treated larvae. Salivary glands were dissected from late third instar larvae that had been heat-shocked at 37°C for 30 minutes, and polytene chromosomes were stained with the JIL-1 antibody. Unlike anti-Pol II0, JIL-1 did not appear to localize preferentially to the heat shock induced puffs. However overall increased JIL-1 antibody staining intensity was observed on heat shocked polytene chromosomes (data not shown).

JIL-1 staining pattern on polytene chromosomes was distinct from that of phosphorylated histone H3 at serine 10: Phosphorylation of histone H3 at serine 10 has been linked to chromosome condensation and segregation during mitosis in vivo. (WEI et al., 1999). However, it is also reported that phosphorylation of histone H3 at this site occurs rapidly in response to growth factors, phorbol esters, okadaic acid and protein synthesis inhibitors (MAHADEVAN et al., 1991). Furthermore, nucleosomes that have histone H3 phosphorylated at serine 10 are also especially susceptible to hyperacetylation (BARRATT et al., 1994). JIL-1 is able to phosphorylate histone H3 in vitro and is associated with chromosomes during the entire cell cycle (JIN et al., 1999). It is therefore important to determine whether there is a correlation between co-localization of JIL-1 and phosphorylation of histone H3 at serine 10. To test this, we did double-label immunostaining of the polytene chromosomes using anti-phosphorylated histone H3 at serine 10 and anti-JIL-1 antibodies. As shown in Figure 6, although there were many overlapping sites for JIL-1 and phosphorylated histone 3 at serine 10, two significant differences existed between the banding pattern of anti-JIL-1 and that of anti-phosphorylated histone H3 at serine 10. The first apparent difference is that JIL-1 is highly enriched in male X chromosomes compared to the rest of the genome (Figure 6b), but anti-phosphorylated histone H3 at serine 10 stains autosomes and the male X chromosome with the same intensity (Figure 6a). The second difference is that, unlike JIL-1, phosphorylated serine 10 of histone H3 was significantly
accumulated in the developmental puff region (2B5) as indicated by arrow in Figure 5B, which is in agreement with its reported correlation with active gene transcription. These observations suggest that JIL-1 is not the kinase responsible for phosphorylation of histone H3 at serine 10. This conclusion is further supported by the fact that the histone H3 that has been phosphorylated by JIL-1 in an in vitro assays can not be recognized by anti-phosphorylated histone H3 at serine 10 (Yanming Wang, personal communication).

DISCUSSION

In this study, we report that JIL-1 maps to a well-defined region of polytene chromosomes, 68A4-5, and our data suggest that it is a nonlethal gene in this region. Molecular characterization of JIL-1 shows that JIL-1 is expressed in all development stages of Drosophila. Western analysis suggests that JIL-1 is differentially modified posttranslationally during development. Transgenic embryos carrying a JIL-1 ribozyme showed reduced viability during late embryogenesis. The overlap of JIL-1 immunostaining pattern with that of Pol II A, Pol II O and phosphorylated histone H3 at serine 10 implies that JIL-1 is related to gene transcription. The observation that JIL-1 is not significantly enriched in highly transcriptionally active puffs suggests that JIL-1 play a regulatory role in defining functional chromatin domains for gene transcription.

JIL-1 is a functionally important, yet, nonessential gene mapped in 68A4-5 region. By a series of deficiency mapping and rescue experiments, JIL-1 can not be assigned to any previous reported lethal complementation group. We think JIL-1 may be a nonlethal gene because this region had been mutated to saturation by two research groups using several different mutagens. It is not unusual that functionally important genes may have no lethal phenotypes because there may be multiple genes that have similar functions. For example, in yeast, both TFIID and SAGA complex are important for gene activation and transcription.
However, no universal defects in transcription were observed by either inactivation of yeast TAF145, a central scaffold subunit of TFIID, or GCN5, the catalytic subunit of SAGA complex. One of the explanations is that SAGA and TFIID function might be redundant (STEVEN HAHN, 1998). From the ubiquitous expression of JIL-1 during Drosophila development and its broad association with chromosomes both in interphase and mitosis (JIN, et al. 1999), we think JIL-1 may play a very general role in remodeling of chromatin structure for chromatin replication and regulation of gene expression. In these complicated cellular processes, JIL-1 may be only a small part of the machinery and manifest its role in one aspect more than others. One possibility would be that JIL-1 is involved in gene dosage compensation in male Drosophila X chromosomes as suggested by colocalization with MSL complexes. (JIN, unpublished result). That JIL-1 may be a functionally important but redundant gene is further supported by the result of JIL-1 ribozyme studies. That transgenic flies carrying JIL-1 ribozyme showed mild disruption of late embryogenesis upon heat shock suggests partial deletion of JIL-1 may effect the overall transcription level of the genome. Future studies will examine the cellular and developmental requirements of JIL-1 in greater detail.

**JIL-1 may define discrete chromatin domains which are competent for gene transcription.** Double labeling of polytene chromosomes using anti-JIL-1, anti-Pol IIA antibodies and anti-JIL-1, anti-Pol II0 antibodies respectively reveals that many JIL-1 sites are coincident with Pol IIA and Pol II0 sites. This co-localization suggests that JIL-1 proteins are associated with transcriptionally active regions. However, a fair number of JIL-1 sites are distinct from Pol IIA sites and Pol II0 sites. JIL-1 does not accumulate within heat shock-induced puffs or within developmental puffs in which genes are under active transcription; instead, JIL-1 antibodies show stronger immunostaining at the edge of these puffs. Our explanation for this observation is that JIL-1 is not directly involved in the process
of transcription. Rather it is involved in initiation of gene activation by defining competent chromatin domains for gene expression possibly through phosphorylation of histone H3 (JIN et al., 1999). This situation also happens with H4Ac16, an acetylated histone H4 isoform that is linked to active gene transcription. H4Ac16 is not enriched in very actively transcribing heat shock and developmental puffs and shows weak staining within these puffs just as JIL-1 does (TURNER et al., 1992). Interestingly, both JIL-1 and H4Ac16 are enriched in the highly transcribing male X-chromosomes. And recently, we found the JIL-1 banding pattern is almost identical to that of H4Ac16 (JIN, unpublished result). This colocalization strongly implies that, besides H4Ac16, JIL-1 may also contribute to activation of gene transcription by remodeling chromatin structure. In the future, it will be our goal to find the in vivo substrate of JIL-1 and, if it is histone H3, the residue being phosphorylated.

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LITERATURE CITED


FIGURE LEGENDS

Figure 1. JIL-1 maps to Drosophila polytene chromosome 68A4-5 region.

(A) JIL-1 is mapped to polytene chromosome 68A by in situ hybridization. Df(3L)lxd8 and Df(3L)vin3 do not include the JIL-1 locus so that both paired parental chromosomes are hybridized by JIL-1 probes. Df(3L)vin2 uncovers the JIL-1 locus so that only one parental chromosome is hybridized by JIL-1 probes. Interestingly, Df(3L)H9 removes only the 3' end of the JIL-1 gene but not the 5' end. (B) JIL-1 is further localized to a very small region 68A4-5 by mapping with Df(3L)vin3, Df(3L)vin4, Df(3L)vin5, Df(3L)lxd6 and Df(3L)h76. Black bar represents the part of chromosome deleted in that particular deficiency strain. Plus sign indicates that JIL-1 locus is uncovered in that deletion. Minus sign indicates that JIL-1 locus is not uncovered in that deletion. The small region between two vertical dash lines is the 68A4-5 region in which the JIL-1 locus is located. (C) The JIL-1 locus does not correspond to any previously reported lethal complementation groups or genes. Lethal complementation groups - l(3)D, l(3)J, l(3)E and a P element induced lethal mutant - l(3)0123g01239 are uncovered by either Df(3L)lxd8 or Df(3L)h76.

Figure 2. Scheme of JIL-1 Rescue Experiment.

Full length JIL-1 cDNA was cloned into EcoRI and NotI sites of the germline transformation vector pCaSpeR-hs70 and introduced into the fly genome through standard P element transformation. Several different transgenic lines were obtained and homozygous lines were established. Through a series of crosses, JIL-1 transgenes were introduced into candidate JIL-1 mutant background. The next generation was heat shocked every day to determine whether there was rescue or not. None of the lethal complementation groups in 68A4-5 region were rescued.
Figure 3. Northern Blot and Developmental Western Blot Analysis of JIL-1.

(A) JIL-1 transcripts were identified as a single band of approximately 6.5 kb. (B) JIL-1 was expressed ubiquitously during entire development of Drosophila. In early embryogenesis, JIL-1 migrated as a triplet or doublet band. From late embryogenesis to adult, it changed to a single band on Western Blot.

Figure 4. JIL-1 Ribozyme was able to mildly affect Late Embryogenesis.

(A) JIL-1 ribozyme construct. JIL-1 ribozyme is a hammer-head RNA catalytic domain flanked by two JIL-1 antisense RNA sequences from kinase domain I. This 43 bp long fragment was synthesized, cloned into pCaSpeR under a strong conditional promoter - heat shock 70 promoter and introduced into flies. Heat shock of transgenic flies can rapidly induce the expression of JIL-1 ribozyme. Expressed JIL-1 ribozyme will bind to endogenous JIL-1 mRNA and cut them at the specific site. The remaining mRNA molecules will be degraded by a double strand specific RNase. (B) Three different groups of flies, wild type, homozygous JIL-1 ribozyme flies and mutant JIL-1 ribozyme flies, were subjected to heat shock treatment at 37° or kept at 25° as control. Heat-shocked wild type flies had almost the same hatch rate as non-heat shocked flies did. Heat-shocked JIL-1 ribozyme flies hatched out with 16% less than the non-heat-shocked JIL-1 ribozyme flies. Heat-shocked mutant JIL-1 ribozyme flies had a 9% lower hatch rate than the non-heat-shocked mutant JIL-1 ribosome. Bars with vertical line represent hatch rates at 37°. Bars with crosshatch stand for hatch rates at 25°.

Figure 5. Immunostaining pattern of JIL-1, Pol IIA and Pol III on polytene chromosomes.

Left panels (A-C): JIL-1 and Pol IIA staining. (A) A male X-chromosomes stained with JIL-1 antibodies and FITC-conjugated secondary antibodies. (B) The same chromosome
spread stained with Pol IIA antibodies and TRITC-conjugated secondary antibodies. (C) Merged image of A and B. Many JIL-1 binding sites can also be found in Pol IIA sites as shown by the yellow or orange shading. There is a general agreement between two banding patterns along the chromosome. But, there are also many JIL-1 bands and Pol IIA bands distinct from each other. (D-F): JIL-1 and Pol II0 staining. (D) Polytenes chromosomes stained by JIL-1 antibodies and FITC-conjugated secondary antibodies. (E) The same polytene chromosomes stained by Pol II0 antibodies and TRITC-conjugated secondary antibodies. (F) Merged image of D and E. Some JIL-1 sites are coincident with that of Pol II0 site as yellow bands. However, compared to that Pol II0s are enriched in major development puffs such as 2B5, 74EF and 75B (indicated by arrow), JIL-1 shows very weak staining within puff region. Also it is noticed that Pol II0 antibodies stained the autosomes and male X chromosome with the same intensity, and JIL-1 stained the male X chromosomes much stronger than autosomes.

Figure 6. JIL-1 is not the kinase responsible for phosphorylation of histone H3 at serine 10.

(A). A male X chromosome stained by antibodies against phosphorylated histone H3 at serine 10 and TRITC-conjugated secondary antibodies. (B) The same chromosome stained by JIL-1 antibodies and FITC-conjugated secondary antibodies. (C) Merged image of A and B. JIL-1 banding pattern was overlapped with, yet is distinct from anti-phosphorylated histone H3 at serine 10. Antibodies against phosphorylated histone H3 at serine 10 strongly stained the development puff-2B5 and showed the same staining intensity on autosomes and male X chromosome.

Table 1. Hatch rates of heat shock treated and non-heat shock treated embryos from wild type, homozygous JIL-1 ribozyme and mutant JIL-1 ribosome flies.
Fig. 1
hs70 promoter → JIL-1 cDNA

P element transformation

Transgenic flies

P[JIL-1,w+] l(3)D
P[JIL-1,w+], TM3,Sb,Ser

P[JIL-1,w+] l(3)E
P[JIL-1,w+], TM3,Sb,Ser

P[JIL-1,w+] l(3)J
P[JIL-1,w+], TM3,Sb,Ser

P[JIL-1,w+] l(3)01239
P[JIL-1,w+], TM3,Sb,Ser

heat shock at 37°C

P[JIL-1,w+] l(3)D
P[JIL-1,w+], l(3)D

P[JIL-1,w+] l(3)E
P[JIL-1,w+], l(3)E

P[JIL-1,w+] l(3)J
P[JIL-1,w+], l(3)J

P[JIL-1,w+] l(3)01239
P[JIL-1,w+], l(3)01239

No rescue
No rescue
No rescue
No rescue

Fig. 2
Fig. 3

A
Northern

B

JIL-1 Developmental Western

6.5 Kb

0 - 3 hr
3 - 6 hr
6 - 24 hr
Larval
Pupae
Adult
A

JIL-1 mRNA

5' m^7 GpppG

G-C
A-U
U-A
A-T
A-T
C-G
G-C
G-C
G-C
U-A
G-C
G-C
U-A
C-A

AAA\text{n}3'

Ribozyme

antisense

catalytic domain

antisense

cleavage site

B

Fig. 4
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<tr>
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<th>Heat shocked</th>
<th>Not heat shocked</th>
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<tr>
<td></td>
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Table 1
JIL-1 defines a new family of serine-threonine kinases

JIL-1 is one of the antigens recognized by a monoclonal antibody, mAb2A, which has a dynamic distribution pattern during the cell cycle (Johansen, 1996; Johansen et al., 1996). Using this antibody to screen a Drosophila expression library, partial clones from two genes were identified, one of which corresponded to JIL-1. Sequence analysis indicates JIL-1 encodes a putative protein kinase with two tandem kinase domains flanked by unique NH2- and COOH-termini. Both kinase domains show homology to serine-threonine kinases. Besides the two kinase domains, there are several interesting motifs found within the JIL-1 protein sequence. The NH2-terminal domain contains an asparagine-rich region, an alanine-rich region and a bipartite nuclear localization signal. There are also three PEST-like sequences, and PEST motif has been implicated in targeting proteins for rapid turnover (Rodgers et al., 1986). Two of the PEST-like sequences are found in the NH2-terminus and the third is found in the inter-kinase domain region.

The entire structure of JIL-1 is similar to kinases of the RSK family, which also have two tandem serine-threonine kinase domains (Jones et al., 1988). However, phylogenetic analysis places JIL-1 with the newly identified MSK1 (Deak et al., 1998), MSK2 and RSKB kinases (Pierrat et al., 1998) into one monophyletic clade with 95% bootstrap value. Thus, it suggests that JIL-1, along with MSK1, MSK2 and RSKB, may define a family of serine-threonine kinases distinct, yet closely related to the RSK kinase and S6 kinase families.
**JIL-1 is expressed throughout development as indicated by Northern and Western analysis**

JIL-1 encodes a 6.5 kb mRNA which is detected as a single band on a Northern blot. Also, a single reading frame was identified from 13 overlapping cDNAs from embryonic and ovary-specific libraries. Developmental Westerns indicate that JIL-1 protein is expressed throughout Drosophila development. JIL-1 is expressed as a doublet or triplet band migrating at approximately 150-160 kD in early embryogenesis. From late embryonic to adult stages, it resolves as a single band corresponding to the higher band in early developmental stages. The change of the size of the JIL-1 gene product on developmental Western blots may reflect that JIL-1 is developmentally regulated by post-translational modifications.

**JIL-1 is a functional kinase which phosphorylates histone H3 in vitro**

Sequence analysis predicts that JIL-1 is a putative protein kinase. To test whether JIL-1 has kinase activity, JIL-1 immunoprecipitated from S2 cell lysate was tested in vitro for kinase activity and found to autophosphorylate itself.

Because histone H3 phosphorylation has been reported to be correlated with active gene transcription and chromatin condensation upon mitosis, JIL-1 was tested for its ability to phosphorylate bovine histone H3 in vitro. And indeed, JIL-1 is found to be able to phosphorylate bovine histone H3 in vitro. Bovine histone H3 and Drosophila histone H3 are highly conserved with only 3 amino acid difference in the histone fold domain and COOH terminus. The amino acid sequence in the N terminal tail of histone H3 in both species, which is important for interaction with DNA and is the targets for post-translational
modifications, is almost identical except that alanine 31 is substituted for a proline. Therefore JIL-1 is expected to be able to phosphorylate Drosophila histone H3 in vitro.

Further analysis showed that JIL-1 is not the kinase responsible for phosphorylation of histone H3 at serine 10, which has been shown to be necessary for chromosome condensation and segregation (Wei et al., 1999), and correlated with active gene transcription (Mahadevan et al., 1991; Barratt et al., 1994). Western blot analysis of histone H3 phosphorylated by JIL-1 showed no immunoactivity to antibodies against phosphorylated histone H3 at serine 10 (Y. Wang, personal communication). Furthermore, the banding pattern of the phosphorylated histone H3 at serine 10 on polytene chromosomes did not correspond to the JIL-1 pattern in immunostained Drosophila polytene chromosome by antibodies.

**JIL-1 may define discrete chromatin domains which are competent for gene expression**

Immunostaining of polytene chromosomes using JIL-1 antibodies shows a broad JIL-1 immunostaining banding pattern on Drosophila polytene chromosomes. These banding patterns are repeatable and insensitive to RNAse treatments, which suggest JIL-1 is not targeted to the polytene chromosomes by binding RNA components on chromatin. Double labeling with Hoechst 33258, a DNA dye, demonstrates that JIL-1 binding sites are complementary to Hoechst bands. Hoechst bands are mainly regions of highly condensed DNA. Therefore, JII-1 immunostaining bands are correspond to interband regions of polytene chromosomes, which contain predominantly active genes. This observation suggests JIL-1 function might be related to regulation of gene expression. To further study the role of JIL-1 in gene transcription, we carried out double-labeled immunostaining of polytene chromosomes using JIL-1 antibodies, anti-hypo/nonphosphorylated carboxyl terminal
domain of RNA polymerase (Pol IIA) and anti-hyperphosphorylated carboxyl terminal domain of RNA polymerase (Pol IIO) antibodies. JIL-1 shows an overlapping yet distinct staining pattern with Pol IIA and Pol IIO. Especially noteworthy, JIL-1 is not enriched in the highly transcribed puff regions which are rich in Pol IIA and Pol IIO; instead JIL-1 accumulates at the edges of these puffs.

**JIL-1 associates with chromosomes throughout the cell cycle**

To view the distribution pattern of JIL-1 in living cells, GFP-JIL-1 transgenic flies were established. GFP tagged JIL-1 fusion proteins are correctly and continuously expressed under hs83 promoter as a 180 Kd protein band revealed by Western blot. The molecular weights for GFP and JIL-1 are approximately 20Kd and 160Kd respectively. Examining the localization of GFP-JIL-1 in the interphase cell, JIL-1 was found to be exclusively within the nucleus and formed a fibrous meshwork-like structure that is colocalized with DNA. Furthermore, GFP-JIL-1 shows a banding pattern in salivary gland polytene chromosomes and approximately two-fold higher concentrations on male X chromosomes, which is in agreement with JIL-1 antibody nuclei and polytene chromosomal staining. This finding confirms that JIL-1’s preferential localization to interbands of polytene chromosomes and male X chromosomes as detected by antibodies reflects its true distribution pattern, and was not due to the greater accessibility of antibodies into loosely packaged interbands relative to the condensed regions or more diffuse male X chromosomes relative to autosomes.

To further understand the localization of JIL-1 during mitosis, the dynamic movement of GFP-JIL-1 was imaged in living *Drosophila* syncytial blastoderm using confocal microscopy. GFP-JIL-1 is found associated with chromosomes throughout the cell cycle. This observation suggests JIL-1 may also play a role in mitosis.
**JIL-1 is implicated in gene dosage compensation in *Drosophila* through MSL complexes**

By comparing immunostaining of polytene chromosomes in males and females, JIL-1 was found to localize to male X chromosomes with about two fold higher levels relative to autosomes and female X chromosomes. This observation raises the possibility that JIL-1 may be related to gene dosage compensation in *Drosophila*. To further pursue this possibility, double-labeled immunostainings of male polytene chromosomes using anti-JIL-1, and either anti-H4Ac16, anti-MSL1 or anti-MSL3 antibodies were performed. The binding sites of JIL-1 along male X chromosomes are almost identical with that of H4Ac16, MSL1 and MSL3 (Figure 2A-C). Also MSL1 and MSL3 proteins can be co-immunoprecipitated by JIL-1 antibodies from S2 cell extracts (Y. Wang, unpublished results). It is proposed that gene dosage compensation is induced by MSL complexes binding to male X chromosomes and recruiting histone acetyltransferase to increase the appearance of H4Ac16 on male chromosomes (see Introduction). Thus, the implication of JIL-1 in gene dosage compensation may be related to its role in the MSL complexes. To further understand whether the appearance of JIL-1 in male X chromosomes is dependent on MSL complexes or not, immunostaining of X chromosomes from both female flies carrying MSL2 transgene and from homozygous msl1 or msl3 mutant female flies carrying MSL2 transgene using the JIL-1 antibody were performed. Ectopically expressed MSL2 proteins can assemble functional MSL complexes in female flies and induce gene dosage compensation (Kelley et al., 1995). The increased binding of JIL-1 on the X chromosome is observed in these MSL2 transgenic female flies, but is missing in the homozygous msl1 or msl3 mutant female flies carrying the MSL2 transgene (Figure 2D-G). These results indicate that the increased binding of JIL-1 on male X chromosome is dependent on the presence of MSL complexes.
Fig. 2 JIL-1 is implicated in gene dosage compensation. A) - C). Merged images of double immunostaining of the male X chromosomes using pairs of antibodies against H4Ac16 and JIL-1; MSL1 and JIL-1; MSL3 and JIL-1, respectively. The yellow or orange shades represent overlapped sites. Green bands represent JIL-1 bands. Red bands represent H4Ac16 in (A), MSL1 in (B) and MSL3 in (C). D) and E). JIL-1 immunostaining of female X chromosomes in flies carrying the MSL2 transgene. E) and G). The preferential binding of JIL-1 appears in female flies carrying the MSL2 transgene and one copy of msl1 mutant gene (D) or one copy of msl3 mutant gene (F), but, disappear in the sibling females carrying the MSL2 transgene and homozygous for the msl1 mutant (E) or for the msl3 mutant (G).
JIL-1 may represent a redundant yet functionally important gene

JIL-1 was mapped to a small region in the Drosophila polytene chromosomes encompassing 68A4-5. This region has been very likely mutated to saturation by different mutagens and two research groups (Campell et al., 1986; Crosby et al., 1986; Staveley et al., 1990). However, the JIL-1 gene could not be assigned to any previously reported lethal complementation groups in this region by deficiency mapping and rescue experiments. Thus, JIL-1 may be a redundant gene.

A model for JIL-1 function and future work

Based on the above results, JIL-1 is proposed to be a protein kinase implicated in potentiating gene transcription possibly by phosphorylation of histone H3 and JIL-1 may provide a direct link between cellular signal transduction pathways and chromatin remodeling. However, it is also possible that JIL-1 may play a role in regulation of gene transcription by phosphorylation of certain important transcription factors or transcription coactivators.

The preferential localization of JIL-1 in interbands regions of polytene chromosome and the approximately two fold higher level on the dosage-compensated X chromosome relative to the autosomes and female X chromosomes suggest JIL-1 is implicated in up-regulation of gene expression. This positive regulation of gene expression by JIL-1 may be achieved by establishing competent chromatin domains for gene transcription. We do not favor a model in which JIL-1 activity is required during the transcription process itself, as JIL-1 is not highly enriched in transcriptionally active puffs in polytene chromosome and, instead, it shows strong staining at the edges of puffs. JIL-1 can phosphorylate histone H3 in
vitro. Therefore it is possible that JIL-1 phosphorylates the N terminus of histone H3 in vivo and weakens the interactions of nucleosomal DNA with histones so that chromatin structure is disrupted. A similar situation also occurs with H4Ac16, which has been convincingly linked to active gene transcription. Like JIL-1, H4Ac16 is not enriched in highly transcribed puff regions either, but shows strong staining at edges of polytene chromosomal puffs (Turner et al., 1990, 1992). The almost identical banding pattern of JIL-1 and H4Ac16 on male X chromosomes further supports this hypothesis. To test that JIL-1 is indeed involved in potentiation of gene transcription by phosphorylation of histone H3, future work will be directed toward determining whether histone H3 is the in vivo JIL-1 substrate. To address this question, the phosphorylation sites of JIL-1 on histone H3 will be mapped, antibodies against the synthesized peptides carrying this specific phosphorylated residue will be raised and used to perform the immunostaining of male polytene chromosomes. If this antibody reveals the same preferential binding of male X chromosomes as JIL-1 antibody does, it will provide strong evidence that JIL-1 is involved in up-regulation of gene transcription through histone phosphorylation. Another possible role of JIL-1 in up-regulation of gene expression is that it may regulate the activity of some ubiquitous transcription factors or coactivators such as CREB, CBP, HMG proteins, and HAT (histone acetyltransferase) by phosphorylation because of the global immunostaining pattern of JIL-1 on polytene chromosomes. This interpretation can also explain the global polytene chromosome immunostaining data.

JIL-1 may provide a direct link between cellular signal transduction and chromatin remodeling. Phylogenetic analysis indicates that JIL-1 defines a new family of protein kinases together with MSK1 and RSKB. Previous reports show that both MSK1 and RSKB may be involved in a mitogen-activated pathway as they can be directly activated by MAPK and both of them can phosphorylate CREB. MSK1 also has been shown to phosphorylate histone 2B in vitro. As a member of the same family, it is very likely that JIL-1 is within the
same or a similar signal transduction pathway. Considering that JIL-1 is associated with chromatin throughout the cell cycle, it may provide a direct link between mitogenic pathway and remodeling of chromatin. The observation that JIL-1 itself is subject to regulation by post-translational modification at different development stages supports such a role of JIL-1 in relaying developmental signals to gene transcription. To pursue this possibility, the post-translationally modified sites on JIL-1 will be mapped and site-directed mutagenesis will be employed to determine the consequences of such changes of the JIL-1 gene on transcriptions of the reported genes. The proteins responsible for these modifications may be identified through conserved motif recognition, yeast two-hybridization screens and other genetic and biochemical means.

The observation that JIL-1 is associated with chromatin during mitosis raises the possibility that JIL-1 may also play a role in mitosis. Many chromatin remodeling proteins are found both in gene transcription activation and repression, such as SWI/SNF complex (Travers, 1999). This paradoxical role of chromatin remodeling proteins can be explained by the newly proposed bidirectionality mechanism for chromatin remodeling complexes (see Introduction), which suggest that the process of chromatin remodeling is required in both unfolding and folding nucleosomal arrays and higher order chromatin structure. The exact role of chromatin remodeling complexes in different states of the cell cycle is dependent on the protein context within the complexes, which varies in response to cellular signals. One major future effort will be to find the protein complexes with which JIL-1 interacts. It is very possible that JIL-1 may be a component of some multi-subunit complexes that work as transcriptional co-activators or co-repressors.

Genetic analysis and rescue experiments suggest that JIL-1 may be a redundant gene. However, from the staining pattern of JIL-1 on polytene chromosomes and imaging of GFP-JIL-1 fusion protein during mitosis, JIL-1 seems to play a very general role both in mitosis
and in interphase transcription possibly through chromatin remodeling, as manifested in gene dosage compensation. It is reasonable that there are multiple genes and pathways that have similar functions as JIL-1 does because the potential function of JIL-1 may be so fundamental to the cell. For example, genetic studies in yeast have shown that SRB/mediator, the SAGA histone acetylase complex, the SWI/SNF complex and, possibly, TFIID have overlapping functions (Hampsey and Reinberg, 1999). In the yeast, TAFs (TATA box binding protein associated protein factor) have been shown to be dispensable for activation in both in vivo and in vitro, apparently replaced by the SRB/mediator complexes (Walker, et al., 1996; Moqtaderi, et al., 1996). Functional redundancy also reflects that many TAF units, such as GCN5, are both shared by SAGA and TFIID complexes (Hampsey and Reinberg, 1999).

Also in Drosophila, mutants of either roxl or roxl alone, genes encoding RNA components specifically appearing on male X chromosome, do not show male specific lethality (Coops et al., 1998). However, removal of both roxl and roxl genes leads to the lethality (Franke and Baker, 1999).

In the future, it is still a major goal to find a JIL-1 null mutant for carrying out genetic studies. Considering the possibility that JIL-1 itself may be redundant, it may prove more successful to screen putative JIL-1 mutants for enhanced phenotypes of hypomorphic msl mutants. For example, the P element induced mutant mle GNT.T: Ivir \ HA! male flies show 50% lower viability compared to their sisters (Richter et al., 1996). A genetic screen can be carried out by looking for enhancers for this mutant. Another genetic screen could be designed based on the observation that removal of one copy of MSL1 suppresses the viability defects of female flies carrying ectopically expressed MSL2 protein. In this case, mutants that remove the suppressive effect of one copy of MSL1 on the viability defects of females carrying ectopically expressed MSL2 gene (Bashaw and Baker, 1996; Keliey et al., 1995) could be analyzed for potential JIL-1 mutations.
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