DOI: 10.1534/genetics.103.018721

The Direct Interaction Between ASH2, a Drosophila Trithorax Group Protein, and SKTL, a Nuclear Phosphatidylinositol 4-Phosphate 5-Kinase, Implies a Role for Phosphatidylinositol 4,5-Bisphosphate in Maintaining Transcriptionally Active Chromatin

Mimi K. Cheng and Allen Shearn[1]

Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218

Manuscript received June 10, 2003
Accepted for publication March 19, 2004

ABSTRACT

The products of trithorax group (trxG) genes maintain active transcription of many important developmental regulatory genes, including homeotic genes. Several trxG proteins have been shown to act in multimeric protein complexes that modify chromatin structure. ASH2, the product of the Drosophila trxG gene *absent, small, or homeotic discs 2* (*ash2*) is a component of a 500-kD complex. In this article, we provide biochemical evidence that ASH2 binds directly to Skittles (SKTL), a predicted phosphatidylinositol 4-phosphate 5-kinase, and genetic evidence that the association of these proteins is functionally significant. We also show that histone H1 hyperphosphorylation is dramatically increased in both *ash2* and *sktl* mutant polytene chromosomes. These results suggest that ASH2 maintains active transcription by binding a producer of nuclear phosphoinositides and downregulating histone H1 hyperphosphorylation.

THE homeotic genes of the Antennapedia complex and bithorax complex specify the segmental identities of the fruit fly *Drosophila melanogaster* (Duncan 1987; Kaufman *et al.* 1990). During early embryogenesis, the initial expression patterns of homeotic genes are established by the gap and pair rule genes (Akam 1987). After gastrulation, expression of the gap and pair rule genes subsides, and the products of the Polycomb group (PcG) and trithorax group (trxG) genes maintain the proper expression patterns of the homeotic genes. PcG proteins are required for the maintenance of homeotic gene repression, while trxG proteins are required for the maintenance of homeotic gene activation (Kennison 1995; Simon 1995). PcG and trxG proteins are thought to act in large multimeric protein complexes that modify chromatin structure, organizing it into either a "closed" or an "open" conformation (Mahmoudi and Verrijzer 2001). In addition to regulating homeotic genes, PcG and trxG proteins regulate many other genes, implying that the faithful maintenance of active or repressed states may be a general mechanism of development (Francis and Kingston 2001).

The trxG gene *absent, small, or homeotic discs 2* (*ash2*) was found in a screen for late larval/early pupal lethals with imaginal disc abnormalities (Shearn *et al.* 1971). Homozygous *ash2* mutants exhibit homeotic transformations characteristic of loss-of-function mutants in homeotic genes (Shearn *et al.* 1987). For example, the haltere and third-leg imaginal discs of *ash2 trans*-heterozygotes show reduced accumulation of the homeotic gene product, Ultrabithorax, and the first leg discs show complete loss of Sex combs reduced (LaJeunesse and Shearn 1995). *ash2* mutants display intergenic noncomplementation with other trxG gene mutants (Shearn 1989). In addition, *ash2* mutants display pattern formation abnormalities of the legs and wings (Adamson and Shearn 1996). The predicted ASH2 protein contains a target sequence for early degradation (PEST sequence; Rogers *et al.* 1986), a domain of unknown function (SPRY domain; Ponting *et al.* 1997), and a bipartite nuclear localization signal (NLS-BP; Robbins *et al.* 1991). The nuclear localization signal is functional as ASH2 is localized in nuclei of salivary gland cells (Adamson and Shearn 1996). Most significantly for this report, ASH2 contains a PHD finger, a putative double zinc finger involved in mediating protein-protein interactions and modifying chromatin structure (Aasland *et al.* 1995) and implicated in functioning as a nuclear phosphoinositide receptor (Gozani *et al.* 2003). Biochemical studies reveal that ASH2 is a subunit of a 500-kD multiprotein complex (Papoulas *et al.* 1998). Additional components of the complex have yet to be determined in Drosophila. However, biochemical purification of the SET1 protein complex in *Saccharomyces cerevisiae* reveals that one of the proposed subunits, Bre2p, contains a SPRY domain and another subunit, Saf41p, contains a PHD finger. It has been proposed that Bre2p and Saf41p together constitute a bipartite functional homolog of ASH2 (Nagy *et al.* 2002). The SET1 complex contains

[1]*Corresponding author:* Department of Biology, Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218.
E-mail: bio_cals@jhu.edu

SET1, a SET domain-containing protein that has been shown to methylate lysine 4 of histone H3 (Miller *et al.* 2001; Roguev *et al.* 2001; Nagy *et al.* 2002). Human and *Schizosaccharomyces pombe* versions of this complex have ASH2 homologs that contain both a SPRY domain and a PHD finger; these complexes have also been shown to methylate lysine 4 of histone H3 (Roguev *et al.* 2003; Wysocka *et al.* 2003). The human ASH2 homolog is 47% identical to Drosophila ASH2 (Ikegawa *et al.* 1999).

Phosphoinositol lipids in the cytoplasm play important roles in growth, differentiation, and vesicular secretion. Phosphatidylinositol 4-phosphate (PtdIns[4]P or PIP) is phosphorylated by phosphatidylinositol 4-phosphate 5-kinase (PIP5K) to become phosphatidylinositol 4,5-bisphosphate (Ptd[4,5]P2 or PIP2; Boronenkov and Anderson 1995; Ishihara *et al.* 1996). PIP2 is a second messenger thought to modulate the functions of cytoskeletal regulatory proteins such as profilin, coilin, fascin, and gelsolin (Janmey 1994). PIP2 also regulates vesicular trafficking and platelet activation (Toker 1998). Phospholipase C hydrolyzes PIP2 to produce the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP3). DAG activates protein kinase C (PKC), while IP3 releases calcium from intracellular stores (Rana and Hokin 1990). In addition, PIP2 is converted into phosphatidylinositol 3,4,5-trisphosphate, which activates some PKC isoforms (Toker *et al.* 1994). Phosphoinositide metabolism is also involved in signal transduction and cytoskeleton regulation via interaction with the Rho family of small G proteins (Chong *et al.* 1994; Ren *et al.* 1996). An interaction has also been implicated between phosphoinositides and receptor tyrosine kinases (Cochet *et al.* 1991). There are two types of PIP5Ks, PIP5KI and PIP5KII, with distinct biochemical properties (Anderson *et al.* 1999). PIPKIs prefer to phosphorylate PI-4-P to PI-4,5-P2 (Anderson *et al.* 1999), while the preferred substrate of PIPKII is PI-5-P rather than PI-4-P (Rameh *et al.* 1997). PIP5KI has been shown to be required for vesicular secretion in PC12 cells (Hay *et al.* 1995), while PIP5KII may be involved in vesicular trafficking in budding yeast (Yamamoto *et al.* 1995).

Phosphoinositides are also present in the nucleus (Divecha *et al.* 1993). There is growing evidence that members of the phosphoinositide pathways are involved in post-transcriptional modification and chromatin-mediated gene regulation. Biochemical experiments have shown the association of phosphatidylinositol (PI), phosphatidylinositol phosphate kinase (PIPK), and DAG activities with the nuclear matrix (Payrastre *et al.* 1992). Multiple isoforms of PIPKs localize to the nucleoplasm and are concentrated at nuclear speckles containing mRNA-processing components (Boronenkov *et al.* 1998). PIP2 was also detected at these speckles, consistent with its production by PIPKs localized to these sites (Boronenkov *et al.* 1998). Genetic evidence has implicated nuclear phosphoinositides and their hydrolysis products in the export of mRNA via the nuclear pore complex (York *et al.* 1999). PIP2 is a necessary component of the pre-mRNA splicing machinery (Osborne *et al.* 2001). A Dictyostelium nuclear phosphatidylinositol phosphate kinase is required for developmental gene expression (Guo *et al.* 2001). Nuclear inositol 1,4,5-trisphosphate kinase in yeast also has a role in transcriptional control (Odom *et al.* 2000). Phospholipids are able to bind histones and nonhistone chromosomal associated proteins (Manzoli *et al.* 1977). PtdIns[3]P and PtdIns[5]P were shown to bind to the PHD fingers of the chromatin-associated protein ING2 and several other proteins (Gozani *et al.* 2003). PIP2 stabilizes the association of the SWI/SNF-like BAF complex with chromatin and the nuclear matrix (Zhao *et al.* 1998), and it binds histone H1, which leads to the inhibition of histone-H1-mediated repression on RNA polymerase II activity (Yu *et al.* 1998).

The Drosophila gene *skittles* (*sktl*) encodes a putative PIP5KI, which is required for cell viability and germline and bristle development; *sktl* mutations affect the ovary, dorsal appendage, egg, and wing (Knirr *et al.* 1997; Hassan *et al.* 1998). In this article, we show that ASH2, a trxG protein, and SKTL bind directly to each other *in vitro* and *in vivo* and that *sktl* mutations enhance the homeotic transformation phenotype of *ash2* mutations. We also report that histone H1 hyperphosphorylation within euchromatin is dramatically increased on *ash2* and *sktl* mutant polytene chromosomes. These results support a model in which PIP2 plays a role in maintaining transcriptionally active chromatin via histone H1 modification.

MATERIALS AND METHODS

Yeast two-hybrid screen: Full-length *ash2* was PCR amplified from cDNA LD31680 (Research Genetics, Huntsville, AL) and subcloned into the TA cloning vector (Invitrogen, San Diego). A construct with ASH2 fused to the DNA-binding domain of GAL4 was generated by subcloning a *Nde*I-*Sal*I fragment into pAS1-CYH2. pAS1-CYH2:ASH2 was transformed into the yeast strain Y190 (*MAT***a** gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 + URA3:GAL → lacZ, LYS2:GAL → HIS3 cyh^r) and transformants were selected with SC-Trp plates. Yeast transformants containing pAS1-CYH2:ASH2 were transformed with a Drosophila third instar larval cDNA library inserted in the pACTII vector containing the activation domain of GAL4. Transformants were selected with SC-Leu, His, Trp, +3-AT plates. Clones positive for β-galactosidase activity were isolated and sequenced.

GST pull-down: Regions of *ash2* were PCR amplified from cDNA LD31680 (Research Genetics) and subcloned into the TA cloning vector (Invitrogen). Constructs encoding in-frame fusions of ASH2 regions to glutathione *S*-transferase (GST) were generated by subcloning *Eco*RI fragments into pGex-2TK (Amersham, Buckinghamshire, UK). Full-length *sktl* was PCR amplified from cDNA LP06742 (Research Genetics) and subcloned into the TA cloning vector (Invitrogen). 6His-SKTL was generated by subcloning a *Kpn*I fragment into pRSETB (Invitrogen). Fusion proteins were expressed in 200-ml cultures of *Escherichia coli* DH5α strain and solubilized in 1.5% sarkosyl, 1 mm dithiothreitol (DTT), and 0.1 mg/ml lysozyme

by sonication. Insoluble proteins were removed by centrifugation. A total of 50 μl of glutathione sepharose beads (Pharmacia) was added to the cleared supernatant to purify the GST fusion proteins. Bead-bound fusion proteins were washed three times in binding buffer (20 mm Hepes-KOH, 2.5 mm MgCl2, 10% glycerol, 1 mm phenylmethylsulfonyl fluoride, 1 mm DTT, 200 mm KCl, 0.08% NP-40). Supernatant of 6His-SKTL was added to the beads and incubated at 4° overnight. After washing the beads three times in binding buffer, SDS-PAGE sample buffer was added, and samples were incubated at 95° for 5 min. GST-ASH2 bound proteins were analyzed by SDS-PAGE and immunoblotted with antibody against 6His (Upstate Biotechnology, Lake Placid, NY).

Generation of *P*-element transformants expressing FLAG epitope-tagged SKTL: *sktl* was PCR amplified from cDNA LP06742 (Research Genetics) and subcloned into the TA cloning vector (Invitrogen). A *Not*I-*Kpn*I restriction fragment was inserted into the pUAST-FLAG vector, which produces a FLAG epitope-tagged SKTL protein under the control of GAL4. This construct was injected into *yw; Dr/TMS, Sb Δ2-3* embryos.

Immunoprecipitation: Embryos containing both upstream activator sequence (UAS)-FLAG-SKTL and tubulin-GAL4 transgenes were collected over 24-hr periods. Collected embryos were dechorionated with 50% bleach and homogenized in RIPA(−)1 buffer (50 mm Tris-HCl pH 7.5, 1% NP40, 0.1% sodium deoxycholate, 150 mm NaCl) with aprotinin. Embryo extracts were spun to pellet insoluble material. Supernatants were incubated with M5 FLAG monoclonal antibody bound to protein G-sepharose beads (Amersham) with rocking at 4° overnight. After washing the beads three times in RIPA(−)1, SDS-PAGE sample buffer was added, and the samples were incubated at 95° for 5 min. Immunoprecipitates were fractionated by SDS-PAGE and immunoblotted with antibody against ASH2 (Adamson and Shearn 1996).

Immunofluorescence of polytene chromosomes: Third instar larvae were dissected in PBS + 0.1% Triton X-100. Salivary glands were fixed for 2 min in acetic acid fix (50% glacial acetic acid, 3.7% formaldehyde, 0.1% Triton X-100) and then washed in 45% acetic acid. Fixed salivary glands were pipetted onto poly-l-lysine-coated slides and covered with siliconized coverslips. Polytene chromosomes were spread out by tapping the coverslip with a rubber-tipped hammer and squashed with thumb pressure. Slides were dipped into liquid N_2. Chromosomes were blocked in antibody dilution buffer (PBS + 1% BSA + 0.1% Triton X-100) three times for 30 min and incubated with a 1:35 dilution of primary antibody at 4° overnight. Chromosomes were washed three times for 15 min and incubated with a 1:250 dilution of fluorescence-conjugated secondary antibody at 37° for 30 min. Chromosomes were washed three times for 15 min and mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole. All washes and dilutions were performed in antibody dilution buffer. ASH2 rabbit polyclonal antibody (Adamson and Shearn 1996) was protein A column purified. M5 FLAG mouse monoclonal antibody was from Sigma (St. Louis). Histone H1 mouse monoclonal antibody (Upstate Biotechnology) was generated against nuclei from myeloid leukemia biopsy cells (clone AE-4). Phospho-histone H1 rabbit polyclonal antibody (Upstate Biotechnology) was generated against the hyperphosphorylated isoform of Tetrahymena histone H1 purified by cation-exchange HPLC. Dimethyl-histone H3 (Lys4) rabbit polyclonal antibody (Upstate Biotechnology) was generated against a BSA-conjugated synthetic peptide corresponding to amino acids 1–8 of human histone H3 in which Lys4 was dimethylated.

Immunohistochemistry of imaginal discs: Third instar larvae were dissected, fixed, and immunostained as described previously (LaJeunesse and Shearn 1995). The DAB reaction consisting of 0.5 μg/ml solution of 3,3′ diaminobenzidine and 0.02% hydrogen peroxide resulted in an orangish-brown stain. Samples were developed simultaneously for the same amount of time and then rinsed with PBS several times. Mouse monoclonal Ultrabithorax (UBX) antibody (described by White and Wilcox 1984) was used at a dilution of 1:50.

Immunoblot of salivary glands: Salivary glands from third instar larvae were dissected, homogenized in SDS-PAGE sample buffer, incubated at 95° for 5 min, fractionated by SDS-PAGE, and immunoblotted with histone H1 mouse monoclonal antibody (Upstate Biotechnology). The immunoblot was analyzed by enhanced chemiluminescence (Pierce, Rockford, IL). After analysis with histone H1 antibody, the blot was stripped with 0.5 n NaOH for 5 min, rinsed with water for 5 min, and then immunoblotted with tubulin antibody. Exposures of immunoblots were scanned and quantitated by densitometry.

Drosophila stocks: Drosophila stocks were maintained at 25° on standard cornmeal, molasses, yeast, and agar food containing tegosept and proprionic acid as mold inhibitors. The wild-type strain used was Canton-S. $ash2^{1803}$ ($ash2^{2}$) is a proline-to-threonine point mutation of amino acid 297, $ash2^{703}$ ($ash2^{1}$) is an inversion with one of the breakpoints within the ASH2 coding region, and $ash2^{X2}$ ($ash2^{18}$) is a complete deletion of the ASH2 coding region (Adamson and Shearn 1996; M. K. Cheng and A. Shearn, unpublished results). Heteroallelic *ash2* mutant larvae were generated by crossing $ash2^{703}$ to $ash2^{X2}$. $sktl^{p1409}$ is a *P* element inserted into an intron of *sktl*, $sktl^{\Delta 15}$ is a small deletion resulting from the excision of *p1409*, and $sktl^{\Delta 20}$ is a larger deletion resulting from the excision of *p1409* (Hassan *et al.* 1998). Heteroallelic *sktl* mutant larvae were generated by crossing $sktl^{\Delta 15}$ to $sktl^{p1409}$. $ash1^{RE418}$ ($ash1^{4}$) has an early stop codon resulting in a protein containing only the first 874 amino acids (of 2144), and $ash1^{VV183}$ ($ash1^{22}$) has an early stop codon resulting in a protein with only the first 46 amino acids (Tripoulas *et al.* 1996).

RESULTS

SKTL is a nuclear phosphatidylinositol 4-phosphate 5-kinase: To identify potential components of an ASH2-containing complex, a Drosophila third instar larval cDNA library was screened for ASH2 binding partners using the yeast two-hybrid assay. A fusion of ASH2 to the GAL4 DNA-binding domain by itself did not activate transcription of a *lacZ* reporter gene. Two of the cDNAs that did activate transcription of the reporter gene when combined with the ASH2 fusion were sequenced and found to be identical to a portion of the known sequence of *sktl*. These cDNAs corresponded to amino acids 503–700 of the predicted SKTL protein. The fusion protein encoded by the *sktl* cDNA fragment fused to the GAL4 activation domain also did not activate transcription of the *lacZ* reporter by itself. The gene *sktl* encodes a protein that is 59% identical to a human PIP5KI and 58% identical to a mouse PIP5KI (Hassan *et al.* 1998). SKTL is required for cell and organism viability, as well as for cytoskeletal regulation during sensory structure development and germline development (Hassan *et al.* 1998). The Drosophila genome contains nine genes that are predicted to encode proteins with sequences similar to PIPKs (Adams *et al.* 2000). Four of these gene products, including SKTL, contain a PIP5K domain. A sequence

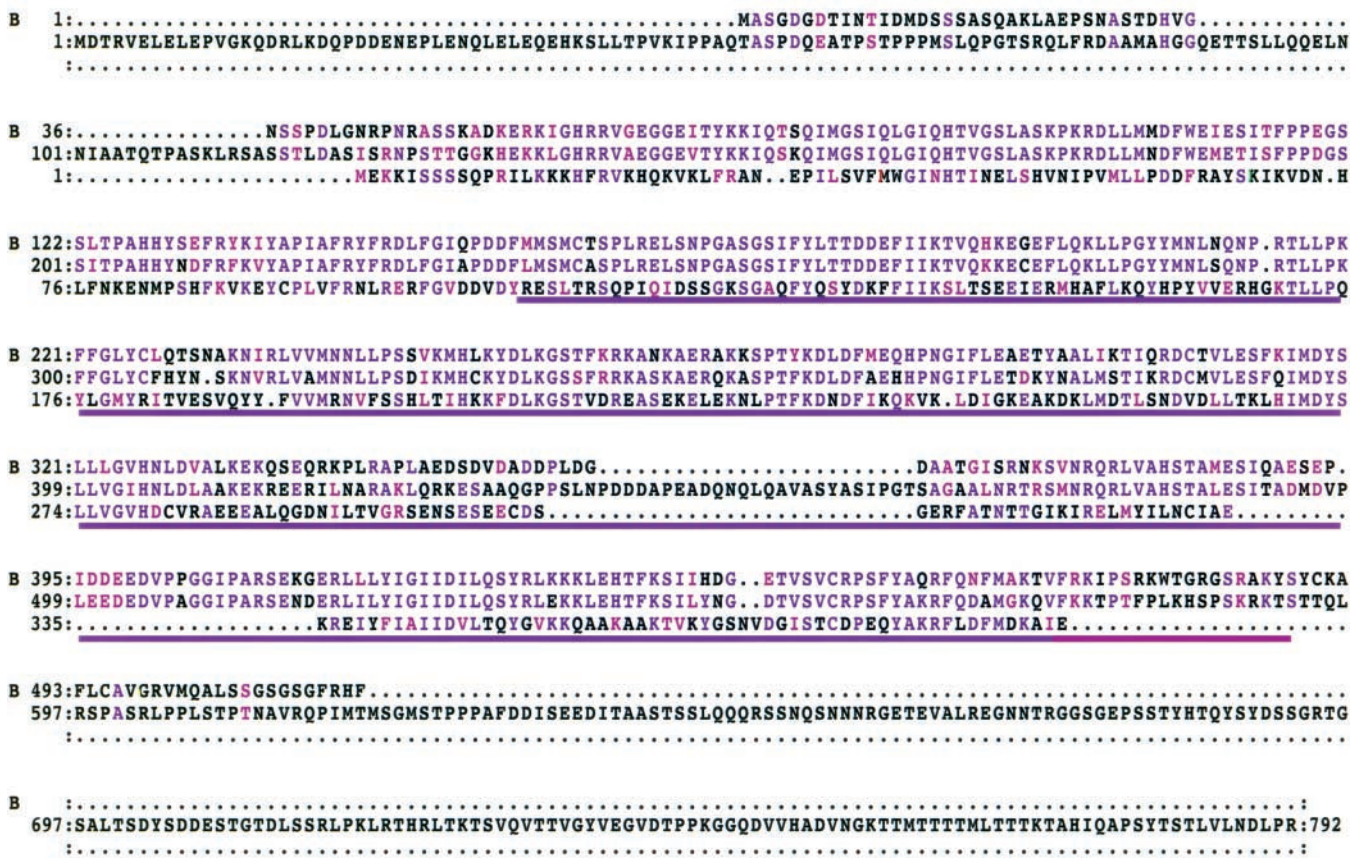

FIGURE 1.—Sequence alignment and comparison of Drosophila PIPKs. The sequences used in this alignment include PIP5K59B, SKTL, and CG17471. The alignment was performed using ClustalW and shaded using Boxshade. Identical amino acids are shown in purple. Similar amino acids are shown in pink. The PIP5K domain is underlined in purple and the NLS of SKTL is underlined in pink.

alignment and comparison of three of these gene products (PIP5K59B, SKTL, and CG17471) is shown in Figure 1. The fourth (EG:52C10.5) does not align with the other three. Of these three, only SKTL has a nuclear localization signal (NLS) according to the PSORTII computer program for the prediction of protein localization sites in cells (http://psort.nibb.ac.jp). SKTL has a PSORTII NLS score of 0.70, while PIP5K59B has a score of −0.22 and CG17471 has a score of 0.47. For comparison, ASH2, which is known to localize to nuclei by immunohistochemistry (ADAMSON and SHEARN 1996), has a PSORTII NLS score of 0.77.

ASH2 binds directly to SKTL *in vitro*: Direct binding between ASH2 and SKTL was confirmed *in vitro* by GST pull-down assays. A series of bacterially expressed fusion proteins containing either full-length ASH2 or regions of ASH2 fused to GST were generated, partially purified, and incubated with a bacterially expressed full-length SKTL tagged with hexahistidine (6His-SKTL). 6His-SKTL protein bound to both the full-length ASH2 fusion (Figure 2B, lane 3) and the fusion containing amino acids 260–404 of ASH2 (Figure 2B, lane 6), as detected by immunoblotting with 6His antibody. 6-His-SKTL did not bind to GST alone (Figure 2B, lane 2) or with the other ASH2 fragments fused to GST (Figure 2B, lanes 4, 5, and 7).

ASH2 physically associates with SKTL *in vivo*: The yeast two-hybrid and GST pull-down results indicate that ASH2 and SKTL can bind directly to each other in yeast and *in vitro*. To address the issue of whether they are associated with each other *in vivo*, co-immunoprecipitation experiments from embryo extracts were performed. To perform these experiments, we constructed a transgenic line containing a UAS promoter fused to FLAG epitope-tagged SKTL (FLAG-SKTL). This construct is able to rescue the lethality of *sktl* mutants when expressed ubiquitously under the control of tubulin-GAL4 (data not shown). This construct leads to accumulation of FLAG-SKTL protein when expressed ubiquitously under the control of a heat-shock protein 70 promoter fused to GAL4 (hsGAL4) if larvae are heat-shocked at 37° for 1 hr and allowed to recover at room temperature for 0.5 hr (data not shown). When FLAG-SKTL-expressing embryo extracts were incubated with the M5 FLAG monoclonal antibody bound to protein G-sepharose, ASH2 was immunoprecipitated, as detected by immunoblotting with ASH2 polyclonal antibody (Figure 3, lane 3). Two ASH2 proteins of 48 and 94 kD are detected in embryos (Figure 3, lane 1). It is of note that only the smaller ASH2 protein was immunoprecipitated. ASH2 was not immunoprecipitated when the identical proce-

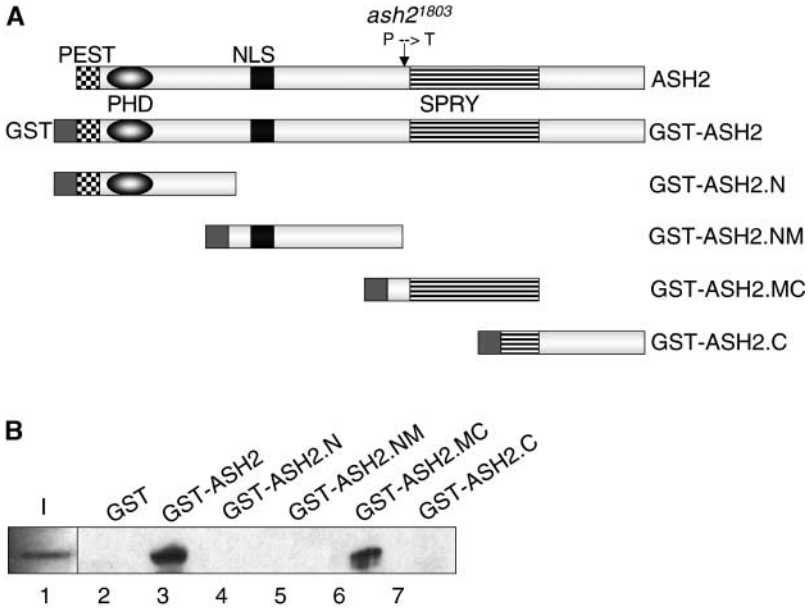

FIGURE 2.—GST fusion constructs and pull-down assay. ASH2 binds to SKTL *in vitro*. (A) Schematic of GST-ASH2 fusion constructs. All constructs consist of glutathione-S-transferase (gray box) fused to the N terminus of a region of ASH2. The full-length ASH2 construct contains amino acids 5–557 of ASH2, GST-ASH2.N contains amino acids 1–130; GST-ASH2.NM contains amino acids 125–270; GST-ASH2.MC contains amino acids 260–404; and GST-ASH2.C contains amino acids 402–557. PEST domain, checkered box; PHD domain, oval; NLS, dark gray box; SPRY domain, striped box. Arrow shows approximate location of *ash2*^{1803} mutation (P297T). (B) GST pull-down assay. Equal amounts of GST and GST-ASH2 constructs were purified and bound to glutathione sepharose beads. The beads were incubated with bacterial supernatant expressing 6His-SKTL. The beads were pelleted, fractionated by SDS-PAGE, and probed with antibody to 6His. A total of 10% of the 6His-SKTL supernatant was loaded in the input lane (I).

dure was performed with wild-type embryo extracts, which contain endogenous SKTL but no FLAG-SKTL (Figure 3, lane 2). ASH2 was also not detected after FLAG-SKTL-containing extracts were incubated with protein G-sepharose only (data not shown).

SKTL does not bind to *ash2* mutant chromosomes: The localization of ASH2 on polytene chromosomes from wild-type larval salivary glands is essential for function. This was shown by examining the chromosomal localization of ASH2 in temperature-sensitive *ash2* mutants. Mutant proteins accumulate at equivalent levels when temperature-sensitive *ash2* mutants are raised at either permissive or restrictive temperatures. However, mutant ASH2 protein localizes only to polytene chromosomes in larvae raised at permissive temperature (M. K. CHENG and A. SHEARN, unpublished results). Immunofluorescence of ASH2 reveals a widespread pattern of accumulation along wild-type chromosome arms (Figure 4A). The chromosome-wide distribution of ASH2 is not reduced and may even be increased on *sktl* mutant (*sktl*$^{\Delta 15}$/*sktl*^{p1409}) chromosomes (Figure 4B), despite the fact that the *sktl* mutant chromosomes have somewhat abnormal morphology. The appearance of the mutant chromosomes is probably due to the fact that the salivary glands were dissected from mutant larvae near their lethal phase. Alternatively, the abnormal morphology may be a real *sktl* mutant phenotype. When third instar larvae with the genotype *yw[UAS-Flag-sktl]/+;hsGAL4/+* were heat-shocked at 37° for 1 hr and allowed to recover at room temperature for 0.5 hr, FLAG-SKTL accumulated in the nucleolus (data not shown) and on polytene chromosomes in a diffuse and speckled manner (Figure 4C). No FLAG signal was detected in *yw[UAS-Flag-sktl]/+;hsGAL4/+* larvae that were not heat-shocked (data not shown). However, when *ash2* mutant larvae (*yw[UAS-Flag-sktl]/+;hsGAL4/+;ash2*^{703}/*ash2*^{X2}) were heat-shocked, there was a complete absence of FLAG-SKTL accumulation on the *ash2* mutant chromosomes (Figure 4D). These results suggest that although ASH2 does not require SKTL protein to bind to chromosomes, SKTL does require ASH2 protein and/or function to bind to chromosomes.

***ash2* and *sktl* display intergenic noncomplementation:** Since we demonstrated that ASH2 and SKTL bind each other both *in vitro* and *in vivo*, we wanted to further investigate the functional significance of this interaction. Three kinds of genetic evidence support the idea that the binding of these proteins to each other is of functional significance. *ash2* heterozygotes, but not *sktl*

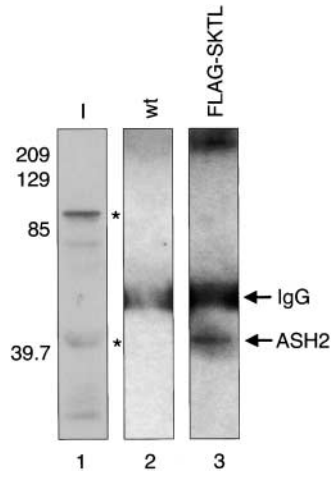

FIGURE 3.—Co-immunoprecipitation of ASH2 from embryo extracts. An extract of either Canton-S wild-type (wt) or transgenic embryos expressing FLAG-tagged SKTL (FLAG-SKTL) under the control of tubulin-GAL4 was incubated with M5 FLAG monoclonal antibody bound to protein G-sepharose beads. Input lane (I) shows 10% volume of wt extract used as starting material for immunoprecipitation; the two ASH2 bands are marked with an asterisk (*). An immunoblot of the immunoprecipitates probed with ASH2 antibody is shown.

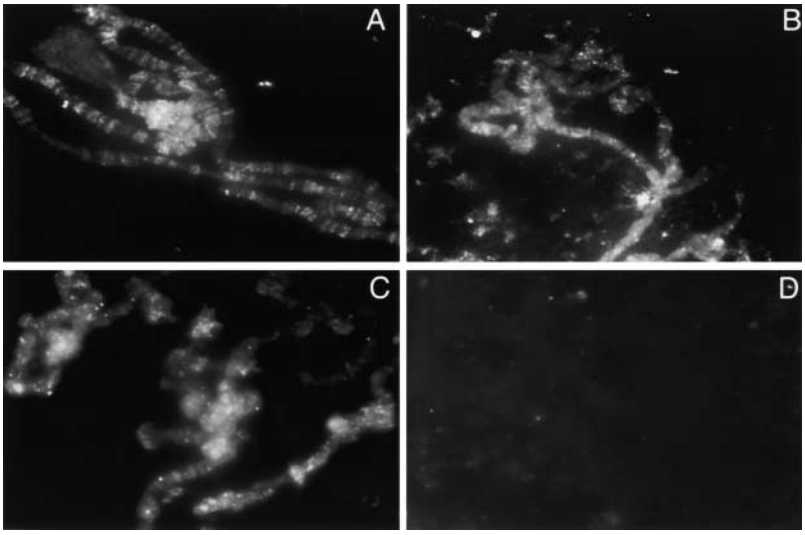

FIGURE 4.—Requirement of ASH2 for SKTL to bind chromosomes. (A) ASH2 localization on Canton-S wild-type salivary gland polytene chromosomes. (B) ASH2 localization on $sktl^{\Delta 15}/sktl^{p1409}$ chromosomes is not affected. (C) FLAG-SKTL localization on *yw[UAS-Flag-sktl]/+;hsGAL4* chromosomes. (D) FLAG-SKTL localization on *yw[UAS-Flag-sktl]/+;hsGAL4/+;*$ash2^{703}/ash2^{X2}$ chromosomes is greatly affected. ASH2 antibody was detected with a Texas Red-labeled secondary antibody. FLAG monoclonal antibody was detected with a FITC-labeled secondary antibody.

heterozygotes, show a low penetrance of homeotic transformations. *sktl/+;ash2/+* double heterozygotes were scored for homeotic transformations and were found to display an increased penetrance of third-leg to second-leg transformations (Figure 5). The penetrance of this phenotype varied depending on which alleles were examined (Table 1). Another trxG mutant, *ash1*, displayed the same homeotic phenotype in combination with *sktl* mutations (Table 1).

In addition to the intergenic noncomplementation seen in the double heterozygotes, larvae heterozygous for *sktl* and *trans*-heterozygous for *ash2* ($sktl^{\Delta 15}/+;ash2^{703}/ash2^{X2}$) were found to have delayed development and died at an earlier stage than *ash2 trans*-heterozygotes (data not shown). Also, the haltere and third-leg discs of *sktl trans*-heterozygotes ($sktl^{\Delta 15}/sktl^{p1409}$) have decreased accumulation of UBX (Figure 6), similar to the decreased UBX accumulation seen in the haltere and third-leg discs of *ash2 trans*-heterozygotes (LAJEUNESSE and SHEARN 1995).

Histone H1 hyperphosphorylation is increased in *ash2* and *sktl* mutants: PIP2, the product of PIPK activity, has been shown to bind histone H1 *in vitro*, leading to transcriptional derepression (YU *et al.* 1998). Also, protein kinase C, which is activated by the PIP2 metabolites DAG and PIP3, phosphorylates histone H1, which leads to chromatin decondensation. Because of these findings that implicate a relationship between PIP2 and its metabolites and histone H1, we wanted to determine if there is any change in histone H1 chromosomal localization or phosphorylation in *ash2* and/or *sktl* mutants. Immunofluorescence reveals a widespread pattern of histone H1 accumulation along wild-type chromosome arms (Figure 7A). This pattern of accumulation is unchanged on chromosomes from $ash2^{703}/ash2^{X2}$ (Figure 7B) and $sktl^{\Delta 15}/sktl^{p1409}$ (Figure 7C). However, the appearance of the fluorescence is reproducibly altered. Immunofluorescence reveals accumulation of hyperphosphorylated histone H1 at a limited number of bands

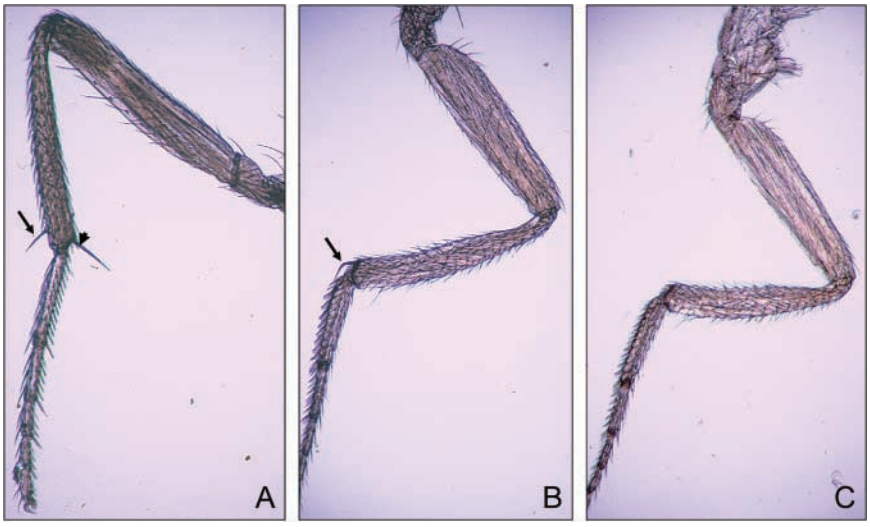

FIGURE 5.—Homeotic transformation of *ash2 sktl* double heterozygotes. (A) Wild-type second leg with characteristic preapical (arrow) and apical (arrowhead) bristles. (B) $sktl^{\Delta 15}/+;ash2^{X2}/+$ third leg showing second-leg transformation by presence of ectopic preapical bristle (arrow). (C) Wild-type third leg with characteristic absence of the preapical and apical bristles.

TABLE 1

Intergenic noncomplementation among *ash1*, *ash2*, and *sktl* mutations

Name of mutation	Penetrance of third-leg to second-leg transformations			
	Canton-S	$sktl^{\Delta 15}$	$sktl^{\Delta 20}$	$sktl^{p1409}$
Canton-S	NA	0 (n = 203)	0 (n = 203)	0 (n = 238)
$ash1^{RE418}$	3.1 (n = 255)	2.5 (n = 197)	5.0 (n = 200)	7.1[a] (n = 184)
$ash1^{VV183}$	0 (n = 139)	3.7[b] (n = 242)	2.4[b] (n = 126)	0 (n = 152)
$ash2^{1803}$	0 (n = 178)	28.6[b] (n = 213)	4.3[b] (n = 140)	0.7 (n = 143)
$ash2^{X2}$	0 (n = 204)	9.4[b] (n = 223)	0 (n = 213)	3.5[b] (n = 200)

Penetrance is the percentage of the number (n) of flies examined with the phenotype. NA, not available.
[a] Penetrance significantly different from Canton-S (wild type) control according to G-test ($P < 0.05$).
[b] Penetrance highly significantly different from Canton-S (wild type) control according to G-test ($P < 0.01$).

along wild-type chromosome arms and extensive accumulation in the nucleolus (Figure 7D). Both $ash2^{703}/ash2^{X2}$ (Figure 7E) and $sktl^{\Delta 15}/sktl^{p1409}$ (Figure 7F) chromosomes show a dramatic increase in hyperphosphorylated histone H1. Many more bands are present on the mutant chromosomes, in addition to the strong nucleolar signal. It is likely that this extensive hyperphosphorylation of histone H1 in the mutants is responsible for the altered appearance of immunofluorescence with antihistone H1. Immunoblot analysis of wild-type and $sktl^{\Delta 15}/sktl^{p1409}$ third instar larval salivary glands (Figure 8) confirmed the immunofluorescence results. An antihistone H1 immunoblot (Figure 8, top) shows similar levels of accumulation of total histone H1 in $sktl^{\Delta 15}/sktl^{p1409}$ larvae as compared to wild type. The same blot was probed with antitubulin as a control and to correct for the amount of protein loaded (Figure 8, bottom). The amount of protein in the hyperphosphorylated and nonhyperphosphorylated histone H1 bands from the wild-type sample together equals the amount in the one hyperphosphorylated histone H1 band from *sktl* mutant salivary glands when corrected for amount of protein loaded. The striking result is that essentially all of the histone H1 in *sktl* mutant salivary glands is hyperphosphorylated. These results demonstrating that lack of ASH2 or SKTL leads to an increase in histone H1 hyperphosphorylation imply that the normal functions of ASH2 and SKTL involve the downregulation of histone H1 hyperphosphorylation.

DISCUSSION

ASH2 physically associates with SKTL: SKTL was identified in our yeast two-hybrid screen as a protein that binds to ASH2. The direct physical association between ASH2 and SKTL, as indicated by the yeast two-hybrid assay, was confirmed *in vitro* by GST pull-down (Figure 2B). Full-length ASH2 (lane 3) and ASH2.MC (lane 6), which contains the entire SPRY domain of ASH2, were sufficient to pull down SKTL. The SPRY domain was named after the two proteins that have this domain, yeast SPla and ryanodine receptor (Ponting *et al.* 1997). Although the function of the SPRY domain is unknown, our GST pull-down results suggest that one of its possible functions is mediating protein-protein interactions.

The physical association between ASH2 and SKTL, as indicated by the yeast two-hybrid assay and GST pull-down, also occurs *in vivo* in Drosophila embryos as shown by co-immunoprecipitation (Figure 3). Antibody against

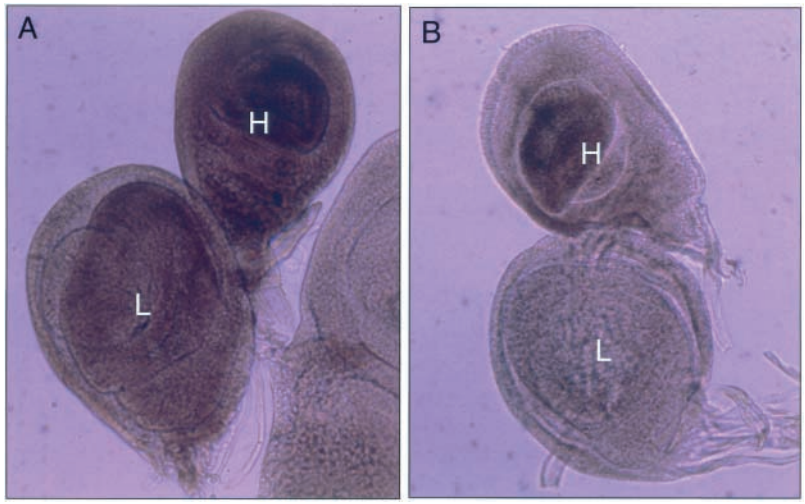

Figure 6.—Reduced UBX accumulation in *sktl* mutants. (A) Wild-type third instar haltere (H) and third-leg (L) discs. UBX accumulates in the haltere and third-leg discs. (B) $sktl^{\Delta 15}/sktl^{p1409}$ third instar imaginal haltere and third-leg discs have reduced UBX accumulation. UBX antibody was detected with an HRP-conjugated mouse secondary antibody and developed with a DAB reaction. All samples shown were dissected, immunostained, developed, and photographed simultaneously.

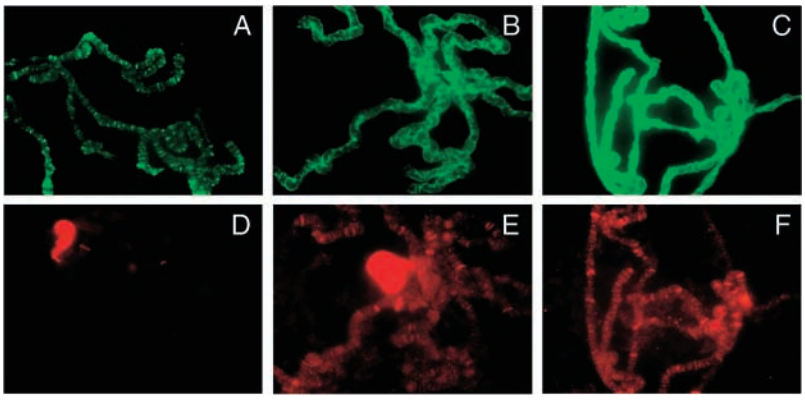

Figure 7.—Histone H1 localization on *ash2* and *sktl* mutant chromosomes. Accumulation of histone H1 was detected by immunofluorescence with FITC-labeled secondary antibody (green). Wild-type salivary gland polytene chromosomes (A) have a different appearance than $ash2^{703}/ash2^{X2}$ (B) and $sktl^{\Delta 15}/sktl^{p1409}$ (C) chromosomes. Accumulation of hyperphosphorylated histone H1 was detected by immunofluorescence with a Texas Red-labeled secondary antibody (red). Wild-type chromosomes (D) have dramatically less accumulation of hyperphosphorylated histone H1 than $ash2^{703}/ash2^{X2}$ (E) and $sktl^{\Delta 15}/sktl^{p1409}$ (F) chromosomes have. All images were taken under identical conditions.

the FLAG epitope was able to immunoprecipitate ASH2 from embryos expressing FLAG-SKTL. This result shows that ASH2 and SKTL are physically associated in a complex. In embryos, the ASH2 antibody recognizes two proteins of different sizes, one ~48 kD and one ~94 kD (Figure 3, lane 1). At other developmental stages (larval to adult), the 48-kD protein is also present but the larger ASH2 protein is ~65 kD (M. K. Cheng and A. Shearn, unpublished results). The 48- and 65-kD proteins are the sizes expected from the translation of two *ash2* transcripts of 1.4 and 2 kb, respectively, which are detectable by RNA blotting (Beltran *et al.* 2003). The 94-kD protein in embryos may result from post-translational modification of the 65-kD protein. Despite the fact that the 94-kD protein is more abundant, only the 48-kD protein is immunoprecipitated by SKTL from embryonic nuclear extracts. Assuming that the 48-kD protein is translated from the 1.4-kb transcript, it would lack the PEST sequence and PHD finger of the full-length ASH2 protein, but would still contain the NLS-BP and SPRY domains. The SPRY domain was found in GST pull-down experiments to be sufficient to bind to SKTL *in vitro*. The 94-kD protein presumably would also contain the SPRY domain, yet it is not immunoprecipitated by SKTL. Perhaps its SPRY domain is modified in some way to prevent physical association with SKTL. ASH2 antibody

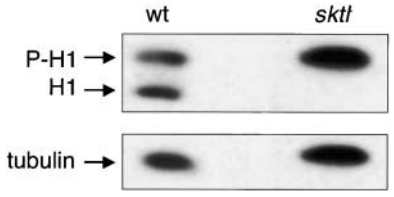

Figure 8.—Histone H1 accumulation in *sktl* salivary glands. Salivary glands from Canton-S wild-type (wt) and $sktl^{\Delta 15}/sktl^{p1409}$ ($sktl^{-}$) third instar larvae were dissected, analyzed by SDS-PAGE, and immunoblotted with histone H1 and tubulin antibodies. (Top) Antihistone H1 immunoblot. Histone H1 antibody recognizes both the phosphorylated (32 kD) and nonphosphorylated (31 kD) forms of histone H1. Nonphosphorylated histone H1 is completely absent in *sktl* mutant salivary glands. (Bottom) Same blot probed with tubulin antibody as a loading control.

was not able to immunoprecipitate FLAG-SKTL from embryos expressing FLAG-SKTL (data not shown). This may be because the ASH2 antibody recognizes the same site on ASH2 that mediates binding with SKTL. The 94-kD ASH2 protein was found to associate with a 500-kD multimeric protein complex (Papoulas *et al.* 1998). SKTL could be a component of this complex. However, it is more likely that SKTL is in a distinct complex with the 48-kD ASH2 protein, because SKTL seems to immunoprecitate only with the 48-kD ASH2 and not with the 94-kD ASH2 found in the 500-kD complex. In *S. pombe*, the ASH2 homolog is present in two distinct complexes (Roguev *et al.* 2003). One complex contains Set1 and the other contains a homolog of Drosophila LID (Gildea *et al.* 2000).

Both ASH2 and SKTL accumulate on polytene chromosomes (Figure 4) and in the nucleolus (data not shown). These results suggest that their physical association and functions involve chromatin and, perhaps, ribosomal DNA transcription. ASH2 accumulates normally on *sktl* mutant chromosomes while SKTL does not accumulate on *ash2* mutant chromosomes (Figure 4). There are two possible explanations for this result. Either SKTL is not made in *ash2* mutants or SKTL requires ASH2 protein and/or function to bind to polytene chromosomes. We have shown that SKTL accumulates to normal levels in *ash2* mutants that express FLAG-SKTL by immunoblot analysis of embryos and larvae (data not shown), so SKTL needs ASH2 protein and/or function to bind to chromosomes. The *ash2* mutant combination we used to represent the null mutant state is a *trans*-heterozygote of an inversion, $ash2^{703}$, and a deletion, $ash2^{X2}$; it does not accumulate either normal-sized *ash2* transcript (Adamson and Shearn 1996) or ASH2 protein (data not shown). Since this mutant lacks ASH2 protein, it necessarily lacks ASH2 function; we cannot distinguish with certainty whether SKTL requires ASH2 protein or function to bind to chromosomes. However, since SKTL binds directly to ASH2, we favor the idea that SKTL requires the ASH2 protein to bind to chromosomes.

It was also found that ASH2 does not accumulate on *ash1* mutant chromosomes, but ASH1 still accumulates on *ash2* mutant chromosomes (M. K. Cheng and A. Shearn,

unpublished results). This suggests that ASH2 needs ASH1 to bind chromosomes. We predict that SKTL will not be able to bind to *ash1* mutant chromosomes, since SKTL requires ASH2 and ASH2 requires ASH1 to bind to chromosomes. This could also explain why we see intergenic noncomplementation between *ash1* and *sktl* mutations (Table 1).

SKTL is a nuclear Drosophila PIP5K: Since SKTL was the only Drosophila PIP5K found to have a NLS (Figure 1) and was shown to accumulate in nuclei (Figure 4), we think that SKTL serves a function distinct from those of other Drosophila PIP5Ks. The cytoplasmic PIP5Ks may be able to bind ASH2 *in vitro*, because they contain protein domains similar to SKTL that ASH2 recognizes. However, since ASH2 and the cytoplasmic PIP5Ks are not localized to the same cellular compartment, we do not expect the other Drosophila PIP5Ks to have a functional physical association with ASH2. The PHD finger of ING2 was shown to function as a nuclear phosphoinositide receptor (Gozani *et al.* 2003), so it is attractive to speculate that the PHD finger of ASH2 can also bind phosphoinositides that can be processed into PIP2 via its interaction with SKTL. It is likely that other members of the nuclear phosphoinositide signaling pathway will have functional interactions with ASH2. As examples, if a *sktl* transcription factor were mutated, then less SKTL would be made or if genes that encoded enzymes that generated SKTL substrates such as PIP or metabolites such as IP3 were mutated, then fewer of these metabolites would be generated. *ash2* mutants might be expected to display intergenic noncomplementation with these mutants as well.

Functional significance of ASH2-SKTL physical association: Several lines of evidence show that the physical association between ASH2 and SKTL is functionally significant. First of all, there is the complete loss of SKTL accumulation on *ash2* mutant chromosomes (Figure 4), suggesting that SKTL requires ASH2 to bind to chromosomes.

The intergenic noncomplementation between *sktl* and *ash2* mutants also shows that the physical association between the two gene products has functional significance. ASH1 and ASH2, like other trxG proteins, play a role in maintaining transcription activation. Reduced UBX accumulation in *sktl* mutants (Figure 6), as well as the intergenic noncomplementation seen with *sktl* mutants in combination with *ash1* or *ash2* (Table 1), suggests a similar role for SKTL in transcription regulation. The PIP2 generated in the nucleus by SKTL activity could be hydrolyzed to DAG and IP3 (Irvine 2000). IP3 can be further phosphorylated to IP4 and IP5, both of which have been shown to activate transcription (Shen *et al.* 2003; Steger *et al.* 2003).

Another result that shows functional significance of the physical association between ASH2 and SKTL is a similar dramatic increase in histone H1 hyperphosphorylation on both *ash2* and *sktl* mutant chromosomes compared to wild-type chromosomes (Figure 8). Histone H1 is thought to be a general repressor of transcription by RNA polymerase II (Croston *et al.* 1991). The presence of histone H1 affects the ability of transcription factors to interact with DNA and is associated with transcription repression, while the removal of histone H1 is associated with transcriptional activation (Laybourn and Kadonaga 1991; Bresnick *et al.* 1992; Juan *et al.* 1994; Schultz *et al.* 1996). Studies in mammals and Tetrahymena have found a correlation between transcriptional activation and increased histone H1 phosphorylation (Dedon *et al.* 1991; Bresnick *et al.* 1992; Dou *et al.* 1999). Dephosphorylated histone H1 bound to chromatin over the mouse mammary tumor virus promoter is thought to restrict chromatin remodeling and transcription factor access (Banks *et al.* 2001). Phosphorylation of histone H1 has also been shown to regulate ATP-dependent chromatin-remodeling enzymes (Horn *et al.* 2002). The effect of phosphorylation is to create a region of negative charge, which may displace histone H1 from chromatin, allowing the binding of specific regulating factors (Dou and Gorovsky 2000). Alternatively, proteins that regulate transcription may recognize the phosphorylated residues (Dou and Gorovsky 2000).

However, histone H1 hyperphosphorylation has the opposite effect and is linked to high chromatin condensation, possibly by allowing the binding of accessory factors (Roth and Allis 1992). During mitosis, histone H1 becomes hyperphosphorylated, which may facilitate the interaction with the DNA minor groove and factors involved in metaphase chromosome condensation (Hohmann 1983; Halmer and Gruss 1996). Therefore, increased histone H1 hyperphosphorylation as observed in *ash2* and *sktl* mutants implies increased chromosome condensation and reduced transcription.

Regulation of transcription activation by the ASH2-SKTL complex: ASH1 has been shown to be able to methylate K4 of histone H3 (Beisel *et al.* 2002; Byrd and Shearn 2003) and *ash1* mutant chromosomes show complete loss of histone H3 K4 methylation (Byrd and Shearn 2003). This result suggests that ASH1 is required for all of the histone H3 K4 methylation that occurs *in vivo*. The *S. cerevisiae* SET1 complex, which contains two subunits that are thought to represent a bipartite functional homolog of ASH2, has also been shown to methylate K4 of histone H3 (Miller *et al.* 2001; Roguev *et al.* 2001; Nagy *et al.* 2002). In Drosophila, if ASH2 were also in a complex that can methylate histone H3 K4, then it would be predicted that *ash2* mutant chromosomes would show a decrease in histone H3 K4 methylation. We did indeed observe a decrease in histone H3 K4 methylation on *ash2* mutant chromosomes (M. K. Cheng and A. Shearn, unpublished results).

During the assembly of nucleosomes, histone acetylation regulates the binding of histone H1 and chromatin condensation (Perry and Annunziato 1989; Ridsdale *et al.* 1990). Displacement of histone H1 is required

prior to acetylation of target genes and activation of transcription, because histone H1 inhibits histone H3 acetylation by hindering the access of histone acetyltransferases to the histone H3 tail (Herrera *et al.* 2000). Herrera *et al.* (2000) predicted that chromatin-remodeling complexes would contain components that modify the interaction of histone H1 with chromatin. ASH2 and SKTL may represent such components. Our results suggest that ASH2 and SKTL are direct binding partners that are associated in a complex. When the ASH2-SKTL complex binds to chromatin, a source of PIP2 (SKTL) is brought to the chromatin. PIP2 can bind to and displace histone H1 and/or be metabolized to IP3 and phosphorylated derivatives. The displacement of histone H1 would prevent its hyperphosphorylation and allow for chromatin decondensation, histone acetylation, and eventually, transcription activation. The presence of IP4 and IP5 would also stimulate transcription.

We thank Stephen Elledge for strains and stocks used in the yeast two-hybrid screen, Hugo Bellen for the *sktl* alleles, Victor Corces and his lab for use of their fluorescence microscope, Evelyn Hersperger for generating the transgenic lines, Grafton Hersperger for technical support and generating the *ash2* alleles, and Bethany Simmons and Nick Bongio for setting up and maintaining certain crosses. We also thank Michelle Beaucher, Charles Na, and Kristin Byrd for their helpful comments on the manuscript. This work was supported by a grant from the National Institute of General Medical Sciences.

LITERATURE CITED

Aasland, R., T. J. Gibson and A. F. Stewart, 1995 The PHD finger: implications for chromatin-mediated transcriptional regulation. Trends Biochem. Sci. **20:** 56–59.

Adams, M. D., S. E. Celniker, R. A. Holt, C. A. Evans, J. D. Gocayne *et al.*, 2000 The genome sequence of *Drosophila melanogaster*. Science **287:** 2185–2195.

Adamson, A. L., and A. Shearn, 1996 Molecular genetic analysis of Drosophila *ash2*, a member of the trithorax group required for imaginal disc pattern formation. Genetics **144:** 621–633.

Akam, M., 1987 The molecular basis for metameric pattern in the *Drosophila* embryo. Development **101:** 1–22.

Anderson, R. A., I. V. Boronenkov, S. D. Doughman, J. Kunz and J. C. Loijens, 1999 Phosphatidylinositol phosphate kinases, a multifaceted family of signaling enzymes. J. Biol. Chem. **274:** 9907–9910.

Banks, G. C, L. J. Deterding, K. B. Tomer and T. K. Archer, 2001 Hormone-mediated dephosphorylation of specific histone H1 isoforms. J. Biol. Chem. **276:** 36467–36473.

Beisel, C., A. Imhof, J. Greene, E. Kremmer and F. Sauer, 2002 Histone methylation by the Drosophila epigenetic transcriptional regulator Ash1. Nature **419:** 857–862.

Beltran, S., E. Blanco, F. Serras, B. Perez-Villamil, R. Guigo *et al.*, 2003 Transcriptional network controlled by the trithorax-group gene *ash2* in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **100:** 3293–3298.

Boronenkov, I. V., and R. A. Anderson, 1995 The sequence of phosphatidylinositol 4-phosphate 5-kinase defines a novel family of lipid kinases. J. Biol. Chem. **270:** 2881–2884.

Boronenkov, I. V., J. C. Loijens, M. Umeda and R. A. Anderson, 1998 Phosphoinositide signaling pathways in nuclei are associated with nuclear speckles containing pre-mRNA processing factors. Mol. Biol. Cell **9:** 3547–3560.

Bresnick, E. H., M. Bustin, V. Marsaud, H. Richard-Foy and G. L. Hager, 1992 The transcriptionally active MMTV promoter is depleted of histone H1. Nucleic Acids Res. **20:** 273–278.

Byrd, K. N., and A. Shearn, 2003 ASH1, a Drosophila trithorax group protein, is required for methylation of lysine 4 residues on histone H3. Proc. Natl. Acad. Sci. USA **100:** 11535–11540.

Chong, L. D., A. Traynor-Kaplan, G. M. Bokoch and M. A. Schwartz, 1994 The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. Cell **79:** 507–513.

Cochet, C., O. Filhol, T. Payrastre, T. Hunter and G. N. Gill, 1991 Interaction between the epidermal growth factor receptor and phosphoinositide kinases. J. Biol. Chem. **266:** 637–644.

Croston, G. E., L. A. Kerrigan, L. M. Lira, D. R. Marshak and J. T. Kadonaga, 1991 Sequence-specific antirepression of histone H1-mediated inhibition of basal RNA polymerase II transcription. Science **251:** 643–649.

Dedon, P. C., J. A. Soults, C. D. Allis and M. A. Gorovsky, 1991 Formaldehyde cross-linking and immunoprecipitation demonstrate developmental changes in H1 association with transcriptionally active genes. Mol. Cell. Biol. **11:** 1729–1733.

Divecha, N., H. Banfic and R. F. Irvine, 1993 Inositides and the nucleus and inositides in the nucleus. Cell **74:** 405–407.

Dou, Y., and M. A. Gorovsky, 2000 Phosphorylation of linker histone H1 regulates gene expression in vivo by creating a charge patch. Mol. Cell **6:** 225–231.

Dou, Y., C. A. Mizzen, M. Abrams, C. D. Allis and M. A. Gorovsky, 1999 Phosphorylation of linker histone H1 regulates gene expression in vivo by mimicking H1 removal. Mol. Cell **4:** 641–647.

Duncan, I., 1987 The bithorax complex. Annu. Rev. Genet. **21:** 285–319.

Francis, N. J., and R. E. Kingston, 2001 Mechanisms of transcriptional memory. Nat. Rev. Mol. Cell Biol. **2:** 409–421.

Gildea, J., R. T. Lopez and A. Shearn, 2000 A screen for new trithorax group genes identified *little imaginal disks*, the *Drosophila melanogaster* homolog of human retinoblastoma binding protein 2. Genetics **156:** 645–663.

Gozani, O., P. Karuman, D. R. Jones, D. Ivanov, J. Cha *et al.*, 2003 The PHD finger of the chromatin-associated protein ING2 functions as a nuclear phosphoinositide receptor. Cell **114:** 99–111.

Guo, K., R. Nichol, P. Skehel, D. Dormann, C. J. Weijer *et al.*, 2001 A *Dictyostelium* nuclear phosphatidylinositol phosphate kinase required for developmental gene expression. EMBO J. **20:** 6017–6027.

Halmer, L., and C. Gruss, 1996 Effects of cell cycle dependent histone H1 phosphorylation on chromatin structure and chromatin replication. Nucleic Acids Res. **24:** 1420–1427.

Hassan, B. A., S. N. Prokopenko, S. Breuer, B. Zhang, A. Paululat *et al.*, 1998 *skittles*, a Drosophila phosphatidylinositol 4-phosphate 5-kinase, is required for cell viability, germline development and bristle morphology, but not for neurotransmitter release. Genetics **150:** 1527–1537.

Hay, J. C., P. L. Fisette, G. L. Jenkins, K. Fukami and T. Takenawa, 1995 ATP-dependent inositide phosphorylation is required for Ca^{2+}-activated exocytosis. Nature **374:** 173–177.

Herrera, J. E., K. L. West, R. L. Schiltz, Y. Nakatani and M. Bustin, 2000 Histone H1 is a specific repressor of core histone acetylation in chromatin. Mol. Cell. Biol. **20:** 523–529.

Hohmann, P., 1983 Phosphorylation of H1 histones. Mol. Cell. Biochem. **57:** 81–92.

Horn, P. J., L. M. Carruthers, C. Logie, D. A. Hill, M. J. Solomon *et al.*, 2002 Phosphorylation of linker histones regulates ATP-dependent chromatin remodeling enzymes. Nat. Struct. Biol. **9:** 263–267.

Ikegawa, S., M. Isomura, Y. Koshizuka and Y. Nakamura, 1999 Cloning and characterization of ASH2L and Ash2l, human and mouse homologs of the Drosophila *ash2* gene. Cytogenet. Cell Genet. **84:** 167–172.

Irvine, R., 2000 Nuclear lipid signaling. Sci. STKE **48:** R1.

Ishihara, H., Y. Shibasaki, N. Kizuki, H. Katagiri and Y. Yazaki, 1996 Cloning of cDNAs encoding two isoforms of 68kD type I phosphatidylinositol-4-phosphate 5-kinase. J. Biol. Chem. **271:** 23611–23614.

Janmey, P. A., 1994 Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. Annu. Rev. Physiol. **56:** 169–191.

Juan, L. J., R. T. Utley, C. C. Adams, M. Vetesse-Dadey and J. L. Workman, 1994 Differential repression of transcription factor binding by histone H1 is regulated by the core histone amino termini. EMBO J. **13:** 6031–6040.

Kaufman, T. C., M. A. Seeger and G. Olsen, 1990 Molecular and genetic organization of the Antennapedia gene complex of *Drosophila melanogaster*. Adv. Genet. **27:** 309–362.

Kennison, J. A., 1995 The Polycomb and trithorax group proteins of *Drosophila*: transregulators of homeotic gene function. Annu. Rev. Genet. **29:** 289–303.

Knirr, S., A. Santel and R. Renkawitz-Pohl, 1997 Expression of the PI4P 5-kinase Drosophila homologue *skittles* in the germline suggests a role in spermatogenesis and oogenesis. Dev. Genes Evol. **207:** 127–130.

LaJeunesse, D., and A. Shearn, 1995 Trans-regulation of thoracic homeotic selector genes of the Antennapedia and bithorax complexes by the trithorax group genes*: absent, small, and homeotic discs 1* and *2*. Mech. Dev. **53:** 123–139.

Laybourn, P. J., and J. T. Kadonaga, 1991 Role of nucleosomal cores and histone H1 in regulation of transcription by RNA polymerase II. Science **254:** 238–245.

Mahmoudi, T., and C. P. Verrijzer, 2001 Chromatin silencing and activation by Polycomb and trithorax group proteins. Oncogene **20:** 3055–3066.

Manzoli, F. A., N. M. Maraldi, L. Cocco, S. Capitani and A. Facchini, 1977 Chromatin phospholipids in normal and chronic lymphocytic leukemia lymphocytes. Cancer Res. **37:** 843–849.

Miller, T., N. J. Krogan, J. Dover, H. Erdjument-Bromage, P. Tempst *et al.*, 2001 COMPASS: a complex of proteins associated with a trithorax-related SET domain protein. Proc. Natl. Acad. Sci. USA **98:** 12902–12907.

Nagy, P. L., J. Griesenbeck, R. D. Kornberg and M. L. Cleary, 2002 A trithorax-group complex purified from *Saccharomyces cerevisiae* is required for methylation of histone H3. Proc. Natl. Acad. Sci. USA **99:** 90–94.

Odom, A. R., A. Stahlberg, S. R. Wente and J. D. York, 2000 A role for nuclear inositol 1,4,5-trisphosphate kinase in transcriptional control. Science **287:** 2026–2029.

Osborne, S. L., C. L. Thomas, S. Gschmeissner and G. Schiavo, 2001 Nuclear PtdIns(4,5)P_2 assembles in a mitotically regulated particle involved in pre-mRNA splicing. J. Cell Sci. **114:** 2501–2511.

Papoulas, O., S. J. Beek, S. L. Moseley, C. M. McCallum, M. Sarte *et al.*, 1998 The *Drosophila* trithorax group proteins BRM, ASH1 and ASH2 are subunits of distinct protein complexes. Development **125:** 3955–3966.

Payrastre, B., M. Nievers, J. Boonstra, M. Breton, A. J. Verkleij *et al.*, 1992 A differential location of phosphoinositide kinases, diacylglycerol kinase, and phospholipase C in the nuclear matrix. J. Biol. Chem. **267:** 5078–5084.

Perry, C. A., and A. T. Annunziato, 1989 Influence of histone acetylation on the solubility, H1 content and DNase I sensitivity of newly assembled chromatin. Nucleic Acids Res. **17:** 4275–4291.

Ponting, C., J. Schultz and P. Bork, 1997 SPRY domains in ryanodine receptors (Ca(2+)-release channels). Trends Biochem. Sci. **22:** 193–194.

Rameh, L. E., K. F. Tolias, B. C. Duckworth and L. C. Cantley, 1997 A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate. Nature **390:** 192–196.

Rana, R. S., and L. E. Hokin, 1990 Role of phosphoinositides in transmembrane signalling. Physiol. Rev. **70:** 115–164.

Ren, X.-D., G. M. Bokoch, A. Traynor-Kaplan, G. H. Jenkins and R. A. Anderson, 1996 Physical association of the small GTPase rho with a 68 kd phosphatidylinositol 4-phosphate 5-kinase in Swiss 3T3 cells. Mol. Biol. Cell **7:** 435–442.

Ridsdale, J. A., M. J. Hendzel, G. P. Delcuve and J. R. Davie, 1990 Histone acetylation alters the capacity of the H1 histones to condense transcriptionally active/competent chromatin. J. Biol. Chem. **265:** 5150–5156.

Robbins, J., S. M. Dilworth, R. A. Laskey and C. Dingwall, 1991 Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell **64:** 615–623.

Rogers, S., R. Wells and M. Rechsteiner, 1986 Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science **234:** 364–368.

Roguev, A., D. Schaft, A. Shevchenko, W. W. M. P. Pijnappel, M. Wilm *et al.*, 2001 The *Saccharomyces cerevisiae* Set1 complex includes an Ash2 homologue and methylates histone 3 lysine 4. EMBO J. **20:** 7137–7148.

Roguev, A., D. Schaft, A. Shevchenko, R. Aasland, A. Shevchenko *et al.*, 2003 High conservation of the Set1/Rad6 axis of histone 3 lysine 4 methylation in budding and fission yeast. J. Biol. Chem. **278:** 8487–8493.

Roth, S. Y., and C. D. Allis, 1992 Chromatina condensation. Does H1 dephosphorylation play a role? Trends Biochem. Sci. **17:** 93–98.

Schultz, T. F., S. Spiker and R. S. Quatrano, 1996 Histone H1 enhances the DNA binding activity of the transcription factor EmBP-1. J. Biol. Chem. **271:** 25742–25745.

Shearn, A., 1989 The *ash1, ash2* and *trithorax* genes of *Drosophila melanogaster* are functionally related. Genetics **121:** 517–525.

Shearn, A., T. Rice, A. Garen and W. Gehring, 1971 Imaginal disc abnormalities in lethal mutants of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **68:** 2695–2698.

Shearn, A., E. Hersperger and G. Hersperger, 1987 Genetic studies of mutations at two loci of *Drosophila melanogaster* which cause a wide variety of homeotic transformations. Roux's Arch. Dev. Biol. **196:** 231–242.

Shen, X., H. Xiao, R. Ranallo, W.-H. Wu and C. Wu, 2003 Modulation of ATP-dependent chromatin-remodeling complexes by inositol polyphosphates. Science **299:** 112–114.

Simon, J., 1995 Locking in stable states of gene expression: transcriptional control during *Drosophila* development. Curr. Opin. Cell Biol. **7:** 376–385.

Steger, D. J., E. S. Haswell, A. L. Miller, S. R. Wente and E. K. O'Shea, 2003 Regulation of chromatin remodeling by inositol polyphosphates. Science **299:** 114–116.

Toker, A., 1998 The synthesis and cellular roles of phosphatidylinositol 4,5-bisphosphate. Curr. Opin. Cell Biol. **10:** 254–261.

Toker, A., M. Meyer, K. K. Reddy, J. R. Falck and R. Aneja, 1994 Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-P_2 and PtdIns-3,4-P_3. J. Biol. Chem. **269:** 32358–32367.

Tripoulas, N., D. LaJeunesse, J. Gildea and A. Shearn, 1996 The Drosophila *ash1* gene product, which is localized at specific sites on polytene chromosomes, contains a SET domain and a PHD finger. Genetics **143:** 913–928.

White, R. A., and M. Wilcox, 1984 Protein products of the bithorax complex in Drosophila. Cell **39:** 163–171.

Wysocka, J., M. P. Myers, C. D. Laherty, R. N. Eisenman and W. Herr, 2003 Human Sin3 deacetylase and trithorax-related Set1/Ash2 histone H3–K4 methyltransferase are tethered together selectively by the cell-proliferation factor HCF-1. Genes Dev. **17:** 896–911.

Yamamoto, A., D. B. DeWald, I. V. Boronenkov, R. A. Anderson and S. D. Emr, 1995 Novel PI(4)P 5-kinase homologue, Fab1p, essential for normal vacuole function and morphology in yeast. Mol. Biol. Cell **6:** 525–539.

York, J. D., A. R. Odom, R. Murphy, E. B. Ives and S. R. Wente, 1999 A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export. Science **285:** 96–100.

Yu, H., K. Fukami, Y. Watanabe, C. Ozaki and T. Takenawa, 1998 Phosphatidylinositol 4,5-bisphosphate reverses the inhibition of RNA transcription caused by histone H1. Eur. J. Biochem. **251:** 281–287.

Zhao, K., W. Wang, O. J. Rando, Y. Xue, K. Swiderek *et al.*, 1998 Rapid and phosphoinositol-dependent binding of the SWI/SNF-like BAF complex to chromatin after T lymphocyte receptor signaling. Cell **95:** 625–636.

Communicating editor: J. Tamkun