A Drosophila Homologue of Sir2 Modifies Position-Effect Variegation but Does Not Affect Life Span

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ABSTRACT

Control of chromosome structure is important in the regulation of gene expression, recombination, DNA repair, and chromosome stability. In a two-hybrid screen for proteins that interact with the Drosophila CREB-binding protein (dCBP), a known histone acetyltransferase and transcriptional coactivator, we identified the Drosophila homolog of a yeast chromatin regulator, Sir2. In yeast, Sir2 silences genes via an intrinsic NAD^+-dependent histone deacetylase activity. In addition, Sir2 promotes longevity in yeast and in *Caenorhabditis elegans*. In this report, we characterize the Drosophila *Sir2* (*dSir2*) gene and its product and describe the generation of *dSir2* amorphic alleles. We found that *dSir2* expression is developmentally regulated and that dSir2 has an intrinsic NAD^+-dependent histone deacetylase activity. The *dSir2* mutants are viable, fertile, and recessive suppressors of position-effect variegation (PEV), indicating that, as in yeast, dSir2 is not an essential function for viability and is a regulator of heterochromatin formation and/or function. However, mutations in *dSir2* do not shorten life span as predicted from studies in yeast and worms.

APPROXIMATELY two meters of DNA are tightly packed into every nucleated cell. The nature of this packed DNA, called chromatin, and the events that control unpacking for the purposes of DNA synthesis, repair, and transcription, are not well understood. In 1937, Alfred Lewy hypothesized that regulation of chromatin structure might directly affect gene expression and speculated that acetylation of lysine residues in histone tails could alter the interaction of the histone octomer with DNA (Pennisi 1997). In this model, neutrally charged acetyl groups are predicted to have lower affinity for the negatively charged DNA, causing relaxation of the chromatin and making it more accessible to transcription factor binding. More recent evidence has directly linked histone acetyltransferases such as p300/CREB-binding protein (CBP) and p300/CBP-associated factor with gene activation (Pennisi 1997). In contrast, histone deacetylation has been correlated with regions of the chromatin that are transcriptionally inactive (Laurenson and Rine 1992). While this model has the appeal of simplicity, it does not adequately describe the acetylation states of all types of chromatin. For example, a number of studies have shown that while transcriptionally silenced heterochromatin is relatively hypoacetylated, acetylation of some of the lysine residues within heterochromatin is required for proper silencing (Braunstein *et al.* 1996). Thus, it seems likely that both histone deacetylase and acetylase activities are required for proper heterochromatin formation.

In a yeast two-hybrid screen for Drosophila CREB-binding protein (dCBP) interacting proteins (Akimaru *et al.* 1997), we identified a Drosophila homolog of yeast Sir2. In yeast, a complex including Sir2, Sir1, Sir3, and Sir4 maintains silencing at the silent mating-type loci HML and HMR. The silencing is specific and is mediated by a heterochromatin-like chromatin structure; the DNA is condensed and resistant to nucleases, and the histones are hypoacetylated when compared to DNA that is packaged in euchromatin (Braunstein *et al.* 1993). Sir2 represses recombination within the reiterated yeast rDNA through the promotion of a condensed chromatin conformation (Gottlieb and Esposito 1989), and nonhomologous end joining, a critical step in DNA repair, requires Sir2 function (Tsukamoto *et al.* 1997). A breakthrough in our understanding of Sir2 function came with the discovery that Sir2 is an NAD^+-dependent histone deacetylase (Tanny *et al.* 1999; Imai *et al.* 2000). This finding directly links heterochromatin structure with deacetylation.

Interestingly, Kaeberlein *et al.* (1999) have also shown that mutations in Sir2 cause premature cell senescence, thus linking Sir2 with a form of aging. Sir2 mutant cells reach senescence early, and it is thought that this is due, in part, to an excessive buildup of extrachromosomal circles (ERCs) that are a product of an increased rate of recombination within the rDNA (Sinclair and Guarente 1997). On the basis of these data,

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Guarente proposed that Sir2 may act as a direct link between metabolism and aging; with a decrease in caloric intake, less NAD^+ is consumed in glycolysis, making more NAD^+ available for Sir2 to function in its silencing role and to repress ERC formation (Guarente 2000). Conversely, in the presence of excess calories, less NAD^+ is available for Sir2, causing a decreased number of cell divisions. According to this model, yeast Sir2 regulates chromosome structure and longevity by promoting regions of silenced DNA via the NAD^+-dependent histone deacetylase activity.

While the conservation of structure and enzymatic function within the Sir2 family suggests that the role of Sir2 in silencing and the regulation of life span is conserved, other lines of evidence suggest that this may not be the case. For example, while Sir2 is well conserved across phylogenetic lines, many of its interaction partners (*e.g.*, Sir3 and -4, RAP-1) are not (Brachmann *et al.* 1995). Furthermore, Sir2 homologs are present in bacteria and bacteria do not have histones, indicating that Sir2 has additional substrates (Tanner *et al.* 2000). Finally, several of the mammalian Sir2 homologs are cytoplasmic, not nuclear (Yang 2000). These findings suggest that the Sir2 enzyme may have a broader role in other organisms. In terms of life span, it is difficult to predict whether Sir2 action would be conserved in postmitotic organisms. In yeast, DNA damage secondary to Sir2 mutations accumulates during cell division. In postmitotic organisms such as Drosophila and *Caenorhabditis elegans*, DNA damage would not accumulate by the same mechanism. However, a recent article has demonstrated that the overexpression of Sir2 in *C. elegans* causes a dramatic increase in life span (Tissenbaum and Guarente 2001).

The interplay of environment and genes on life span is also well studied in Drosophila. Experiments dating back to the 1920s have used *Drosophila melanogaster* to study both the phenotypes of aging (*i.e.*, decreased fecundity and activity level) and the factors that affect life span, including genotype, temperature, starvation, larval density, population density, and parental age (reviewed in Lamb 1978). These studies show that life span is quite sensitive to environmental conditions. Thus, a study of Sir2 function in flies raised under conditions that are known to affect life span will increase our understanding of the role of Sir2 in the maintenance of longevity.

Chromosome structure is also well characterized in Drosophila. Like silenced chromatin from yeast, the centric heterochromatin in flies is condensed, resistant to nucleases, and relatively hypoacetylated (Turner *et al.* 1992). Studies of genes translocated to heterochromatin have provided insight into the factors that control chromatin states. A striking example of this is illustrated by the *white mottled 4* (w^{m4}) mutant in which an inversion on the X chromosome places the *white* gene near the edge of the centromeric heterochromatin and results in an eye phenotype that is white with red patches (Mueller 1930). The clonal nature of this phenotype indicates that, in some cells, the *white* gene is packaged in heterochromatin and silenced while in other cells it is left in a euchromatic environment and is active. This location-specific, variegated expression is called position-effect variegation (PEV). Mutations in genes that either enhance or suppress the w^{m4} phenotype are predicted to control chromosome structure. For example, mutations in factors that maintain an open chromatin state, such as *GAGA* (Farkas *et al.* 1994) and *zeste* (Judd 1995), enhance the w^{m4} phenotype. On the other hand, mutations in the heterochromatin protein, HP1 (Eissenberg *et al.* 1992), and in transcriptional repressors such as *polycomb* (Fauvarque and Dura 1993) suppress the w^{m4} phenotype.

To study the functional significance of a deacetylase (dSir2) and acetyltransferase (dCBP) interaction, we generated and characterized two mutations in the *dSir2* gene. A recent report describes mutations in the Drosophila *Sir2* gene as lethal and dominant suppressors of PEV (Rosenberg and Parkhurst 2002). In this report, we present a molecular and functional characterization of the Drosophila *Sir2* gene, *dSir2*, and demonstrate that, as in yeast, Sir2 is not an essential function. Furthermore, we show that mutations in *dSir2* are recessive modifiers of PEV and thus of the function and/or formation of heterochromatin. In contrast to yeast and worms, we provide evidence that mutations in *dSir2* do not affect life span.

MATERIALS AND METHODS

Fly strains and culture conditions: The *TMSΔ 2-3, CyO, TM2, CyO, P{w+ mC=GAL4-Kr.C}DC3, P{w+ mC=UAS-GFP.S65T}DC7* (referred to as *CyO, Kr-GFP*) and *SM6b, P{ry+ t7.2=eve-lacZ8.0}SB1* (referred to as *SM6b, eve-LacZ*) balancer chromosomes and the w^{m4}, *P{ry+ t7.2=PZ}l(2)05327* cn^1 and *P{w, LacZ}l(2)K14513* (Torok *et al.* 1993) chromosomes used in these studies are from the Bloomington Stock Center and the Berkeley Drosophila Genome Project. They are described in FlyBase (1999) or by Lindsley and Zimm (1992). Flies were cultured on standard Drosophila cornmeal-yeast source media in 8-oz plastic bottles or 28 × 95-mm plastic shell vials. All crosses were reared at 25°.

Cloning and mutagenesis of the *dSir2* gene: Two independent partial *dSir2* cDNA clones were identified in a two-hybrid screen using CREB-binding domain [CBD; amino acid (aa) 825–1043] of dCBP as bait to screen a library made from 0- to 6-hr Drosophila embryos (Akimaru *et al.* 1997). We used one of the partial *dSir2* cDNA clones (bp 1621–3839) as a probe to isolate the full-length cDNA using standard library screening methods at high stringency (Sambrook *et al.* 1989). Six hybridizing phage were isolated from a Drosophila λgt11 head library (provided by P. Salvaterra, City of Hope, Duarte, CA). λphage DNA was purified using a glycerol cushion (Sambrook *et al.* 1989) and digested with *Eco*RI; cDNA fragments were purified and sublconed into Bluescript (KS) (Stratagene, La Jolla, CA). cDNA fragments were sequenced and ligated together on the basis of homology to the known amino acid sequence of yeast Sir2 to create a contiguous cDNA clone. All

sequencing was performed manually (USB 101 Sequenase Kit) or with automated sequencing (ABI Prism 310 Genetic Analyzer) using standard methods. The cDNA sequence was later confirmed using sequence from the genomic clone (see below). Five independent genomic clones were isolated from an EMBL3 Drosophila library (provided by John Tamkun, University of California, Santa Cruz, CA) using a 5′ fragment of the cDNA (bp 270–1508) to make random-primed [α-^{32}P]dCTP-labeled probe. Hybridizing phage were purified, subcloned into Bluescript (using *Sal*I and *Eco*RI), and sequenced as above.

To define the 5′ end of *dSir2*, primer extension assays were performed using an oligonucleotide 188 bp from the 5′ end of the *dSir2* cDNA (bp 301–331, GAGGCGAG AGCGCAAAGC GGAGAGACCGAGG). The oligonucleotide was labeled by incubation with nucleotide kinase and [γ-^{32}P]ATP and hybridized overnight to 25 μg of total RNA isolated from Drosophila embryos. The RNA/primer mix was ethanol precipitated, resuspended, mixed with Superscript enzyme (BRL), and incubated at 42° for 90 min. The reaction was terminated with EDTA; RNase was added, followed by phenol/chloroform extraction and ethanol precipitation. The primer-extended product was resuspended in TE and electrophoresed on a polyacrylamide gel next to a sequencing reaction that was primed with the same oligonucleotide.

dSir2 was mapped to position 34A of chromosome 2L. On the basis of this location, strains with *P* elements inserted near the *dSir2* gene were obtained from the Bloomington Stock Center and the Berkeley Drosophila Genome Project. Using genomic Southern blotting (Sambrook *et al.* 1989) and plasmid rescue (Sullivan *et al.* 2000), we identified two strains, the *l(2)05327, cn* ry+ insertion line and the *l(2)K14513, w*+ insertion line with *P* elements that insert near *dSir2*. To generate partial deletions of *dSir2*, *l(2)05327, cn/SM6b, eve-LacZ; ry^{506} e^{s}* flies were crossed to *Sp/CyO; TMSΔ2-3/TM2* flies and the *l(2)05327, cn/CyO; ry^{506} e^{s}/TMSΔ2-3* females were mated to *ry^{506} e^{s}* males. All of the non-*CyO*, *ry^{506} e^{s}* males that were rosy (indicating loss of the ry+ insertion) were mated to *SM6b, eve-LacZ/Sco; ry^{506} e^{s}* females. The deleted *l(2)05327,cn* chromosome was detected as *l(2)05327, cn/SM6b, eve-LacZ; ry^{506} e^{s}* flies having cinnabar eye color and were saved in the balanced stocks with the *SM6b, eve-LacZ* and *CyO, Kr-GFP* balancers. To generate the second *dSir2* mutation, *w^{1}; l(2)K14513, w+/CyO* flies were mated to *w^{1}; Sp/CyO; TMSΔ2-3/TM2* males and the *l(2)K14523, w+/CyO*; *TMSΔ2-3/+* male offspring were mated to *w^{1}; Sp/CyO* virgin females. All of the *CyO* males that were white eyed (indicating loss of the *w*+ insertion) were crossed to *w^{1}; Sco/CyO* virgin females. The *l(2)K14513, w^{-}* deletion chromosomes were kept as balanced stocks with the *SM6b, eve-LacZ* and *CyO, Kr-GFP* balancers.

Forward primer BN058 (TTCAATCCATATCGGTCGTAG ATG, within *DnaJ-H* coding) and reverse primer BN035 (ATC GCGCATATATGCCATTG) were used to amplify genomic DNA (isolated using the Genomic DNA kit from Gentra Systems, Research Triangle Park, NC). Deletions in *dSir2* that left *DnaJ-H* intact were detected as a PCR amplification product <2.3 kb. To define the deletions exactly, the positive samples and same primers were used to generate PCR products for sequencing (ABI Prism 310 Genetic Analyzer). Large deletions that included these primers would not be detected. In addition, mutant chromosomes were analyzed with *P*-element primers to determine the presence of residual *P*-element sequences. Approximately 100 *l(2)K14513* deletion chromosomes and 40 *l(2)05327, cn* deletion chromosomes were analyzed by PCR. The two deletions, *l(2)05237$^{5.26}$* (*dSir2$^{5.26}$*) and *l(2)K14513$^{4.5}$* (*dSir2$^{4.5}$*), used in this study were chosen because they unambiguously delete *dSir2* and leave *DnaJ-H* intact.

Northern blot and *in situ* hybridization: RNA was prepared from Drosophila embryos using TRI REAGENT (Molecular Research Center, Cincinnati). For the developmental Northern analysis, mRNA was prepared using the small-scale PolyA-tract mRNA isolation system (Promega, Madison, WI). The mRNA was electrophoresed on formaldehyde agarose gels and transferred to Hybond membranes. To make riboprobe, the C-terminal *Eco*RV-*Xho*I (1621–3839) fragment of *dSir2* was subcloned into Bluescript and *in vitro* transcribed using the Promega riboprobe transcription kit. For the Northern blot of the *dSir2* mutant, total RNA was used and the riboprobe was made from an *Eco*RI fragment of dSir2 that was subcloned into Bluescript. The *Eco*RI fragment includes bp 270–1508 and encodes the N-terminal nonconserved portion of *dSir2* plus most of the conserved catalytic core domain; the catalytic core domain is encoded by sequences that include bp 1098-1875. The Northern filters were hybridized with the riboprobe under high-stringency conditions. For *in situ* hybridizations, the C-terminal (1621–3839) clone was used to synthesize a DIG-labeled riboprobe. The first 600 bp of the *dDnaJ-H* open reading frame (ORF) was amplified by PCR using the BN058 forward primer and the more 3′ BN053 reverse primer (CTGTATACTTGACGTATAATAG) and subcloned into Bluescript. The sequence was confirmed and the DIG-labeled riboprobe was synthesized in the same fashion as the *dSir2* riboprobes. RNA *in situ* hybridizations were performed using standard techniques (Sullivan *et al.* 2000).

Production of anti-dSir2 antibody: The *Pvu*II-*Xho*I fragment of *dSir2* was subcloned into pGEX-4T3 (Pharmacia) and used to produce a glutathione S-transferase (GST)-carboxy terminal portion of dSir2 (aa 698–821). The portion of dSir2 used to make antibody is C-terminal to the catalytic core domain. We purposefully excluded the catalytic core domain in our antigen to avoid cross-reactivity with other Sir2 homologs in Drosophila. GST-CTdSir2 was purified from SDS-PAGE gels using the Promega Chromophor system. Antibody to the dSir2 fusion protein was generated by the Pocono Rabbit Farm in Sprague-Dawley rats according to their protocols. Preimmune/immune rat serum was preabsorbed with Drosophila embryos and used at a final dilution of 1:2000 for Western blotting and immunohistochemistry.

Western analyses and whole-mount *in situ* hybridization: Whole flies (embryos, larvae, and adults) were ground with a Teflon pestle in 20 μl/fly of hot 2× SDS-PAGE sample buffer (Sambrook *et al.* 1989). The homogenate was centrifuged for 10 min at maximum speed and 10 μl of supernatant was separated on an 8% polyacrylamide gel. The gel was transferred to Immobilon-P membrane (Millipore, Bedford, MA) and probed with anti-dSir2 antibody using standard procedures (LeGendre 1990). Secondary anti-rat HRP-conjugated antibodies were obtained from Sigma (St. Louis). Immunohistochemistry of embryos and polytene chromosomes was performed using standard methods (Sullivan *et al.* 2000). For the embryos, the dSir2 antibody was used at a 1:2000 dilution while for the polytene analysis it was used at a 1:200 dilution. The anti-dCBP antibody (Bantignies *et al.* 2000) was used at a concentration of 1:1000. All of the biotinylated secondary antibodies were obtained from Vector Scientific. For the polytene analysis, donkey anti-rat rhodamine red X-labeled secondary antibody (Jackson) was used at a 1:100 dilution and the anti-chicken fluorescein isothiocyanate-labeled secondary antibody (Vector) was used at a 1:100 dilution. The polytene images were collected from a Zeiss fluorescent microscope using OpenLab software. Mutant embryos were detected as those not staining for β-galactosidase activity from a cross of *dSir2$^{5.26}$/SM6, eve-LacZ* to *dSir2$^{4.5}$/SM6, eve-LacZ* or were collected from crosses of *trans*-heterozygotes. Mutant larvae were detected as those not expressing GFP from crosses of *dSir2$^{5.26}$/CyO, Kr-GFP* to *dSir2$^{4.5}$/CyO, Kr-GFP*.

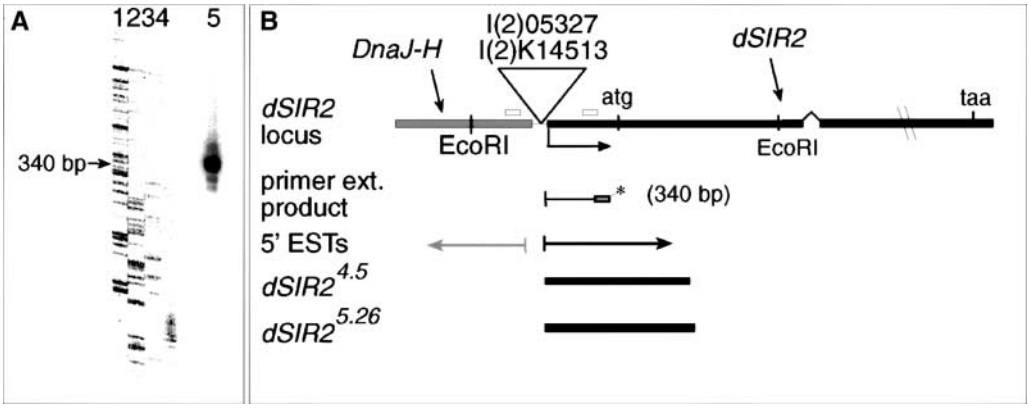

FIGURE 1.—*dSir2* locus. (A) Primer extension assays define the transcription start site of *dSir2*. Lanes 1–4 correspond to the sequencing reaction. Lane 5 contains the primer extension product. The site identified by the primer extension is the same as the transcription start site identified in the 5′ EST sequence from GenBank. (B) Genomic organization of the *dSir2* locus. The solid line in the top row represents the dSir2 transcription unit. The shaded line represents the transcription unit of *DnaJ-H*. *P* elements *l(2)05327* and *l(2)K14523* insert at nucleotide −13. The arrow represents the *dSir2* transcription start site. The open bars designate the oligonucleotides used in the PCR analysis of the deletion mutations. In the second row, the bar with the asterisk represents the ^{32}P-labeled primer extension product generated in the primer extension assay. The length of the primer extended sequence corresponds to the 5′ EST sequences found in GenBank (accession no. LD07439). Two 5′ EST fragments (the shaded bar is *DnaJ-H* and the solid bar is *dSir2*) are represented in the third row with arrowheads. The solid $dSir2^{4.5}$ and $dSir2^{5.26}$ boxes show the extent of the *dSir2* deletion in the two mutant chromosomes.

Deacetylase assays: Recombinant dSir2 was produced in *Escherichia coli* BL21 (DE3) as a C-terminally (his)6-tagged protein. The bacterial expression vector was constructed by inserting the full-length cDNA into pET23d (Novagen) as a PCR product using the forward primer CCAAGCTTCCAT GGTGAAATCAAAACAAAAACATTGGCTG and the reverse primer GGTCTAGAGTCGACCACTGCTGCTAACTGTCCTG GAGGC. Tagged protein was purified from bacterially cleared lysates by sequential Ni-chelate chromatography (QIAGEN, Chatsworth, CA) and anion exchange chromatography (Hi-Trap Q-sepharose, Pharmacia). Protein was dialyzed into 25 mM Tris, pH 8.0, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1 mM dithiothreitol. Protein concentrations were estimated by a Bio-Rad (Richmond, CA) protein assay using BSA as a standard. Nuclear extract from HeLa cells was prepared as previously described (DIGNAM *et al.* 1983). Histone deacetylase assays were performed using a histone deacetylase assay kit (UBI catalog no. 17-281). In this assay a peptide corresponding to the N-terminal sequence of histone H4 was chemically acetylated with 3H-acetate. Tritiated peptide (20,000 dpm; ~500,000 dpm/mg) was incubated in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol with 0.2 mM phenylmethylsulfonyl fluoride and the indicated amount of protein, with or without 500 mM NAD^+ and 50 mM sodium butyrate as indicated, for 3 hr at 37°. Reactions were terminated by the addition of 1 N HCl/0.1 N acetic acid and the released counts were determined by extraction with ethyl acetate and liquid scintillation counting.

Determination of *dSir2* survival curves: Parents for all experimental animals were 3–7 days old from uncrowded cultures. Control and experimental animals were male sibs from an outcross ($dSir2^{4.5}$/*Cyo* flies crossed to $dSir2^{5.26}$/*Cyo* flies) and thus internally controlled for humidity, parental age, and crowding. Under nonstress conditions, flies were given new food every 2 days and individual vials were maintained at 20–30 males per vial until populations fell below 20. These conditions are considered to be nonstress (see LAMB 1978). Under stress conditions the flies were given new food every 4 days and population densities were not optimally maintained. Maintaining populations of flies that are higher or lower than 20–30 in shell vials is considered a stress (see LAMB 1978). Standard *t*-tests were used to determine the differences of the mean and median life spans.

Determination of the *dSir2* effects on PEV: We used a standard protocol to assess the effect of the *dSir2* mutations on PEV (SASS and HENIKOFF 1998). Briefly, 8 days posteclosion, the female progeny from a cross of w^{m4}; $dSir2^{4.5}$/*CyO* females to w^{m4}; $dSir2^{5.26}$/*Sco* males and the reciprocal cross, or from w^{m4}; $dSir2^{5.26}$/*Sco* and w^{m4}; $dSir2^{4.5}$/*Sco* males mated to w^{m4} females, were sorted into five categories on the basis of eye color, with category 1 representing almost completely white eyes and category 5 representing completely red eyes. The intermediate categories represent animals with progressively more pigment. Once scored for eye color, the *dSir2* animals were scored for the presence or absence of *Sco*. Animals carrying the *CyO* balancer were discarded as *CyO* affects PEV. At least 200 flies were scored in each blind experiment and each experiment was done twice. This protocol is at least as accurate as pigment determination protocols, which can exhibit variability due to differences in eye and animal size (SASS and HENIKOFF 1998). The nonparametric Kolmogorov-Smirnov two-sample test was used to determine the differences between the distributions. The χ^2 value was determined for a degree of freedom (d.f.) of 2.

RESULTS

Cloning of dSir2: We isolated and sequenced *dSir2* genomic and cDNA clones. To better define the transcription start site, we performed primer extension assays and produced a 335-bp product that is 144 bp longer than our longest cDNA (Figure 1A). The longest *dSir2* 5′ expressed sequence tag (EST) fragment in the Berkeley Drosophila Genome Project (GenBank accession no. AA941691) is also 144 bp longer than the cDNA, suggesting that the *dSir2* transcription begins at this site. Using these data to define the putative transcription start site, we calculated that the complete cDNA clone is 3.839 kb with an open reading frame of 2.463 kb that begins with an ATG (+440) and ends with a TAA (+2903) followed by a potential polyadenylation signal. The predicted protein is 821 aa with a molecular weight

of 92 kD and an acidic pI of 4.5. Alignment of the genomic and cDNA clones identified a 200-bp intron beginning at nucleotide +1644. Sequencing of the 5′ end of the genomic clone revealed an ORF in the direction opposite to that of the *dSir2* gene. The predicted size of the ORF is 389 amino acids (FlyBase 1999) and the sequence is very similar to the bacterial DnaJ chaperonin protein. A Berkeley Drosophila Genome Project 5′ EST (accession no. AA941379) predicts a transcription start site for the *dDnaJ-homolog* (*dDnaJ-H*) at −583 relative to the *dSir2* transcription start site.

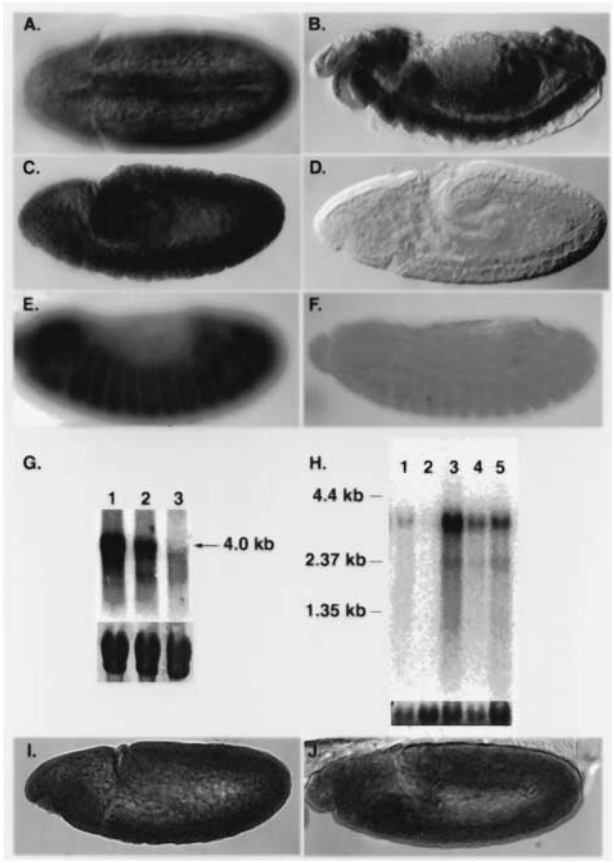

Figure 2.—Expression of *dSir2* mRNA during Drosophila development. (A–F) Expression of *dSir2* transcript in whole-mount embryos. (A–C) The expression pattern of *dSir2* during early development. (D) An embryo from the sense RNA control experiment. (E and F) From a separate experiment comparing the expression of *dSir2* transcripts in Canton-S (E) with $dSir2^{5.26}/dSir2^{4.5}$ embryos (F). The dSir2 transcript is absent from mutant embryos. (G and H) Northern analysis of dSir2 mutants. (G) A Northern analysis of total RNA probed with a radiolabeled antisense *dSir2* probe. (Top) Lane 1 contains RNA from a Canton-S (wild-type) embryo, lane 2 contains mRNA from $dSir2^{4.5}/CyO$ and $dSir2^{5.26}/CyO$ embryos, and lane 3 contains RNA from $dSir2^{5.26}/dSir2^{4.5}$ embryos. (Bottom) Represents the same filter stained with methylene blue to show the relative amounts of RNA in each lane. In comparison to the RNA from Canton S embryos (lane 1), the amount of the 4.0-kb *dSir2* transcript is decreased in heterozygous mutant embryos (lane 2) and is absent in homozygous mutant embryos (lane 3). (H, top) mRNA from embryos collected at different times during the first 24 hr of development. The blot was probed with radiolabeled antisense *dSir2*: lane 1, 0–2 hr; lane 2, 2–4 hr; lane 3, 4–8 hr; lane 4, 8–12 hr; lane 5, 12–24 hr. (H, bottom) The same filter, stained with methylene blue, to show relative amounts of RNA in each lane. An RNA marker (GIBCO/BRL, Gaithersburg, MD) was run alongside the mRNA and the sizes and position of the marker bands are indicated on the left. (I and J) Embryos stained with an antisense *DnaJ-H* probe. (I) A Canton-S embryo. (J) A $dSir2^{5.26}/dSir2^{4.5}$ embryo. These data show that the *DnaJ-H* transcript is still present, despite deletion of the neighboring *dSir2* gene.

Generation of dSir2 mutants: We mapped *dSir2* to the left arm of chromosome 2 at position 34A5-6 by *in situ* polytene chromosome hybridization (not shown). Using genomic Southern analysis and plasmid rescue, we identified two *P*-element lines, *l(2)05327* (ry^+) and *l(2)K14513* (w^+), which have *P* elements inserted at −13 relative to the putative *dSir2* transcription start site (Figure 1A). We found that the second-site lethals carried by these chromosomes are not due to the insertions in the *dSir2* gene. The *l(2)05327/l(2)K14513 trans*-heterozygotes are viable and fertile as are the *trans*-heterozygous *dSir2* mutants, which lack any detectable dSir2 product (described below). We generated partial deletions of *dSir2* by imprecise excisions of the mobilized *P* elements and screened genomic DNA from the deletion strains by PCR and Southern analysis. We identified one mutant from each screen: $l(2)05237^{5.26}$ ($dSir2^{5.26}$) and $l(2)K14513^{4.5}$ ($dSir2^{4.5}$), which deleted the *dSir2* coding sequence and left the *dDnaJ-H* coding sequence intact (Figure 1B). The $dSir2^{4.5}$ deletion includes nucleotides −16 to +759 and the $dSir2^{5.26}$ deletion includes nucleotides −16 to +859. The $dSir2^{5.26}/dSir2^{4.5}$ *trans*-heterozygous mutants are viable, fertile, and phenotypically normal. We used the $dSir2^{5.26}/dSir2^{4.5}$ *trans*-heterozygotes in all of the *dSir2* analyses because the parental chromosomes carry second-site lethal mutations.

Expression of *dSir2* mRNA during Drosophila development: To determine the size, level, and expression pattern of *dSir2* transcript, we analyzed the *dSir2* RNAs in Northern analyses and *in situ* hybridization of wild-type and *dSir2* mutant embryos (Figure 2). Because many of the important developmental events in Drosophila occur within the first 24 hr of embryogenesis, we collected embryos from different stages within this period. In Northern analyses, the antisense riboprobe hybridized to an ~4-kb transcript in embryos throughout embryogenesis (Figure 2H). The *dSir2* transcript was first detected in 0–2 hr embryos, which indicates that these transcripts are maternally derived. Expression of *dSir2* during cellular blastoderm and gastrulation decreases (2–4 hr) and then increases dramatically during germ-band elongation and morphogenesis (4–8 hr). The $dSir2^{5.26}/dSir2^{4.5}$ mutant embryo RNA does not contain *dSir2* transcript in Northern blots (Figure 2G) or whole-mount embryos (Figure 2, D and F). The *dSir2* deletions remove the 5′ end of the *dSir2* coding sequence and end ~300 bp before the core domain of dSir2 begins. Thus, it is conceivable that in the mutants a truncated product that contains the core domain is

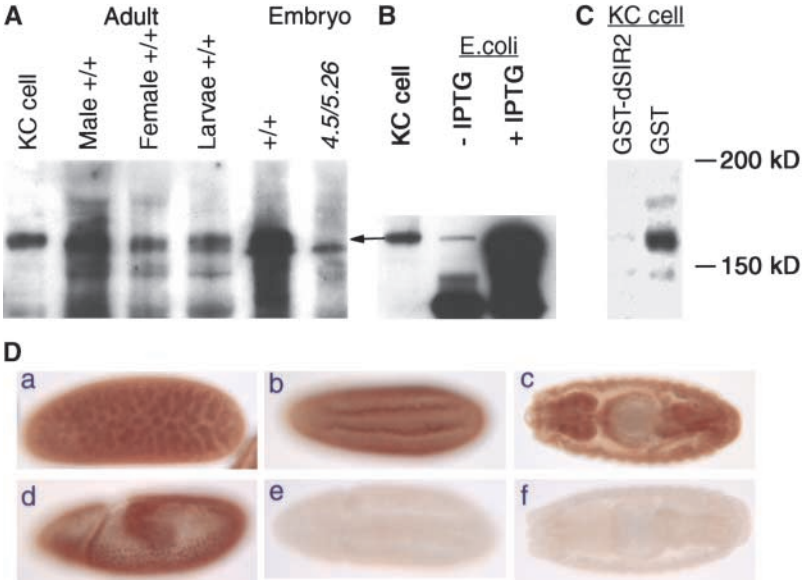

FIGURE 3.—Expression of dSir2. (A) dSir2 antibody was used to probe Western blots of whole-cell extracts. Kc cell lanes: protein from 1/5 of a 10-cm plate of cells lysed in RIPA buffer. Fly extracts from equal amounts of homogenized tissue were loaded into each lane as indicated. A 125-kD band is present in Kc cells, wild-type adults, larvae, and embryo extracts. A slightly smaller, background band is present in both wild-type and mutant embryo extracts. It is not a consistent band. The upper 125 kD is absent in the $dSir2^{4.5}/dSir2^{5.26}$ embryos. (B) Western blot analysis of bacterially produced dSir2. Lane 1 is a positive control (Kc cell extract), lane 2 is extract from bacteria grown without isopropyl thiogalactoside, and lane 3 is the extract from induced bacterial cultures. (C) The dSir2 antibody is specific: we mixed the dSir2 antibody with an excess of either the GST-dSir2 fusion protein or GST alone. The GST-dSir2 protein competes the antibody so that it no longer recognizes the 125-kD band in Western blots of Kc cells. (D) Wild-type (a–d) and $dSir2^{4.5}/dSir2^{5.26}$ (e and f) embryos stained with dSir2 antibody at a 1:2000 dilution (anterior is left). Wild-type embryos express dSir2 during syncitial and cellular blastoderm stages, at high levels in the nuclei surrounding the morphogenic furrows during gastrulation, and in the germ band. Later, the expression is primarily detected in the CNS. The $dSir2^{4.5}/dSir2^{5.26}$ embryos do not express dSir2 at any stage.

produced. To rule out this possibility, we hybridized the Northern blot with a probe that includes most of the core domain coding sequences (the core domain is from 1085 to 1885 and the C-terminal end of the probe ends at 1514) and determined that this probe could not detect an RNA species in the mutant animals. The $dSir2^{5.26}/dSir2^{4.5}$ mutant embryos probed with an antisense *dDnaJ* RNA have a normal distribution of the *dDnaJ* transcript compared to wild-type embryos, demonstrating that mutant phenotypes associated with $dSir2^{5.26}/dSir2^{4.5}$ mutants are specifically due to lesions in the *dSir2* gene and not to changes in the pattern of *dDnaJ* expression (Figure 2, I and J). The levels of *dDnaJ* expression in the *dSir2* mutants may be somewhat different from wild type; however, they are identical to the wild-type levels of *dDnaJ* expression as detected by *in situ* hybridization.

Characterization of the dSir2 protein: We generated a polyclonal antibody in rats against a C-terminal dSir2 (aa G522-V821)-GST fusion protein. The antibody specifically recognizes a 125-kD protein in extracts from Kc cells and from adult, larval, and embryonic tissues (Figure 3A). The dSir2 antibody is specific: when titrated with GST-dSir2 protein, the antibody no longer recognizes the 125-kD band in Western blots of Kc cells (Figure 3C). However, incubating the dSir2 antibody with GST alone does not affect the ability of the antibody to detect dSir2 (Figure 3C). The predicted molecular weight of dSir2 is 92 kD, which is 33 kD less than the band seen on immunoblots. This difference is most likely a reflection of the acidic pI rather than a post-transcriptional modification because bacterially expressed dSir2 is also 125 kD (Figure 3B). As expected, the 125-kD band is not detected in the $dSir2^{5.26}/dSir2^{4.5}$ embryo extract (Figure 3A).

dSir2 is detected throughout development and, unlike some of the mammalian Sir2 proteins, is primarily nuclear (Figure 3D, b and d). However, at certain times during development (cellular blastoderm), dSir2 is excluded from the nucleus; at other times, it is both cytoplasmic and nuclear (late in embryogenesis). dSir2 is present during syncitial and cellular blastoderm at high levels in the nuclei surrounding the morphogenic furrows during gastrulation (Figure 3D, b) and in the germ band (Figure 3D, d). Later, its expression is primarily detected in the central nervous system (CNS; Figure 3D, c).

In addition, dSir2 localizes both to heterochromatin and to discrete bands within euchromatic regions of polytene chromosomes (Figure 4). To show the specificity of the dSir2 antibody binding, we also stained dSir2 mutant chromosomes with the dSir2 antibody. As a positive control in these experiments, we also labeled the $dSir2^{5.26}/dSir2^{4.5}$ chromosomes with an anti-dCBP antibody. As shown in Figure 4, C and D, there is no detectable dSir2 staining of the *dSir2* mutant chromosome while the dCBP staining is quite robust.

These data suggest that dSir2 is regulated not only in a temporal- and tissue-specific fashion but also at the level of subcellular localization. The antibody was used to determine the expression of dSir2 in the $dSir2^{4.5}/dSir2^{5.26}$ mutant embryos (Figure 3D, e and f). No dSir2 was detected in these animals at any stage of development. Because the antibody was produced against the C-terminal portion of dSir2 and because the deletions remove most of the N-terminal portion of dSir2, we believe it is unlikely that any truncated products are

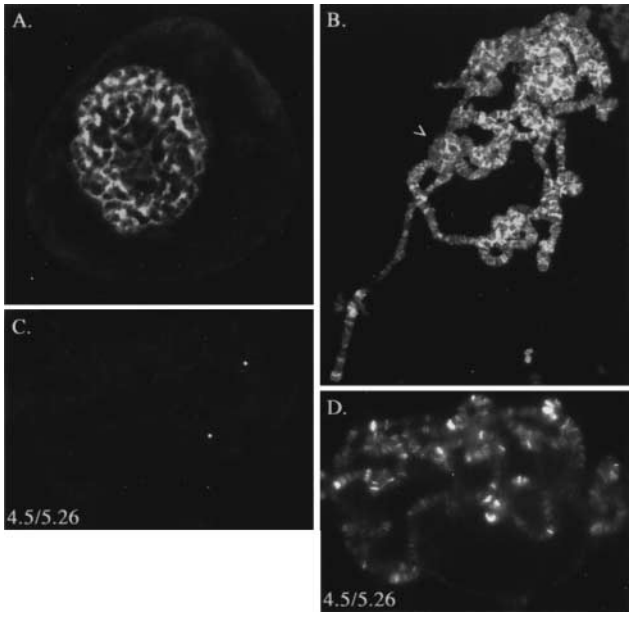

FIGURE 4.—dSir2 localizes to chromatin. (A and B) Salivary gland tissue from wild-type larvae stained with anti-dSir2 antibody. (A) Salivary gland cell shows specific nuclear staining. (B) dSir2 localizes to both heterochromatin (arrowhead) and discrete bands in euchromatic regions of polytene chromosomes. (C and D) A single salivary gland nucleus from the $dSir2^{5.26}/dSir2^{4.5}$ mutant is stained with both anti-dSir2 and anti-dCBP antibodies. (C) dSir2 staining is absent from the mutant chromosomes. (D) dCBP staining is normal on the same polytene chromosomes and serves as a positive control.

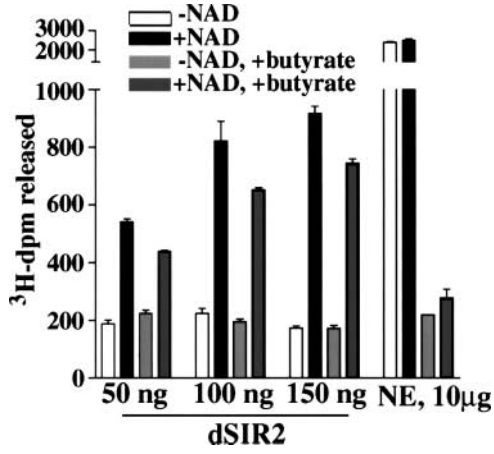

FIGURE 5.—dSir2 is an NAD-dependent deacetylase. Release of ethyl acetate extractable ^{3}H-product from ^{3}H-histone H4 peptide by recombinant dSir2 protein or HeLa-cell nuclear extract (NE) in the absence or presence of NAD^+ (500 μM) or sodium butyrate (50 mM). Background counts (in the absence of added protein) extracted were 200 dpm. $N = 3$; error bars indicate SEM.

produced. Results of the Northern analysis are also consistent with these findings.

Bacterially expressed dSir2 is a NAD^+-dependent histone deacetylase: On the basis of homology to other Sir2 homologs, we predicted that dSir2 would have intrinsic NAD^+-dependent deacetylase activity. Purified, bacterially expressed dSir2 released ^{3}H-dpm from acetylated histone H4 peptide in a NAD^+-dependent manner and the activity was not inhibited by the HDAC inhibitor sodium butyrate. This result is consistent with the observation that the histone deacetylase (HDAC) inhibitor tricostatin A does not inhibit ySir2 (IMAI *et al.* 2000; Figure 5). Furthermore, these data are consistent with a study showing that dSir2 overexpressed in S2 cells has an intrinsic NAD^+-dependent deacetylase activity that is not inhibited by tricostatin A or by sodium butyrate (BARLOW *et al.* 2001). In contrast, HDAC activity present in HeLa-cell nuclear cell extract is NAD^+ independent and completely butyrate sensitive.

***dSir2* modifies PEV:** On the basis that dSir2 and ySir2 are deacetylases, that ySir2 is required for heterochromatin formation, and that dSir2 is detected in heterochromatin, we determined whether mutations in *dSir2* would suppress PEV. We used the w^{m4} chromosome as the assay system for PEV. *dSir2* mutations are recessive suppressors of PEV, causing an increase of red patches in the eyes of w^{m4}; $dSir2^{5.26}/dSir2^{4.5}$ animals as compared to those of the w^{m4}; *dSir2/Sco* or w^{m4} controls (Figure 6). The data presented in Figure 6 represent two sets of experiments with four different backgrounds and illustrate the effect of background on the expression of w^{m4}. Nevertheless, the distribution of the *dSir2* heterozygotes shows that they do not have a dominant effect on PEV. For example, in the first set of crosses where w^{m4}; $dSir2^{4.5}/Sco$ and w^{m4}; $dSir2^{5.26}/Sco$ are compared with w^{m4}; $dSir2^{4.5}/dSir2^{5.26}$ and w^{m4}; $dSir2^{5.26}/dSir2^{4.5}$, the w^{m4}; *dSir2* heterozygotes have the same distribution ($\chi^2 = 1.05$, $0.5 < P < 0.7$) and the two sets of w^{m4}; *dSir2 trans*-heterozygotes have the same distribution ($\chi^2 = 0.387$, $0.8 < P < 0.9$). However, the two sets of w^{m4}; *dSir2* heterozygotes are significantly different from the two sets of w^{m4}; *dSir2 trans*-heterozygotes (χ^2's for pairwise comparisons = 13.02, 11.9, 23, and 18.27, $P < 0.01$). In the second set of crosses, the w^{m4}; *dSir2 trans*-heterozygotes are compared with w^{m4}; *Sco*/+. The eye color distributions are not significantly different (χ^2's for pairwise comparisons are 7.5, 1.29, 4.44, 5.66, 1.78, and 7.5, $P > 0.02$, 0.5, 0.1, 0.05, 0.3, and 0.02, respectively). The $dSir2^{5.26}/dSir2^{4.5}$ *trans*-heterozygotes do not affect the wild-type *w* phenotype and thus do not affect *w* expression in the w^{m4} chromosome. This result suggests that, in the absence of *dSir2*, heterochromatin does not form and/or function properly. By using the $dSir2^{5.26}/dSir2^{4.5}$ *trans*-heterozygotes we ensure that the mutant phenotypes are not due to recessive mutations on the $dSir2^{5.26}$ and $dSir2^{4.5}$ chromosomes.

***dSir2* and life span:** The overexpression of Sir2 in yeast and *C. elegans* increases life span. In yeast, mutations in Sir2 shorten life span (KAEBERLEIN *et al.* 1999). To determine whether loss of *dSir2* function could also decrease life span, we assessed the life span of flies with

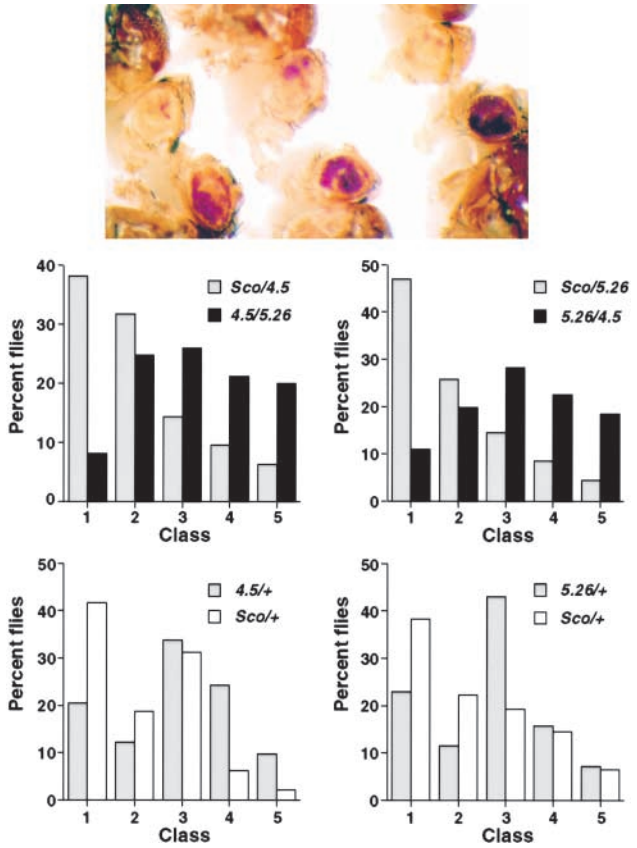

FIGURE 6.—*dSir2* mutations modify PEV. (Top) w^{m4} animals that are heterozygous ($dSir2^{5.26}/Sco$, top row) or homozygous ($dSir2^{4.5}/dSir2^{5.26}$, bottom row) for *dSir2* mutations. (Bottom) The effect of *dSir2* mutations on the distribution of eye color within populations. The graphs show that *dSir2* mutations are recessive suppressors of w^{m4} PEV.

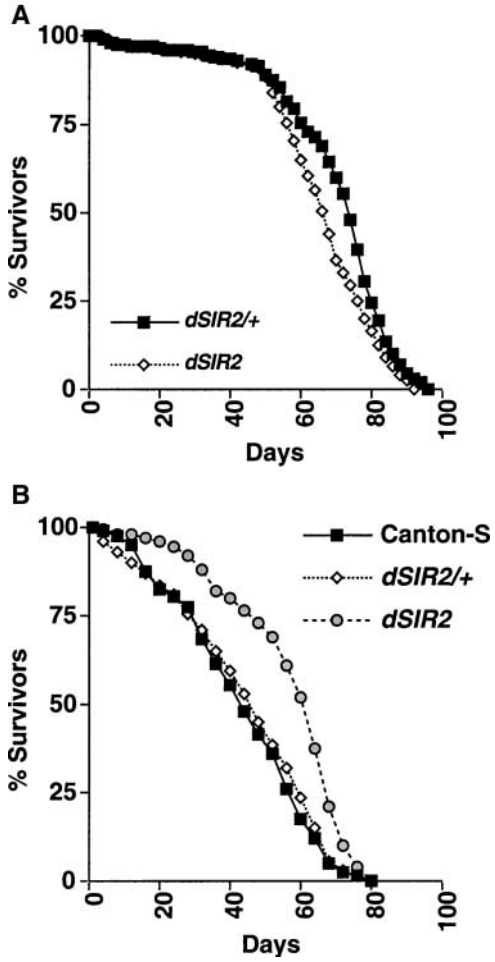

FIGURE 7.—Effect of *dSir2* mutations on life span. (A) Male flies maintained under standard environmental conditions. (B) Male flies maintained under stress conditions.

one, two, or no copies of *dSir2* under stress and nonstress environmental conditions (Figure 7). A total of 668 $dSir2^{-}/+$ and 638 $dSir2^{5.26}/dSir2^{4.5}$ male flies in eight simultaneous experiments were maintained under nonstress conditions. The average median life span of the $dSir2^{5.26}/dSir2^{4.5}$ males is 62.53 ± 4.98 days, slightly less that the average median life span of the *dSir2*- heterozygous males, which is 71.28 ± 5.01 days. This difference is significant ($P = 0.0035$). The average life span of the $dSir2^{5.26}/dSir2^{4.5}$ males is 85.75 ± 6.27 days while the average life span of the *dSir2* heterozygous controls is 91.25 ± 5.23 days. This difference is not significantly different ($P = 0.0776$).

A total of 555 Canton-S, 571 $dSir2^{-}/+$, and 551 $dSir2^{5.26}/dSir2^{4.5}$ flies in four simultaneous experiments were assessed under stress conditions. The results of the stress test show that the median life spans of the Canton-S (41.42 ± 5.8 days) and $dSir2^{-}/+$ (45.01 ± 2.0 days) controls are not significantly different ($P = 0.557$). Similarly, the average life spans of the Canton-S (76 ± 5.7 days) and $dSir2^{-}/+$ (79 ± 3.8 days) controls are not significantly different ($P = 0.414$). As under nonstress conditions, the mean life spans among the three genotypes are not significantly different (Canton-S *vs. dSir2*, $P = 0.2459$; *dSir2/+ vs. dSir2*, $P = 0.6131$). However, in this case, the median life span of the $dSir2^{5.26}/dSir2^{4.5}$ flies, 60.96 ± 2.03 days, is somewhat longer than the median life spans of the controls, and this difference is significant (*dSir2/+ vs. dSir2*, $P = 0.0206$; Canton-S *vs. dSir2*, $P = 0.0007$).

DISCUSSION

Characterization of *dSir2* and its product: We have cloned and characterized the *D. melanogaster* homologs of the yeast *Sir2* gene. On the basis of primer extension, 5′ EST clones, and Northern analyses, we predict that the transcription start site is 440 bp 5′ of the *dSir2* open reading frame. However, at the predicted transcription start site, consensus sequences (*i.e.*, TATA, CAAT, initiator, downstream promoter element) are not present. Thus, it is likely that the transcription start site for the *dSir2* gene is nonclassical. It is also significant that ~600

bp upstream of the putative *dSir2* initiation start site an open reading frame in the opposite direction encodes a homolog of the bacterial chaperonin molecule, DnaJ (Iliopoulos *et al.* 1997). We call this gene *dDnaJ-H* (*H* for homolog). It is not known if *dSir2* and *dDnaJ-H* share promoter elements; however, this close spatial relationship between the two genes makes mutational analysis more complicated. For example, a recent article identifies an EP line (2300), which is predicted to overexpress dSir2 and enhances the SCA1 overexpression phenotype of neural degeneration (Fernandez-Funez *et al.* 2000). It is also possible, however, that enhanced neural degeneration in the EP line is due to disruption of *dDnaJ-H*, especially given that a mutation in another DnaJ homolog is identified in Fernandez-Funez *et al.*'s screen as a suppressor of the phenotype. In our mutagenesis we screened for *dSir2* mutations that left the *dDnaJ-H* sequence intact and did not affect *dDnaJ-H* expression.

Expression of *dSir2* is developmentally regulated. *dSir2* mRNA is present at the early stages of development (0–2 hr) and downregulated between 2 and 4 hr in development. High levels of *dSir2* zygotic expression begin between 4 and 8 hr after fertilization at germ-band elongation. In the embryo, expression is ubiquitous and highest in the cephalic furrow, dorsal lateral folds, ventral furrow, germ band, epidermis, and CNS. dSir2 is most often nuclear, but during cellular blastoderm is excluded from the nucleus and during germ-band retraction is detected equally in the nucleus and cytoplasm. The significance of this level of regulation is not known. However, both the pattern of expression and the subcellular regulation is strikingly similar to that of dCBP protein (Ludlam *et al.* 2002) and is also consistent with the idea that dSir2 and dCBP form a complex that is active during development.

The predicted amino acid sequence of dSir2 is 823 aa in length, making it the longest known Sir2 homolog (Frye 1999). The core domain, which encodes the deacetylase activity, is the only region of dSir2 that is conserved with ySir2 (Sherman *et al.* 1999). We have demonstrated that bacterially expressed dSir2 has an intrinsic NAD^+-dependent deacetylase activity. Outside the core domain, there is no homology to any known proteins, making it difficult to speculate on the function of these domains. Within the Drosophila genome, five genes contain significant homology to the yeast Sir2. Of the five genes, *dSir2* is the most similar to yeast Sir2 (Frye 2000). This is an important fact when comparing mutant phenotypes across phylogenetic lines.

dSir2 mutant phenotype: To study the mutant phenotype of *dSir2* mutations, we generated mutations on two unrelated chromosomes that deleted a large portion of *dSir2* cDNA but left *dDnaJ-H* sequences intact. The $dSir2^{5.26}/dSir2^{4.5}$ *trans*-heterozygous animals do not express a *dSIR2* transcript or dSir2 at any time during development. The RNA probes and anti-dSir2 antibodies could have detected any truncated product but they did not. We also assessed *dDnaJ-H* expression in whole-mount embryos to ensure that we had not disrupted *dDnaJ-H* coding or regulatory sequences. We used flies that were heterozygous for the two mutant chromosomes to characterize the *dSir2* mutant phenotype so that the second-site lethals on the parental chromosomes would not affect the phenotype. We found that, as in yeast, loss of *dSir2* does not cause lethality. These data contradict a recent report stating that mutations in *dSir2* are recessive lethals (Rosenberg and Parkhurst 2002). Both studies used the *P{ry+t7.2=PZ}l(2)05327 cn*[1] chromosome, which we found to carry second-site lethal mutations. Our data show that *dSir2* mutations are viable and we suggest that the lethal phenotypes described are due, at least in part, to these additional mutations.

In yeast and *C. elegans*, Sir2 affects life span. In yeast, Sir2 mutants reach senescence more quickly than wild-type cells; in *C. elegans*, duplication of the Sir2 gene causes a dramatic extension of life span. Our data do not support these previous findings. When we assessed the effect of *dSir2* mutants on life span we saw no significant difference between $dSir2^{5.26}/dSir2^{4.5}$ *trans*-heterozygotes and $dSir2^{-}/+$ heterozygotes. To sensitize the system and detect a more subtle effect of dSir2 on life span, we determined whether *dSir2* mutations affect life span under more stressful conditions. Surprisingly, the $dSir2^{5.26}/dSir2^{4.5}$ flies had a slightly longer median life span than the median life span of the *dSir2* heterozygotes and Canton-S controls, although the average life spans among the genotypes were not significantly different. The significance of the slight increase in the median life span of the *dSir2* mutants is not clear. It may suggest that dSir2 is a negative regulator for stress-related metabolic processes or transcription that in turn may affect vitality. However, it is important to note that this effect does not increase the average life span of the *dSir2* mutant flies. Thus, these data argue that the effect of Sir2 on life span is not conserved in flies. While other *Sir2*-like genes in Drosophila might affect life span, the *Sir2* gene described here is most like the *Sir2* from yeast and worms that has been shown to affect life span. On the basis of our data, it is likely that dSir2 functions as a transcription and chromatin-remodeling factor that regulates the expression of many genes and thus would not have a specific effect on life span. However, it may be that, in flies, dSir2 acts with other Sir2 family members to elicit an effect on life span. In this case, its effects might be detected only in animals mutant for the other Sir2 proteins.

On the other hand, we found that *dSir2* mutations are recessive suppressors of PEV, consistent with the model that Sir2 is involved in heterochromatin regulation across phylogenetic lines. Furthermore, we found that *dCBP* mutations dominantly suppress PEV (S. Smolik, unpublished observation), suggesting that dSir2 and

dCBP may act together to control the pattern of heterochromatin histone acetylation. For example, dCBP may be inactivated by acetylation and dSir2 may be required to deacetylate dCBP for proper heterochromatin function and/or formation. Alternatively, because CBP is known to acetylate proteins other than histones (*e.g.*, p53; reviewed in Goodman and Smolik 2000), dCBP may facilitate heterochromatin formation through modification of other proteins in the complex. The fact that *dSir2* mutations are recessive suppressors of PEV while dCBP mutations are dominant modifiers of PEV suggests that dCBP's role in maintaining heterochromatin formation or function is more dosage sensitive. It is possible that dSir2 helps to stabilize heterochromatin but is not absolutely required for its formation. dSir2 antibodies can co-immunoprecipitate dCBP from Drosophila Kc cells (B. Newman, unpublished observation), demonstrating that dSir2 and dCBP interact *in vivo*. Dosage studies of dSir2 and dCBP on PEV will clarify the nature of the dSir2-dCBP interaction. It will also be important to determine whether the deacetylase activity of dSir2 and the acetyltransferase activity of dCBP are important for their functions in heterochromatin formation and/or activity.

In summary, we present the cloning and characterization of *dSir2*. We show that dSir2 is developmentally regulated and a NAD^+-dependent histone deacetylase. From genetic assays, we show that dSir2's role in silencing is conserved across phylogenetic lines and is not involved in the regulation of life span, as has been previously reported in other organisms.

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