Spreading of a Corepressor Linked to Action of Long-Range Repressor Hairy[▽]

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Transcriptional repressor proteins play key roles in the control of gene expression in development. For the *Drosophila* embryo, the following two functional classes of repressors have been described: short-range repressors such as Knirps that locally inhibit the activity of enhancers and long-range repressors such as Hairy that can dominantly inhibit distal elements. Several long-range repressors interact with Groucho, a conserved corepressor that is homologous to mammalian TLE proteins. Groucho interacts with histone deacetylases and histone proteins, suggesting that it may effect repression by means of chromatin modification; however, it is not known how long-range effects are mediated. Using embryo chromatin immunoprecipitation, we have analyzed a Hairy-repressible gene in the embryo during activation and repression. When inactivated, repressors, activators, and coactivators cooccupy the promoter, suggesting that repression is not accomplished by the displacement of activators or coactivators. Strikingly, the Groucho corepressor is found to be recruited to the transcribed region of the gene, contacting a region of several kilobases, concomitant with a loss of histone H3 and H4 acetylation. Groucho has been shown to form higher-order complexes *in vitro*; thus, our observations suggest that long-range effects may be mediated by a “spreading” mechanism, modifying chromatin over extensive regions to inhibit transcription.

Transcriptional repression plays central roles in developmental gene regulation, providing the temporal and spatial specificity required for complex expression patterns. In *Drosophila melanogaster*, the Hairy transcriptional repressor directs the patterning of segmental pair-rule stripes in the blastoderm embryo and in later stages directs neuronal differentiation (22, 41, 36, 51). Hairy and related transcription factors belong to a conserved metazoan family of Hairy Enhancer of Split (HES) proteins involved in cell fate decisions in neurogenesis, vascular development, mesoderm segmentation, and myogenesis (8, 12). Understanding the molecular basis by which these proteins exert their functions will shed light on the transcriptional regulatory mechanism of multiple developmental processes.

An important functional distinction between different repressors is their ability to interfere with proximally or distally located activators. In *Drosophila*, Hairy can inhibit the activities of activators located over 1 kbp away, leading to its characterization as a long-range repressor (3). In contrast, short-range repressors are limited to interfering with activators bound within ~100 bp (16). The limited range of short-range repressors appears to be well adapted to the architectures of the regulatory regions that they control. In the *Drosophila* embryo, short-range repressors, such as Knirps and Giant, repress the modular enhancers controlling pair-rule genes, such as *even-skipped* and *hairy*. The independent activity of

such enhancers is guaranteed by the local action of the repressors; if enhancers are brought into artificially close proximity, or if binding sites for short-range repressors are moved close to the transcriptional start site, unwanted cross-regulation can occur (45, 2).

In contrast to the insights gained about short-range regulation, the molecular logic of circuits controlled by long-range repressors is less obvious. At the *Drosophila achaete* gene, the long-range repressor protein Hairy binds at –300 bp, 50 bp 5' of a cluster of activator proteins, a position from which short-range repressors would also presumably work well (51). Similarly, the Hairy homolog HES1 binds to its own promoter at four sites 20 to 170 bp from the transcriptional start site (49). Dorsal protein-regulated ventral repression elements from *Drosophila zen*, *tld*, and *dpp* genes can similarly act over long distances, but at least in the case of the *zen* ventral repression element, the activators bind immediately 5' of the repression element (24). Thus, it is not clear if the long range of activity in these instances is essential to the normal regulatory function. Perhaps the strength of repression of Hairy is the most important feature, which is only incidentally associated with long-range effects.

Hairy/E(spl) proteins possess a conserved basic helix-loop-helix DNA binding domain and effector domains that include motifs important for interaction with corepressors (12). Hairy interacts physically and genetically with the following three corepressors: Groucho, the C-terminus-binding protein (CtBP), and the Sir2 histone deacetylase (37, 39, 40). The C terminus of Hairy contains a WRPW motif that directly contacts the Groucho corepressor, and the removal of the motif compromises the activity of Hairy. A motif adjacent to the Groucho-interacting region binds to the CtBP corepressor. Hairy protein has been shown to possess CtBP-mediated re-

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pression activity in certain circumstances; however, CtBP has also been suggested to play an antagonistic role in repression by Hairy, because the binding of Groucho and CtBP might be mutually exclusive and the removal of the CtBP-interacting motif has a less drastic effect on repression than the removal of the Groucho motif (58). The histone acetylase Sir2 interacts with Hairy through its DNA binding domain, and genetic interactions between *hairy* and Sir2 have been reported (40).

The whole-genome mapping of binding sites for Hairy and cofactors indicates that at many loci, Hairy is not associated with all three cofactors. In fact, Hairy was rarely found to colocalize with regions bound by Groucho, while colocalization with CtBP was observed in a majority of cases (5). These studies indicate that Hairy may associate with specific cofactors in a context-dependent manner, perhaps invoking different modes of transcriptional regulation. A limitation of these studies is that the physical resolution is limited so that it is not known whether Hairy and the corepressor proteins are in direct contact or if in some cases other transcription factors might recruit these cofactors. In addition, it is not known for most loci whether the observed binding event is functional. Thus, while genetic and physical interactions hint at potential complexity, the activity of Hairy and its set of possible corepressors is not understood at a molecular level. To better understand molecular mechanisms of long-range repression, we have employed a novel approach to measure the activity of the Hairy repressor on a highly defined system in the *Drosophila* embryo.

Using transgenic lines containing a transcriptional switch that can be repressed uniformly in the embryo, we have analyzed the recruitment of activators, coactivators, corepressors, and histone modifications associated with Hairy repression. The results show that repression does not require the displacement of the activators or coactivators; rather, it is associated with the binding and spreading of the Groucho corepressor and the histone deacetylase Rpd3 throughout the coding region of a *lacZ* reporter. In addition, Hairy repression is associated with a marked decrease in histone acetylation levels and an increase in total histone occupancy.

MATERIALS AND METHODS

Transcriptional switch system. A Hairy-repressible transcriptional switch, pC2L5U2L, was constructed by placing binding sites for a LexA-Hairy fusion and the yeast Gal4 activator (upstream activation sequence [UAS]) into the P-element transformation vector pC4PLZ (54), 55 bp from the basal *transposase-lacZ* reporter and 350 bp from the divergently transcribed mini *white* reporter. Based on the architecture of previous modules shown to be effectively repressed in the embryo (27), the 212-bp regulatory region contains two LexA binding sites inserted 5' and two LexA sites 3' of five high-affinity UAS sites derived from a modified *UAS-lacZ* plasmid (26). Oligonucleotides bearing two LexA binding sites (DA-721/722) were cloned 5' of five tandem UAS sites into NotI/HindIII sites and 3' into an SphI site (DA-641/642) in pBluescript SK+ (5' GCG GCC GC CTG TAT ATA TAT ACA GCA TCT AGA ACC TGT ATA TAT ATA CAG AAG CTT GCC TGC AGG T [CGG AGT ACT GTC CTC CGA G]₅ CGG AGA CTC TAT CAT GG CTG TAT ATA TAT ACA GCA GGT ACC TGC TGT ATA TAT ATA CAG CAT GC 3', where binding sites for LexA and Gal4 are bold and restriction sites are underlined), and this element was inserted as a NotI/SphI fragment into pC4PLZ. The construct was introduced into the third chromosomes of flies by P-element-mediated germ line transformation (46). An autoactivating stock was then generated by crossing and recombining the reporter line to a ubiquitous *daughterless* Gal4 driver (Bloomington, IN; database number 5460). Finally, a heat shock-inducible LexA-Hairy fusion (*hsp70 LexA-Hairy*) was recombined onto the same chromosome. This gene was created by joining a

BamHI/KpnI fragment containing a Kozak sequence, initiator ATG, and coding sequence for the entire LexA protein (amino acid residues 1 to 202) in frame to a KpnI/XbaI fragment containing the portion of Hairy C-terminal to the DNA binding domain (residues 93 to 337) and introducing it into the BglII and XbaI sites of pCaSper-hs (38), (5' GGA TCC ACC AAA ATG AAA ... TGG CTG GAA TTC CCG GGC CGG GGT ACC GCA GCC ... TGG TAG TCT AGA 3', where coding sequences for LexA and Hairy are bold and restriction sites are underlined). The resulting line, containing all three transgenes on the third chromosome carried over with a TM3 Sb balancer, behaves as a transcriptional switch in which the default state is "on" before heat shock and "off" after induction of the LexA-Hairy repressor. The *pKruppel-lexA-Hairy* gene in Fig. 1B was constructed by exchanging the Gal4 DNA binding domain for the LexA domain in the pKreg vector (34) via restriction digestion with BamHI/KpnI and ligation of a BamHI/KpnI LexA fragment PCR amplified from the pLexA vector (18) by using DA-645 (5' GCG GAT CCA CCA AAA TGA AAG CGT TAA CGG CCA GG 3') and DA-646 (5' CGG GGT ACC CCG GCC CGG GAA TTC CAG CCA GTC GC 3'). The reporter in Fig. 1B containing LexA binding sites 3' of the *rho* and *twi* enhancers was constructed by removing the Giant binding sites in the *gt-55* vector (20) by SphI digestion and the insertion of DA-641/2 (5' CTG TAT ATA TAT ACA GCA GGT ACC TGC TGT ATA TAT ATA CAG CCA TG 3') containing two LexA binding sites.

In situ hybridization and antibody staining of *Drosophila* embryos. Embryos were fixed for in situ hybridization and stained using a digoxigenin-UTP-labeled RNA probe antisense to *lacZ* (46). Antibody staining for LexA-Hairy expression was done using a mixture of three mouse monoclonal antibodies raised against LexA (2 µg of YN-lexA-2-12, 4.3 µg of YN-lexA-6-10, and 2 µg of YN-lexA-16-7), a gift from S. Triezenberg (48).

Formaldehyde cross-linking of embryos for chromatin immunoprecipitation. For chromatin immunoprecipitation assays, embryos (0.25 to 0.5 g) were collected from plastic laying bottles for 3 h and aged for 2 h at room temperature. Low-level expression of the heat shock construct was observed in some cases, but it was not enough to repress *lacZ* expression. Embryos were then either immediately fixed or heat shocked (20 min) by floating the plates in a 38°C water bath and recovered for various lengths of time at room temperature. Embryos were collected and dechorionated with bleach. For single cross-linking, embryos were fixed for 20 min with vigorous shaking in a 50-ml Corning tube in 10 ml 3% formaldehyde fixing buffer (50 mM HEPES [pH 7.6], 1 mM EDTA, 0.5 mM EGTA, 100 mM NaCl, with formaldehyde [catalog number 2106-01; J. T. Baker] added immediately before use from a 37% stock) and 30 ml heptane. Embryos were then centrifuged at 2,000 × g in a clinical centrifuge, the supernatant was removed, and the cross-linking reaction was stopped with 25 ml stop buffer (0.125 M glycine, 0.01% Triton X-100 in phosphate-buffered saline [1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, 18 mM KH₂PO₄, pH 7.4]) while the tube was shaken vigorously for 30 min. Embryos were centrifuged as described above and immediately processed for chromatin or flash frozen and stored at -75°C.

Double cross-linking of embryos for chromatin immunoprecipitation. Embryos were collected, heat shocked, and dechorionated as described above and then placed in a 50-ml Corning tube with 8 ml of phosphate-buffered saline and freshly prepared 25 mM dithiobis(succinimidyl propionate) (DSP; catalog number 22585; Pierce) cross-linking solution in dimethyl sulfoxide (with a final concentration of 5 mM DSP and 20% dimethyl sulfoxide). Embryos were shaken vigorously for 30 min and centrifuged at 2,000 × g, and the supernatant was removed and cross-linked with formaldehyde as described above.

Preparation of chromatin from whole embryos. A total of 0.25- to 0.5-g cross-linked embryos was washed in 10 ml embryo wash buffer (10 mM HEPES [pH 7.6], 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate, 0.02% sodium azide) for 10 min with vigorous agitation, centrifuged at 2,000 × g at 4°C, resuspended in 5 ml of sonication buffer (10 mM HEPES [pH 7.6], 1 mM EDTA, 0.5 mM EGTA, 0.1% Triton X-100), and transferred to a 15-ml Corning tube. A proteinase inhibitor tablet (catalog number 11836153001; Roche) was added, and the embryos were sonicated for 30 s (100% duty cycle) with a 1-min cooling interval 12 times by using a Branson sonicator. With each pulse, the output was gradually increased from setting 1 to setting 7 to avoid foaming. Crude chromatin was aliquoted into microcentrifuge tubes and centrifuged at 16,000 × g for 15 min at 4°C, and without disturbing the pellet, the supernatant was transferred to a 15-ml Corning tube. An equal volume of 2× radioimmunoprecipitation assay (RIPA) buffer (2% Triton X-100, 280 mM NaCl, 20 mM Tris-HCl [pH 8.0], 2 mM EDTA [pH 8.0], 0.2% sodium dodecyl sulfate [SDS]) was added. For Groucho immunoprecipitation experiments from double-cross-linked embryos, the SDS was omitted. Chromatin was precleared by adding 10 µl/ml of a 50% slurry containing an equal mixture of agarose beads coupled to protein A and protein G (catalog numbers 16-125 and 16-266; Upstate) previously washed three times with 1× RIPA buffer. Chromatin was then aliquoted into microcentrifuge

tubes in 1-ml fractions and either flash-frozen and stored at -75°C or immediately immunoprecipitated.

Immunoprecipitation. Immunoprecipitations were carried out by overnight incubation on a rotary mixer of 1 ml of precleared chromatin at 4°C with the antibody. At the same time, protein A and protein G agarose beads were mixed in equal proportions, washed three times with $1\times$ RIPA buffer and then incubated overnight with 0.1 mg/ml bovine serum albumin and 0.2 mg/ml salmon sperm DNA in $1\times$ RIPA buffer. For Groucho immunoprecipitation experiments, 20 μg of rabbit anti-mouse immunoglobulin G (IgG) bridging antibody was added and the tube was incubated for an additional 1 to 2 h. Before the addition of mixed protein A and protein G agarose beads, chromatin-antibody reaction mixtures were centrifuged at $16,000\times g$ for 15 min, 900 μl of the supernatant was transferred to a new tube, and 40 μl of 50% slurry of blocked protein A and protein G beads was added and incubated for 4 h on a rotary mixer. Beads were centrifuged at $80\times g$ for 1 min, washed (0°C) three times with 1-ml portions of low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl [pH 8.0], 150 mM NaCl), three times with high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl [pH 8.0], 500 mM NaCl), once with LiCl buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-Cl [pH 8.0]), and twice with Tris-EDTA (10 mM Tris [pH 8.0], 1 mM EDTA). For Groucho immunoprecipitation experiments using double-cross-linked chromatin, six washes were performed with $1\times$ RIPA buffer lacking SDS (1% Triton X-100, 140 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Chromatin was eluted at room temperature in a rotary mixer with 250 μl elution buffer (1% SDS, 0.1 M monobasic NaHCO_3 [pH 8.0]) for 15 min. Beads were centrifuged, and the supernatant was transferred to a screw cap microcentrifuge tube; a second elution was performed, supernatants were combined, 25 μl of 4 M NaCl was added, and cross-links were reversed overnight at 65°C . In parallel, 200 μl of input chromatin (20%) was mixed with 300 μl elution buffer and incubated overnight at 65°C as input titration controls. Then, 10 μl 0.5 M EDTA [pH 8.0], 20 μl 1 M Tris-Cl [pH 6.5], 1 μl 10-mg/ml RNase A, and 1 μl 20-mg/ml proteinase K were added and incubated for 2 h at 42°C . DNA was extracted with 500 μl of phenol-chloroform, 400 μl of the aqueous phase was placed in a new tube, and DNA was precipitated with 15 μg GlycoBlue pellet paint (Ambion), 44 μl 3 M NaO acetate (pH 5.2), and 444 μl isopropanol. Tubes were incubated (at room temperature to prevent SDS precipitation) for 1 h and centrifuged at $16,000\times g$ for 15 min, and pellets were carefully washed with 0.5 ml 70% ethanol and dried at 65°C under vacuum for approximately 15 to 30 min and then resuspended in 50 μl purified water for PCR analysis.

Using the simple formaldehyde cross-linking protocol, we sometimes obtained cross-linking of Groucho to the transcribed region but never to the promoter-proximal sequences. This signal appears to be specific because Groucho is never detected on other regions. Using the double-cross-linking protocol, we found that Groucho, when detected, was also associated with the promoter in addition to downstream regions. Even using this protocol, we were not always successful in detecting Groucho, which is consistent with the reported difficulty of detecting transcriptional cofactors indirectly bound to the DNA (28).

Sequential immunoprecipitation. The first immunoprecipitation was carried out as described above until the DNA was eluted from the agarose beads. A total of 500 μl of the eluted sample was transferred to a 15-ml Corning tube and diluted 10-fold with 4.5 ml RIPA buffer minus SDS. The second antibody was then added, and the tubes were incubated at 4°C overnight. A total of 40 μl of a 50% slurry of blocked protein A and protein G beads was added and incubated for 4 h on a rotary mixer at 4°C . Tubes were then centrifuged at $1,000\times g$ in a clinical centrifuge, the supernatant was removed, and the beads were transferred to 1.5-ml Eppendorf tubes. Washing, cross-link reversal, and DNA purification were done as previously described.

Antibodies for chromatin immunoprecipitation. We used the following antibodies: nonspecific mouse IgG (10 μg ; Upstate), rabbit anti-LexA (3 μg ; Upstate), rabbit anti-Gal4-TA (5 μg ; Santa Cruz), rabbit anti-dGCN5 (2 μl ; J. Workman), rabbit anti-dAda3 (1 μl ; J. Workman), mouse monoclonal anti-Groucho (50 and 100 μl ; Iowa Hybridoma Bank), rabbit anti-HDAC1 (1, 2, and 4 μl ; Abcam), rabbit anti-H3 (1 μl ; Abcam), rabbit monoclonal anti-mono-/di-/trimethyl histone H3 K4 (1 μl ; Upstate), rabbit anti-acetyl histone H4 (5 μl ; Upstate), rabbit anti-dimethyl histone H3 K27 (5 μl ; Upstate), rabbit anti-acetyl histone H3, and rabbit anti-mouse IgG (20 μg ; Upstate).

PCR analysis. Two-microliter samples of immunoprecipitated DNA were analyzed on a gradient 96 RoboCycler with platinum hot-start polymerase (Invitrogen). The primers used were DA-1027 (white forward, 5' ATA CAG GCG GCC GCG GAT CTG AT 3'), DA-1028 (white reverse, 5' AGA TAG CGG ACG CAG CGG CGA A 3'), DA-942 (promoter forward, 5' ATC AGA TCC GCG GCC GCC TGT AT 3'), DA-943 (promoter reverse, 5' CGT CCG CAC ACA ACC TTT CCT CTC 3'), DA-865 (+1 kb forward, 5' CGG GCG CTG GGT

CGG TTA CG 3'), DA-873 (+1 kb reverse, 5' GGT GCC GCT GGC GAC CTG C 3'), DA-948 (+2 kb forward, 5' AAC CGT CAC GAG CAT CAT CC 3'), DA-949 (+2 kb reverse, 5' ATT CAT TCC CCA GCG ACC AG 3'), DA 1012 (+4 kb forward, 5' CGG TCG CTA CCA TTA CCA GT 3'), and DA 1013 (+4 kb reverse, 5' ATT GTA ACA GTG GCC CGA AG 3'). The primers used to amplify intergenic regions were DA-954 and DA-955 (X intergenic forward and reverse, 5' CAC AGT GGA CAC ATA CCA TAG 3' and 5' CGG AAA ATA TCA GTG CGA AAG 3', respectively) and DA-960 and DA-961 (chromosome 3 intergenic forward and reverse, 5' GTT GAG AAT GTG AGA AAG CGG 3' and 5' CGA AAA AGG AGA AGG CAC AAA G 3', respectively).

The densitometric analysis of gel images was performed using ImageJ software. The gel images shown in Fig. 2 to 5 were background subtracted, and the brightness and contrast were adjusted so that the input titrations in experiments using multiple chromatin samples matched as closely as possible. The statistical significance of changes during activation and repression were determined using a one-tailed *t* test, with the assumption of equal levels of variance between the populations. A change was determined to be statistically significant when the *P* value was <0.05 .

RESULTS

Construction and characterization of a regulated Hairy-repressible transgene. Our objective was to study the activity of Hairy in the context of embryo development, but due to the spatially and temporally limited action of the endogenous Hairy protein, genes in a relatively small percentage of the total nuclei of the embryo were repressed by Hairy at any point in time. Therefore, we engineered a gene-regulatory system that allowed us to place a constitutively expressed activator on the promoter and induce the expression of a LexA-Hairy fusion protein in a facultative manner, converting the embryo from an "all on" to an "all off" state (Fig. 1A). The target gene contains five binding sites for the Gal4 activator, flanked by two pairs of LexA binding sites to accommodate the repressors; similar configurations had already been tested and shown to be repressed by endogenous Hairy protein (27).

To test the ability of LexA-Hairy to repress a Gal4-activated reporter in transgenic embryos, we used a *Kruppel* enhancer to express LexA-Hairy in a central stripe in embryos containing a *lacZ* reporter regulated by the Gal4 activator or endogenous enhancers (Fig. 1B). Embryos expressing the LexA-Hairy gene driven by the *Kruppel* enhancer showed a wide swathe of understained nuclei in the central region, indicating that the repressor is functional (Fig. 1B). We therefore constructed an inducible form of the LexA-Hairy transgene under the control of the *hsp70* promoter, allowing various levels of induction by titration of heat shock conditions. As measured by in situ hybridization, *lacZ* transcript levels in the entire embryo dropped markedly after even a short (5-min) heat shock. Successively longer heat shocks resulted in the complete loss of *lacZ* staining (Fig. 1C and D). In typical experiments, the percentage of embryos showing strong staining dropped from almost half to less than 1% 1 hour after the induction of the repressor, indicating that the repression was effective in the vast majority of nuclei and embryos. Embryos aged for 2 hours after the heat shock induction showed the restoration of *lacZ* expression, indicating that the repression is reversible (Fig. 1D). Heat shock treatment had no effect on embryos carrying the reporter gene and the activator in the absence of the LexA-Hairy repressor protein, indicating that heat shock itself does not interfere with the transcription of this gene (Fig. 1C, lower panels). The expression of endogenous pair-rule genes (*ftz*, *eve*, *run*) in the embryo was unaffected by the induction of

FIG. 1. Hairly-regulated gene system in the *Drosophila* embryo. (A) Three transgenes were combined onto a single chromosome to create a regulated on/off system: a *lacZ* reporter containing Gal4 sites, the Gal4 activator driven by the *daughterless* (*Da*) enhancer for broad expression in the embryo, and a heat-inducible LexA-Hairy construct. *Gro*, Groucho. (B) Repression by the LexA-Hairy protein expressed in a central domain under the *Krüppel* enhancer. (Upper images) *lacZ* reporter construct shown in panel A; (lower images) reporter activated by the *rho* and *twi* enhancers, with LexA binding sites at the promoter. In both cases, a central swathe of repression demonstrates the effect of the LexA-Hairy chimera. (C) Expression of *lacZ* and LexA-Hairy in blastoderm embryos. Embryos containing the transgenes shown in panel A were heat shocked (HS) for various times and fixed for in situ analysis of *lacZ* expression (top panel) or antibody staining of the LexA-Hairy protein (middle panel). As LexA protein accumulates, *lacZ* mRNA decreases. Heat shock has no effect on *lacZ* in embryos lacking the LexA-Hairy protein (two lower images of embryos). (D) Repression and reactivation of transgenes in embryos after heat shocks of various durations. A 5-min induction of the LexA-Hairy protein is sufficient to cause a significant loss of *lacZ* mRNA within 30 min. RNA levels remain low at 60 min and show recovery after 120 min (at this point, most embryos have aged to the germ band extended stage). Longer heat shocks (10 and 20 min) show similar recovery kinetics. Embryos are shown anterior to the left, with the dorsal surface up.

the LexA-Hairy repressor, indicating a lack of off-target or squelching effects (not shown).

Cooccupancy of the activator and repressor during gene repression. After establishing the efficacy of the system, large-scale collections were carried out, and 3- to 5-h-old embryos were treated with formaldehyde prior to chromatin preparation for immunoprecipitation reactions. We initially sought to detect the regulatory proteins that bound the promoter region. As expected, the Gal4 protein was detected at the promoter only in lines carrying the *da-Gal4* driver and the *lacZ* reporter gene (Fig. 2A, top two gels). LexA-Hairy was readily detected at the promoter region only in the presence of the *lexA-hairy* transgene. A very strong signal was detected at the promoter after the heat shock induction; a weak signal was also sometimes detected prior to heat shock, which is likely due to the background expression of the LexA-Hairy protein, although not at levels sufficient to inhibit transcription or to be detected by antibody staining (Fig. 1C). As a confirmation of the specificity of this interaction, neither Hairly nor Gal4 was found to associate with a nonspecific intergenic region on the X chromosome (Fig. 2A).

Strikingly, the appearance of the LexA-Hairy repressor at the promoter did not preclude the association of the Gal4 activator, indicating that the repressor appears not to rely on the displacement of the activator for its activity (Fig. 2A, fourth and fifth gels). A robust signal for the Gal4 protein was evident 30 min after the induction of the repressor, at a time point

when most *lacZ* mRNA had disappeared from the embryo (Fig. 1D and 2A). The Gal4 signal does not represent the reassembly of active promoter complexes at this 30-min time point, because the embryos continue to show repression even at 60 min (Fig. 1D). The persisting Gal4 signal also does not represent material bound to a separate population of unrepressed promoters, because virtually all of the embryos are uniformly repressed at this stage. To further test the Gal4 and LexA-Hairy cooccupancy of the promoter, sequential chromatin immunoprecipitations were carried out. Chromatin was immunoprecipitated with anti-LexA or anti-Gal4 antibodies and reimmunoprecipitated by anti-Gal4 antibodies or nonspecific IgG. The specific enrichment of chromatin in the sequentially immunoprecipitated LexA/Gal4 sample provides direct evidence for cooccupancy by these proteins (Fig. 2B). The signal for Gal4 and LexA was strongly concentrated over the promoter region; considerably weaker signals were detected further 3' within the open reading frame, possibly because of the compact topology of the promoter region or the presence of a small fraction of larger chromatin fragments of >500 bp (Fig. 2A; discussed with Fig. 4 below). With regard to levels of Gal4 binding, the quantitation of the chromatin immunoprecipitations showed a possible modest reduction in Gal4 binding during repression, but the difference does not appear to be statistically significant (Fig. 2C). This apparent reduction may represent reduced binding or epitope masking, but the de-

FIG. 2. (A) Promoter occupancy by the Gal4 activator and LexA-Hairy repressor measured by chromatin immunoprecipitation. In the top group, no detectable Gal4 or LexA-Hairy protein was detected with chromatin prepared from a line containing only the *lacZ* reporter gene, as expected. The gel on the second line of the first group contains a strong signal for the Gal4 protein, from chromatin containing the activated reporter. These embryos were heat shocked and recovered to parallel the treatment used for LexA-Hairy-containing strains. In the bottom group, results are from lines containing all three transgenes prior to and directly after the induction of the LexA-Hairy repressor. Prior to induction, a signal for Gal4 is visible at the promoter, as well as a weak LexA-Hairy signal due to low-level expression. After a 20-min heat shock and 30-min recovery, a strong signal for LexA-Hairy is visible, in addition to the Gal4 signal. Both signals remain visible after 60 min of recovery. LexA-Hairy signals drop by 120 min. No signals are seen at the intergenic region on chromosome X. Hs and hs, heat shock; da, *daughterless*. (B) Sequential chromatin immunoprecipitation of embryos expressing LexA-Hairy and Gal4. Low levels of chromatin were recovered in immunoprecipitations in which anti-LexA- or anti-Gal4-enriched material was reprecipitated by nonspecific IgG. As expected, double immunoprecipitations with anti-Gal4 or anti-LexA antibodies recovered significantly more material, as did the sequential LexA/Gal4 immunoprecipitation. For panels A and B, input titrations are shown for each chromatin preparation (2%, 1%, 0.5%,

crease is in itself not sufficient to account for the almost complete repression of *lacZ* expression in the embryos.

Transcriptional coactivators remain associated with the repressed promoter. The persistence of the activators at the promoter indicates that Hairy does not block their access, raising the question of why the promoter is not activated by Gal4 in this situation. We reasoned that the repressor's exclusion of coactivators might prevent Gal4 from having a stimulatory effect on the promoter. The Gal4 activation domain has been reported to recruit the SAGA coactivator complex (29, 6); therefore, we carried out immunoprecipitations using antibodies against the Ada3 and Gcn5 subunits of SAGA (Fig. 3). A promoter-localized signal for both of these proteins was detected in lines carrying the Gal4 activator (Fig. 3A, compare the top two gels), which is consistent with the recruitment of the complex to the active promoter by Gal4. After the induction of the Hairy repressor, the coactivators remained detectable at the promoter at a point in time at which *lacZ* gene expression in the embryo has ceased (Fig. 3A, fourth and fifth gels). The binding of these coactivators was not detected at a nonspecific intergenic locus (Fig. 3A, lower gels). The quantitation of Gcn5 and Ada3 levels showed only slight changes in occupancy during repression (Fig. 3B). Thus, Hairy apparently does not exclude either the activator or the coactivator to effect the repression of the promoter.

Association of Groucho and Rpd3 corepressors with wide tracts of the repressed gene. We tested whether the Hairy corepressor Groucho was recruited to the gene during repression (Fig. 4A). Using formaldehyde-cross-linking techniques, Groucho was detected after the induction of the Hairy repressor. No signal was detected at the promoter; however, strong signals were evident at regions 3' of the promoter at +1 kbp and +2 kbp, and an attenuated signal was detected at +4 kbp (Fig. 4B). Groucho was not found to cross-link to an intergenic region, indicating that the signal is specific to the Hairy-repressed gene (Fig. 4B). A similar pattern of cross-linking was obtained with the Rpd3 histone deacetylase, with strongest signals observed at +1 kbp and +2 kbp. This deacetylase has been identified as a Groucho-interacting protein in biochemical assays (7).

The detection of Groucho using this cross-linking protocol was varied; therefore, we tested a double-cross-linking procedure recently described for the cross-linking of cofactors in *Saccharomyces cerevisiae*, which involves a two-step treatment employing the bifunctional cross-linker DSP first, followed by

0.25%, 0.125%, and 0.0625%). At left, results of quantitative PCRs are shown, and at right, heat shock and recovery conditions are shown (heat shock was done at 37°C for 20 min, with recovery at room temperature). The presence or absence of the Gal4 protein and the heat shock-inducible *LexA-Hairy* transgene is noted by + or -, respectively. (C) Quantitation of LexA-Hairy and Gal4 levels at promoters before and after repression. Shown are percentages of recovery from three or more chromatin immunoprecipitation experiments (mean values and standard deviations are indicated). Black bars represent chromatin from strains lacking LexA-Hairy and Gal4, white bars represent chromatin from strains containing both LexA-Hairy and Gal4 (with only Gal4 expressed), and gray bars represent strains with both Gal4 and LexA-Hairy expressed.

FIG. 3. Promoter occupancy by the SAGA coactivator constituents Gcn5 and Ada3 as measured by chromatin immunoprecipitation. (A) Protein occupancy of the *lacZ* reporter promoter region in different states. The top gel shows no signal for these coactivators on the promoter lacking Gal4 activators, as expected. In the second gel, both proteins are detected on the promoter in a Gal4 activator-containing strain. Subsequent panels show continued SAGA component occupancy in strains in which the LexA-Hairy repressor was induced. No signals are seen in the intergenic region on chromosome X. Input titrations are shown for each chromatin preparation (2%, 1%, 0.5%, 0.25%, 0.125%, and 0.0625%). At left, results of quantitative PCRs are shown, and at right, heat shock and recovery conditions (heat shock was done at 37°C for 20 min, with recovery at room temperature). The presence or absence of the Gal4 protein and the heat shock-inducible *LexA-Hairy* transgene is noted by + or –, respectively. Hs and hs, heat shock; da, *daughterless*. (B) Quantitation of Gcn5 and Ada3 levels at promoters before and after repression. Shown are percentages of recovery from three or more chromatin immunoprecipitation experiments for Ada3 or from four or more experiments for Gcn5 (mean values and standard deviations are indicated). Black bars represent chromatin from strains lacking LexA-Hairy and Gal4, white bars represent chromatin from strains containing both LexA-Hairy and Gal4 (with only Gal4 expressed), and gray bars represent strains with both Gal4 and LexA-Hairy expressed.

formaldehyde (57). Using this procedure, we were again able to detect Groucho on downstream regions of the *lacZ* reporter gene, with strong signals detected up to +4 kbp from the transcriptional initiation site. With this method, a strong signal

FIG. 4. Occupancy of the promoter and transcribed regions of the *lacZ* gene by Groucho and Rpd3 corepressors during repression by LexA-Hairy. (A) Schematic diagram of a LexA-Hairy-regulated gene, showing the portions amplified. w+, mini-white gene. (B) PCR analysis of formaldehyde-cross-linked chromatin immunoprecipitated from embryos that were induced to express the LexA-Hairy repressor for 20 min and aged for 30 min. As expected, no signal was seen for the nonspecific IgG precipitation. A strong promoter-localized signal was detected for LexA-Hairy, with weaker signals detected for distal regions of the gene. Signals for the Rpd3 histone deacetylase (results with 1, 2, and 4 μ l of Rpd3 antibody are shown) and the Groucho corepressor (results with 50 and 100 μ l of Groucho antibody are shown) were detected at +1 kbp and +2 kbp, with a weak signal for Groucho (Gro) at +4 kbp. No cross-linking was detected for an intergenic region on chromosome 3. Levels of input titration are shown at the right (2%, 1%, 0.5%, 0.25%, 0.125%). (C) PCR analysis of immunoprecipitated DSP-formaldehyde-cross-linked chromatin. LexA and Gal4 were detected at the promoter, with weaker signals detected in 3' regions. In contrast to the results shown in panel B, a strong Groucho signal was detected at the start of the *white* gene (–350 bp), at the promoter, and throughout the transcribed region. No cross-linking was detected for an intergenic region on chromosome X. Groucho was not detected on chromatin samples derived from embryos that were not heat shock induced for LexA-Hairy or that were heat shocked but lacked the *lexA-hairy* gene (not shown). Levels of input titration are shown at the right (10%, 2%, 1%, 0.5%, 0.25%).

was also detected over the promoter region, perhaps reflecting the longer reach of the DSP cross-linker (~12 Å) (Fig. 4C). Again, no Groucho was found at a nonspecific intergenic region on chromosome 3, demonstrating the specificity of the signal. The LexA-Hairy protein in this experiment is also detected more weakly at regions within the transcribed region, suggesting that there are protein contacts with sites distal to the binding sites within the promoter. The extended signal is

not simply the result of very long chromatin fragments, because the average chromatin size in this experiment is less than 500 bp and the Groucho signal seen in Fig. 4B is centered over the transcribed region, not the promoter. These widespread contacts of Groucho with the transcribed region of the gene are consistent with a model proposed by Courey and Jia (9) in which the multimerization of protein through the N-terminal domain forms an extended "spread" conformation that permits the protein to influence multiple regions of the DNA, which is consistent with the long-range activity of Hairly (47).

We tested whether Hairly recruits Groucho to upstream regions in a pattern similar to that seen on the transcribed locus. More-distal regions could not be specifically sampled because of the presence of three copies of the *white* gene on the different P-element vectors in these strains. However, PCR primers specific to the promoter and the upstream mini *white* transgene were used to amplify proximal regions 5' of the promoter. These experiments showed the recruitment of Groucho in chromatin prepared using the double-cross-linking protocol (Fig. 4C).

We sought to detect the CtBP and Sir2 corepressors at this gene, but we did not reliably detect signals above the background. Sir2 interaction with Hairly has been mapped to the DNA binding domain, which is absent in this LexA-Hairy chimeric protein; thus, it is not surprising that no signal was detected for this protein (40). The negative result regarding CtBP is not very conclusive, because this particular antibody may not be suitable for immunoprecipitations. Nonetheless, repressors such as Hairly can utilize at least a subset of corepressors for the regulation of individual target promoters, and perhaps CtBP is not recruited to this gene (5).

Chromatin modification associated with activation and the repression of the target gene. In light of the numerous connections between chromatin modifications and gene regulation and the association of the deacetylase Rpd3 with the reporter, we tested the effects of gene repression on histones and chromatin modifications in the promoter region (Fig. 5). Chromatin was prepared from embryos carrying only the reporter gene (unactivated state), embryos with both the reporter and the activator (activated state), and embryos with the reporter, activator, and repressor, induced and aged for 30 to 60 min (repressed state) (Fig. 5A). The recruitment of Gal4 to the reporter gene was associated with an overall reduction of levels of histones, measured with anti-H3 antibody (Fig. 5A, first and second gels, and 5B, third group of bars). The levels of histone H3 and H4 acetylation relative to that of total histone H3 were elevated, consistent with the appearance of SAGA subunits at the promoter (Fig. 2 and 5A, first and second gels, and 5B, first and second groups of bars). Also associated with the activation of the gene is a relative decrease in histone H3 K27 methylation (Fig. 5A, first and second gels, and 5B, fourth group of bars). The induction of LexA-Hairy and the repression of the gene are associated with an increase in overall H3 levels and a relative decrease in H3 and H4 acetylation levels, consistent with the recruitment of histone deacetylases to the gene (Fig. 5A, second and third gels, and 5B). By these measures, the effect of the repressor is the opposite of that of the activator. However, the drop in relative H3 K27 methylation levels is not reversed by the recruitment of the repressor, indicating an association of this modification with the activation, but not

FIG. 5. (A) Chromatin remodeling, assayed by chromatin immunoprecipitation. Total histone levels, acetylation, and methylation were assayed on the reporter gene in unactivated, activated, and repressed states, as well as with an unactivated reporter gene with the repressor bound. The top two panels demonstrate the effect of Gal4 activators on the promoter; in the presence of Gal4 (second gel), overall histone H3 levels drop, as do H3 K27 methylation levels, while relative acetylated histone H4 and H3 levels increase. In the presence of the LexA-Hairy repressor (compare the second and third gels), 30 min after induction, total H3 levels increase modestly and relative H3 and H4 acetylation levels drop. In this experiment, H4 acetylation levels were more affected. Relative levels of H3 K27 methylation remained low in the repressed state. The binding of the repressor to the nonactivated gene (compare the first and fourth gels) did not affect total histone H3 occupancy or H3 K27 methylation and had only modest effects on relative acetylation. Below, relative levels of histone modifications at an intergenic locus on chromosome X are unchanged by the treatments. Immunoprecipitations were carried out with antibodies that recognize total histone H3, K9/14 acetylation of histone H3, K5/8/12/16 acetylation of histone H4, or K27 dimethylation of histone H3. Levels of input titration are shown at the right (10%, 2%, 1%, 0.5%, 0.25%, 0.125%). Hs and hs, heat shock; da, *daughterless*. (B) Quantitation of chromatin modifications at the promoter during activation and repression. Levels of acetylation, methylation, and histones were determined using densitometry analysis. Signals were normalized to the unactivated state. Shown are the average values and standard deviations of results from at least three biological replicates. Signals for AcH3, AcH4, and H3 K27 methylation (K27 me) are divided by the signal value of H3 to take into account differences in total histone levels. The statistical significance of chromatin changes was determined by a one-tailed *t* test with an alpha of 0.05. Activation is associated with a strong increase in relative H3 and H4 acetylation levels (P values = $8.1\text{E}-04$ and $1.6\text{E}-04$), as well as a decrease in relative K27 methylation levels and H3 occupancy (P values = 0.0033 and $2.5\text{E}-06$). Repression is associated with a significant loss of H3 and H4 acetylation (P values = $1.3\text{E}-05$ and $1.6\text{E}-06$), as well as a slight increase in H3 occupancy (P value = $6.2\text{E}-04$). No changes in relative K27 methylation levels were observed during repression (P value = 0.43).

repression, pathways (Fig. 5B). None of these effects were noted on a nonspecific intergenic locus, indicating that the modifications were specific to the reporter gene (Fig. 5A, lower gels). In addition, heat shock alone did not visibly alter chromatin signals, indicating that the heat induction regimen alone was not accountable for these changes (Fig. 5A, lower gels).

Previous studies of endogenous Hairy protein indicated that it is unable to repress a distal enhancer if it binds in a regulatory region lacking activators, possibly because Hairy cannot access a domain that has not been subject to remodeling induced by activators, the so-called “hot chromatin” model (35). Simulating this situation, we performed chromatin immunoprecipitations for a strain lacking the Gal4 activator protein and found that the Hairy chimera is able to access the promoter (Fig. 5A, fourth gel). This result suggests that there is no requirement for locally acting activators to recruit this form of Hairy; however, the promoter context may be more permissive than distal enhancer regions, or the endogenous Hairy protein may be subject to more-stringent requirements for DNA binding. The binding of the repressor did not affect total histone H3 levels or K27 methylation and had only modest effects on histone acetylation levels (most frequently a slight decrease in relative levels of histone H3 acetylation) (Fig. 5A, first and fourth gels).

In light of the long-range contacts seen for the Groucho corepressor, we measured histone levels and histone acetylation levels on the coding region of the reporter gene (Fig. 6). Changes in H3 and H4 acetylation in response to repression were observed up to 1 kb 3' of the transcriptional initiation site, but little change was observed at +2 kbp and +4 kbp (Fig. 6A and B, white and gray bars). Total H3 histone occupancy was observed to drop in response to activation, but these levels were little changed at +1 kbp, +2 kbp, and +4 kbp upon subsequent repression (Fig. 6C). Taken together, these results suggest that Hairy induces local changes in histone acetylation and occupancy but that certain features induced by activation, such as H3 K27 promoter demethylation and total H3 occupancy, are not completely reversed by repression. Activator-associated promoter H3K4 methylation is similarly unaffected by repression (data not shown).

DISCUSSION

Despite the intensive study of Hairy and other HES proteins in development, little is known of the mechanism of this model long-range repressor. Thus, we lack a full understanding of how distinct corepressors may be implicated in different developmental circuitry or the logic of regulatory regions controlled by this protein. Our finding that Groucho associates with a Hairy-repressed locus over the distance of several kilobases provides a possible mechanism that explains how Hairy can influence the activities of activator sites over a great distance. The molecular nature of the repressed locus may involve “spreading,” as suggested by Courey and Jia, involving progressive deacetylation and binding to hypoacetylated histones (9, 47).

In support of this model, Groucho binds hypoacetylated histone H3 and H4 tails, and mutations in its N-terminal oligomerization domain block its repression activity in vivo (7, 47). The recruitment of histone deacetylases may thus enhance

FIG. 6. Chromatin changes occurring on the transcribed region of the gene during activation and repression as assayed by chromatin immunoprecipitation. The statistical significance of chromatin changes was determined by a one-tailed *t* test with an alpha of 0.05. (A) Relative histone H3 K9/14 acetylation levels (normalized to that of H3) at +1, +2, and +4 kbp. A strong increase in relative H3 acetylation at +1 kbp is observed with the Gal4 activator present (P value = 0.027), and this acetylation decreases after the induction of the repressor (P value = 0.016). No large changes at +2 and +4 kbp were observed during activation (P values = 0.091 and 0.13) or repression (P values = 0.29 and 0.37). (B) Relative histone H4 K5/8/12/16 acetylation levels (normalized to that of H3) at +1, +2, and +4 kbp. Relative H4 acetylation at +1 kbp was observed to increase after activation (P value = 0.026). However, no statistically significant change in relative H4 acetylation at +1 kbp was observed during repression (P value = 0.059). Similarly, no changes in relative H4 acetylation were observed during repression at +2 and +4 kbp (P value = 0.17 and 0.22). A slight increase in relative H4 acetylation during activation was detected at +4 kbp (P value = 0.014) but not at +2 kbp (P value = 0.095). (C) Total relative histone H3 occupancy on the reporter during activation and repression. Overall levels decreased in the activated state relative to those of the unactivated gene throughout the open reading frame (P values at +1, +2, and +4 kbp were 0.0041, 0.035, and 0.016, respectively) and are not much further affected during repression (P values at +1, +2, and +4 kbp were 0.054, 0.50, and 0.29, respectively). Shown are the average values and standard deviations from at least three biological replicates. Signals have been normalized to those of the unactivated state.

Groucho binding to adjacent histones in a positive-feedback loop. A similar mechanism has been suggested for Tup1, the Groucho homolog in yeast (14). As with Groucho, histone deacetylases have been shown to be crucial for Tup1 repression (10). Moreover, Tup1 also has an affinity for hypoacetylated amino-terminal histone tails, and mutations or deletions of the tails cause the derepression of Tup1 targets (21, 53). As

with the Hairy repressor here, Tup1 does not change the methylation status of target genes, and the deletion of histone methyltransferases does not affect Tup1-mediated repression (50). This suggests that methylation marks may not need to be reversed to achieve repression but they may facilitate ready reactivation seen upon the depletion of Hairy. Regarding the extent of association of Tup1 with target genes in yeast, chromatin immunoprecipitation studies have yielded conflicting pictures. Tup1 has been reported to interact with the *a*-cell-specific *STE6* gene only at the promoter or over a distance from 1 to 3.5 kbp, encompassing the entire gene (13, 55, 11). This discrepancy may be due to differences in cross-linking or immunoprecipitation conditions, reflecting the difficulty in analyzing indirectly bound factors. Indeed, in our study, promoter interactions by Groucho were observed only with the use of a double-cross-linking protocol.

Models of transcriptional repression include direct competition, local "quenching" (displacement or interference with activators), and interactions with the basal machinery (1). Our finding that activators and coactivators are still present under conditions in which the gene is repressed by Hairy suggests that the third model might apply here. The Gal4 activator might represent a particularly stably bound protein, as it does not show the high rate of exchange noted for other transcriptional activators (33). Thus, it is possible that Hairy-mediated repression does interfere with the binding of some activators on endogenous loci. However, repression can be quite effective even in the absence of activator displacement, perhaps by targeting the basal machinery, similarly to Tup1-mediator interactions seen in yeast (30, 17, 19). The promoter-proximal location of the repressor in our system might bias the system to such interactions, but this arrangement is physiologically relevant, as Hairy is found in such proximal locations on endogenous genes. In addition, the LexA-Hairy repressor is also active when bound at -2 kbp, indicating that promoter proximity is not required for activity (L. Li, unpublished observations). Interestingly, a recent chromatin immunoprecipitation survey of enhancers targeted by the Snail short-range repressor suggests that this repressor can be bound to inactive enhancers simultaneously with activators, raising the possibility that short-range repression might also involve direct interactions with the basal machinery (56).

The extensive contacts of Groucho over the repressed locus are strongly reminiscent of the extended nucleoprotein structures deposited on regions repressed by stable, heritably acting systems such as Polycomb group (PcG) proteins in animals and Sir proteins in silent-mating-type loci and subtelomeric regions of yeast. There, chromatin regions are modified and inhibited for the formation of productive transcription complexes (31, 32, 42). Indeed, the association of activators and components of the transcriptional machinery with repressed loci in these systems mirrors the continued binding of activators and coactivators in the system that we study here, suggesting that the limiting factor for transcription occurs at a later stage (43, 25). What sort of inhibitory interaction might be involved in this case? A number of recent reports have raised the possibility that repressed, or nonactivated, promoters feature RNA polymerase II that is blocked for elongation, similar to the paused polymerase found at the *hsp70* locus under noninducing conditions (15, 52). Wang and colleagues found that RNA poly-

merase II is not displaced from the *slp1* gene upon repression with Runt, a Groucho-binding protein (52). It is possible that Groucho itself, through contacts with histone proteins and/or the recruitment of deacetylases such as Rpd3, establishes a chromatin environment that is inhibitory for transcriptional elongation. However, we were unable to obtain reliable signals for RNA polymerase II at this promoter, precluding a definitive statement about polymerase occupancy in activated and repressed states.

A difference between the repression complexes assembled by the Hairy repression domain and by these other proteins is the transience of the effect; while PcG regulation is linked to epigenetic modifications that allow repression to persist for an extended time when PcG proteins are depleted, the regulation that we see here is readily reversed upon the loss of the LexA-Hairy repressor (4). Similar effects are observed with elements regulated by the endogenous Hairy protein; enhancers bearing Dorsal and Twist activator sites that are repressed by Hairy in the blastoderm embryo are reactivated minutes later in the germ band extended stage (M. Kulkarni, unpublished). Thus, Hairy appears to be designed for highly effective but readily reversible repression, which may be useful in particular developmental settings.

In contrast to a model of linear spreading, an alternative picture of Groucho interaction that is consistent with our observations is that the corepressor may be tethered to the promoter region, forming larger multimeric complexes around which proximal and distal portions of the gene are wrapped (the "turban" model). Groucho would indirectly contact the promoter region via Hairy binding and make direct histone contacts with more-distal regions. The selective cross-linking of Groucho to promoter regions treated only with the additional DSP cross-linker is consistent with such a picture (Fig. 4). This model may also explain why we often detect downstream interactions, albeit weak ones, of the LexA-Hairy repressor, particularly when employing the more extensive double-cross-linking protocol. In either case, Groucho itself may be important for interfering with activities of transcription factors or the transcription of distant loci. Both of these models suggest that the extensive spread or extensive contacts of Groucho is mechanistically linked to transcriptional repression; however, it is possible that Groucho's extensive contacts with downstream regions are not the main effector of Hairy-mediated repression. Promoter-proximal activities of Groucho, or of other Hairy corepressor proteins, may play the decisive role in dictating long-range effects. Extensive experimental evidence indicates that Groucho plays a key role in the repression mediated by Hairy; therefore, it seems parsimonious to assume that Groucho activity on the repressed gene is important for repression. In support of a "turban" model of repression, a recent study of the human Groucho homolog Grg3 showed that the recruitment of Grg3 to chromatin induces the formation of a highly condensed structure in vitro (44). In addition, in vivo recruitment of Grg3 by FoxA resulted in Grg3 being detected by chromatin immunoprecipitation analysis up to distances of 1 kbp from the FoxA binding site (44).

Our study demonstrates that repression by Hairy is associated with histone deacetylation, which is certainly consistent with the nature of cofactors associating with this protein. Interestingly, this modification appears to be restricted to regions

close to the repressor binding sites, which in our configuration places them close to the transcriptional initiation site. How might this be related to the long-range effects mediated by Hairy? One possibility is that Hairy, regardless of where it is bound, induces characteristic changes on chromatin close to the transcriptional start site, which would induce a dominant (and hence long-range) effect on target genes. Alternatively, the local chromatin deacetylation may reflect the reversal of promoter-localized histone acetylases (e.g., SAGA), and acetylation levels on other portions of the gene are already too low to show robust deacetylation. A third possibility is that other Hairy-induced chromatin modifications that are not assayed here are more extensive than the deacetylation.

Our study strongly supports a model for Hairy repression that involves contacts between the Groucho corepressor and extended regions of the silenced gene, providing a basis for the long-range repression observed for this protein that is independent of activator displacement. Interesting questions for future studies are how Groucho spreading is limited and whether specific chromatin signals modulate this activity. In addition, such extended repression complexes might be specific to subsets of Hairy targets. A recent study identified a mutation in *gro* that blocks multimerization but not repression of some genes, suggesting that this cofactor is likely to employ distinct activities at different genes (23). In addition, genomic surveys indicate that Hairy is likely to associate with distinct cofactors at different loci. Future work will focus on identifying the roles of individual cofactors of this repressor at genes that represent the diversity of Hairy targets in *Drosophila*.

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