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18. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
19. Cells were lysed in 20 mM tris-HCl buffer (pH 8.0) containing 100 mM NaCl, 0.5% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, aprotinin (2 μg/ml), leupeptin (2 μg/ml), antipain (2 μg/ml), and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 15 min at 4°C. After centrifugation at 10,000*g* for 10 min, proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. After incubation with nonfat milk (5%) in TBST [tris-HCl (pH 7.5), 100 mM NaCl, and 0.05% Tween 20], the membranes were incubated with anti-Chk2 or anti-HA (BAbCO), and then with horseradish peroxidase–conjugated secondary antibodies (Promega), and detected by enhanced chemiluminescence (Amersham).
20. Cell extracts were incubated with anti-Chk2 and protein A beads (Pharmacia) for 1 hour at 4°C. The precipitated beads were washed four times with 20 mM tris-HCl (pH 8.0), 0.1% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, aprotinin (2 μg/ml), leupeptin (2 μg/ml), antipain (2 μg/ml), and 1 mM PMSF and then three times with 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, and 10 mM $MnCl_2$. Kinase reactions contained immunoprecipitated endogenous Chk2 bound to protein A beads and GST-Cdc25 substrates in 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 40 μM adenosine triphosphate (ATP), and 15 μCi [γ-^{32}P]ATP for 30 min at 30°C. Proteins were separated by SDS-PAGE and visualized by Coomassie blue staining and autoradiography. ^{32}P incorporation into GST-Cdc25 substrates was quantitated with a PhosphorImager (Molecular Dynamics).
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dMi-2, a Hunchback-Interacting Protein That Functions in *Polycomb* Repression

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Early in *Drosophila* embryogenesis, gap gene products directly repress transcription of homeotic (HOX) genes and thereby delimit HOX expression domains. Subsequently, Polycomb-group proteins maintain this repression. Currently, there is no known molecular link between gap and Polycomb-group proteins. Here, dMi-2 is identified as a protein that binds to a domain in the gap protein Hunchback that is specifically required for the repression of HOX genes. Genetic analyses show that dMi-2 participates in both Hunchback and Polycomb repression in vivo. Hence, recruitment of dMi-2 may serve as a link between repression of HOX genes by Hunchback and Polycomb proteins.

The design of animals depends on spatially restricted expression of HOX genes (*1*). In the early *Drosophila* embryo, segmentation gene products that are locally expressed delimit the domains of HOX gene expression (*2*, *3*). Gap proteins, such as Hunchback (Hb), bind directly to regulatory sequences of HOX genes and repress their transcription in cells outside of HOX expression domains (*4*, *5*). Although HOX genes need to be continuously repressed in these cells and in their descendants, gap proteins are only transiently available. The role of the Polycomb-group (PcG) gene products is to maintain repression of HOX genes throughout development (*1*, *6*–*8*). To identify proteins that may act as a molecular link between the Hb repressor and PcG proteins, we used Hb protein as a bait in a yeast two-hybrid screen.

Using LexA-Hb as bait, we isolated cDNAs representing six different genes (*9*). In interaction tests with various unrelated LexA baits, proteins encoded by three of the six cDNAs interacted exclusively with Hb (Fig. 1A). Among these proteins, the hip76 clone product exhibited the strongest interaction with Hb. We isolated multiple cDNA clones (*10*) that span a complete open reading frame (ORF) encoding a 1982–amino acid protein with high sequence similarity to the human autoantigen Mi-2 (*11*). We refer to the *Drosophila* protein as dMi-2. dMi-2 contains five conserved sequence motifs (*11*) that are also present in the two human Mi-2 proteins and in two *Caenorhabditis elegans* ORFs (*12*): two chromodomains (*13*), a DNA-stimulated adenosine triphosphatase (ATPase) domain (*14*), two PHD finger motifs (*15*), a truncated helix-turn-helix motif resembling the DNA-binding domain of c-myb (*16*), and a motif with similarity to the first two helices of an HMG domain (*17*).

To map the dMi-2–interacting domain in Hb, we generated Hb fragments and tested them for dMi-2 interaction in yeast two-hybrid assays (Fig. 1B). dMi-2 interacted very strongly with sequences overlapping the D domain, a stretch of amino acids that is conserved between Hb proteins of different insect species (*18*). Mutations in the D box cause extensive derepression of HOX genes of the Bithorax complex (BXC) (*2*) (see below). Both D box alleles are premature termination codons, suggesting that the D domain and its COOH-terminal flanking sequences are critical for repression of BXC genes (*19*). Our interaction tests (Fig. 1B) show that this protein portion of Hb interacts with dMi-2. In vitro binding assays with bacterially expressed dMi-2 and Hb proteins confirmed that these proteins bind directly to each other (*20*). Thus, dMi-2 binds to a portion of Hb that appears to be critical for repression of BXC genes.

In situ hybridization to polytene chromosomes revealed that *dMi-2* maps to subdivision 76D (*21*). In a screen for zygotic lethal mutations in this region, we identified five complementation groups (*21*).

To test whether any of these five complementation groups encode dMi-2, we sequenced the *dMi-2* coding regions of several

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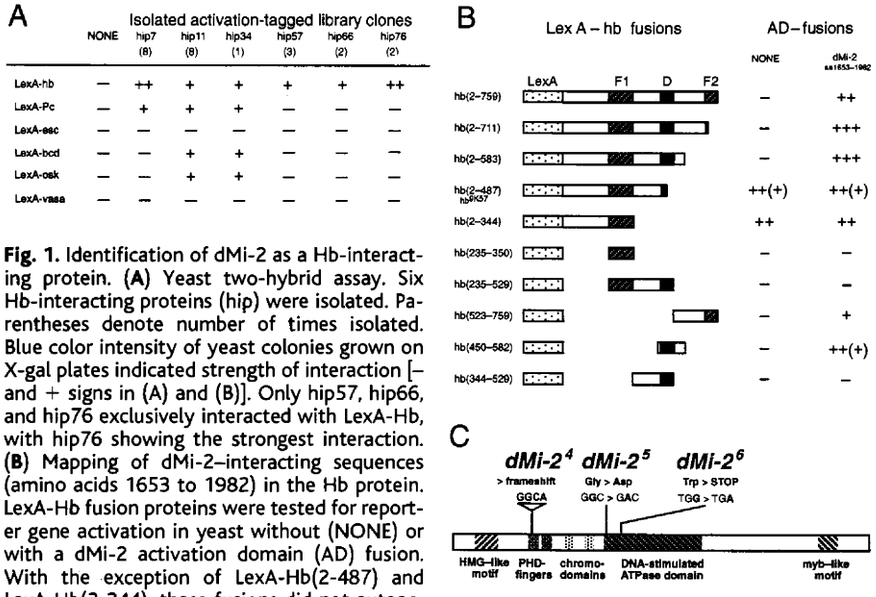


Fig. 1. Identification of dMi-2 as a Hb-interacting protein. (**A**) Yeast two-hybrid assay. Six Hb-interacting proteins (hip) were isolated. Parentheses denote number of times isolated. Blue color intensity of yeast colonies grown on X-gal plates indicated strength of interaction [– and + signs in (A) and (B)]. Only hip57, hip66, and hip76 exclusively interacted with LexA-Hb, with hip76 showing the strongest interaction. (**B**) Mapping of dMi-2–interacting sequences (amino acids 1653 to 1982) in the Hb protein. LexA-Hb fusion proteins were tested for reporter gene activation in yeast without (NONE) or with a dMi-2 activation domain (AD) fusion. With the exception of LexA-Hb(2-487) and LexA-Hb(2-344), these fusions did not autoactivate transcription (NONE). Repression assays (*29*) demonstrated that all LexA-Hb fusion proteins bind to LexA operator sites in yeast nuclei. The D domain (black box) together with sequences directly COOH-terminal to it is sufficient to bind to dMi-2. F1, finger domain 1; D, D domain; F2, finger domain 2. (**C**) Lesions present in dMi-2 alleles. *dMi-2⁴* shows an insertion of 4 base pairs after codon 398 that results in a frameshift and consequently a predicted premature termination within the first PHD finger domain. In *dMi-2⁵*, a strictly conserved Gly (Gly^{737}) that is present in all ATPase domains of the SWI2/SNF2 family is substituted by Asp; in *dMi-2⁶*, the base substitution changes a Trp codon in the ATPase domain (Trp^{801}) into a termination codon.

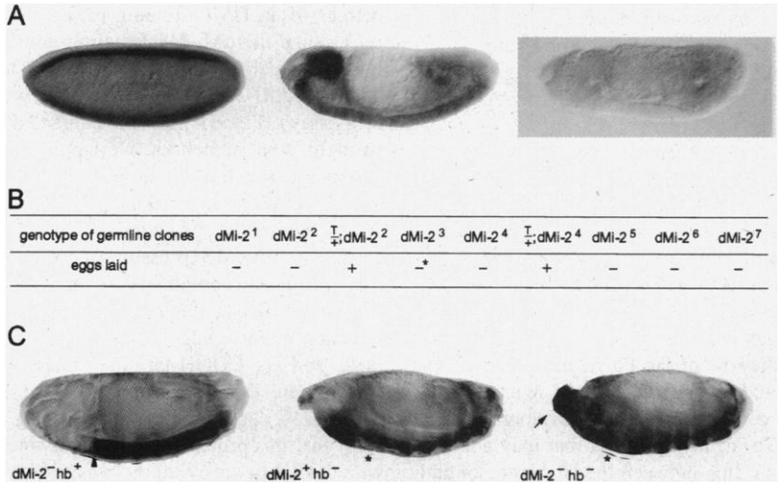


Fig. 2. Expression and function of dMi-2 in germ cells and in embryos. (**A**) Embryos from a *Df(3L) kto2* stock (the deletion removes *dMi-2*) hybridized with a digoxygenin-labeled dMi-2 antisense RNA probe. Up to the blastoderm stage (left), all embryos were labeled uniformly; this RNA is therefore maternally deposited. In 12-hour-old *Df(3L) kto2* homozygotes, the maternal dMi-2 RNA became undetectable (right). Zygotic expression of dMi-2 in wild-type embryos (middle). (**B**) No eggs were obtained from *dMi-2* mutant germ cells (* denotes that rare abnormal eggs were laid). *dMi-2²* and *dMi-2⁴* mutant germ cells carrying a dMi-2 transgene ("T") (*23*) developed into normally shaped eggs, but no embryos were obtained. (**C**) Synergy between *hb* and *dMi-2*. Eleven- to thirteen-hour-old embryos stained with an antibody to Ubx (*2*). *dMi-2⁴* mutants (left) showed wild-type morphology and *Ubx* expression (arrowhead indicates the anterior Ubx boundary in parasegment 5); hb^{9K57} mutants (middle) showed derepression of Ubx anterior to the morphological gap (asterisk) (*3*). *dMi-2⁴* hb^{9K57} double mutants (right) showed much more extensive derepression (arrow) anterior to the gap. Embryos are oriented with the anterior to the left and the dorsal side up.

alleles (*22*). All three sequenced alleles of one of the complementation groups showed individual base changes within conserved domains of dMi-2 (Fig. 1C). The identification of the *dMi-2* gene by these molecular lesions is further supported by a rescue test with a dMi-2 transgene (*23*).

dMi-2 homozygotes survived until the first or second larval instar. Mutant embryos and larvae showed no obvious mutant phenotypes. Specifically, expression of BXC genes such as *Ultrabithorax* (*Ubx*) and *Abdominal-B* (*Abd-B*) was completely normal in these mutant embryos (Figs. 2C and 3, A and B). This normal expression may be due to maternally deposited dMi-2 RNAs or proteins that persist through subsequent development. Consistent with this, we found that all early embryos from a *dMi-2* deletion stock (including those lacking the gene) showed the same high levels of dMi-2 RNA (Fig. 2A).

We thus attempted to generate embryos from mutant *dMi-2* germ cells (*23*). However, germ cells that are mutant for any of the seven tested *dMi-2* alleles failed to develop (Fig. 2B). This failure can be rescued by a dMi-2 transgene (Fig. 2B) (*23*), demonstrating that *dMi-2* is essential for the development of germ cells. We therefore could not generate embryos that lack dMi-2 protein.

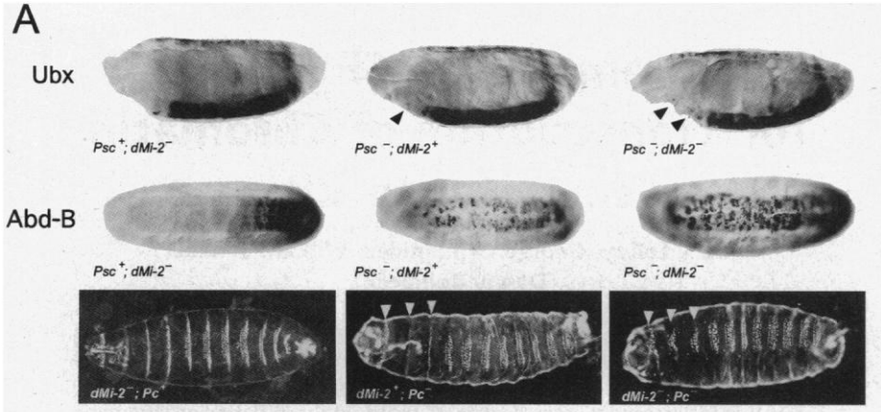
Next we tried to detect a genetic interaction between *dMi-2* and *hb*. hb^{9Q} mutants [carrying a premature stop codon upstream of the first finger domain (*19*)] showed only slight anterior derepression of *Ubx* in embryos because of perdurance of maternal *hb* products (*2*, *18*). hb^{9K57} mutants (carrying a D box lesion) showed more extensive anterior derepression of *Ubx* (Fig. 2C); this mutant protein is thought to have dominant-negative effects on the persisting maternal wild-type product (*2*). *dMi-2⁴* hb^{9K57} double mutants showed much more extensive derepression of *Ubx* than hb^{9K57} mutants (Fig. 2C). Similarly, *dMi-2⁴* hb^{9Q} double mutants showed more extensive derepression than hb^{9Q} mutants alone. These results demonstrate a synergy between *hb* and *dMi-2* that is consistent with our finding that dMi-2 binds to Hb. Furthermore, it provides strong evidence that *dMi-2* functions in the repression of BXC genes.

We next tested whether dMi-2 protein participates in PcG repression. As in the case of dMi-2, maternally deposited PcG product often rescues homozygous mutant PcG embryos to a considerable extent (*1*, *6*–*8*). Extensive derepression of HOX genes can be observed if such homozygous embryos are also mutant for another PcG gene (*7*). We thus examined embryos homozygous for the PcG gene *Posterior sex combs* (*Psc*) and *dMi-2* and found that *Ubx* and *Abd-B* were derepressed more extensively in this double mutant than in *Psc* homozygotes alone (*24*) (Fig. 3A). A similar result was found if *dMi-2* was combined with other PcG muta-

tions (*24*); these double mutants consistently led to much enhanced homeotic transformations compared with the single PcG mutants (Fig. 3A). Thus, there is a synergy between *dMi-2* and PcG genes. dMi-2 behaves like the PcG mutations *Enhancer of Polycomb* and *Suppressor 2 of zeste*, neither of which on their own cause a homeotic phenotype but do so in combination with other PcG mutations (*8*). This suggests that dMi-2 functions in PcG repression.

Next we examined imaginal discs for derepression of HOX genes as well as the phenotypes of their adult derivatives. Clonal analysis suggested that *dMi-2* is required for the survival of somatic cells (*23*). We therefore tested whether dMi-2 mutations exhibited gene-dosage interactions with PcG mutations. Whereas larvae heterozygous for *Polycomb* (*Pc*) mutations showed slight derepression of *Ubx* (Fig. 3B), larvae transheterozygous for both *Pc* and *dMi-2* mutations showed more extensive derepression (Fig. 3B). Furthermore, derepression of the HOX gene *Sex combs reduced* (*Scr*) in the second and third leg discs of *Pc* heterozygotes results in the formation of a first leg structure, the sex comb, on the second and third legs (*25*). The extent of this homeotic transformation reflects the number of cells that misexpress Scr protein. We found that this homeotic transformation was far stronger in *dMi-2/Pc* transheterozygotes than in adults heterozygous for *Pc* alone (Fig. 3C), which is consistent with more extensive derepression of *Scr* in the double mutant. These results are further evidence that *dMi-2* acts together with PcG proteins to repress HOX genes.

Previous studies led us to propose that Hb directly or indirectly recruits PcG proteins to DNA to establish PcG silencing of homeotic genes (*5*, *26*). Our present data suggest that dMi-2 might function as a link between Hb and PcG repressors. Although dMi-2 contains two motifs with similarity to DNA-binding domains (the myb and HMG domains), dMi-2 does not seem to bind to DNA on its own. Therefore, Hb may recruit dMi-2 to DNA. *Xenopus* Mi-2 was recently purified as a subunit of a histone deacetylase complex (*27*) with nucleosome remodeling activity. In yeast and in vertebrates, several transcription factors repress transcription by recruiting histone deacetylases (*28*). It is possible that in *Drosophila*, nucleosome remodeling and deacetylase activities of a dMi-2 complex—recruited to homeotic genes by Hb—may result in local chromatin changes that allow binding of PcG proteins to the nucleosomal template. Alternatively, the proposed hb–dMi-2 complex might directly bind a PcG protein and recruit it to DNA. Finally, the involvement of dMi-2 in PcG silencing suggests that this process may involve deacetylation of histones.



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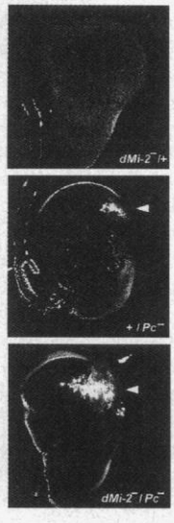

C

mutation X	mut.X/Pc^4	Pc^4/Bal	enhancement
dMi-2^1	291	49	5.9
dMi-2^2	320	107	3.0
dMi-2^3	250	104	2.4
dMi-2^4	168	58	2.9
dMi-2^5	186	86	2.2
dMi-2^6	267	144	1.9
dMi-2^7	120	40	3.0
dMi-2^8	157	44	3.6
dMi-2^9	393	169	2.3
Pcl^{10}	824	134	6.1
Trl^{62}	120	121	1.0
Trl^3	48	91	0.5
Trl^{P2088}	275	267	1.0
l(3)76BD d^3	11	29	0.4
l(3)76BD d^4	244	202	1.2
l(3)76BD o^1	97	157	0.6
l(3)76BD o^2	88	112	0.8
l(3)76BD s^1	66	146	0.5
l(3)76BD s^2	45	133	0.3
l(3)76BD t^1	20	58	0.3

Fig. 3. *dMi-2* and PcG genes synergize to repress homeotic genes. (**A**) Thirteen- to sixteen-hour-old embryos stained for Ubx (top row, side views) or Abd-B protein (middle row, ventral views of central nervous system). No derepression of either *Ubx* or *Abd-B* is seen in *dMi-2* mutants. Many more cells misexpress Ubx and Abd-B in *Psc dMi-2* double mutants compared with *Psc* mutants (arrowheads). Not all cells that misexpress Ubx are seen in these focal planes. The embryonic cuticle pattern of *dMi-2* mutants is indistinguishable from wild-type cuticles (bottom row, left). Stronger homeotic transformations of the thoracic denticle belts (arrowheads) were observed in *dMi-2 Pc* double mutants compared with *Pc* mutants (the Pc^{e9} mutation used here is a hypomorph). *dMi-2* alleles were *dMi-2*^{4} (top row), *dMi-2*^{6} (middle row), and *dMi-2*^{9} (bottom row). (**B**) Derepression of *Ubx* in wing discs of third instar larvae with the indicated genotype. In *dMi-2* heterozygotes, like in wild-type, Ubx protein is not expressed in wing discs. Wing discs of *Pc* heterozygotes show derepression of *Ubx* (arrowhead) that is enhanced in *dMi-2/Pc* transheterozygotes (on average, three times as many cells express Ubx). (**C**) Homeotic transformations due to derepression of *Scr*. Each number in the second and third columns is the total number of sex comb teeth on second and third legs of 20 sibling males of the genotypes indicated. The ratio of the numbers in the second and third columns shows the enhancement caused by the mutation X. All *dMi-2* mutations enhance the transformation comparable to a *Pcl* mutation. Mutations in *Trithorax-like* (*Trl*), the gene encoding the GAGA factor, do not enhance this transformation.

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21. Biotinylated probes were used for in situ hybridization on salivary gland chromosomes. Fine mapping of dMi-2 in the 76-dalton region was done by polymerase chain reaction (PCR) on single embryos from deficiency stocks as described by J. A. Knoblich and C. F. Lehner [*EMBO J.* **12**, 65 (1993)]. *dMi-2$^{4\text{-}9}$*, *l(3)76BDd^3*, *l(3)76BDd^4*, *l(3)76BDo^1*, *l(3)76BDo^2*, *l(3)76BDo^4*, *l(3)76BDo^5*, *l(3)76BDs^1*, *l(3)76BDs^2*, and *l(3)76BDt^1* were all ethylmethane sulfonate (EMS)–induced [E. B. Lewis and F. Bacher, *Dros. Inf. Service* **43**, 193 (1968)] on the same isogenized *red e* chromosome. *dMi-2^1* was isolated in a different screen (J. Kennison, unpublished data); *dMi-2^2* and *dMi-2^3* are P-element–induced mutations that correspond to l(3)A154.3M3 and l(3)j3D4, respectively. Details on the mapping of these mutations can be found at www.sciencemag.org/feature/data/984280.shl and in flybase.
22. Larvae that were homozygous for *l(3)76BDd^4*, *l(3)76BDo^2*, *l(3)76BDo^5*, *l(3)76BDt^1*, *dMi-2^4*, *dMi-2^5*, and *dMi-2^6* were identified by the *red* marker mutation on the mutant chromosome. We isolated DNA from such larvae and amplified the genomic DNA spanning the *dMi-2* ORF from codons 26 to 1982 by PCR. Four overlapping subfragments covering this interval were amplified by PCR and subcloned into Bluescript, and two independent clones were sequenced in each case. We only found sequence alterations in the case of the *dMi-2* alleles (see text). We note that the exon or exons containing the 132-nt 5′ untranslated region and first few codons appear to be separated from the other exons by a very large intron because we were unable to amplify a genomic fragment with appropriate primers.
23. *dMi-2$^{1\text{-}7}$* were each recombined onto an *FRT2A* chromosome. Germ line clones were induced with the Flp *ovo^{D1}* system. A rescue transgene that expresses the dMi-2 protein under the control of the *armadillo* promoter (*arm-dMi-2*) was constructed by substitution of the *Pc* cDNA in *arm-Pc* [J. Müller, S. Gaunt, P. A. Lawrence, *Development* **121**, 2847 (1995)] with a full-length *dMi-2* cDNA. For the rescue test, we generated females *hs-flp/+*, *arm-dMi-2/+*, and *dMi-2^4 FRT2A/ovo^{D1}FRT2A*. The *armadillo* promoter is strongly active in the germ line, and the *arm-dMi-2* transgene rescued the germ-cell lethality due to *dMi-2* mutations. However, *dMi-2* homozygotes or transheterozygotes carrying the transgene were not rescued to adults. Somatic clones were induced in the first larval instar with the Flp/FRT system and appropriately marked chromosomes. No *dMi-2* mutant clone tissue was found in the adult epidermis compared with wild-type control clones. This suggests that dMi-2 mutant cells either die or are eliminated because of "cell competition." Imaginal discs carrying marked *dMi-2* mutant clones were stained with antibodies to Ubx or Abd-B, but no misexpression of these genes was detected. We found that 96 hours after clone induction the *dMi-2* mutant clones were substantially smaller (often they had completely disappeared) compared with the wild-type "twin spot" clones induced by the same recombination event. The same results were obtained with several *dMi-2* alleles.
24. For double-mutant combinations of *dMi-2* with PcG genes, we used *Psc^{e24}*, *Pc^{XT109}*, *Pcl^{D5}*, and an EMS-induced hypomorphic *Pc* mutation, *Pc^{e9}*. *dMi-2^9* homozygotes were identified by the linked *red* marker mutation. *dMi-2* homozygous embryos were identified with balancer chromosomes carrying a LacZ marker gene. *dMi-2^4/Pc^{XT109}* larvae were identified with appropriately marked balancer chromosomes. Standard procedures were used for staining with antibodies to Ubx, Abd-B, and β-galactosidase.
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Requirement of RSF and FACT for Transcription of Chromatin Templates in Vitro

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Transcription of naked DNA in vitro requires the general transcription factors and RNA polymerase II. However, this minimal set of factors is not sufficient for transcription when the DNA template is packaged into chromatin. Here, a factor that facilitates activator-dependent transcription initiation on chromatin templates was purified. This factor, remodeling and spacing factor (RSF), has adenosine triphosphate–dependent nucleosome-remodeling and spacing activities. Polymerases that initiate transcription with RSF can only extend their transcripts in the presence of FACT (facilitates chromatin transcription). Thus, the minimal factor requirements for activator-dependent transcription on chromatin templates in vitro have been defined.

Intense biochemical efforts have resulted in the purification of a minimal set of factors necessary for transcription of class II genes in vitro. This minimal transcription system consisting of the general transcription factors (GTFs) TFIIB, TFIID, TFIIE, TFIIF, and TFIIH and RNA polymerase II (RNAPII) was established with assays that reconstituted accurate transcription from class II promoters on naked DNA templates (*1*, *2*). In vivo, the DNA template is organized by histones into chromatin. The minimal RNAPII transcription system cannot transcribe DNA that is packaged into chromatin (*3*). However, transcription can be reconstituted on chromatin templates with nuclear extracts in the presence of an activator, suggesting that accessory factors are present in crude nuclear extracts that facilitate RNAPII transcription from chromatin templates (*4*).

Different adenosine triphosphate (ATP)–dependent nucleosome-remodeling complexes have been isolated. The NURF (nucleosome-remodeling factor) and ACF (ATP-utilizing chromatin assembly and remodeling factor) complexes were purified from *Drosophila* nuclear extracts and have been shown to facilitate activator-dependent transcription on chromatin templates (*5*, *6*). Several other ATP-dependent chromatin remodeling enzyme complexes have been purified from different organisms: SWI/SNF from yeast, human, and *Drosophila*; RSC (remodels the structure of chromatin) from yeast; and CHRAC (chromatin accessibility complex) from *Drosophila* (*7*). Biochemical studies with the human and yeast SWI/SNF complexes have suggested that it may

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