MOLECULAR AND CELLULAR BIOLOGY, Feb. 2002, p. 1218–1232 Vol. 22, No. 4
0270-7306/02/$04.00+0 DOI: 10.1128/MCB.22.4.1218–1232.2002

The AT-Hook Protein D1 Is Essential for *Drosophila melanogaster* Development and Is Implicated in Position-Effect Variegation

Nathalie Aulner,[1]† Caroline Monod,[1] Guillaume Mandicourt,[1] Denis Jullien,[1] Olivier Cuvier,[2] Alhousseynou Sall,[1] Sam Janssen,[2] Ulrich K. Laemmli,[2] and Emmanuel Käs[1]*

Laboratoire de Biologie Moléculaire Eucaryote, CNRS UMR 5099, 31062 Toulouse Cedex, France,[1] *and Department of Molecular Biology, University of Geneva, CH1211 Geneva 4, Switzerland*[2]

Received 24 September 2001/Returned for modification 15 November 2001/Accepted 26 November 2001

We have analyzed the expression pattern of the D1 gene and the localization of its product, the AT hook-bearing nonhistone chromosomal protein D1, during *Drosophila melanogaster* development. D1 mRNAs and protein are maternally contributed, and the protein localizes to discrete foci on the chromosomes of early embryos. These foci correspond to 1.672- and 1.688-g/cm^3 AT-rich satellite repeats found in the centromeric heterochromatin of the X and Y chromosomes and on chromosomes *3* and *4*. D1 mRNA levels subsequently decrease throughout later development, followed by the accumulation of the D1 protein in adult gonads, where two distributions of D1 can be correlated to different states of gene activity. We show that the EP473 mutation, a P-element insertion upstream of D1 coding sequences, affects the expression of the D1 gene and results in an embryonic homozygous lethal phenotype correlated with the depletion of D1 protein during embryogenesis. Remarkably, decreased levels of D1 mRNA and protein in heterozygous flies lead to the suppression of position-effect variegation (PEV) of the *white* gene in the *white*-mottled (w^{m4h}) X-chromosome inversion. Our results identify D1 as a DNA-binding protein of known sequence specificity implicated in PEV. D1 is the primary factor that binds the centromeric 1.688-g/cm^3 satellite repeats which are likely involved in *white*-mottled variegation. We propose that the AT-hook D1 protein nucleates heterochromatin assembly by recruiting specialized transcriptional repressors and/or proteins involved in chromosome condensation.

In recent years, HMG-I/Y proteins have received considerable attention since the identification of their role in promoting the assembly of multiprotein complexes ("enhancesomes") involved in transcriptional activation or of functional nucleoprotein complexes, such as the human immunodeficiency virus type 1 (HIV-1) preintegration complex. These diverse biological functions have been recently reviewed (43). HMG-I/Y, now referred to as HMGA1a and HMGA1b and collectively as HMGA proteins (see reference 43 for nomenclature), interacts with DNA via repeated AT hook motifs which specifically recognize AT-rich sequences. This motif, which binds the deep and narrow minor groove of dA•dT sequences, constitutes a nonclassical example of a DNA-binding domain which can recognize a remarkably broad spectrum of sequences based on structural constraints rather than those imposed by specific nucleotide arrays (44). The AT hook domain is repeated three times in members of the HMGA family of mammalian proteins (44). HMGA1a and HMGA1b constitute the archetypes for multi-AT hook (MATH) proteins that were initially characterized as a component of alpha-satellite arrays of the African green monkey (53). In spite of remarkable advances in our understanding of how HMGA might be implicated in regulatory mechanisms affecting gene expression, its localization in satellite repeats found in heterochromatin and its role there have not been analyzed in detail.

A recent survey reported on the existence of upwards of 100 proteins from different species containing the AT-hook DNA-binding domain (4). Its remarkable evolutionary conservation and its presence in a large number of different proteins suggest that it fulfills an important role in targeting varied protein functional domains to AT-rich sequences of the genome, such as those found in scaffold-associated regions (SARs) (34), satellite repeats (35, 36, 53), or upstream gene regulatory regions (58). It is also found as single or moderately repeated units associated with other DNA-binding domains (10), including zinc fingers (7; GenBank accession no. AJ002056 [unpublished data]). In this case, the AT hook might provide a secondary association with a neighboring AT-rich sequence, possibly stabilizing an interaction primarily mediated by another DNA-binding domain.

While HMGA proteins are highly conserved in vertebrates, the only known similar MATH protein found in *Drosophila melanogaster* is the D1 nonhistone chromosomal protein (2). D1 was one of the first sequence-specific DNA-binding proteins identified in *Drosophila* (28) and was reported to be associated in vitro with the 1.688-g/cm^3 satellite III (SATIII) repeats (2, 35) located in the centromeric heterochromatin of the *Drosophila* X-chromosome (27). Interestingly, this highly repeated satellite possesses an alpha-like sequence related to repeats found in mammalian species, including the African green monkey satellite that serves as a major binding site for HMGA proteins (35). Based on this localization, the D1 protein might thus be the orthologue of HMGA in *Drosophila*, where it could be implicated in similar functions. However, few

* Corresponding author. Mailing address: LBME-CNRS UMR 5099, 118 route de Narbonne, 31062 Toulouse Cedex 4, France. Phone: 33 (0) 5 61 33 59 59. Fax: 33 (0) 5 61 33 58 86. E-mail: kas@ibcg.biotoul.fr.

† Present address: Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, St. Louis, MO 63104.

studies have addressed the possible role of D1 in this organism. While its DNA-binding characteristics have been analyzed (36), its cDNA has been cloned (5), and the protein has been tentatively localized (1, 2), nothing is known of its developmental expression profile and of its biological function. Such studies have been hindered by the lack of mutations affecting the D1 gene.

Two studies have addressed the localization of D1 in *Drosophila* polytene chromosomes (1, 2), confirming a predominant association with heterochromatin but yielding few functional correlates. The precise localization of the protein has been difficult to ascertain due to the under-replication of satellite sequences in polytene nuclei. More recently, immunolocalization of D1 in embryos showed that it is associated with chromatin from the earliest stages of development (45). In vitro binding studies have established specific binding of D1 to SATIII repeats and to the 1.672-g/cm^3 AATAT simple-sequence satellite I (SATI) repeats (36). Dispersed SATIII-related sequences are also found in euchromatin (18), and these sequences could function as potential targets for D1. Thus, centromeric heterochromatin might serve primarily as a storage site from which, depending on the concentration of the protein, D1 might be dispatched to a select number of targets in euchromatin.

Such a potential dual targeting of D1 could reflect different cellular functions. Its association with alpha-satellite heterochromatin suggests a possible role in long-range chromosome architecture and/or heterochromatin-mediated transcriptional inactivation. In the case of mammalian HMGA1a/1b, its localization to alpha-satellite repeats could reflect a similar function in chromatin structure and function, while its presence in euchromatin would fit its proposed general role in transcriptional activation. A dual localization of HMGA and D1 proteins in both heterochromatin and euchromatin might thus be attributed to the existence of distinct nuclear subpopulations of these proteins. Such a possibility is in agreement with our finding that three populations of HMGA1a/1b of distinct properties exist in mammalian cells (3).

We have recently reported on a role for HMGA1a in general transcriptional activation in early mouse embryos (8), an effect directly attributable to the interaction of its three AT hooks with AT-rich target sequences. Interestingly, HMGA1a is the progenitor for the MATH-20 protein, a synthetic sequence containing 20 AT hooks repeated in tandem, which has been shown to have profound effects on chromosome structure and dynamics (54) and, more recently, on gene expression. In the latter case, targeting MATH-20 expression to *Drosophila* eyes resulted in a strong suppression of position-effect variegation (PEV) of the *white* gene in the *white*-mottled (w^{m4h}) inversion (23), which places the gene near SATIII repeats (56). More recently, synthetic polyamides that target the minor grove of AT-rich sequences with remarkable specificity were shown to induce gain-of-function phenotypes in *Drosophila* (29, 30). In this case, treatment of flies with P9, a molecule specific for SATI and SATIII sequences, led to a suppression of w^{m4h} PEV. The suppression of w^{m4h} PEV by both MATH-20 and P9 could be due to the displacement of an endogenous factor involved in heterochromatin-mediated repression, thus promoting higher expression levels of the *white* gene. Because SATIII DNA repeats are likely to mediate the inactivation of the *white* gene in the w^{m4h} inversion, D1 is a prime candidate for being this factor.

To test this hypothesis, we implemented a systematic study of D1 expression and localization during *Drosophila* development, seeking functional correlates that might relate it to heterochromatin-mediated effects on transcription. We report here that D1, a maternally contributed protein, is indeed primarily associated with SATI and SATIII heterochromatin throughout the cell cycle during embryonic and larval stages and is essential to *Drosophila* development. In support of the hypothesis that D1 associated with X-chromosome SATIII repeats might be the factor primarily responsible for the PEV observed in the w^{m4h} inversion, we show that the EP473 P-element insertion affects the expression of the D1 gene and results in the suppression of w^{m4h} PEV.

MATERIALS AND METHODS

Fly strains and culture. Flies were grown at 22°C on standard cornmeal-glucose-yeast medium. An OregonR laboratory stock was used as a wild-type control strain. The balanced EP(3)0473/TM3Twist(LacZ) fly strain, designated EP473 in this study, was obtained from the Rorth EP collection (48). A stock of EP473 heterozygotes balanced over TM3 or TM6b was crossed to wild-type flies. The resulting F_1 progeny were allowed to mate and yielded F_2 flies, of which one quarter were EP473 homozygous embryos which failed to develop. These were analyzed from 1 to 4 days after the hatching of the F_2 wild-type and heterozygous progeny (see Fig. 2). Alternatively, EP473 stocks were allowed to mate, yielding viable heterozygotes and nonviable homozygous progeny carrying two TM3 balancer chromosomes or two EP473 alleles. The latter were recovered by forced hatching in bleach as described in the text, and their identities were confirmed by β-galactosidase staining (see below).

To make a P-element construct carrying the D1 gene under the control of the hsp70 promoter, the D1 cDNA was inserted in the multiple cloning site of a pINDY vector carrying a mini-*white* marker provided by L. Seroude (51). A D1 cDNA encoding a truncated form of the protein lacking the 66 C-terminal amino acids, D1ΔE, was similarly cloned into pINDY. The DNA sequence encompassing the D1 genomic region (GenBank accession number DMU56393) is that reported by Glover et al. (unpublished data). Sequence information for the EP473 insertion (accession number AQ02S091) was obtained from Janos Szidonya (Szeged, Hungary). The D1 cDNA sequence used to construct D1 transgenes was according to GenBank sequence accession number JO472S. A *white*$^-$ laboratory strain was used as recipient for injections. P-element-mediated germ line transformation was done using standard procedures (49). For each construct, multiple independent lines were established, and the chromosomal location of the transgene was determined by standard genetic analysis using balancer chromosomes. Expression of the D1 or D1ΔE transgenes on the X chromosome and on chromosome *2*, respectively, was induced by two 1-h heat shocks (37°C) during early pupation of flies cultured at 18°C.

The w^{m4h} inversion [ln(I)wm^{4h}] was used to score the effects of the EP473 insertion and of D1 transgenes on *white* expression. Eye pigments from 5-day-old female flies were extracted and measured as previously described (23), using samples of 30 heads from at least three independent crosses. Pigment values contributed by the mini-*white* gene carried by one of the parents were subtracted from the value measured in the progeny. Results were subjected to the χ^2 test and the differences shown in Fig. 7 were statistically significant ($P < 0.05$). We found no effect of the TM3 balancer chromosome on the phenotype of w^{m4h} flies under the conditions used (data not shown). The eye colors of the parents were also measured with or without prior heat shock, yielding no significant difference in eye color. Photographs were taken using Leica MZFLIII binoculars equipped with a Spot Insight Color charge-coupled device (CCD) camera. Partial rescues were performed after recovery of EP473 homozygotes from EP473/TM6b (*tubby*) heterozygotes carrying a heat shock-inducible D1 transgene on the X chromosome. EP473/EP473 embryos were grown at 22°C with two daily 1-h heat shocks or continuously at 30°C, yielding larvae beyond the normal late embryonic stage of lethality. Non-*tubby* larvae were never recovered from stocks that did not carry the HS-D1 transgene.

Northern blot analysis. Messenger RNAs were purified from each developmental stage shown in Fig. 1 by using the Straight A's mRNA isolation system (Novagen). Samples containing 2 μg of poly(A)$^+$ RNA were separated on a 1% agarose/formaldehyde gel and transferred onto a Hybond N$^+$ filter (Amersham).

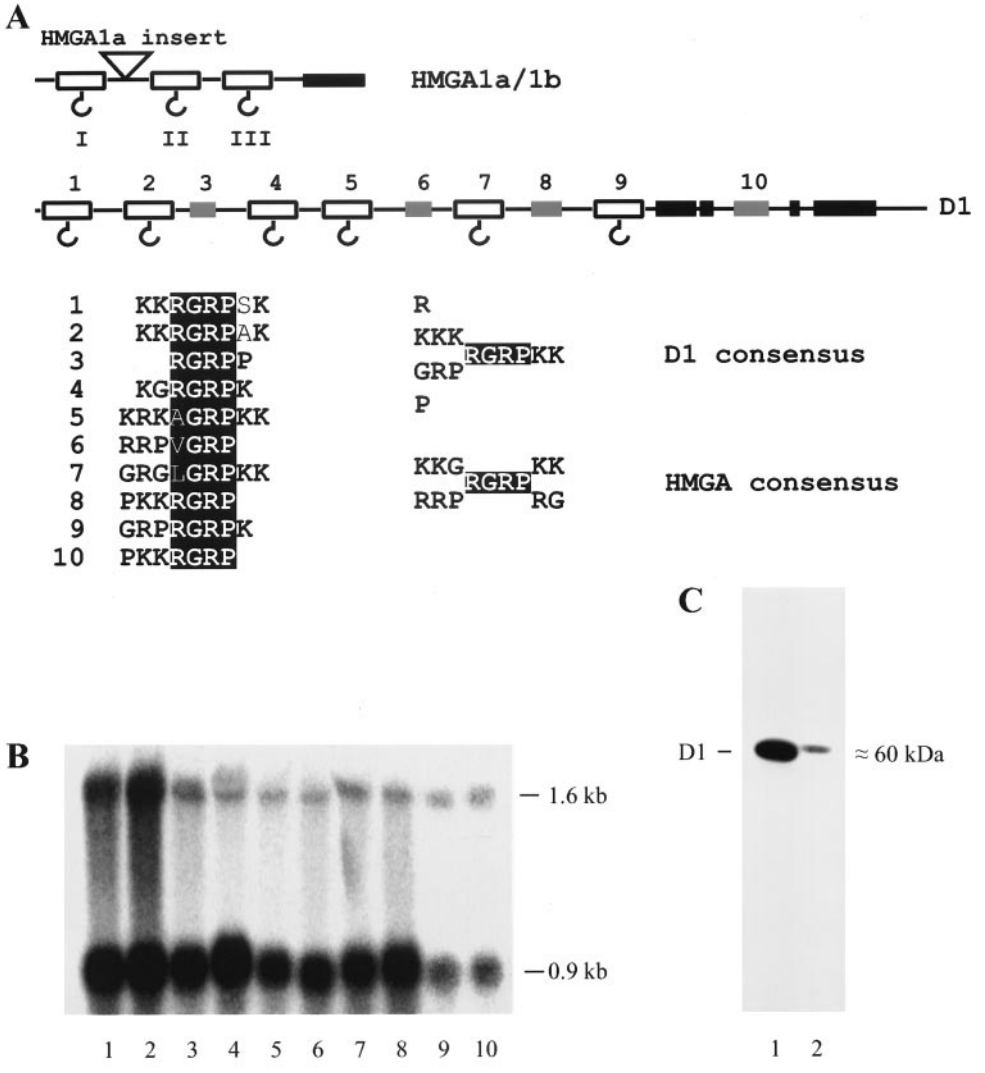

FIG. 1. Developmental expression profile of the D1 gene. (A) Diagrams representing the primary structures of the 11-kDa HMGA1a and the 37-kDa D1 proteins. HMGA proteins contain three AT hooks (I, II, and III) represented by boxes with a hook and a short acidic C-terminal domain (black box). An 11-amino-acid insert generated by alternative splicing of the HMGA mRNA is absent in HMGA1b and present in HMGA1a. The AT hook-like sequences of D1 (5) are numbered from 1 to 10 and are represented by boxes with hooks for full-length or near full-length motifs and by gray boxes for shorter sequences containing the conserved GRP core motif. Acidic C-terminal domains are represented by filled boxes. The corresponding AT hook sequences are shown below the map and can be used to extrapolate a D1 AT hook consensus. The sequence of the human HMGA AT hook consensus is also shown. (B) poly(A)$^+$ mRNA from flies at various developmental stages was hybridized to labeled D1 and RPL17 probes which detect 1.6- and 0.9-kb bands, respectively. poly(A)$^+$ mRNA was purified from 0- to 2-h embryos (lane 1), 0- to 10-h embryos (lane 2), 10- to 20-h embryos (lane 3), first-, second-, and third-instar larvae (lanes 4 to 6, respectively), pupae (lane 7), adult males (lane 8), adult females (lane 9), and dissected ovaries (lane 10). (C) Western blot of protein extracts prepared from Kc cells (lane 1) or 0- to 20-h embryos (lane 2) probed with an antibody raised against the D1 protein.

The blot was hybridized to the ^{32}P-labeled D1 whole cDNA or to the RPL17 probe (42) as previously described (31).

Western blotting, immunostaining, and in situ hybridization. A rabbit polyclonal antibody was raised against the recombinant D1 protein and affinity purified against a preparation of recombinant D1ΔE. Nuclei from Kc tissue-culture *Drosophila* cells and protein extracts from whole embryos or first-instar larvae were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5 to 15% acrylamide gradient gel and transferred onto a Hybond C-super membrane (Amersham). Filters were blocked for 1 h in 5% nonfat milk in TBS and incubated overnight at 4°C with the affinity-purified primary antibody at a 1:100 dilution in 2% nonfat milk–0.05% Tween 20 in TBS. The secondary antibody was an alkaline phosphatase-conjugated anti-rabbit antibody (Promega) used at a dilution of 1:5,000.

In situ D1 immunostaining was performed on embryos, dissected larvae, and adult gonads, fixing for 20 min in 4% formaldehyde according to standard procedures. After a 1-h preincubation in PAT (phosphate-buffered saline [PBS], 1% bovine serum albumin [BSA], 0.1% Triton X-100), the primary antibody (anti-D1; 1:200 dilution) was added and incubation was allowed to proceed overnight at 4°C in the same buffer. Two different procedures were used to reveal the primary antibody. For peroxidase staining, biotinylated anti-rabbit antibodies and related reagents were purchased from Vector Laboratories. For immunofluorescence staining, fluorescein isothiocyanate (FITC)- or tetramthyl rhodamine isocyanate (TRITC)-coupled anti-rabbit antibodies (Sigma) were used at a dilution of 1:5,000. DNA was detected by propidium iodide staining (10 μg/ml) in the last wash following RNase treatment (1 mg/ml in PBS for 2 h at 37°C) or with DAPI (4′,6′-diamidino-2-phenylindole) added to the final mounting medium at 50 ng/ml. Staining with fluorescein-labeled olygopyrrole Lex9F (30) was performed in solution P (PBS containing 1 mM $MgCl_2$ and 0.1% Triton X-100) for 10 min at room temperature at a final concentration of 200 nM. Samples were then washed five times in solution P before mounting in the presence of DAPI.

A 1-kb cDNA corresponding to the D1-coding region cloned in the pBSKS vector was linearized with *Hin*dIII and transcribed with T7 RNA polymerase to generate a digoxigenin-labeled antisense RNA probe. EP473 flies were aged for 10 h at 20°C before use in experimental procedures. EP473 homozygous embryos were recovered from a balanced stock carrying the β-galactosidase gene. The D1 mRNA was detected according to the procedure described previously (57), and *Twist*-driven β-galactosidase expression was revealed by peroxidase staining as described above.

Brains from third-instar larvae were dissected in 0.7% saline and incubated for 2 h in saline containing 10 μM colchicine. Following a 15-min hypotonic shock in 0.5% sodium citrate, brains were fixed for 1 min in 45% acetic acid, squashed, and processed for D1 immunostaining as above, except that incubation with the primary antibody was performed for 2 h at room temperature. Samples were stained with Lex9F and mounted as above. Fixation with 4% formaldehyde yielded similar results, but the shorter acetic acid fixation was preferred in these experiments because of better preservation of chromosome morphology.

Confocal microscopy was performed on a Zeiss LSM410 apparatus using 510-to-525-nm and 590-nm filters with argon (488 nm) and helium/argon (543 nm) lasers for excitation, respectively. Images were recorded using the LSM software and processed using Adobe Photoshop. Conventional fluorescence microscopy was performed on a Leica DMRB microscope equipped with UV, FITC, and TRITC filters, using a 100× objective (NA 1.25). Images were captured using a CoolSnap cooled CCD camera and processed in Photoshop.

RESULTS

Temporal and spatial regulation of D1 gene transcription. While the D1 protein of *D. melanogaster* has been known for many years (1, 2) and the cDNA encoding it has been cloned and characterized (5), its function is not known. Based on the presence of repeated AT-hook motifs, D1 might be the functional homologue of the mammalian HMGA proteins (Fig. 1A). As the HMGIA gene is preferentially expressed in proliferating undifferentiated cells and largely absent in terminally differentiated tissues (22), the D1 gene of *D. melanogaster* might similarly exhibit a temporal or spatial regulation of its expression. We addressed this question by performing Northern blot analyses using *Drosophila* mRNAs extracted at different developmental stages. As shown in Fig. 1B, a probe corresponding to the D1 cDNA detected a single 1.6-kb mRNA species in all samples tested. Using the RPL17 ribosomal protein mRNA that is expressed at roughly constant levels throughout development as an internal control (42), we observed varying levels of D1 mRNA starting with 10-h embryos (lanes 3 to 10). Before 10 h, high levels of the transcript could be detected, particularly at 0 to 2 h of development (lanes 1 and 2). Because zygotic transcription has not begun at this early stage, we conclude that these D1 mRNAs are maternally contributed.

In late (10 to 20 h) embryos, D1 mRNA levels decreased sharply (lane 3) and remained low throughout larval development, in pupae and in adult males (lanes 4 to 8). This decrease might be due to a general decrease in D1 gene transcription, or to its restriction to a small subset of tissues. Indeed, the higher mRNA levels detected in adult females (lane 9) could be correlated to preferential expression in the ovaries (lane 10). This suggests that spatial, as well as temporal, regulation of D1 gene transcription can account for the variations observed in D1 mRNA levels during development.

Using a polyclonal rabbit antibody raised against the recombinant D1 protein, we performed Western blot analyses to ascertain the presence of the protein in *Drosophila* embryos. A sample containing total embryonic proteins was electrophoresed alongside a nuclear extract prepared from *Drosophila* Kc tissue culture cells. In both cases, our antibody detected a single band with an apparent molecular mass of 55 kDa, consistent with the known abnormal mobility of the D1 protein in SDS gels (Fig. 1C). These bands comigrated with the purified, bacterially expressed, recombinant protein (data not shown), although a small difference in mobility could be observed when comparing the recombinant protein and that detected in Kc nuclei with the endogenous species found in embryos, the latter migrating with a slightly reduced mobility (compare lanes 1 and 2 of Fig. 1C and see Fig. 2C).

D1 protein is essential for *Drosophila* development. The maternal contribution of D1 mRNA suggested the possibility that D1 might play a role during *Drosophila* development. A functional analysis of D1 has been hampered by the lack of mutations or deletions in the 85D1/D2 cytological region harboring the D1 coding sequence. However, a systematic P-element-mediated mutagenesis of the third chromosome of *Drosophila* (48) recently yielded a P-element insertion in this region. Based on the reported homozygous lethal phenotype and molecular data indicating an insertion point very close to the D1-encoding gene, we obtained the corresponding mutant, denoted EP473, and analyzed it for evidence of a defect in D1 expression.

As shown in Fig. 2A, the P-element insertion site maps to the 5′ noncoding region of the D1 gene, at position −66 relative to the 5′ end of the mRNA. Another transcript, *Pumilio*, is located downstream of the D1 gene (6), approximately 3 kb from the insertion point, but the reported EP473 phenotype does not match that of *pumilio* mutants, which present defects chiefly in oogenesis. The EP473 insertion has been characterized as an embryonic lethal in that no homozygous larvae are ever recovered from a cross. This suggests that D1 is required for normal embryonic development, assuming that it is the only gene affected in the EP473 strain. We verified that mobilization of the P element resulted, in most cases (>90%), in a clean excision and restoration of a wild-type phenotype, thus establishing that the lethal phenotype was indeed due to the insertion. Furthermore, partial rescue of the embryonic lethal phenotype was ascertained by the recovery of non-*tubby* homozygous EP473 larvae from crosses of EP473 heterozygotes carrying a TM6b chromosome (see Materials and Methods), an effect which could be shown to require the presence of an inducible D1 transgene (data not shown). Thus, the only defect attributable to the EP473 insertion implicates expression of the D1 gene.

To characterize the mutant phenotype further, we set out to collect late homozygous embryos for analysis (see Materials and Methods) and processed them for the presence of D1 mRNA and protein. To our surprise, dechorionation of these presumably dead embryos resulted in vigorously swimming first-instar larvae that failed to develop any further. EP473 homozygotes thus complete embryogenesis but do not hatch or develop further. The unhatched embryos or first-instar larvae could be kept for as long as 3 to 4 days and still yield swimming larvae after being placed in dilute bleach for a few seconds. This places the defect at relatively late stages of embryonic development, which are most likely attained by use of a maternally provided pool of D1 mRNA and protein (Fig. 1). We could detect no obvious abnormalities beyond the failure of the embryos to hatch and develop further and the observation that

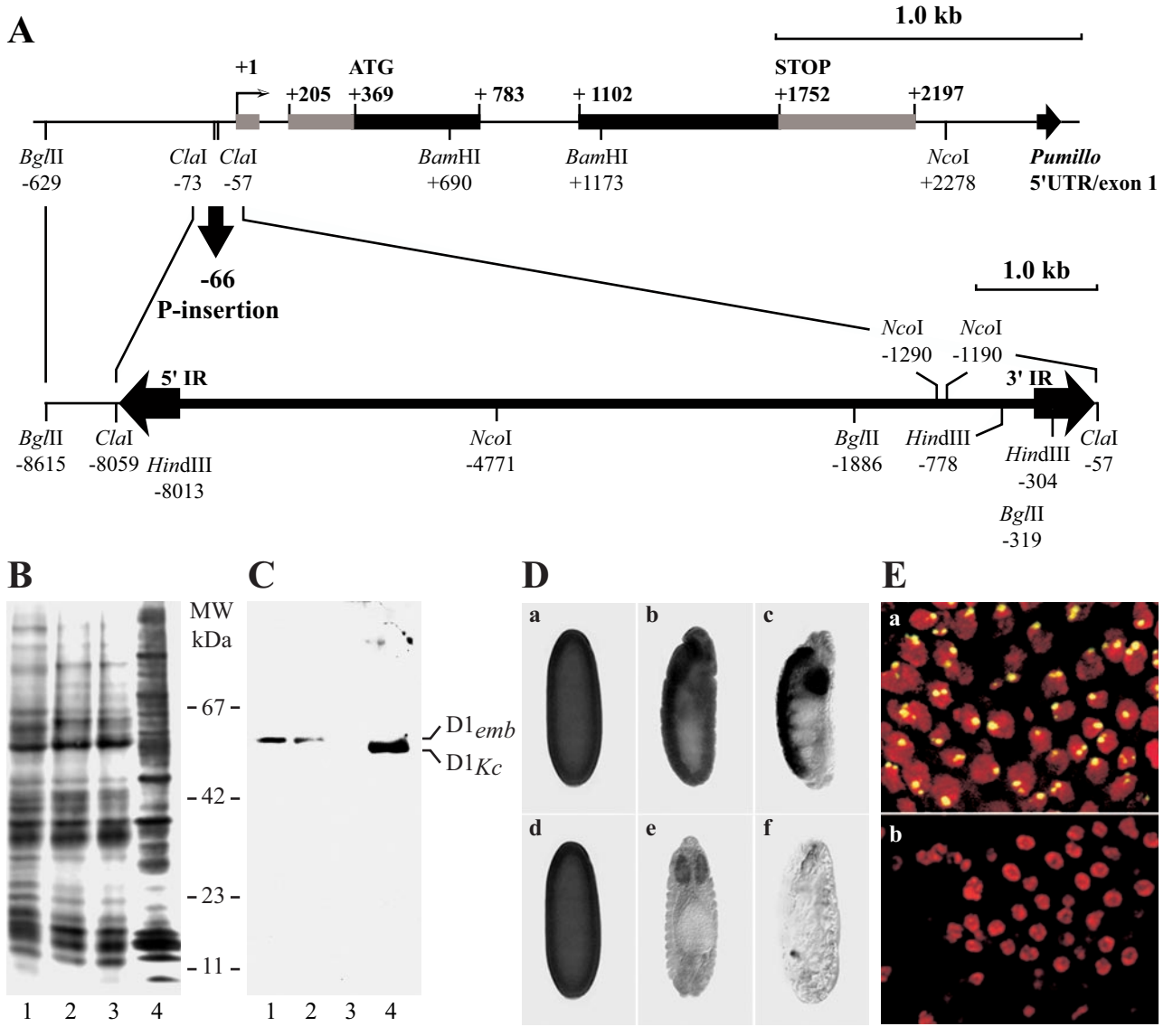

FIG. 2. The EP473 P-element insertion results in down-regulation of D1. (A) Map of the genomic D1 locus. Sequence coordinates are given relative to the start of the D1 mRNA (+1). Exons are represented by black boxes and 5′ and 3′ untranslated regions are in gray. Restriction enzyme sites shown are those used to characterize the EP473 P-element insertion and excisions thereof. The P-element insertion diagrammed below the map occurs at position −66. The thick line indicates the extent of the transposon bounded by 5′ and 3′ inverted repeats (IR; divergent filled arrows). (B) Coomassie blue-stained gel loaded with equivalent amounts of total proteins from wild-type, heterozygous, or homozygous EP473 mutant first-instar larvae and from Kc nuclei (lanes 1 to 4, respectively). A duplicate gel was subjected to Western blot analysis as described above (C). The positions of the D1 bands observed in embryos and tissue-culture cells are indicated to the right. (D) In situ hybridizations to D1 mRNA were performed with wild-type (a to c) and homozygous (d to f) EP473 embryos identified as described in Materials and Methods. (E) Wild-type larvae and homozygous EP473 force-hatched first-instar larvae (see text for details) were processed for DNA staining (red) and D1 immunolabeling (green). Merged signals are shown for wild-type and mutant larval nuclei. Note the smooth appearance of the nuclei in the absence of D1, the smaller space occupied by chromatin and the overall absence of differentially condensed regions of chromatin relative to wild-type nuclei. Bar = 5 μm.

the EP473 homozygous larvae obtained after forced hatching in bleach did not grow further and all died within 24 h.

Taken together, these data suggested a transcriptional defect induced by the EP473 insertion, most likely a down-regulation of the D1 gene or/and alteration of its regulation. We verified this possibility by performing in situ hybridization and Western blot experiments to detect possible changes in D1 mRNA and protein levels in heterozygous and homozygous EP473 embryos. In agreement with the hypothesis that the P-element insertion affects expression of the D1 gene, protein levels were found to be decreased by 30 to 40% in heterozygous first-instar larvae (Fig. 2C, compare lanes 1 and 2). We were also able to test for the presence of the D1 protein in late homozygous embryos or first-instar larvae: in this case, D1 protein levels were barely detectable relative to wild-type or heterozygous flies (lane 3). Note that, in both cases, the migration of the D1-positive band detected in embryos and larvae was again found to be altered relative to that in nuclear extracts from Kc tissue-culture cells. This difference in mobilities is most likely due to a posttranslational modification of D1 in

vivo that is absent ex vivo or lost during the preparation of nuclei from tissue culture cells.

We also performed in situ hybridizations against D1 mRNA in embryos at various stages of development. As can be seen from the examples shown in Fig. 2D, wild-type early embryos (panels a to c) stained very strongly for D1 transcripts, in agreement with the maternal contribution of the mRNA. Later during development, as accumulated D1 mRNA levels decreased (Fig. 1B), the hybridization signal became weaker and less ubiquitously distributed and a preferred localization to the embryonic nervous system was particularly evident at late stages (c) but was not investigated further. Figure 2D, panels d to f, show D1 mRNA hybridization patterns in EP473 homozygotes. While D1 transcripts were readily detected in heterozygotes (data not shown), levels were markedly lower in homozygotes, where a strong signal could be observed through early embryonic stages (d) but dropped precipitously in older embryos (e) and was no longer detectable in embryos about to hatch (f). These results are consistent with the hypothesis that the effect of the P-element insertion in the 5′ noncoding region of the D1 gene is primarily transcriptional, with the maternal contribution of D1 transcripts accounting for the positive signal seen in early homozygous embryos and its dilution at later stages of embryogenesis.

We confirmed the absence of D1 protein in EP473 homozygotes by performing immunolocalization experiments using our antibody raised against the protein. Figure 2E shows merged DNA/D1 signals for wild-type larvae and EP473 homozygotes processed at a time when they are still alive. The latter were found to be negative for D1 staining, confirming the effect of the P-element insertion on the presence of D1 protein at this terminal stage of viability. Interestingly, a comparison of nuclei from wild-type larvae and homozygous mutants revealed clear differences in their morphologies. In EP473 mutants devoid of D1 protein, nuclei were markedly smaller, possibly indicative of under-replication, and their chromatin was distributed as relatively smooth masses forming a circle with a hole at its center. Evidence of differences in the degree of chromatin condensation was largely absent from these structures. These changes in nuclear morphology in EP473 mutants are presently under investigation.

Localization of D1 protein in early embryos. In an effort to understand the function(s) of the D1 protein that might be essential during *Drosophila* development, we first set out to analyze in detail its cellular localization at different developmental stages. D1 was previously reported to localize predominantly at the chromocenter of polytene chromosomes, suggesting that it is associated with heterochromatin (1, 2). This conclusion was indeed supported by the recovery of D1 protein in nucleosomes from SATIII repeats (35) and by its preferential binding to SATI and SATIII sequences in vitro (36).

As shown in Fig. 2E, the D1 protein was found to localize to an average of two large foci in first-instar larvae. We used a similar approach to determine its distribution in early embryos, hoping to correlate the lethality associated with lack of D1 to a defined localization pattern. As shown in Fig. 3, the anti-D1 serum revealed that the protein localized to discrete areas in interphase/prophase nuclei of precellularization embryos (panels a and b), indicating a preferential association of large amounts of the protein with specific loci. Most nuclei contained an average of two to eight foci, with no protein detected elsewhere in the nucleoplasm. Careful examination revealed that some dots most likely corresponded to several coalesced smaller foci (compare Fig. 3a and b).

We next asked whether D1 remained associated with mitotic chromosomes. In these early embryos, the protein was found to remain bound to DNA in all mitotic figures analyzed (Fig. 3c to e), in contrast to other proteins known to interact with heterochromatin (12, 33). However, the distribution of the protein was found to undergo changes as nuclei proceeded through division. The D1 foci observed in interphase/prophase nuclei (Fig. 3a and b) first coalesced into larger areas (c). As chromosomes proceeded through mitosis, multiple discrete foci again appeared, forming an array of dots (d and e), some of which were clearly located on different chromosomes (d). Following completion of chromosome segregation, the D1 immunolocalization pattern returned to one similar to that shown in Fig. 3a over the course of the next interphase. In the photographs shown in Fig. 3d and e, D1 foci that do not localize to centromeres near the spindle poles can be seen to extend along a single chromosome. They most likely correspond to multiple D1 association sites on the Y chromosome of male embryos (see Fig. 5).

We also analyzed the localization of D1 following the cellularization that occurs at division cycle 14. At this stage, parallel to the onset of zygotic transcription, the D1 signal redistributed to two major foci in both interphase and mitotic nuclei (Fig. 3f and i, respectively). Confocal sections taken through the embryo (h and k) showed that the D1 signal localized to its periphery, a characteristic of heterochromatin (21, 26) and heterochromatin-associated proteins (13, 33). No D1 could be detected in the subapical portion of the embryonic nuclei that contains the bulk of euchromatin (g and j). After cycle 14, the localization of D1 remained fairly invariant, although overall levels of the protein decreased, consistent with the exhaustion of maternal mRNA and protein pools and reduced levels of accumulated transcripts. During interphase, most of the D1 signal was found to localize to an average of one or two large foci (see below), a pattern similar to that seen in larvae (Fig. 2E, panel a) and examined in more detail below. This transition from multiple foci to an average of two prominent foci might reflect the onset of somatic pairing of homologues after cellularization.

D1 protein is predominantly associated with SATI and SATIII in vivo. Results shown in Fig. 3 suggest that the D1 protein is primarily associated with heterochromatin. Such a localization might indicate that, in vivo, D1 interacts with the AT-rich satellite DNA sequences it is known to bind in vitro (36). To confirm this localization, we used a highly specific FITC-labeled polyamide, Lex9F (30), to determine more precisely the nature of the sequences D1 is bound to in embryonic nuclei. In the experiments described next, whole-mount embryos were immunostained for D1, followed by staining with Lex9F for SATI and SATIII repeats. DAPI staining was then used to label DNA. Figure 4 shows the Lex9F and D1 signals observed in embryonic nuclei and mitotic chromosomes. All of the D1 foci were found to correspond precisely to discrete Lex9F-bright sites associated with the simple-sequence SATI and 359-bp SATIII repeats found on the different chromosomes of *D. melanogaster*. In all cases, the dynamic distribution

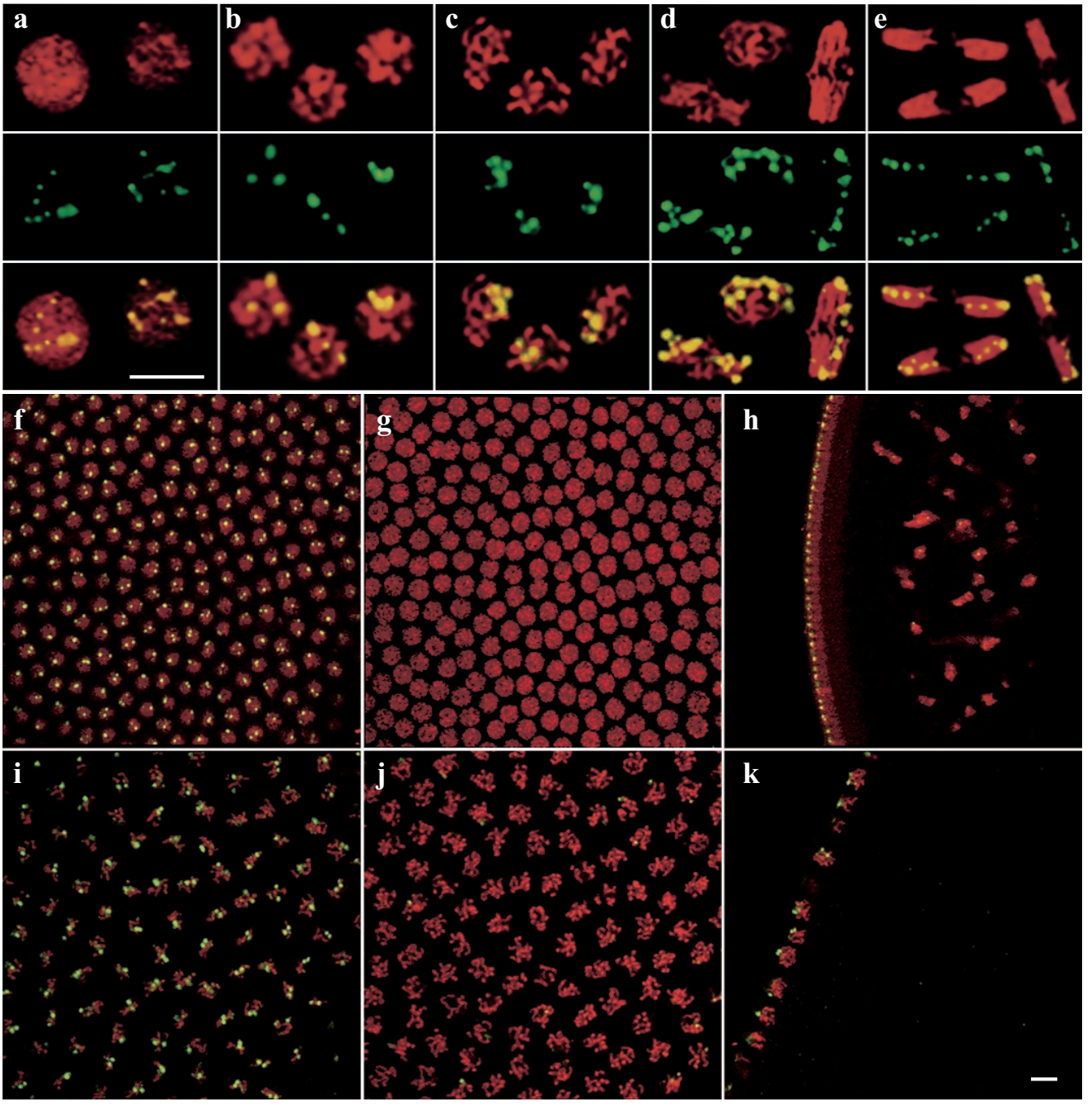

FIG. 3. Localization of D1 protein during early embryonic cell cycles and localization in heterochromatin. Embryos at the synctitial preblastoderm stage were processed for confocal microscopy after immunolabeling with the anti-D1 antibody. D1 was revealed using a fluorescein-coupled antibody (green signal) and DNA was stained with propidium iodide (red signal). (a to e) Distribution of D1 as chromosomes progress through the cell cycle (a, interphase; b, early prophase; c, prometaphase; d, early/middle anaphase; e, late anaphase). Bar = 5 μm. Confocal sections taken through embryos during interphase (f to h) or mitosis (i to k) show that D1 localizes exclusively to centromeric heterochromatin at cellularization. Images taken at the apical pole of the embryos (f and i), through the embryos (g and j), and at their periphery (h and k) show that no D1 can be detected in euchromatin. Bar = 5 μm.

of these repeats during the cell cycle was precisely followed by D1. Again, D1 and Lex9F foci could be sometimes be observed to extend outside of centromeric regions along the length of a chromosome (Fig. 4, rows 4 and 5). These most likely correspond to D1 protein associated with multiple SATI repeats on the Y chromosome of males. It is worth noting that not all DAPI-bright regions stained for Lex9F and D1, indicating that the signals detected correspond to genuine SATI and SATIII repeats, rather than to generally AT-rich regions. We conclude from these results that the bulk of D1 protein is bound to SATI and SATIII repeats in vivo.

We further mapped D1 binding sites using larval neuroblasts

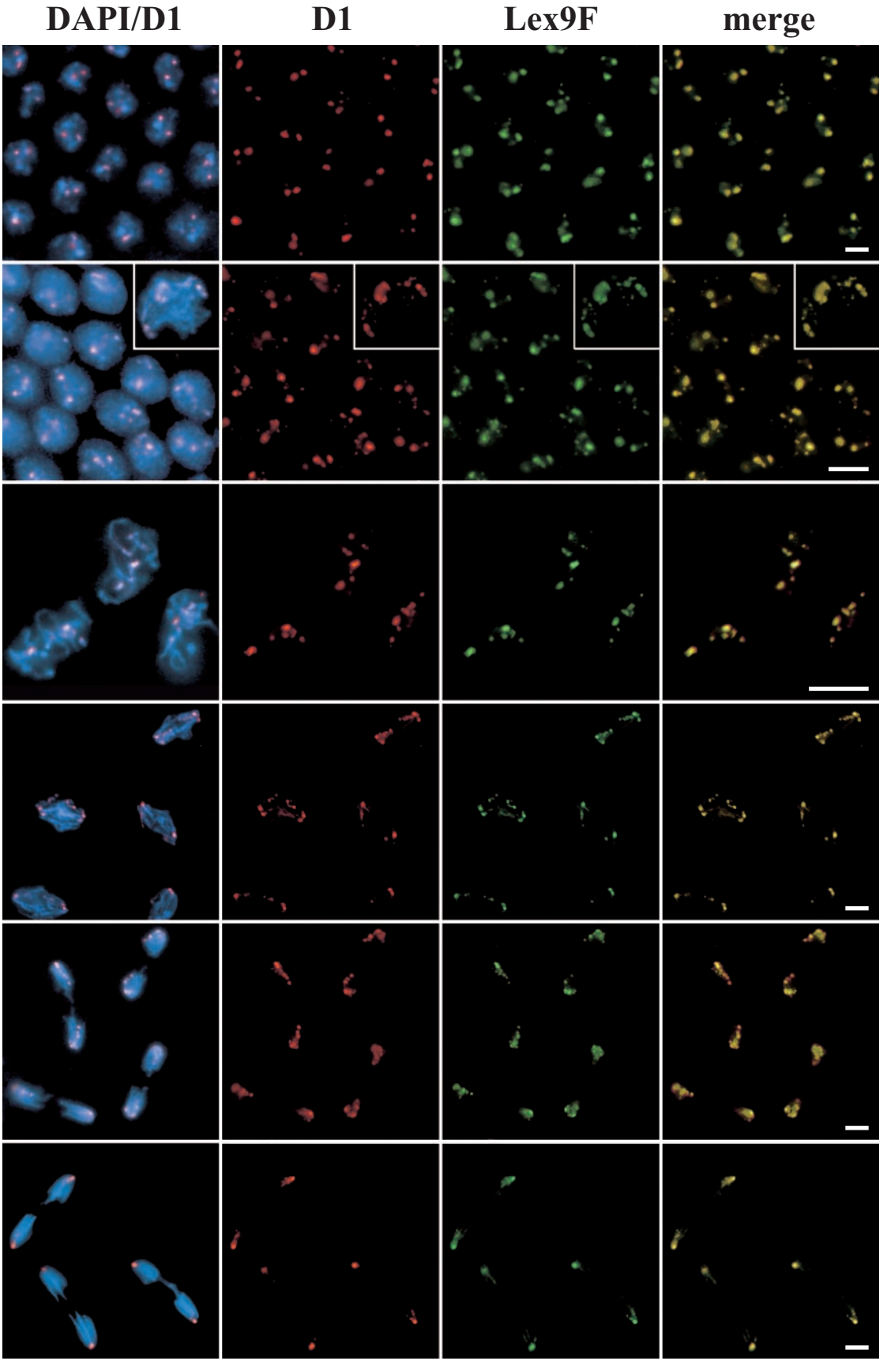

FIG. 4. D1 colocalizes with SATI and SATIII repeats in vivo. D1 (red signal) was detected in whole-mount early embryos stained with DAPI (blue) and Lex9F (green). Each row of photographs shows nuclei and chromosomes from embryos at different stages of the cell cycle. The signals shown are identified above each column and illustrate the strict colocalization of D1 with Lex9F, which specifically detects SATI and SATIII repeats. Bars = 5 μm.

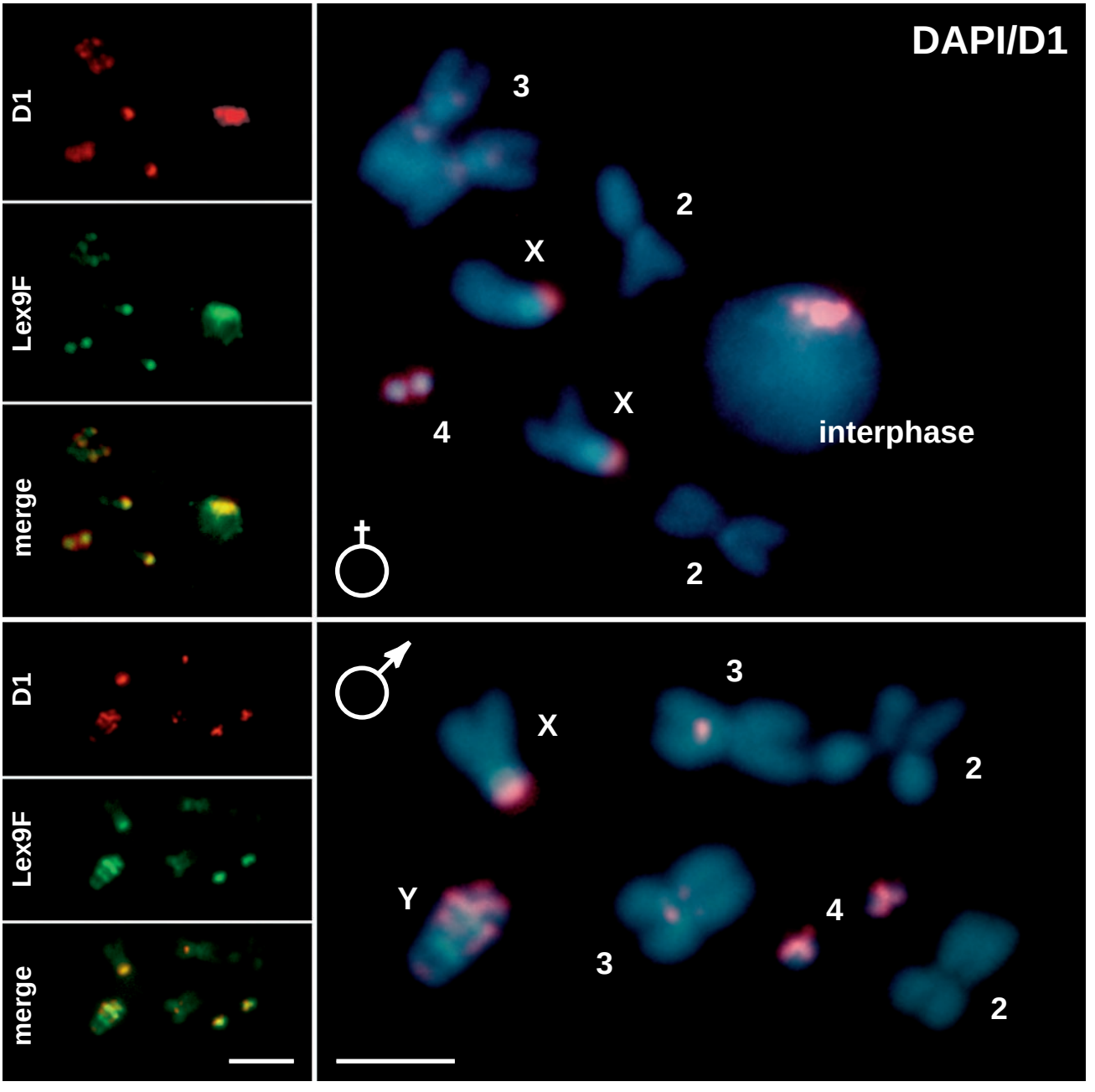

FIG. 5. Mapping of D1 binding sites on metaphase chromosomes. Metaphase chromosomes from larval neuroblasts were stained for D1, DNA, and Lex9F as described above. Chromosome sets are shown for both female and male brains. A single interphase nucleus can also be seen. The DAPI, D1, and Lex9F signals shown are as labeled on the photographs. D1 associates with discrete regions of all chromosomes except chromosome 2, consistent with the known localization of SATI and SATIII repeats. The colocalization of D1 with SATIII repeats in the centromeric region of the X chromosome is particularly evident. Bars = 5 μm.

in which the high proportion of mitotic cells can be used to assign a signal on individual chromosomes. As can be seen in Fig. 5 for both female and male metaphase chromosome sets, D1 was also associated with mitotic chromosomes at this stage of development, and the D1 and Lex9F signals were again precisely colocalized and could be assigned to the centromeric region of the X chromosome. Fainter signals could be seen in the centromeric region of chromosome *3*, while chromosome *4* was almost completely decorated by the antibody, as well as large regions of the Y chromosome in males. In the latter case, we note that the distinctive banded pattern of the Lex9F signal only partially overlapped with the D1 signal. These binding sites are in complete agreement with the known location of SATI and SATIII repeats (9, 38, 59). Taken together with previous mapping data (17), we conclude from these results that D1 is clearly associated with SATI sequences on the Y chromosome and on chromosomes *3* and *4*, and with SATIII sequences in the centromeric region of the X chromosome. Neither D1 nor Lex9F signals could be detected on chromosome *2*, which is devoid of SATI and SATIII repeats (38). The

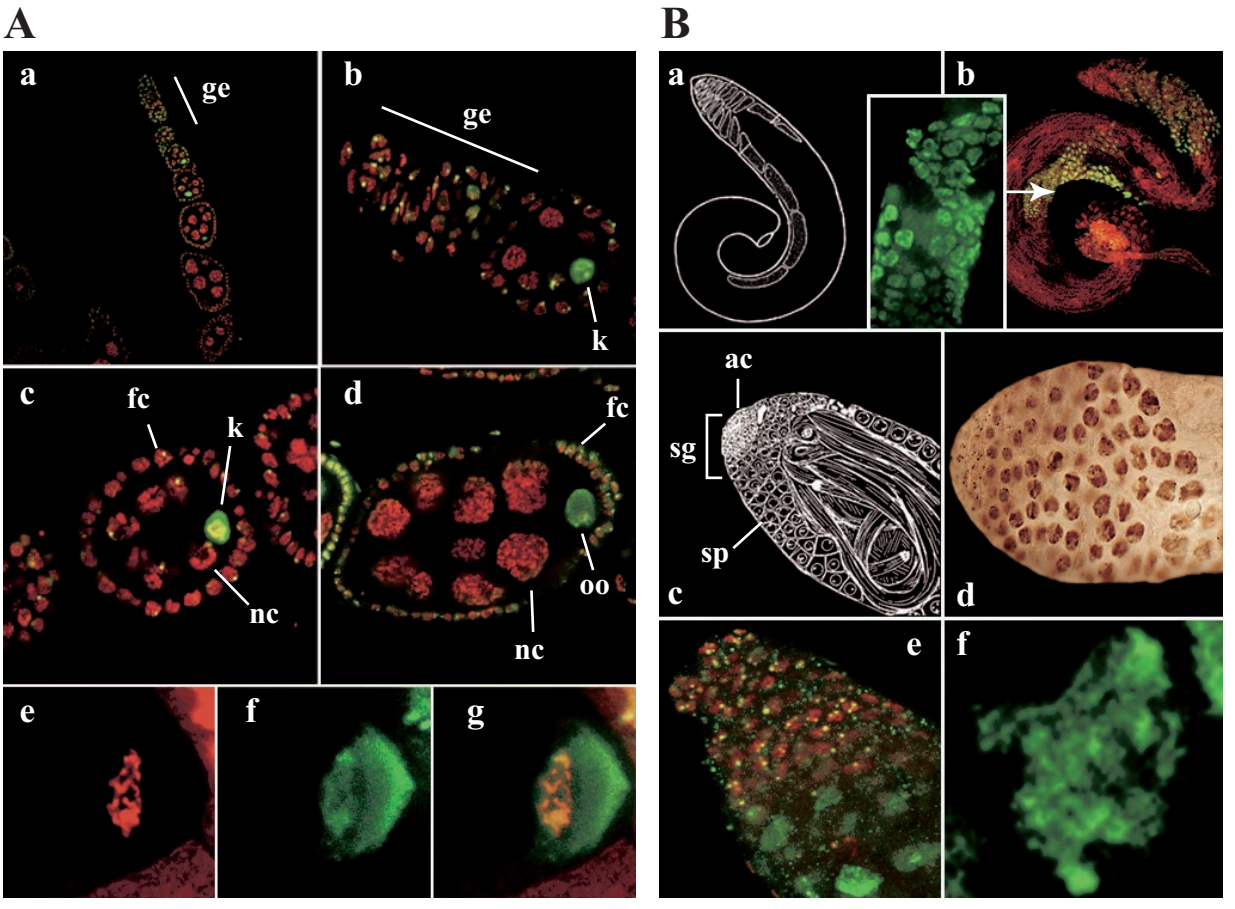

FIG. 6. Dynamic redistribution of D1 during gametogenesis. Adult gonads were stained for DNA and D1 protein and processed for confocal microscopy. (A) Different views of female ovaries: global and more detailed views of the anterior part of an ovariole (a and b, respectively) showing the germarium, ovarian chambers (c and d), and DNA, D1, and merged images of a late-stage oocyte (e, f, and g, respectively). The germarium (ge), follicle cells (fc), nurse cells (nc), the karyosome (k), and oocyte (oo) are indicated. A similar analysis is shown for male testes (B). D1 was revealed by immunofluorescence as described above or by peroxidase staining (d). A diagram adapted from one by Lindsley and Tokuyasu (37) is shown (a) and represents the first half-gyre of a testis with the different spermatocyte cysts that form single-cell layers near the testicular walls. A whole gonad is shown (b) together with a close-up view of the D1 signal in cells from the central part of the testis (top inset). In the germarium (d and e and diagrammed in c), D1 localizes to bright foci in both apical cells (ac) and spermatogonia (sg). Cells situated just below correspond to spermatocytes (sp) in which the protein has redistributed to fill most of the nucleoplasm (e, lower area; f, magnification of panel e).

top of Fig. 5 also shows a single interphase nucleus in which the D1 and Lex9F signals merge in a single region. This was found to be generally the case, and D1 binding sites that map to SATI and SATIII heterochromatin located on different chromosomes were consistently found to coalesce into a single large and discrete area during interphase (14). Occasionally, two foci could be distinguished (data not shown), and they resembled the bright foci seen in embryonic nuclei and in other larval tissues. This suggests that SATI and SATIII repeats on different chromosomes share a common compartment during interphase and that D1-containing heterochromatin might be generally restricted to such a subnuclear localization.

Distribution of D1 protein in gonads. The results presented above are consistent with a predominant association of the D1 protein with pericentric heterochromatin. Such a localization suggested a possible correlation with transcriptional inactivation. *Drosophila* testes and ovaries contain cells at all stages of gametogenesis. These cells differ in their morphology and the regions of the gonads they occupy, and can be readily identified. Most importantly, they also differ in their transcriptional state as a function of their differentiation stage. As shown in Fig. 1B, significantly increased levels of D1 mRNA were found to accumulate in adult females, where most of the signal could be attributed to ovaries (compare lanes 9 and 10). In males (lane 8), D1 might also preferentially localize to the testes. For these reasons, we immunolocalized D1 in isolated ovaries and testes, where a distinct localization might be correlated to the transcriptional state of the different cells that can be identified in the gonads.

Confocal images in Fig. 6A show different views of female ovaries. In the global view of the anterior part of an ovariole (Fig. 6A, panel a), D1 localized to foci in the germarium. In contrast, in the more detailed view of a germarium (Fig. 6A, panel b), the karyosome stained homogeneously for D1. Close-up views of stage-3 ovarian chambers (Fig. 6A, panels c and d, respectively) showed continued dense staining of the

karyosome while D1 localized to bright foci in follicle cells. Nurse cells, which are derived from the same progenitor as the oocyte, were largely negative for D1 after they became polyploid (Fig. 6A, panel d). DNA, D1 and merged images of a late-stage oocyte (Fig. 6A, panels e, f, and g, respectively) revealed two populations of D1 in this transcriptionally inactive cell: one localized to the chromosomes while the other most likely represents a pool of stored protein. The absence of D1 staining in certain regions of the oocyte (Fig. 6A, panel f) makes it unlikely that the signal localized over chromosomes is simply due to high background D1 levels in this cell type. Thus, the distribution of D1 observed in transcriptionally active and inactive cells lends support to the hypothesis that a broad redistribution of D1 onto bulk chromatin correlates with transcriptional repression while, in active cells, most of the protein localizes to discrete foci that may correspond to D1 storage sites in heterochromatin. This implies that the spatial distribution of the D1 protein can be regulated and shifted between distinct populations of AT-rich binding sites.

We performed similar experiments with male testes, where maturing sperm cells also occupy specific regions of the gonad and go through developmental stages that lead to terminal differentiation and transcriptional repression. D1 was found to be present in all types of maturing spermatocytes (Fig. 6B, panel b). Again, it was found to assume a distinctive distribution at the apical pole of the testes. In the spermatogonia containing the germinal stem cells and the somatic cells of the testis and shown in Fig. 6B, panels d and e, the protein appeared as dense foci in the top layer of cells. In contrast, transcriptionally repressed maturing spermatocytes situated below showed a broad distribution of the D1 protein extending throughout the nucleoplasm (Fig. 6B, inset and panel f).

In summary, this analysis of the localization of D1 in adult gonads establishes two important points. The first one is that the transcriptional and differentiated states of maturing gametes can be correlated with a localization of D1 to a small number of discrete foci as opposed to a homogenous dispersion onto chromosomes. In addition, these data demonstrate that the localization of D1 can indeed change as a function of development and/or transcriptional state. In the latter case, whether this phenomenon is a cause or an effect remains difficult to establish. At any rate, our results indicate that the D1 chromosomal protein is not restricted to a single family of DNA target sequences—such as the SATI or SATIII repeats—but can redistribute to much broader regions of the genome as a function of development. This property is that expected of a protein implicated in the general modulation of chromatin structure and function.

The EP473 insertion is a haplo-suppressor of PEV. The data shown in Fig. 6 suggest that D1 might play a role as a general transcriptional repressor or corepressor. This hypothesis is consistent with the predominant localization of D1 in SATI and SATIII heterochromatin. SATIII repeats are located in the centromeric region of the X chromosome (11), a region which exerts strong repressive effects on gene expression. A well-known system of X-chromosome heterochromatin-mediated transcriptional inactivation is that of the PEV associated with the *white*-mottled (w^{m4h}) inversion which moves the *white* gene responsible for the dark red eye color of wild-type flies from its normal position in cytological region 3C to region 20 at the proximal end of the X chromosome (56). This results in the variegated inactivation of *white* in its new location, where it is sensitive to enhancement or suppression by modifiers of PEV (19, 47), including the artificial MATH20 protein (23), and P9, an olygopyrrole related to Lex9 and its fluorescent derivative Lex9F and similarly specific for SATI and SATIII sequences (29).

To test whether the EP473 insertion might similarly modify the variegating w^{m4h} phenotype, we performed a cross between w^{m4h} flies and EP473 heterozygotes and analyzed the progeny for changes in eye color. As shown in Fig. 7, adult w^{m4h} flies carrying a single EP473 allele had markedly darker eyes relative to w^{m4h} controls. If decreased expression of the D1 gene is indeed directly responsible for this suppression of PEV, overexpression of the gene should have the opposite effect—enhancement of PEV. We constructed transgenic lines expressing an inducible D1 transgene under the control of a heat shock promoter. These lines were crossed to w^{m4h} flies and the progeny were examined for eye color after heat shock. In support of our conclusion that the EP473 allele of D1 is a suppressor of PEV, overexpression of the D1 transgene enhanced PEV, yielding flies with markedly reduced eye color over the light orange background due to expression of the mini-*white* reporter gene present on the D1 construct (Fig. 7). In this case, we noted that induction of the D1 transgene consistently resulted in a decrease of both w^{m4h} expression and of the mini-*white* gene carried on the P element inserted in the X chromosome. One possibility is that reduced expression of w^{m4h} in the presence of elevated D1 protein levels leads to *trans*-inactivation of the mini-*white* gene.

These results identify the EP473 insertion as a haplo-suppressor of w^{m4h} PEV and suggest that the D1 protein can affect gene expression as a functional component of SATIII heterochromatin. As shown next, this effect most likely requires interactions between D1 and other proteins. We tested the effect of the overexpression of D1ΔE, a D1 transgene deleted of its C-terminal acidic amino acids that might constitute a protein/protein interaction domain. This deletion does not affect DNA binding to SAT III repeats in vitro (data not shown). In this case, induction of D1ΔE expression led to suppression of w^{m4h} PEV, an effect opposite that of elevated D1 levels and similar to that of reduced D1 protein levels in EP473 heterozygotes (Fig. 7). These results suggest that the modulation by D1 of the functional properties of heterochromatin, as measured by its effects on w^{m4h} expression, requires both binding to AT-rich satellite repeats and interaction with protein partners.

DISCUSSION

We have shown that the D1 nonhistone chromosomal protein, an essential protein of *D. melanogaster*, is a modifier of w^{m4h} PEV. D1 thus constitutes the first example of a DNA-binding protein of known specificity and precise localization that is involved in heterochromatin-mediated transcriptional inactivation. D1 most likely exerts this effect by interacting with the SATIII repeats in X-chromosome centromeric heterochromatin. So far, we have found no clear evidence for the D1-dependent modification of PEV at other loci. The EP473 insertion was found to suppress only slightly the variegation of the mini-*white* gene in the *Heidi* rearrangement on chromo-

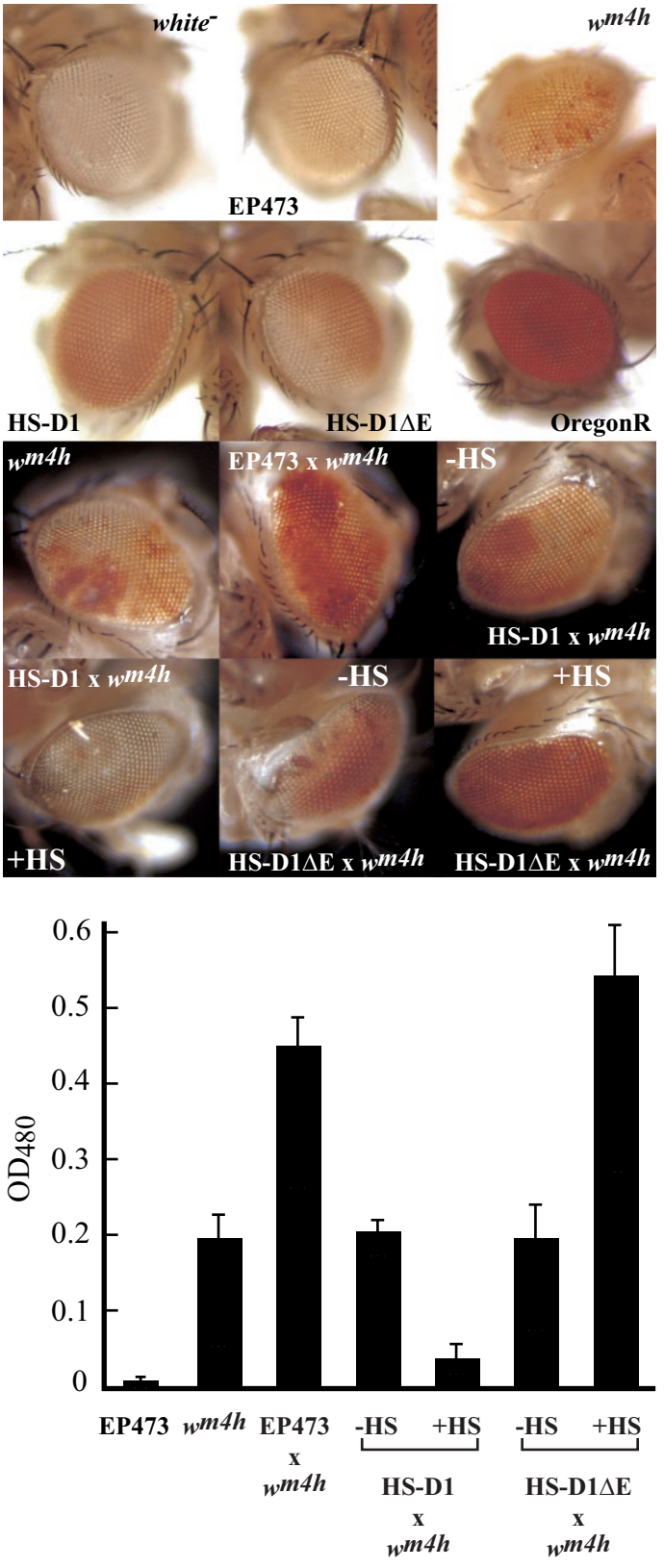

FIG. 7. D1 is a modifier of w^{m4h} PEV. (Top two rows) Representative eyes from 5-day-old female parents used in these studies: *white*$^-$, EP473, w^{m4h}, HS-D1, HS-D1ΔE, and a wild-type OregonR stock. The light eye color contributed by the mini-*white* gene carried by EP473 and the HS-D1 and HS-D1ΔE P elements, ranging from pale yellow to pale orange, did not interfere with analysis of the modification of PEV. (Bottom two rows) Eye color of representative 5-day-old females obtained from the following: w^{m4h} control, EP473 × w^{m4h}, HS-D1 × w^{m4h} without (−HS) or with (+HS) heat shock, and HS-D1ΔE × w^{m4h} without or with heat shock. Flies recovered from the crosses shown in each photograph and in the graph below have the following full genotypes: EP473 × w^{m4h}: w^{m4h}/w^-; EP473/TM6b. HS-D1 × w^{m4h}: w^{m4h}/w^- HS-D1. HS-D1ΔE × w^{m4h}: w^{m4h}/w^-; HS-D1ΔE/+. The genotypes of the parents are indicated in Materials and Methods. Corresponding eye pigment levels (see Materials and Methods) are indicated in the graph below and correspond to mean values ± standard errors from three independent measurements.

some *2* (52). No discernible effects could be observed in the case of the BL1 and BL2 mini-*white* insertions on chromosome *3* and on the Y chromosome, respectively (39). As D1 binds SATIII sequences more weakly than SATI repeats in vitro (36), it is possible that these differential effects reflect a preferential loss of D1 from SATIII repeats when protein levels are reduced.

D1 is essential for embryonic development. *Drosophila* cells accumulate only moderate amounts of D1 transcripts, early embryos being a notable exception. Indeed, the considerably stronger D1 mRNA signal observed in 0- to 2-h embryos is consistent with a strong maternal contribution of the message and the markedly increased levels of D1 transcripts observed in adult females can be almost completely accounted for by their accumulation in the ovaries. This is also reflected at the protein level, with the existence of two distinct populations of D1 protein in mature gametes: one is associated with the chromosomes of the oocyte while the other likely represents a pool of stored protein (Fig. 6A).

This maternal contribution is in agreement with our characterization of the embryonic lethal phenotype associated with the EP473 allele, which suggests that D1 is an essential gene required throughout embryonic development. The simplest explanation for the lethality we observe is that embryogenesis proceeds until D1 reaches a subthreshold level, following exhaustion of depleted maternal pools that cannot be replenished, as suggested by the results shown in Fig. 2. The eventual arrest of development thus correlates with a complete absence of D1 transcripts and protein (Fig. 2D and E), while induction of a D1 transgene under the control of a heat shock promoter can partially overcome this lethality (data not shown; see Materials and Methods). This suggests a role for D1 later during development as this rescue did not yield adult flies.

The AT hook-bearing D1 protein serves to establish and/or enhance PEV. Our analysis of the phenotype associated with the EP473 P-element insertion suggests that the D1 protein serves to establish and/or enhance PEV associated with X-chromosome centromeric heterochromatin, a conclusion fully supported by the effects of the expression of an inducible D1 transgene (Fig. 7). DNA binding is not sufficient for this function since overexpression of D1ΔE, a protein with a deleted acidic C-terminal domain but fully competent for DNA binding, has opposite effects. Thus, the recruitment of other proteins via the C-terminal domain of D1 may be required for D1-mediated transcriptional silencing.

In support of this hypothesis, analysis of the in vivo expression of the synthetic MATH-20 protein, a 20-AT-hook-bearing derivative of HMGA1a lacking a C-terminal acidic domain, showed that it is a strong suppressor of w^{m4h} PEV (23). The similar effects of the EP473 insertion and of MATH-20 expression raise the possibility that, as in the case of D1ΔE, expression of MATH-20 in *Drosophila* behaves as a dominant-negative mutation of D1, thus accounting for suppression of w^{m4h} PEV. Similarly, P9, a molecule related to the Lex9 ligand used in this study to map D1 binding sites in vivo, targets SATI and SATIII repeats (29) and induces PEV-modifying effects similar to those of EP473, MATH-20, and D1ΔE. These effects are correlated with the opening of SATIII chromatin in vitro, as measured by the P9-dependent increase in accessibility to topoisomerase II cleavage (30). These results can be explained by a displacement of D1 protein from SATIII repeats by D1ΔE, MATH-20, and P9 (25, 29, 30), which would support the hypothesis that D1 might be the factor primarily responsible for the PEV observed in the w^{m4h} inversion. In this case, D1 would target SATIII repeats which it binds preferentially and recruit other factors involved in the assembly of heterochromatin. The biological function(s) of D1 would then be largely determined by the nature of the partners recruited via its C-terminal domain.

Among other known modifiers of PEV, two in particular have been well characterized: Su(var)3–7 (46) and Su(var)2–5 (20) are mutations that both suppress w^{m4h} PEV. The corresponding genes have been cloned and their gene products, HP-1 in the case of Su(var)2–5, have been characterized and shown to interact with one another (12). A recent study of HP-1 has shown that it binds DNA and nucleosomes in vitro but, as in the case of Su(var)3–7, without a demonstrable specificity (60). Thus, these proteins lack a specific DNA-binding function that may explain their presumed localization near X-chromosome centromeric heterochromatin, where w^{m4h} expression is modulated. While future experiments will help establish whether pathways of PEV modification by HP-1, Su(var)3–7, and D1 are shared or distinct, our preliminary results indicate that the enhancement of w^{m4h} PEV by the overexpression of a Su(var)3–7 transgene can be suppressed by expression of a D1ΔE transgene (N. Aulner et al., unpublished data). D1 might thus interact with Su(var)3–7 and HP-1, recruiting them to AT-rich regions of the genome where they would exert their function.

D1 and transcriptional silencing. A role for D1 in heterochromatin-mediated silencing is in line with our results showing that a redistribution of D1 to the chromatin of maturing gametes correlates with the known extinction of gene expression during differentiation of oocytes and spermatocytes. The localization of D1 can thus shift between different genomic interaction sites over the course of gamete maturation. In this case, D1 becomes associated with the entire chromatin contents of transcriptionally inactive mature gametes (Fig. 6), while it is restricted to a small number of foci or absent in transcriptionally active cells from the same lineage and in the somatic cells found in the germarium. This phenomenon constitutes a particularly striking example of spreading of the protein from SATI and SATIII repeats to euchromatin, concomitant with the shutdown of transcription. The modification of w^{m4h} PEV by D1 reported here may thus reflect a more general role for D1 in transcriptional silencing. It is therefore possible that, as in the case of other heterochromatin-associated proteins such as HP-1 or GAGA factor (33), the biological function of D1 is not solely exerted in the context of satellite heterochromatin, although SATI and SATIII repeats constitute major binding sites throughout much of *Drosophila* development.

The genome-wide redistribution of D1 observed in gametes can be rationalized in terms of its known DNA-binding specificity. Stretches as short as four or five dA•dT base pairs can be recognized by AT hook motifs (43), and generally AT-rich genomic regions that contain many such sequences might constitute preferential targets. This is indeed the case for the SATI and SATIII repeats specifically bound by D1 (36) and, perhaps more generally, for AT-rich scaffold/matrix-associated regions,

of which the approximately 11-Mbp SATIII array at the proximal end of the X-chromosome constitutes an extreme example (32). Given the small size of a minimal binding site, much of the genome would be expected to be recognizable by the protein and might interact with D1. The biological function(s) of D1 might thus be extremely sensitive to the intranuclear concentration of the protein, which might in turn affect its chromosomal localization. Indeed, the modification of PEV by a single EP473 allele correlates with a moderate decrease in the levels of D1 protein (only 30 or 40%; Fig. 2C). Alternatively, the DNA-binding activity of D1 might be directly regulated, perhaps by posttranslational modification, as in the case of HMGA proteins. Our Western blot analysis revealed a difference in the electrophoretic mobility of D1 in whole embryos relative to tissue culture cells (Fig. 2C), and a recent study has confirmed that D1 cycles between dephosphorylated and phosphorylated states during development, high levels of phosphorylation being associated with oogenesis and early embryogenesis (45). Future experiments will address this question in more detail, particularly in view of the association of D1 with bulk chromatin in mature gametes.

How D1-mediated assembly of heterochromatin might affect expression of nearby genes remains unclear. It has been proposed that assembly of heterochromatin-specific multiprotein complexes can expand into neighboring euchromatin to silence gene expression. According to this spreading model, expansion or retraction of heterochromatin would be sensitive to the concentration of D1 and of other components of SATIII heterochromatin. In the case studied here, a decrease in the concentration of D1 would drive a retraction of heterochromatin accompanied by an increased expression of w^{m4h}. This could result from down-regulation of D1 expression, as in the case of the EP473 insertion, or from expression of a competitor unable to recruit other proteins, such as MATH-20 or D1ΔE. As levels of the D1 protein vary during *Drosophila* development, contraction/retraction cycles might normally influence the expression of genes located in the proximal region of the X chromosome.

However, results obtained from several experimental systems argue against a simple linear expansion model of heterochromatin-mediated transcriptional repression (14, 41, 55). In these cases, available data support a spatial model of inactivation in which heterochromatin would constitute a discrete nuclear compartment largely inaccessible to factors required for the expression of genes normally located in euchromatin (40). The colocalization of D1-associated satellites found on different chromosomes in interphase nuclei (Fig. 5) is consistent with the existence of such compartments. A spatial model could also explain why certain genes that reside in heterochromatin and whose expression requires the presence of proteins such as HP1 become silenced if relocalized to euchromatin (40). Heterochromatin—a term derived from largely morphologic and cytogenetic observations—would thus represent a specialized nuclear compartment, characterized by a selective accessibility to transcriptional activators or repressors recruited by proteins of which D1 would be one example in the case of SATI and SATIII repeats. While the results of our studies do not permit us to distinguish between spreading and spatial models of gene silencing, the original description of the phenotype of the w^{m4h} inversion, the effects observed in this system by numerous investigators and our own observations suggest that the D1 protein acts far upstream in the processes leading to heterochromatin-mediated transcriptional repression.

Finally, we cannot exclude that D1 may also participate in the assembly of specialized structures outside of heterochromatin, by interacting with other arrays of AT-rich sequences, such as those found in SARs/MARs (24) that form the bases of chromatin loops (50) or flanking the Boundary Elements (BE) described for *Drosophila* (16). Indeed, recent results indicate that D1 specifically associates with certain BEs and interacts directly with BEAF32, the protein which mediates the activity of BEs, through its acidic domain (15). Interestingly, D1 can direct the association of BEAF32 to AT-rich sequences devoid of cognate binding sites. D1 might thus serve as a regulator of BE function by affecting the ratio of BEAF32 associated with BEs or unproductively sequestered in AT-rich satellite sequences. According to this model, the D1-dependent modification of PEV reported here would largely reflect a deregulation of boundary activity leading to heterochromatinization. We are presently investigating this possibility.

ACKNOWLEDGMENTS

We thank Leonora Poljak, François Payre, and Michèle Crozatier for discussions and Dale Dorsett and Joel Eissenberg for comments on the manuscript. We are grateful to our colleagues at the Centre de Biologie du Développement (CBD, Toulouse, France) for advice and use of *Drosophila* facilities and to Eric Drier (Cold Spring Harbor Laboratory) for help in quantitative assays of PEV. We also thank David Villa for help in the preparation of the figures, Joshua Käs for expert technical assistance, and Ezra Käs for a critical reading of the manuscript. We are particularly grateful to the *Drosophil*a Stock Centre and to Peter Maroy, Department of Genetics and Molecular Biology, Szeged, Hungary, for maintaining and distributing fly stocks. We also thank the referees of the manuscript for constructive criticism and useful suggestions.

This work was supported by grants from the Centre National de la Recherche Scientifique (CNRS), the Ligue Nationale Contre le Cancer and the Association pour la Recherche sur le Cancer (ARC, grants no. 9284 and 5698). N. Aulner and D. Jullien gratefully acknowledge graduate fellowships from the ARC.

REFERENCES

1. **Alfageme, C. R., G. T. Rudkin, and L. H. Cohen.** 1976. Locations of chromosomal proteins in polytene chromosomes. Proc. Natl. Acad. Sci. USA **73:**2038–2042.
2. **Alfageme, C. R., G. T. Rudkin, and L. H. Cohen.** 1980. Isolation, properties and cellular distribution of D1, a chromosomal protein of *Drosophila*. Chromosoma **78:**1–80.
3. **Amirand, C., A. Viari, J. P. Ballini, H. Rezaei, N. Beaujean, D. Jullien, E. Käs, and P. Debey.** 1998. Three distinct sub-nuclear populations of HMG-I protein of different properties revealed by colocalization image analysis. J. Cell Sci. **111:**3551–3561.
4. **Aravind, L., and D. Landsman.** 1998. AT-hook motifs identified in a wide variety of DNA-binding proteins. Nucleic Acids Res. **26:**4413–4421.
5. **Ashley, C. T., C. G. Pendleton, W. W. Jennings, A. Saxena, and C. V. Glover.** 1989. Isolation and sequencing of cDNA clones encoding *Drosophila* chromosomal protein D1. A repeating motif in proteins which recognize at DNA. J. Biol. Chem. **264:**8394–8401.
6. **Barker, D. D., C. Wang, J. Moore, L. K. Dickinson, and R. Lehmann.** 1992. *Pumilio* is essential for function but not for distribution of the *Drosophila* abdominal determinant *Nanos*. Genes Dev. **6:**2312–2326.
7. **Barthelemy, I., L. Carramolino, J. Gutierrez, J. L. Barbero, G. Marquez, and A. Zaballos.** 1996. zhx-1: a novel mouse homeodomain protein containing two zinc-fingers and five homeodomains. Biochem. Biophys. Res. Commun. **224:**870–876.
8. **Beaujean, N., C. Bouniol-Baly, C. Monod, K. Kissa, D. Jullien, N. Aulner, C. Amirand, P. Debey, and E. Käs.** 2000. Induction of early transcription in one-cell mouse embryos by microinjection of the non-histone chromosomal protein HMG-I. Dev. Biol. **221:**337–354.

9. **Bonaccorsi, S., and A. Lohe.** 1991. Fine mapping of satellite DNA sequences along the Y chromosome of *Drosophila melanogaster*: relationships between satellite sequences and fertility factors. Genetics **129:**177–189.
10. **Bourachot, B., M. Yaniv, and C. Muchardt.** 1999. The activity of mammalian brm/SNF2alpha is dependent on a high-mobility-group protein I/Y-like DNA binding domain. Mol. Cell. Biol. **19:**3931–3939.
11. **Carlson, M., and D. Brutlag.** 1977. Cloning and characterization of a complex satellite DNA from *Drosophila melanogaster*. Cell **11:**371–381.
12. **Cleard, F., M. Delattre, and P. Spierer.** 1997. SU(VAR)3–7, a *Drosophila* heterochromatin-associated protein and companion of HP1 in the genomic silencing of position-effect variegation. EMBO J. **16:**5280–5288.
13. **Cleard, F., M. Matsarskaia, and P. Spierer.** 1995. The modifier of position-effect variegation Suvar(3)7 of *Drosophila:* there are two alternative transcripts and seven scattered zinc fingers, each preceded by a tryptophan box. Nucleic Acids Res. **23:**796–802.
14. **Csink, A. K., and S. Henikoff.** 1996. Genetic modification of heterochromatic association and nuclear organization in *Drosophila*. Nature **381:**529–531.
15. **Cuvier, O., C. M. Hart, E. Käs, and U. K. Laemmli.** 2001. Identification of a multicopy chromatin Boundary Element at the borders of silenced chromosomal domains. Chromosoma, in press.
16. **Cuvier, O., C. M. Hart, and U. K. Laemmli.** 1998. Identification of a class of chromatin boundary elements. Mol. Cell. Biol. **18:**7478–7486.
17. **Dernburg, A. F., J. W. Sedat, and R. S. Hawley.** 1996. Direct evidence of a role for heterochromatin in meiotic chromosome segregation. Cell **86:**135–146.
18. **DiBartolomeis, S. M., K. D. Tartof, and F. R. Jackson.** 1992. A superfamily of *Drosophila* satellite related (SR) DNA repeats restricted to the X chromosome euchromatin. Nucleic Acids Res. **20:**1113–1116.
19. **Eissenberg, J. C.** 1989. Position effect variegation in *Drosophila*: towards a genetics of chromatin assembly. BioEssays **11:**14–17.
20. **Eissenberg, J. C., T. C. James, D. M. Foster-Hartnett, T. Hartnett, V. Ngan, and S. C. Elgin.** 1990. Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **87:**9923–9927.
21. **Foe, V. E., and B. M. Alberts.** 1983. Studies of nuclear and cytoplasmic behaviour during the five mitotic cycles that precede gastrulation in *Drosophila* embryogenesis. J. Cell Sci. **61:**31–70.
22. **Giannini, G., L. Di Marcotullio, E. Ristori, M. Zani, M. Crescenzi, S. Scarpa, G. Piaggio, A. Vacca, F. A. Peverali, F. Diana, I. Screpanti, L. Frati, and A. Gulino.** 1999. HMGI(Y) and HMGI-C genes are expressed in neuroblastoma cell lines and tumors and affect retinoic acid responsiveness. Cancer Res. **59:**2484–2492.
23. **Girard, F., B. Bello, U. K. Laemmli, and W. J. Gehring.** 1998. *In vivo* analysis of scaffold-associated regions in *Drosophila*: a synthetic high-affinity SAR binding protein suppresses position effect variegation. EMBO J. **17:**2079–2085.
24. **Hart, C. M., and U. K. Laemmli.** 1998. Facilitation of chromatin dynamics by SARs. Curr. Opin. Genet. Dev. **8:**519–525.
25. **Henikoff, S., and D. Vermaak.** 2000. Bugs on drugs go GAGAA. Cell **103:**695–698.
26. **Hiraoka, Y., D. A. Agard, and J. W. Sedat.** 1990. Temporal and spatial coordination of chromosome movement, spindle formation, and nuclear envelope breakdown during prometaphase in *Drosophila melanogaster* embryos. J. Cell Biol. **111:**2815–2828.
27. **Hsieh, T., and D. Brutlag.** 1979. Sequence and sequence variation within the 1.688 g/cm3 satellite DNA of *Drosophila melanogaster*. J. Mol. Biol. **135:**465–481.
28. **Hsieh, T., and D. L. Brutlag.** 1979. A protein that preferentially binds *Drosophila* satellite DNA. Proc. Natl. Acad. Sci. USA **76:**726–730.
29. **Janssen, S., O. Cuvier, M. Muller, and U. K. Laemmli.** 2000. Specific gain- and loss-of-function phenotypes induced by satellite-specific DNA-binding drugs fed to *Drosophila melanogaster*. Mol. Cell **6:**1013–1024.
30. **Janssen, S., T. Durussel, and U. K. Laemmli.** 2000. Chromatin opening of DNA satellites by targeted sequence-specific drugs. Mol. Cell **6:**999–1011.
31. **Jullien, D., M. Crozatier, and E. Käs.** 1997. cDNA sequence and expression pattern of the *Drosophila melanogaster* PAPS synthetase gene: a new salivary gland marker. Mech. Dev. **68:**179–186.
32. **Käs, E., and U. K. Laemmli.** 1992. *In vivo* topoisomerase II cleavage of the *Drosophila* histone and satellite III repeats: DNA sequence and structural characteristics. EMBO J. **11:**705–716.
33. **Kellum, R., J. W. Raff, and B. M. Alberts.** 1995. Heterochromatin protein 1 distribution during development and during the cell cycle in *Drosophila* embryos. J. Cell Sci. **108:**1407–1418.
34. **Laemmli, U. K., E. Käs, L. Poljak, and Y. Adachi.** 1992. Scaffold-associated regions: *cis*-acting determinants of chromatin structural loops and functional domains. Curr. Opin. Genet. Dev. **2:**275–285.
35. **Levinger, L., and A. Varshavsky.** 1982. Selective arrangement of ubiquitinated and D1 protein-containing nucleosomes within the *Drosophila* genome. Cell **28:**375–385.
36. **Levinger, L. F.** 1985. D1 protein of *Drosophila melanogaster*. Purification and AT-DNA binding properties. J. Biol. Chem. **260:**14311–14318.
37. **Lindsley, D. L., and K. T. Tokuyasu.** 1980. Spermatogenesis, p. 225–294. *In* M. Ashburner and T. R. F. Wright (ed.), The genetics and biology of *Drosophila*, 2nd ed. Academic Press, London, United Kingdom.
38. **Lohe, A. R., A. J. Hilliker, and P. A. Roberts.** 1993. Mapping simple repeated DNA sequences in heterochromatin of *Drosophila melanogaster*. Genetics **134:**1149–1174.
39. **Lu, B. Y., C. P. Bishop, and J. C. Eissenberg.** 1996. Developmental timing and tissue specificity of heterochromatin-mediated silencing. EMBO J. **15:**1323–1332.
40. **Lu, B. Y., P. C. Emtage, B. J. Duyf, A. J. Hilliker, and J. C. Eissenberg.** 2000. Heterochromatin protein 1 is required for the normal expression of two heterochromatin genes in *Drosophila*. Genetics **155:**699–708.
41. **Martin-Morris, L. E., A. K. Csink, D. R. Dorer, P. B. Talbert, and S. Henikoff.** 1997. Heterochromatic trans-inactivation of *Drosophila white* transgenes. Genetics **147:**671–677.
42. **Noselli, S., and A. Vincent.** 1992. The *Drosophila melanogaster* ribosomal protein L17A-encoding gene. Gene **118:**273–278.
43. **Reeves, R., and L. Beckerbauer.** 2001. HMGI/Y proteins: flexible regulators of transcription and chromatin structure. Biochim. Biophys. Acta **1519:**13–29.
44. **Reeves, R., and M. S. Nissen.** 1990. The A•T-DNA-binding domain of mammalian high mobility group I chromosomal proteins. J. Biol. Chem. **265:**8573–8582.
45. **Renner, U., S. Ghidelli, M. A. Schafer, and J. R. Wisniewski.** 2000. Alterations in titer and distribution of high mobility group proteins during embryonic development of *Drosophila melanogaster*. Biochim. Biophys. Acta **1475:**99–108.
46. **Reuter, G., M. Giarre, J. Farah, J. Gausz, A. Spierer, and P. Spierer.** 1990. Dependence of position-effect variegation in *Drosophila* on dose of a gene encoding an unusual zinc-finger protein. Nature **344:**219–223.
47. **Reuter, G., and P. Spierer.** 1992. Position effect variegation and chromatin proteins. BioEssays **14:**605–612.
48. **Rorth, P., K. Szabo, A. Bailey, T. Laverty, J. Rehm, G. M. Rubin, K. Weigmann, M. Milan, V. Benes, W. Ansorge, and S. M. Cohen.** 1998. Systematic gain-of-function genetics in *Drosophila*. Development **125:**1049–1057.
49. **Rubin, G. M., and A. C. Spradling.** 1982. Genetic transformation of *Drosophila* with transposable element vectors. Science **218:**348–353.
50. **Saitoh, Y., and U. K. Laemmli.** 1994. Metaphase chromosome structure: bands arise from a differential folding path of the highly AT-rich scaffold. Cell **76:**609–622.
51. **Seroude, L.** 1995. Ph.D. thesis. Université Paul Sabatier, Toulouse, France.
52. **Seum, C., A. Spierer, M. Delattre, D. Pauli, and P. Spierer.** 2000. A GAL4-HP1 fusion protein targeted near heterochromatin promotes gene silencing. Chromosoma **109:**453–459.
53. **Strauss, F., and A. Varshavsky.** 1984. A protein binds to a satellite DNA repeat at three specific sites that would be brought into mutual proximity by DNA folding in the nucleosome. Cell **37:**889–901.
54. **Strick, R., and U. K. Laemmli.** 1995. SARs are *cis* DNA elements of chromosome dynamics: synthesis of a SAR repressor protein. Cell **83:**1137–1148.
55. **Talbert, P. B., and S. Henikoff.** 2000. A reexamination of spreading of position-effect variegation in the *white-roughest* region of *Drosophila melanogaster*. Genetics **154:**259–272.
56. **Tartof, K. D., C. Hobbs, and M. Jones.** 1984. A structural basis for variegating position effects. Cell **37:**869–878.
57. **Tautz, D., and C. Pfeifle.** 1989. A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. Chromosoma **98:**81–85.
58. **Thanos, D., and T. Maniatis.** 1992. The high mobility group protein HMG I(Y) is required for NF-kappa B-dependent virus induction of the human IFN-beta gene. Cell **71:**777–789.
59. **Wollenzien, P., P. Barsanti, and J. E. Hearst.** 1977. Location and underreplication of satellite DNA in *Drosophila melanogaster*. Genetics **87:**51–65.
60. **Zhao, T., T. Heyduk, C. D. Allis, and J. C. Eissenberg.** 2000. Heterochromatin protein 1 binds to nucleosomes and DNA *in vitro*. J. Biol. Chem. **275:**28332–28338.