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Molecular Cloning and Characterization of a Transcription Factor for the *copia* Retrotransposon with Homology to the BTB-Containing Lola Neurogenic Factor

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By transfection experiments, we previously identified a 72-bp enhancer sequence within the *Drosophila copia* retrotransposon which is involved in the control of the transcription level of this mobile element in cells in culture. Gel shift assays with nuclear extracts from *Drosophila hydei*-derived DH-33 cells further demonstrated specific interactions of at least two nuclear factors with this enhancer sequence. Using this sequence as a probe for the screening of an expression cDNA library that we constructed from DH-33 cells RNA, we have isolated a cDNA clone encoding a 110-kDa protein with features common to those of known transcription factors; these include a two-zinc-finger motif at the C terminus, three glutamine-rich domains in the presumptive activation domain of the protein, and an N-terminal domain which shares homology with the Bric-à-brac, Tramtrack, and Broad-Complex BTB boxes. The precise DNA recognition sequence for this transcription factor has been determined by both gel shift assays and footprinting experiments with a recombinant protein made in bacteria. The functionality of the cloned element was demonstrated upon transcriptional activation of *copia* reporter genes, as well as of a minimal promoter coupled with the identified target DNA sequence, in cotransfection assays in cells in culture with an expression vector for the cloned factor. Southern blot and nucleotide sequence analyses revealed a related gene in *Drosophila melanogaster* (the *lola* gene) previously identified by a genetic approach as involved in axon growth and guidance. Transfection assays in cells in culture with *lola* gene expression vectors and in situ hybridization experiments with *lola* gene mutants finally provided evidence that the *copia* retrotransposon is regulated by this neurogenic gene in *D. melanogaster*, with a repressor effect in the central nervous systems of the embryos.

Virus-like retrotransposons share structural and functional homologies with the proviral form of vertebrate retroviruses and form a large and widespread family of mobile genetic elements present in all eukaryotic genomes studied so far. The Ty1 element in *Saccharomyces cerevisiae* (for a review, see reference 3), the *copia* element in *Drosophila melanogaster* (for a review, see reference 20), and the IAP sequences in mammals (for reviews, see references 30 and 33) belong to this family of dispersed elements, for which it has been formally demonstrated (Ty1 [4] and IAP [30]) that transposition occurs via the reverse transcription of a full-length genomic RNA intermediate of the element. However, little is known about the factors and cellular genes which control the transposition frequencies of these mutagenic agents. Actually, a key step in this control, as a consequence of the transposition mechanism, is transcription, as shown for the yeast Ty1 and the mammalian IAPs, for which induction of transcription at a high level was demonstrated to result in high transposition frequencies (1, 4). Transcription factors involved in the regulation in yeast of the Ty1 element have been partially characterized (34, 35) and, in *D. melanogaster*, homeoproteins have recently been demonstrated to regulate both positively and negatively the transcription of the *copia* (and to some extent of the 412) mobile elements (see references 7 and 18). In an attempt to characterize more extensively the host factors involved in the regulation of expression of transposable elements in vivo, we have selected the *copia* element as a model element; with the Ty1 element in *S. cerevisiae*, this transposon represents the archetype of the virus-like retrotransposons found in all higher eukaryotes, including humans, and together with its *Drosophila* host constitutes an adequate system to study the interactions that should exist between these potentially mutagenic, parasite-like elements and their host.

The *copia* element is a widespread retrotransposon found in most species of the *Drosophila* genus, with a few exceptions (e.g., *Drosophila hydei*) which, therefore, provide, de facto, control or virgin genomes. *copia* is a 5.4-kb provirus-like element with two 276-bp long terminal repeats (LTRs) which has previously been entirely sequenced (39). Its two major transcripts, which are 5.2 and 2.1 kb long, have been extensively characterized both in vivo during the different stages of fly development, for several natural populations, and in the *D. melanogaster*-derived cell lines S2 and Kc (15, 18, 41, 46). They code for putative proteins showing strong homologies with the GAG and POL products of vertebrate retroviruses; *copia*-containing plasmids produce virus-like particles which can be easily detected by electron microscopy when introduced into cells devoid of endogenous *copia* element (DH-33 cells [derived from *D. hydei*]) (58), and these virus-like particles possess reverse transcriptase activity (38).

As observed for the yeast Ty1 element (24, 35), downstream to its 5′LTR, in a 145-bp 5′ untranslated region (5′UTR), *copia* possesses distinct elements essential for its transcriptional regulation (for a review, see reference 7). One of them

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has been previously identified as disclosing strong homologies with the simian virus 40 core enhancer (39, 51), and in a previous work we have demonstrated that this sequence, which can be reduced to a 72-bp domain containing two 22-bp repetitions with dyad symmetry, behaves as an enhancer in reporter plasmids when associated either with the *copia* LTR in its native configuration or with a heterologous minimal promoter (8). Actually, the enhancer effect of this 72-bp sequence is much larger (i.e., as much as a 500-fold increase in transcription level) when the reporter plasmids are introduced into *D. hydei*-derived DH-33 cells than that when they are introduced into *D. melanogaster*-derived S2 or Kc cells. We have further demonstrated that whole-cell extracts prepared from DH-33 cells specifically contain a transcriptional factor which binds to the 72-bp minimal enhancer element described above and is not present in S2 cell extracts. This factor most probably binds to the enhancer sequence with a stoichiometry of two molecules per molecule of DNA, as could be expected from the presence of the two 22-bp repeats within the enhancer sequence, and is possibly responsible, among other factors, for the high transcription level of *copia* in the DH-33 cells. To identify a factor(s) which specifically binds the *copia* enhancer sequence, we have now established a cDNA library in λ phages starting with poly(A)$^+$ RNAs from DH-33 cells. In this study, the multimerized 72-bp element, which was used as a target to screen the library for expression, allowed us to clone a 3.5-kb cDNA with coding capacity. The putative protein encoded by this cDNA has a theoretical molecular mass of 110 kDa and displays a BTB box, several glutamine-rich and acidic regions, and a two-zinc-finger domain, which is reminiscent of the structure of the recently cloned Tramtrack, Broad-Complex, and Bric à brac transcription factors (17, 26, 29). By footprinting and gel shift experiments, we have characterized the precise DNA target of the cloned factor in the *copia* enhancer and have demonstrated that this minimal target sequence is sufficient to activate transcription of a heterologous promoter in the presence of an expression vector for the factor, in cotransfection assays with S2 cells. A related gene is also present in *D. melanogaster*, as revealed by Southern blot hybridization, and the identified factor discloses strong similarities with the product of a recently cloned 4.7-kb cDNA from the *D. melanogaster lola* gene, which is involved in axonal growth and guidance (25). This characterization finally allowed us to provide evidence, using *lola* mutants, that *lola* is involved in the regulation of *copia* in *D. melanogaster.*

MATERIALS AND METHODS

DNA constructs. Enh1, Enh2, Hom1, and Hom2 oligonucleotides have been previously described (7); they were used to PCR amplify from the *copia*-containing pBScopia plasmid (8) a 58-bp fragment containing the consensus homeoprotein binding sites and a 72-bp fragment containing the two dyad symmetries of the enhancer region. The following three couples of complementary oligonucleotides corresponding to sequences within the 72-bp enhancer (see Fig. 5A) were also synthesized, reassociated at room temperature after a 2-min treatment at 95°C, and purified on 5% polyacrylamide gels: I-cop1, 5′-AAATAGCATTTTTTCACATTCT; I-cop2, 5′-AGAATGTGAAAAAATGCTATTT; II-cop1, 5′-CATTTTTTCACATTCTTGTGAAATAG; II-cop2, 5′-CTATTTCACAAGAATGTGAAAAAATG; III-cop1, 5′-TCTTGTGAAATAGCTTTTTTTTTCACATTCT; and III-cop2, 5′-AGAATGTGAAAAAAAAAGCTATTTCACAAGA.

(i) Construction of the *copia* LTR-*lacZ* responder plasmids. The two *copia* LTR-derived responder plasmids (LTR-*lacZ* and LTR-5′UTR-*lacZ*) have been described previously (8).

(ii) Construction of chloramphenicol acetyltransferase (CAT) responder plasmids. The pD-33CAT plasmid (22) was linearized with *Pst*I, blunt ended, phosphatase treated, and ligated to single and multiple copies of a 22-mer I-cop oligonucleotide encompassing the 5′ terminus of the 72-bp enhancer sequence (pD-33CAT-I-cop), a 31-mer III-cop oligonucleotide encompassing the core of the enhancer sequence (pD-33CAT-III-cop), or a 72-mer corresponding to the complete enhancer domain of *copia* (pD-33CAT-72-cop). The pAE1970AluCAT plasmid containing the adult Adh gene enhancer and the distal promoter of the alcohol dehydrogenase gene has been described elsewhere (22).

(iii) Construction of eukaryotic expression vectors for 9.2.1AB and Lola. The expression vector for 9.2.1AB was constructed by inserting a 3,522-bp *Not*I-*Afl*II Klenow enzyme-treated fragment from the pSK-9.2.1AB-rescued plasmid (obtained by the Exassist helper phage system according to the manufacturer's instructions [Stratagene]) at the unique, phosphatase-treated, *Eco*RV site of the pAct5C-PPA plasmid (27). The pAct 5C-lola 4.8 and pAct 5C-lola 8.13 expression vectors, for the long form and the short form of the Lola protein, respectively (25), were constructed by inserting 3,816- and 2,052-bp *Eco*RI Klenow enzyme-treated fragments from clones 4.8 and 8.13 (both gifts from E. Giniger) at the *Eco*RV site of pAct5C-PPA as described above.

(iv) Construction of a His-tagged 9.2.1AB prokaryotic expression vector. The $(His)_6$-tagged fusion protein expression vector was constructed by using the pET19b plasmid (Novagen), which was opened with *Xho*I, blunt ended, and ligated to a 414-bp *Bsa*BI blunt-ended fragment from pSK$^-$9.2.1AB corresponding to the 5′ end of the 9.2.1AB cDNA; the remaining part of 9.2.1AB was inserted as a *Spe*I-*Kpn*I fragment (from pSK$^-$9.2.1AB) blunt ended at the *Kpn*I site into the above intermediate construct opened at the homologous *Spe*I site and at a *Bam*HI blunt-ended site in the pET19b plasmid polylinker.

Production and purification of the His-tagged protein. Synthesis of the recombinant His-tagged 9.2.1AB protein was induced in *Escherichia coli* BL21 cells grown in Luria-Bertani medium with 2 mM isopropyl-thio-β-galactopyranose (IPTG). The denatured recombinant protein (in 6 M urea) was purified with a nickel-chelate affinity resin according to the specifications of the manufacturer (Novagen) and dialyzed at 4°C in phosphate-buffered saline containing 1 mM $ZnCl_2$ and decreasing concentrations of urea (from 6 to 0 M urea). In the last dialysis steps with low urea concentrations, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT) were added in the dialysis buffer. Protein concentrations were measured by the Bradford method (Bio-Rad).

In vitro transcription and translation. DNA (1 μg) from the pSK$^-$9.2.1AB plasmid linearized at the unique *Afl*II site was transcribed in a final volume of 25 μl with 36 U of T3 RNA polymerase (Promega), 500 μM m7G(5′)ppp(5′)G (Pharmacia), and nucleoside triphosphates. Approximately 1 μg of RNA products was translated with 50 μl of rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine. The translation products were analyzed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE), and radiolabeled peptides were detected upon autoradiography of the gels.

Cells and transfections. DH-33 cells from *D. hydei* (53) and Schneider 2 (S2) or Kc cells from *D. melanogaster* (21, 45) were grown in Schneider medium (Gibco) with 10% fetal calf serum (Gibco) at 25°C. Transient transfection assays were performed by the calcium phosphate procedure, with 7×10^6 cells, 1 μg of responder plasmid, 4 μg of effector plasmid, and 5 μg of either pAct5C-PPA (27) or Bluescript vector (Stratagene) for completion to 10 μg.

β-Galactosidase assays. β-Galactosidase activities of the responder plasmids were measured 2 days posttransfection, after protein extraction as described elsewhere (49), by using a spectrophotometric assay with chlorophenol red-β-D-galactopyranoside (CPRG; Boehringer) as a β-galactosidase substrate. In a standard experiment, 10 to 15 μl of protein extract (0.5 mg/ml) and 4.5 mM CPRG were used. β-Galactosidase activities are expressed as optical density units per microgram of protein extract per minute.

CAT assays. Two days posttransfection, cells (approximately 7×10^6) were resuspended in 150 μl of 250 mM Tris-HCl (pH 8.0) and then lysed by three cycles of freezing (in liquid N_2) and thawing (at 37°C). Lysates were incubated at 60°C for 10 min to inactivate endogenous acetylases, and the debris was then removed by centrifugation. CAT assays were performed at 37°C for 30 min in a final volume of 70 μl containing 30 μl of lysate plus 40 μl of a premix containing 28 μl of 250 mM Tris-HCl (pH 8.0), 0.5 mg of *N*-acetyl-coenzyme A (Boehringer) per ml, and 0.15 μCi of [^{14}C]chloramphenicol (NEN). [^{4}C]chloramphenicol and its acetylated products were separated by thin-layer chromatography. The extent of conversion of chloramphenicol to its acetylated form was quantitated with a Bio-Imaging analyzer (model BAS1000; Fuji, Tokyo, Japan).

Nucleic acids. Genomic DNAs from various *Drosophila* species (*D. melanogaster*, *D. virilis*, *D. simulans*, and *D. hydei*) were extracted as described elsewhere (31), with in addition two phenol and one chloroform-isoamyl alcohol extractions prior to ethanol precipitation. DNA samples digested with appropriate restriction enzymes were electrophoresed on 1% agarose gels in TBE buffer and blotted onto Hybond N$^+$ nylon filters (Amersham) in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Southern blots were probed with 15 ng of random-primed (Multiprime kit [Amersham]) α-^{32}P-labeled DNA fragments in 15 ml of hybridization buffer (a 548-bp *Bst*NI-*Esp*I fragment from the pSK$^-$9.2.1AB plasmid or a 756-bp *Pvu*II fragment from the pEG 183 plasmid containing the cDNA encoding the short product of the *lola* gene [gift from E. Giniger]). Southern blots were prehybridized for 60 min and hybridized for 15 h in Church solution (7% SDS, 1 mM EDTA [pH 8.0], 0.5 M $NaPO_4$ [pH 6.8], no bovine serum albumin [12]) at 65°C. Filters were washed once in 0.5× SSC–0.1% SDS for 15 min at 65°C, once in 0.1× SSC–0.1% SDS for 15 min at 65°C, and once in 0.1× SSC–0.1% SDS for 15 min at 68°C.

Total cellular RNAs from S2 and DH-33 cells were prepared by the guanidinium method (11). Poly(A)$^+$ RNAs were purified on oligo(dT) cellulose (Collaborative Research; type 3). A 5-μg amount of poly(A)$^+$ RNAs was fractionated by electrophoresis in 1% (wt/vol) formaldehyde-agarose gel in 1× morpho-

linepropane sulfonic acid buffer and transferred to Hybond N nylon filters (Amersham) in 0.15 M NH_4 acetate buffer. Northern (RNA) blots were probed with the 548-bp *Bst*NI-*Esp*I fragment used for genomic DNA analysis. Prehybridization, hybridization, and wash conditions were as for Southern blots.

Construction and screening of the DH-33 λZAP-cDNA expression library. The *D. hydei* DH-33 cell cDNA library was constructed in the λ Uni-ZAP XR expression vector according to the manufacturer's instructions (λZAP-cDNA synthesis kit [Stratagene]) by using 10 μg of poly(A)$^+$ RNAs from DH-33 cells for cDNA synthesis; recombinant phages were encapsidated with the Gigapack II packaging extract (Stratagene). The library contained approximately 10^6 independent clones and had an average insert size of 1.7 kb.

Screening of the library was performed with a multimerized DNA probe; the 72-bp fragment containing the transcription factor(s) binding sites was first amplified by PCR with the two previously described Enh1 and Enh2 primers (7), and after phosphorylation of the termini, this fragment was multimerized with T4 DNA ligase (as many as six fragments were ligated, as controlled by migration of the labeled probe on a 10% polyacrylamide gel) and was [α-^{32}P]dCTP labeled by random priming (Amersham). The probe was purified on a Sephadex G-50 column (specific activity of the probe: 4×10^8 cpm/μg), and screening of the library was by standard procedures (36, 50). Each replica filter (BA-S 85 [Schleicher and Schuell]) was individually incubated in 10 ml of hybridization buffer overnight at 4°C with gentle shaking. The hybridization buffer was the same as the one described for mobility shift assays, with 0.25% nonfat dried milk and 10 μg of sonicated, denatured salmon sperm DNA (Pharmacia) per ml in addition. Positive clones were subcloned twice, and Bluescript SK(−) phagemids containing cDNA inserts were excised from the plaque-purified λ Uni ZAP XR clones and rescued by using the ExAssist helper phage according to the manufacturer's instructions. The double-stranded cloned cDNA insert from phagemid 9.2.1 (rescued in SOLR cells) was entirely sequenced by using a Thermosequenase fluorescence labeled primer cycle sequencing kit (Amersham) and an Applied Biosystems 373 DNA sequencer.

A 500-bp *Pst*I fragment of the partial 9.2.1 cDNA clone obtained by the screening described above was then [α-^{32}P]dCTP labeled and used as a probe to screen the cDNA library by DNA-DNA hybridization. Replica filters were incubated overnight at 65°C in Church solution. Filters were then washed in 0.1× SSC–0.1% SDS at 70°C for 20 min. Positive phages were purified twice and excised as described above, and the one with the largest insert (9.2.1AB) was entirely sequenced as described above.

Whole-cell extracts. Cells were washed three times in ice-cold phosphate-buffered saline and pelleted by centrifugation at $1,000 \times g$. The pelleted cells were then resuspended in 4 vol of buffer A containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 5 mM DTT and were incubated at 0°C for 20 min. Cells were then fragmented in a Dounce homogenizer (8 to 10 strokes with pestle B), and 4 vol of extraction buffer B (50 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 2 mM DTT, 25% sucrose, 50% glycerol, 1 mM PMSF) was added. One volume of saturated ammonium sulfate $(NH_4)_2SO_4$ was added slowly (final concentration, 10% of saturation), and the extracts were rocked gently at 4°C for 20 min and then were centrifuged at $230,000 \times g$ for 3 h. The pellet was discarded, and ammonium sulfate (0.33 g/ml of supernatant) was added, together with 1 N hydroxide sodium (1 μl of NaOH per g of ammonium sulfate). The extracts were rocked at 4°C for 30 min and centrifuged at $15,000 \times g$ for 20 min. The pellets were resuspended in 0.8 vol of EPN buffer containing 50 mM Tris-HCl (pH 8.0), 40 mM $(NH_4)_2SO_4$, 6 mM $MgCl_2$, 2 mM DTT, 0.1 mM EDTA, 17% glycerol, and 1 mM PMSF. The cell extracts were dialyzed overnight at 4°C in the same buffer. Protein concentrations were determined by the Bradford method (Bio-Rad). Heparin column fractionation of the DH-33 whole-cell extracts was performed with increasing KCl concentrations (from 100 to 800 mM) according to the manufacturer's specifications (Pharmacia). The column eluates were dialyzed overnight at 4°C in phosphate-buffered saline containing 1 mM PMSF and 1 mM DTT.

Mobility shift assays. Mobility shift assays were performed essentially as previously described (8). Reaction mixtures (20 μl) contained 10 μl of binding buffer (40 mM HEPES [pH 7.9], 200 mM KCl, 2 mM DTT, 0.2 mM EDTA, 10 mM $MgCl_2$, 40% glycerol), 0.5 ng of γ-^{32}P-labeled oligonucleotide probe, 2 μg of poly(dI-dC), and 15 to 35 μg of whole-cell extracts. Probes and proteins were incubated for 30 min at room temperature. When indicated, unlabeled double-stranded oligonucleotides were added in competition assays (Sp1 site [5′-GATCGATCGGGGCGGGGCGATC-3′]). Reaction products were separated on 5% polyacrylamide gels (acrylamide-bisacrylamide, 29/1) at room temperature for 2 h and 30 min at 190 V. The gels were dried and autoradiographed. Approximately 2 μg of His-tagged 9.2.1AB protein or 20 μl of heparin column-dialyzed eluates was added to the DNA reaction mixture when indicated.

DNase I footprinting. Approximately 3 μg of a 578-bp *Hin*dIII fragment from the LTR-*lacZ* responder was end labeled by filling in the *Hin*dIII sites with [α-^{32}P]dCTP with the Klenow enzyme. This fragment was then restricted with *Pst*I to remove the 5′-labeled end and was purified on a Sephadex G-75 column. The probe was then run at room temperature on a native 5% polyacrylamide preparative gel and recovered by the crush and soak method (28) and then by phenol extraction and ethanol precipitation. A 250-fmol (95-ng) amount of this fragment was incubated for 30 min at room temperature with various amounts of the purified His-tagged 9.2.1AB protein (25 nM to 3 μM) in 25 μl of incubation buffer [20 mM HEPES (pH 7.9), 100 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 1 mM $ZnCl_2$, 0.1 mM EDTA, 20 μg of poly(dI-dC) per ml, 20 μg of bovine serum albumin per ml, 20% glycerol]. The total volume was adjusted to 50 μl with 25 μl of incubation buffer, and 2 μl of 400 U of DNase I per ml was added; incubation was for 1 min at room temperature in the presence of 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$, and the reaction was stopped upon addition of 140 μl of stop buffer (190 mM sodium acetate, 30 mM EDTA, 0.15% SDS, 65 μg of yeast RNA per ml). DNA was isolated by phenol-chloroform extraction and then by ethanol precipitation and was run on an 8% sequencing gel. A G+A reaction was performed in parallel as described elsewhere (37).

Histochemistry. Immunohistochemistry and RNA tissue in situ hybridization to whole-mount embryos (0- to 24-h embryos) were performed by using two P-element-induced strong alleles of *lola* (*lola*$^-$), which were previously described (25), i.e., *lola*^{5D2} [l(2)5D2] and *lola*^{4D4} [l(2)4D4]. Controls (*lola*$^+$) were with isogenic unrelated P element insertions (from the same experiment that yielded the two *lola* alleles), namely, l(3)3C8 and l(3)3D1. All flies were kindly provided by E. Giniger.

(i) RNA tissue in situ hybridization to whole-mount embryos. Whole-mount RNA tissue in situ hybridization was performed according to the method described elsewhere (54), with a digoxigenin-labeled DNA probe made as suggested by the manufacturer (Boehringer Mannheim) and alkaline phosphatase visualization. The *copia* DNA probe is a 2.5-kb internal *Hin*dIII-*Hpa*I fragment from the pBScopia plasmid previously described (7). Embryos were mounted in Spurr embedding medium (E. F. Fullam, Inc.)

(ii) Immunohistochemistry. Immunostaining was performed as described elsewhere (25), except that embryos were fixed for 20 min in 0.8% paraformaldehyde instead of 4% formaldehyde. The anti-Lola (α-Lola) antibodies used were affinity-purified rabbit polyclonal antibodies (25 [gift from E. Giniger]) raised against the short form of Lola (amino acids 19 to 467 [25]). They were used at a 1:100 dilution. Secondary antibodies were from Vector Laboratories and were preabsorbed against wild-type fly embryos prior to use (50 μl of packed embryos per 500 μl of 50-fold-diluted antibody); final dilution was 1:1,000. Antibody staining was visualized by the ABC method (Vector Laboratories) and embryos were mounted in Spurr embedding medium.

RESULTS

Gel shift assay for protein interactions with the enhancer sequence of the *copia* retrotransposon. To identify proteins interacting with the *copia* enhancer, gel retardation assays were performed with extracts from two cell types, S2 and DH-33. We have previously shown that a protein present in DH-33 whole-cell extracts (a cell line from the *D. hydei* species devoid of the endogenous *copia* element) can specifically bind to the enhancer sequence of the transposon, whereas extracts from S2 cells (from the *D. melanogaster* species with 50 to 100 endogenous *copia* elements) fail to display such a factor (8). This cell-type-specific protein (with a molecular mass of approximately 50 kDa) most probably binds to the *copia* enhancer with a ratio of two molecules per molecule of DNA target (bands with retarded mobility [B1 and B2] [Fig. 1A]). This factor is not present in S2 cells and is, therefore, expected to be responsible for the observed strong differential expression of *copia* between the DH-33 and S2 cell lines. A more extensive analysis of DH-33 cell extracts with gel shift assays (Fig. 1A) disclosed another band (further designated B′) of intermediate mobility between bands B1 and B2. Unlike B1 and B2, this specific band is equally present in S2 cell extracts (Fig. 1B). A fourth band of lower mobility can also be observed, which we have designated as B″, and which is common to S2 and DH-33 extracts but seems, to some extent, to be unspecific, since it is only partially displaced by an excess of unlabeled target DNA (Fig. 1A and C). An experiment involving passage of cellular DH-33 extracts on a heparin column and subsequent elution of bond factors at increasing salt concentrations (Fig. 1C) resulted in a clear-cut separation of the B1, B2, and B′ bands (which, as expected for DNA binding proteins, were eluted at high salt concentration) from the B″ band which eluted at much lower salt concentrations and again was not significantly reduced upon addition of an excess of unlabeled probe. These data suggest that both differential and common factors from S2 and DH-33 extracts can specifically bind to the *copia* enhancer sequence. These results prompted

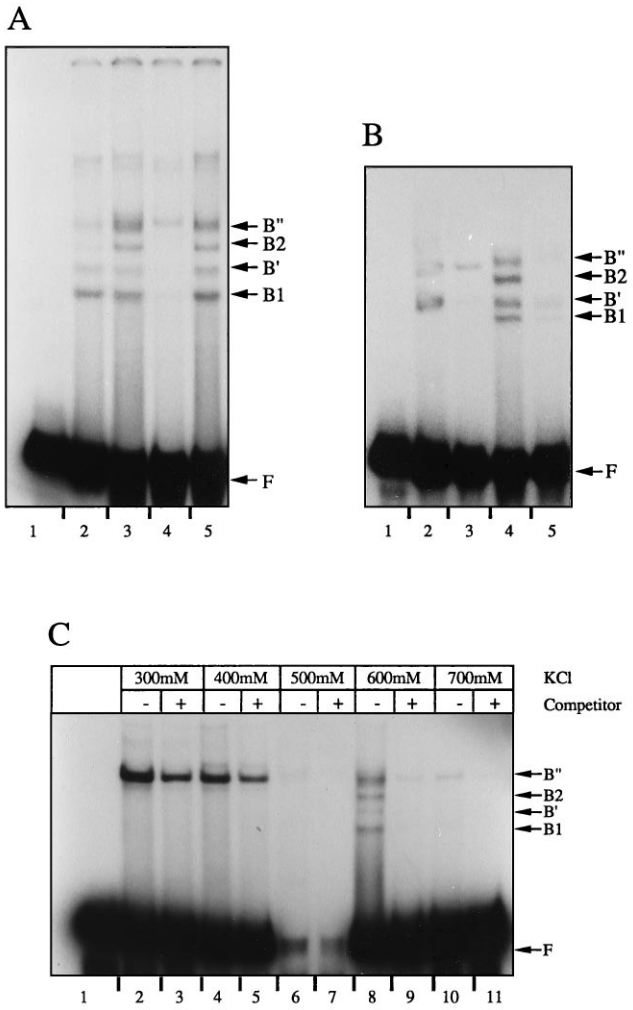

FIG. 1. Mobility shift assay for the interaction between factors from DH-33 and S2 whole-cell extracts and the *copia* enhancer. (A) Gel shift analysis of whole-cell extracts from DH-33 cells with a γ-^{32}P-labeled 72-bp *copia* enhancer probe. Lanes: 1, probe alone (F); 2, 15 μg of extract; 3 to 5, 36 μg of extract. A 25-fold molar excess of either the unlabeled DNA probe or an irrelevant 58-bp DNA (HpRE [see Materials and Methods]) was added in lanes 4 and 5, respectively. B1, B′, B2, and B″ correspond to specific complexes. (B) Comparison of S2 and DH-33 cell extracts; same experimental conditions as for panel A, with 15 μg of whole-cell extracts from S2 (lanes 2 and 3) and DH-33 (lanes 4 and 5) cells. A 25-fold molar excess of unlabeled probe was added in lanes 3 and 5. The specific complexes are indicated as in panel A. (C) Fractionation of DH-33 cell extracts on a heparin column and elution with increasing salt concentrations. Gel shift assays were as for panel A, with 20 μl of dialyzed fractions eluted from a heparin column loaded with DH-33 whole-cell extracts; KCl concentrations at each elution step are indicated (300 to 700 mM); a 50-fold molar excess of unlabeled probe was added in the lanes with odd numbers (except lane 1 [free probe]). Specific complexes are indicated as in panel A.

us to use the *copia* enhancer region as a probe to screen a cDNA library derived from DH-33 cells, in an attempt to characterize both types of transactivating factors.

Identification of a cDNA clone with binding activity to the *copia* enhancer. To isolate cDNAs encoding factors which bind to the *copia* enhancer, we constructed an oligo(dT)-primed cDNA library in λUni-ZAP XR (Stratagene), starting with poly(A)$^+$ RNA from the *D. hydei* DH-33 cell line. As mentioned above, these cells were preferred to S2 cells because they contained both differential and nondifferential binding factors; moreover, it had been shown that the *copia* element is much more heavily transcribed in DH-33 than in S2 cells (8, 51) cells. The DH-33 library was screened by a modification of the method originally described by Singh et al. (36, 50), with a radiolabeled *copia* enhancer multimer as a probe (see Materials and Methods). From a primary screen of 100,000 phage plaques, only one recombinant of nine (further designated 9.2.1) bound specifically to this probe and remained positive in the two subsequent purification steps. The 1.4-kb insert of the recombinant clone was isolated by using the ExAssist helper phage system (Stratagene) and sequenced (see below). It contained an open reading frame starting from the very 5′ end of the cDNA and, accordingly, was most probably 5′ truncated. A 500-bp *Pst*I fragment from the 1.4-kb insert was, therefore, used as a probe to tentatively isolate a complete cDNA upon screening the DH-33 cDNA library by DNA-DNA hybridization. Several recombinant phages were isolated and mapped for the presence of restriction sites of the 9.2.1 cDNA. The largest one, 9.2.1AB, contained a 3.5-kb insert and was entirely sequenced.

Structure of the 9.2.1AB cDNA clone and coding sequence. The complete sequence of the 9.2.1AB cDNA insert is given in Fig. 2A. It contains a 3.0-kb open reading frame, ending with a TAA termination codon, and starting with a consensus initiation codon at position 230. According to data bank analysis, this sequence had never been described before, although it shares some features with previously characterized genes (see below). The coding capacity of this cDNA was tested by an in vitro synthesis experiment with rabbit reticulocyte lysate. As shown in the SDS-PAGE gel in Fig. 2C, a protein with an apparent molecular mass of approximately 170 to 180 kDa was synthesized, which actually is 50% greater than the molecular mass predicted from the sequence, i.e., approximately 110 kDa. The basis for this unusually large difference may involve the numerous acidic residues of the protein (see below) and is reminiscent of that observed for the *tramtrack* gene product, which discloses partial sequence similarities with 9.2.1AB (see below and reference 44).

The presumptive 9.2.1AB cDNA-encoded protein (see overall organization in Fig. 2B) displays the following interesting features, shared by products of previously characterized genes. Firstly, in the C-terminal region, the protein possesses two putative zinc finger domains separated by eight amino acid residues (945-CRHCGKKYRWKSTLRRHENVEC-966 and 975-CPYCTYKAKQRGNLGVHVRKHH-996). These are most probably responsible for the DNA binding activity. In agreement with this putative function, it should be noticed that this region was the one initially isolated upon screening of the cDNA library for expression. In the most N-terminal zinc finger motif (945 to 966), a cysteine residue is substituted for the second histidine residue involved in Zn^{2+} coordination. Such a substitution has previously been described for 2 of 12 zinc finger motifs of the suppressor of hairy wing protein (SuHw [42]) and is also found in the products of several *Drosophila* genes, such as *snail* (5), *serendipity* β (43), *escargot* (56), and *lola* (25). As shown in Fig. 3A, the two zinc finger domains of the presumptive 9.2.1AB protein disclose amino acid similarity with the corresponding domains of BR-CZ2 and p69 Ttk proteins. Some residues (positions 6, 9, and 12 of the zinc finger motif), which are assumed to be involved in DNA recognition, are clearly conserved. Amino acid similarity is even higher when the sequence is compared (Fig. 3A) to the corresponding region of the long product of the *lola* gene (96.5 kDa [25]); the overall amino acid identity is 92%, and all six residues involved in DNA recognition are strictly conserved, namely, the Trp, Thr, and Arg residues at positions 6, 9, and 12,

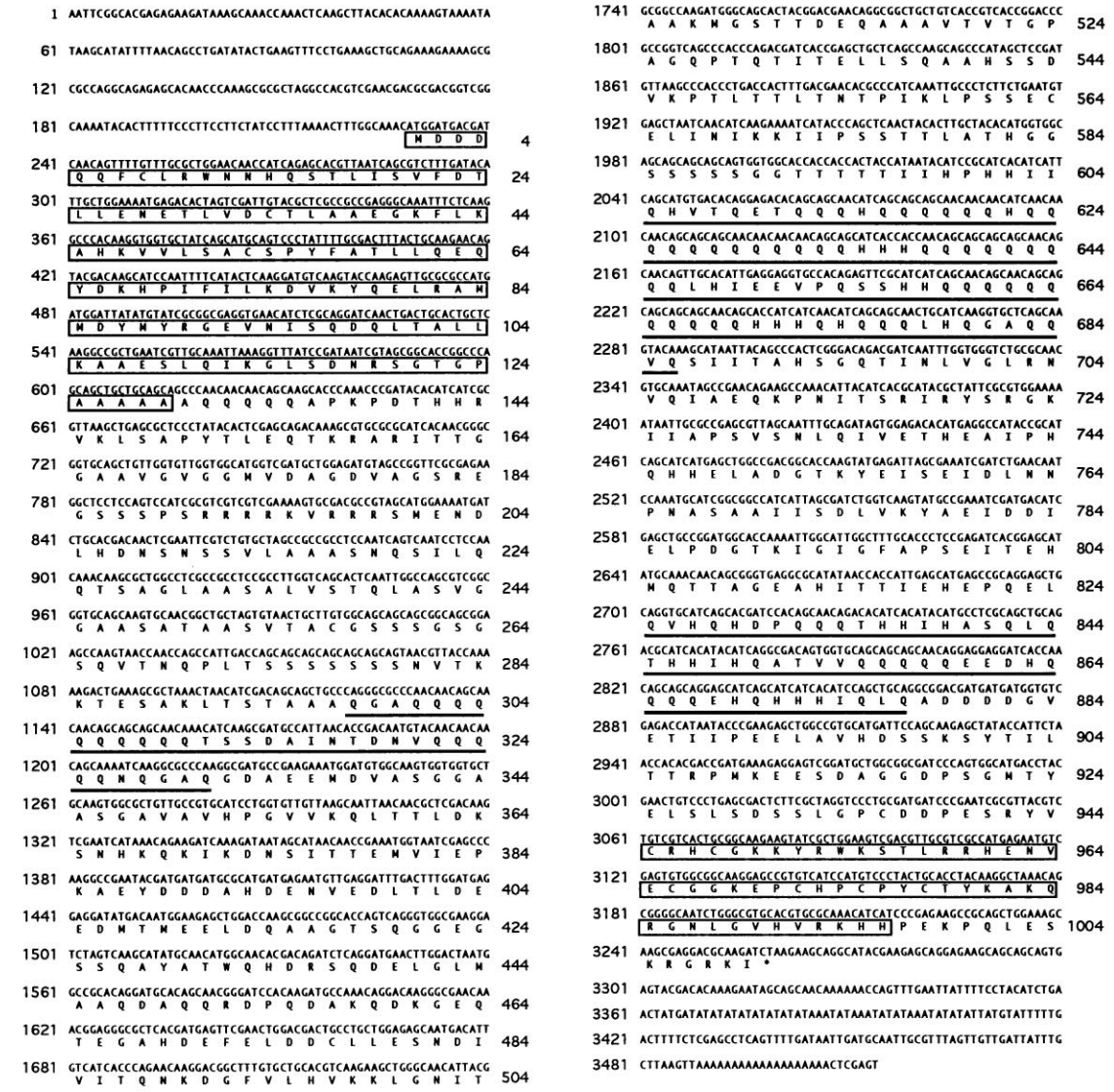

FIG. 2. 9.2.1AB sequence and characteristic features of the protein. (A) Complete cDNA and deduced amino acid sequences of 9.2.1AB, with nucleotide positions on the left and amino acid positions on the right. The BTB domain and the two zinc finger domains are boxed. The three glutamine-rich domains (I, II, and III) are underlined. (B) Schematic organization of the 9.2.1AB product and localization of the predicted motifs; for simplicity, the acidic domains have been omitted. TS, threonine-serine stretches; nls, nuclear localization signal. (C) SDS-PAGE (10% polyacrylamide) analysis of the in vitro transcription plus translation products of 9.2.1AB cDNA. Lanes: 1, rabbit reticulocyte lysate translation products of in vitro transcripts of the 9.2.1AB cDNA; 2, control translation with no RNA; 3, molecular mass markers (Amersham). The major translation product of 9.2.1AB is indicated by an arrow.

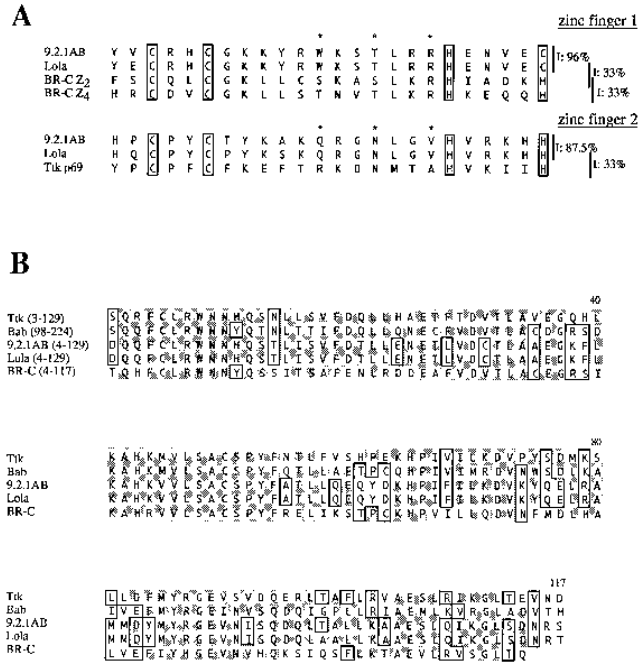

FIG. 3. Comparison of the 9.2.1AB zinc finger and BTB domains with previously characterized sequences. (A) Zinc finger motifs of 9.2.1AB, Ttk p69 (23), BR-C Z_2 (17), BR-C Z_4 (55), and Lola (25). Boxes, homologous cysteine and histidine residues; asterisks, residues involved in DNA recognition. Percentages of identity are indicated on the right. (B) N-terminal region (BTB domain) of 9.2.1AB and other *Drosophila* zinc finger proteins. Numbers are relative to the methionine initiation codon of the genes *ttk* (44), *BR-C* (17), *bab* (60), and *lola* (25); residues are shaded when at least four of five amino acids are conserved and are boxed when two of five amino acids are identical.

respectively, for the first zinc finger motif (945 to 966), and the Gln, Asn, and Val residues at positions 6, 9, and 12, respectively, for the second zinc finger motif (975 to 996). Accordingly, these two proteins possibly recognize the same DNA target.

A second salient feature concerns the presence of three stretches of polyglutamine in the central part of the protein. These domains, designated A (amino acids 298 to 331), B (amino acids 605 to 686), and C (amino acids 822 to 877), from the N- to C-terminal regions of the protein, are, respectively, 53, 63, and 37% glutamine rich. Two of them (the A and the B domains) are preceded by stretches of serine and threonine residues, as observed for the Sp1 factor (14). The 9.2.1AB protein also discloses three acidic domains (amino acids 383 to 413, 453 to 483, and 737 to 787). These three polyglutamine stretches and three acidic domains most probably correspond to the activation domains of the 9.2.1AB protein.

Finally, the N-terminal 120 residues of the protein have significant similarity (Fig. 3B) to the N-terminal region of the proteins encoded by the *Broad Complex* (*BR-C* [49% amino acid identity]), *tramtrack* (*ttk* [62% identity]), and *bric à brac* (*bab* [58% identity]) genes. Compared with the same domain of the *lola* products, the overall identity reaches 98%. This peculiar region has previously been designated the BTB or POZ domain (2, 26). It is involved in protein-protein interactions and mediates homo- and heterodimer formation (2, 10, 13, 16). The protein products of *BR-C*, *ttk*, *bab*, and *lola* are all believed to be transcription factors (6, 17, 25, 29).

The 9.2.1AB protein is a potent and specific transcriptional activator of the *copia* retrotransposon. To determine whether the 9.2.1AB cDNA-encoded protein can specifically activate the transcription of *copia*, we performed cotransfection experiments using *lacZ* reporter plasmids and an expression vector for the 9.2.1AB protein (see Materials and Methods). The previously described LTR-5′UTR-*lacZ* and LTR-*lacZ* responder constructs (8) contain the *lacZ* gene under the control of the *copia* LTR alone (LTR-*lacZ*) or of the LTR with the 5′ enhancer-containing *copia* UTR (LTR-5′UTR-*lacZ*). S2 cells were selected for the cotransfection assays because of the absence of a strong *copia* activator in these cells (8). As illustrated in Fig. 4, the level of expression of the *copia* reporter is enhanced 30-fold in the presence of the 9.2.1AB expression vector. This strong activation is specific to the 5′UTR, since the 9.2.1AB protein has no significant effect on the *copia* LTR alone. We can infer from these data that there is most probably no DNA-binding site for this factor in the *copia* LTR (see below) and that the 9.2.1AB protein can act as a potent and specific transcriptional activator of the *copia* retrotransposon.

Identification of the DNA-binding site for the 9.2.1AB protein in the *copia* 5′UTR. The protein encoded by the 9.2.1AB cDNA was expressed in *E. coli* as a fusion protein (His-tagged 9.2.1AB [see Materials and Methods]). It had to be denatured for its extraction and purification on a nickel-chelate affinity resin, and it was then tentatively refolded (in the presence of

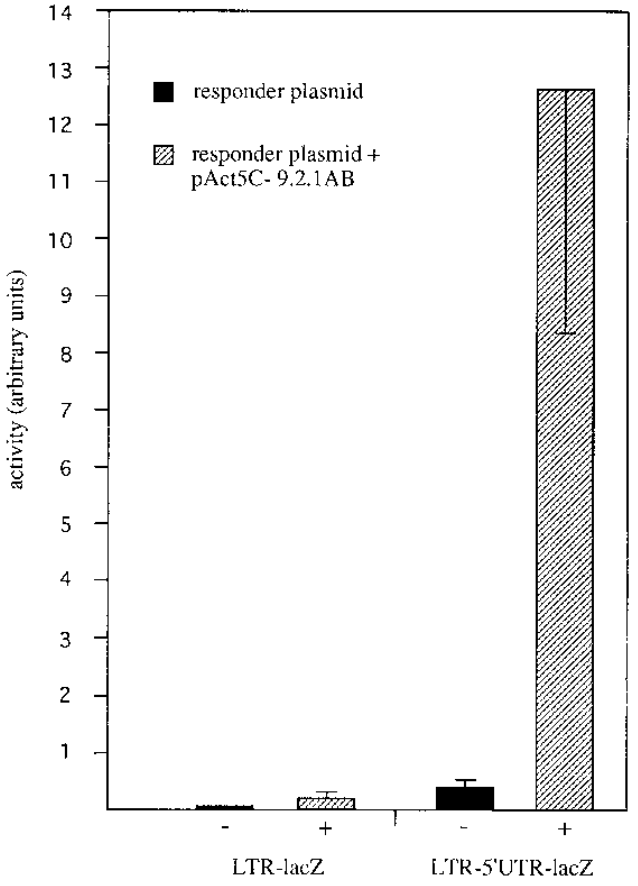

FIG. 4. 9.2.1AB- and 5′-UTR-specific activation of the *copia* retrotransposon. Cotransfection assays were carried out with 1 μg of 9.2.1AB expression vector (pAct5C-9.2.1AB) or a control vector (pAct5C-none) and 4 μg of the *copia* LTR-*lacZ* or *copia* LTR-5′UTR-*lacZ* responder plasmids. Total plasmid DNA concentrations were adjusted to 10 μg with the Bluescript plasmid (Stratagene). β-Galactosidase activities were measured 2 days posttransfection. Each value is the mean of three independent transfection experiments. Error bars, standard deviations.

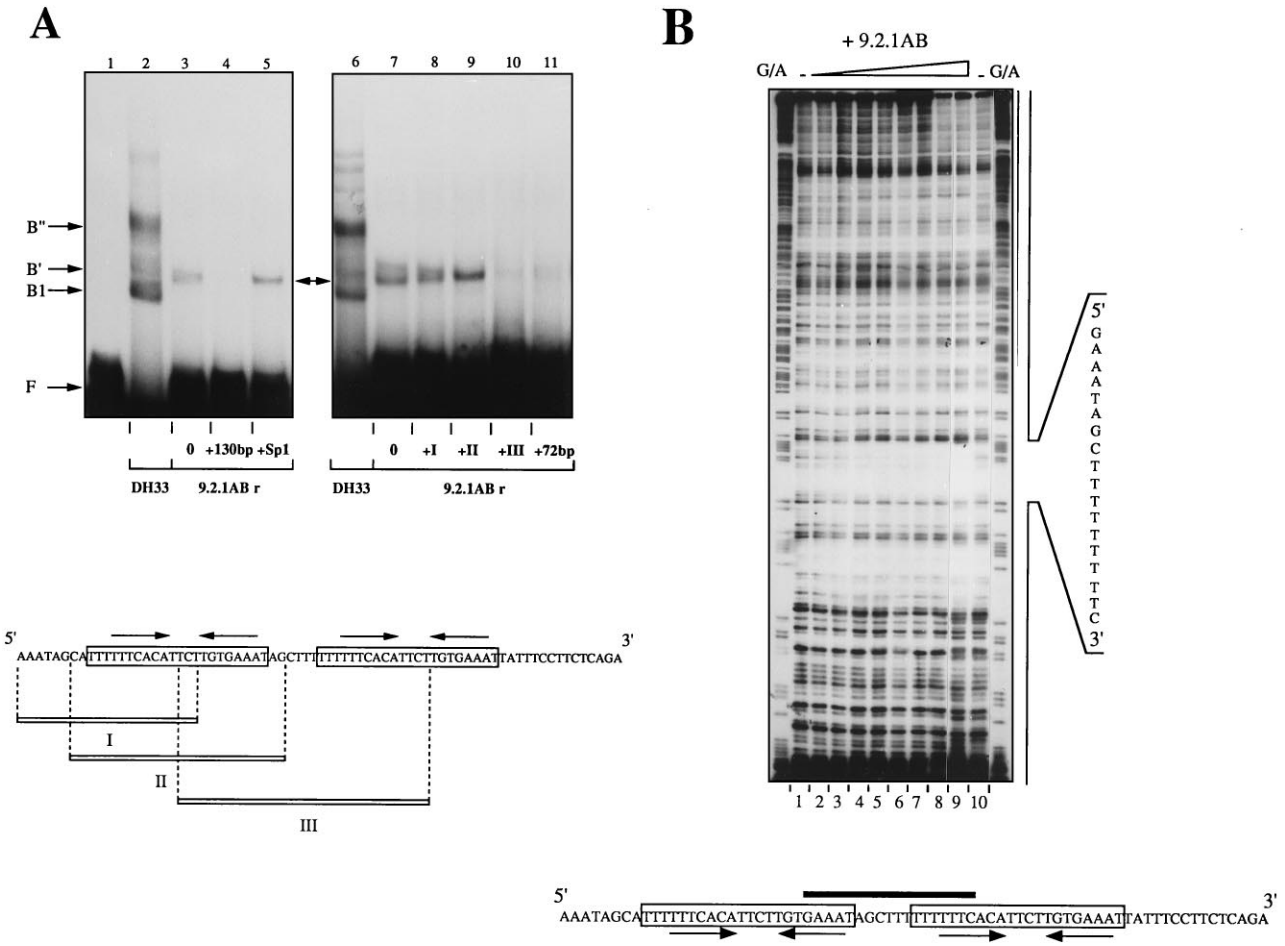

FIG. 5. Characterization of the 9.2.1AB DNA-binding site by mobility shift assays and footprinting experiments. (A) Mobility shift assays of the interaction between the 9.2.1AB purified recombinant protein and the *copia* 130-bp 5′UTR. A 2-μg amount of the His-Tag-purified recombinant protein (9.2.1ABr [lanes 3 to 5 and 7 to 11]) or 15 μg of DH-33 whole-cell extracts as a control (DH33 [lanes 2 and 6]) was tested for the formation of DNA-protein complexes and analyzed on native 5% polyacrylamide gels, as described in Materials and Methods. The specificity of the interactions was assessed by using a 200-fold molar excess of the unlabeled oligonucleotides I-cop (lane 8), II-cop (lane 9), III-cop (lane 10), the 72-bp enhancer region (lane 11), or the 130-bp 5′UTR (lane 4) or a 600-fold molar excess of an unrelated 22-bp double-stranded oligonucleotide (Sp1 site [lane 5]). The sequences and positions of the competitor oligonucleotides within the 72-bp *copia* enhancer are indicated in the lower part of the figure. (B) DNase I footprinting analysis of the *copia* LTR and 5′UTR with purified 9.2.1AB recombinant protein. Footprinting reactions were carried out as described in Materials and Methods on a 565-bp *Pst*I-*Hin*dIII fragment containing the entire *copia* LTR and 5′UTR. A G+A sequencing reaction was run in parallel. The amounts of purified protein used were as follow: lanes 1 and 10, none; lane 2, 25 nM; lane 3, 50 nM; lane 4, 100 nM; lane 5, 200 nM; lane 6, 400 nM; lane 7, 800 nM; lane 8, 1.5 μM; lane 9, 3 μM. The sequence of the protected domain in the *copia* 5′UTR is indicated on the right and positioned in the scheme below (bold line).

zinc [see Materials and Methods]) to restore DNA-binding activity. The recombinant protein was first tested for its specific DNA-binding activity by gel shift experiments, with the 130 bp of the *copia* 5′UTR as a probe. A series of unlabeled double-stranded oligonucleotides, overlapping the 72-bp enhancer sequence, were also tested in competition assays (oligonucleotides I-cop, II-cop, III-cop, and 72 bp [see Materials and Methods]) to more precisely characterize the binding domain of the 9.2.1AB protein. As illustrated in Fig. 5A, a band of retarded mobility can unambiguously be observed in the presence of 9.2.1AB, which comigrates, as a first approximation, with the previously described B′ complex. This band corresponds to a specific interaction of the recombinant protein with the *copia* 5′UTR, since it is no more observed in the presence of an excess of unlabeled 72- or 130-bp 5′UTR enhancer sequence. Furthermore, addition of the oligonucleotides mentioned above discloses a competition specifically with the III-cop oligonucleotide, which corresponds to a sequence located between the two 22-bp repeats in the enhancer domain.

The precise DNA-binding site of 9.2.1AB was further characterized by footprinting experiments with the whole *copia* LTR plus 5′UTR, and increasing amounts of the recombinant 9.2.1AB protein (Fig. 5B). In agreement with the mobility shift assays and the cotransfection experiments in S2 cells, these experiments revealed only one protected domain, located in the *copia* 5′UTR between the two 22-bp repeats, with the following sequence: 5′-GAAATAGCTTTTTTTTTC-3′. Interestingly, another region located just 5′ to it and with a closely related sequence (5′-GAAATAGCATTTTTTC-3′) is not protected by the protein, thus strengthening the specificity of the recognition site for the cloned factor.

The identified DNA-binding site is sufficient to confer 9.2.1AB response to a heterologous basal promoter. The 9.2.1AB binding site-dependent enhancement of transcription was assessed in vivo by using S2 cells and a heterologous basal promoter. A series of reporter plasmids were constructed, in which synthetic sequences containing variable numbers of minimal 9.2.1AB binding sites (III-cop), 72-bp *copia* enhancer

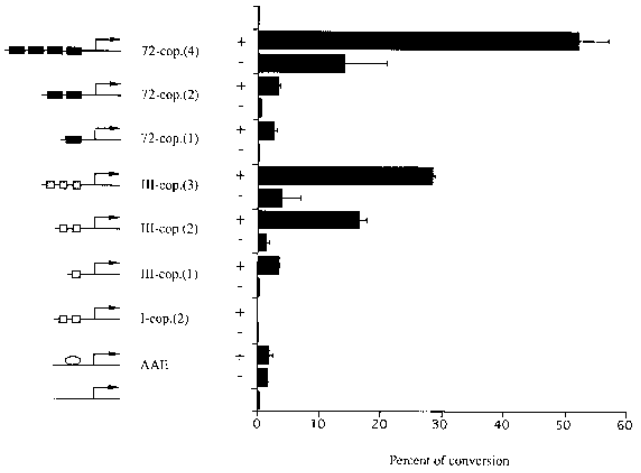

FIG. 6. 9.2.1AB- and binding site-dependent activation of transcription. The basal Adh promoter-CAT responder plasmid (22) containing (or not containing) single or multimerized copies (numbers indicated in parentheses and schematized by boxes) of the I-cop, III-cop, or 72-bp sequences (see Fig. 5) or the adult Adh gene enhancer (AAE) as a reference were cotransfected with (+) or without (−) 4 μg of the pAct 5C-9.2.1AB expression vector in S2 cells; 1 μg of responder plasmid was used, and total plasmid DNA concentration was adjusted to 10 μg with pAct 5C-PPA. CAT activity was measured as indicated in Materials and Methods. Each value is the mean of three independent transfection experiments. Error bars, standard deviations.

sequences, or irrelevant oligonucleotide (I-cop) were placed 5′ to an Adh basal promoter (nucleotide −33) and a CAT reporter gene (22). These plasmids were cotransfected (or not) with the pAct-9.2.1AB expression vector into S2 cells, and the CAT activities were quantitated 2 days posttransfection. As illustrated in Fig. 6, a significant stimulation (7- to 18-fold) of the III-cop target promoter was observed in the presence of the pAct-9.2.1AB expression vector, and similar relative levels of induction (4- to 24-fold) were observed with the 72-bp enhancer sequence, as expected. Conversely, the pAct-9.2.1AB expression vector had no effect on the reporter containing the I-cop sequence, located at the 5′ end of the 72-bp enhancer region (for the exact location of this sequence, see Fig. 5A), as well as on an enhancer sequence unrelated to *copia* (the adult Adh enhancer) or the Adh basal (−33) promoter alone. Finally, as expected for enhancer sequences, the levels of expression of the plasmids containing either the III-cop or the 72-bp sequences were found to increase with the number of 9.2.1AB binding sites in the reporters. Interestingly, this was also observed for the control levels of expression in the absence of expression vector, therefore suggesting that S2 cells contain an endogenous transactivating factor(s) for *copia* (possibly one of the *lola* products [see below and Discussion]), also acting at the level of the identified 9.2.1AB DNA-binding and transactivating site.

The *9.2.1AB* gene in *D. hydei* is structurally related to the *lola* gene in *D. melanogaster*. As previously mentioned (Fig. 3A and B), there exists a very high sequence conservation between Lola and 9.2.1AB within their N- and C-terminal domains (92 and 98%, respectively), despite the large phylogenetic distance (>40 million years) between *D. hydei* and *D. melanogaster*. Actually, Lola is the closest relative to 9.2.1AB according to searches in the available databases, with an overall similarity index of 60%. A DotPlot analysis of both sequences (Fig. 7) further reveals a third domain (positions 378 to 455 in 9.2.1AB) with 96% identity between the two gene products. However, the remaining domains of the encoded proteins are more distantly related, but a similar situation has been described for unambiguously homologous genes such as *engrailed* or *bicoid*, among distinct *Drosophila* species (32, 48); domains with high sequence conservation were found embedded within regions of poor similarity, further resulting in proteins of distinct lengths. To analyze further the possible relationship between the *9.2.1AB* and *lola* genes, genomic DNAs from both *D. hydei* and *D. melanogaster* were restricted with a series of enzymes and hybridized with a probe encompassing the above-mentioned common internal domain (a 548-bp *Bst*NI-*Esp*I fragment from the 9.2.1AB cDNA). This domain was selected as a probe rather than the BTB or zinc finger domains, since these are also found in several transcription factors. With this probe, as illustrated in Fig. 8A, single bands (except possibly with *Bgl*I restriction) were detected in *D. hydei*, suggesting that the *9.2.1AB* locus is a single copy gene. Interestingly, this 9.2.1AB-derived probe also revealed a single-copy gene in *D. melanogaster* genomic DNA, even under conditions of high stringency washing of the Southern blots (same conditions as those for *D. hydei* DNA). After dehybridization, the same blot was reprobed with a specific 756-bp *Pvu*II fragment from the *lola* gene, under the same stringency conditions for hybridization and washes. This *lola* fragment shares less than 45% nucleotide identity with the corresponding region from the *9.2.1AB* gene according to the alignment method described by Lipman and Wilbur. As seen in Fig. 8B for *D. melanogaster* genomic DNA, the same bands as the ones detected with the 9.2.1AB probe are revealed with the *lola*-specific fragment (and no band can be detected within the *D. hydei* genomic DNA, as expected). These data, therefore, indicate that the *9.2.1AB* gene is most probably a single-copy gene in *D. hydei* and that its closest relative in *D. melanogaster* is *lola*. Interestingly, the 548-bp *Bst*NI-*Esp*I probe from the 9.2.1AB cDNA also revealed single bands within restricted genomic DNAs from *D. simulans* and *D. virilis* flies (not shown), therefore suggesting a conservation of the cloned gene in these other distantly related Drosophila species.

Two gene products have been previously identified for the *lola* gene in *D. melanogaster*, Lola short and Lola long (25). They differ by their C termini, the short one being a zinc finger-deleted version of the long one (see position of the truncation in Fig. 7), and it had been suggested that they result from alternative splicing (25). To compare further the *9.2.1AB* and *lola* genes, RNAs from S2 and DH-33 cells were analyzed by Northern blotting and were hybridized with the 9.2.1AB cDNA probe described above, common to both genes. As illustrated in Fig. 8C, this probe actually revealed several transcripts in the *D. melanogaster*-derived cells and, similarly, in the *D. hydei* cells. Both series of transcripts most probably correspond to alternative splicing events from a single gene, since the same probe revealed only a single gene locus in the Southern blot analysis of Fig. 8A and B.

Functional relationship between *lola* and *9.2.1AB*: Lola regulates *copia* expression. The above analysis strongly suggests that the *lola* gene in *D. melanogaster* is the homolog of *9.2.1AB* in *D. hydei*. Accordingly, we devised two series of experiments to determine whether *lola* regulates *copia* transcription. In a first series of experiments, the two previously cloned *lola* cDNA gene products (25) were tested in a cotransfection assay as in Fig. 4, using expression vectors for these cDNA and the *copia* LTR-5′UTR-*lacZ* reporter gene. As illustrated in Fig. 9 (left), no direct effect of either the Lola long or the Lola short gene product could be detected on the level of expression of the LTR reporter gene, under conditions in which an at least 10-fold enhancement of expression was observed with 9.2.1AB. However, as illustrated in Fig. 9 (right), the Lola long gene

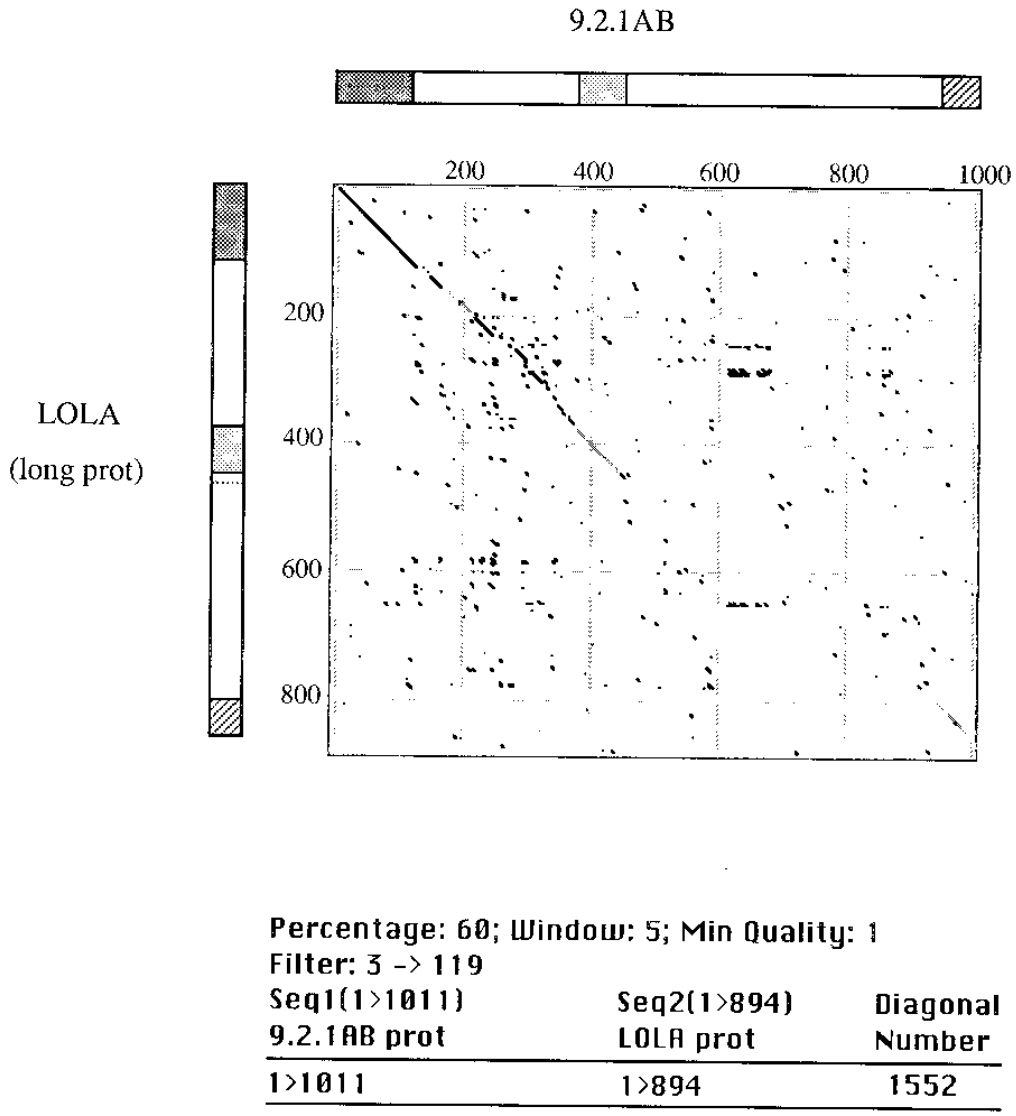

FIG. 7. DotPlot comparison of the 9.2.1AB protein (1,010 amino acids) and the Lola long gene product (894 amino acids). The BTB, a conserved acidic region within the activation domain, and the zinc finger motifs are boxed. The C-terminal end of the Lola short gene product is indicated by a dotted line in the schematic representation of Lola long. The parameters used for alignment are indicated. prot, protein.

product proved to be a potent competitor for the 9.2.1AB-induced activation of *copia* expression, with a complete, dose-dependent inhibition of 9.2.1AB induction. Interestingly, this antagonist effect is not observed with the Lola short gene product lacking the zinc finger DNA-binding domain, therefore strongly suggesting that the inhibition effect is actually due to a direct interaction of the Lola long protein, via its DNA-binding domain, to the *copia* enhancer. This is consistent with the previously mentioned strong similarities between the DNA-binding domains of both genes and provides a first hint for a functional homology between the two genes, although their regulatory activity seems to have diverged during the course of evolution.

Possible regulatory effects of *lola* gene products on *copia* were assessed by an in vivo analysis of *copia* transcription in wild-type and *lola* mutant embryos. Actually, there exist several P-mediated *lola* mutants which disclose strongly reduced (if any) expression of the *lola* gene (25) and therefore provide a unique opportunity to test for the occurrence of *lola*-mediated regulations of *copia* expression. In situ hybridization experiments were, therefore, carried out with a *copia* probe on both wild-type and *lola* mutants, as well as immunolabeling experiments to detect *lola* gene products by using anti-Lola immunopurified antibodies (raised against Lola short [kindly provided by E. Giniger]). As illustrated in Fig. 10, *lola* gene products can be easily detected by immunostaining of wild-type embryos at stages 15 to 17 within the central nervous system (CNS). This labeling, which has been previously characterized (25), is no more observed, as expected, in the *lola* mutants. Interestingly, detection of *copia* transcripts by in situ hybridization reveals a difference between the wild-type and *lola* mutant embryos specifically in this tissue (see panels c to f). This labeling coincides, spatially, with the anti-Lola immunolabeling profile but is inversely correlated with Lola expression; *copia* expression is indeed higher in the mutants than in the controls. This negative regulation could be simply accounted for by assuming expression of a Lola isoform with repressing regulatory effects on *copia* in this tissue. This interpretation

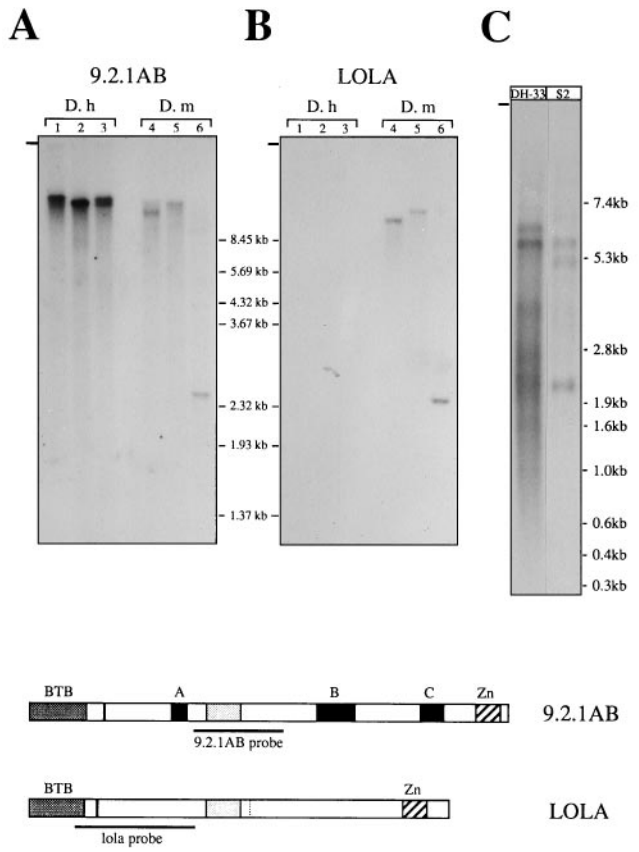

FIG. 8. (A and B) Southern blot characterization of genomic DNAs from *D. hydei* (D.h.) and *D. melanogaster* (D.m.), with 9.2.1AB and *lola* DNA probes. The same blot was hybridized with a 548-bp *Bst*NI-*Esp*I fragment from the 9.2.1AB cDNA (A) or with a 756-bp *Pvu*II fragment from the pEG 183 plasmid (see Materials and Methods) (B). The position of each probe is indicated on the schematic representation of the corresponding gene (same symbols as in Fig. 2B and 7). DNAs from *D. hydei* (lanes 1 to 3) and *D. melanogaster* (lanes 4 to 6) were restricted with *Xba*I (lanes 1 and 4), *Sac*I (lanes 2 and 5), or *Bgl*I (lanes 3 and 6). (C) Northern blot analysis of RNAs from S2 and DH-33 cells. Each lane corresponds to 5 μg of poly(A)$^+$ RNAs. The 548-bp *Bst*NI-*Esp*I fragment from the 9.2.1AB cDNA was used for hybridization.

would actually fit with the effect observed with the Lola long gene product in the above-described S2 cell assay and with the general requirement for transposon shutoff in somatic tissues (see Discussion). Another difference between control and mutant embryos was observed at the level of the gonadal cells for *copia*, with an intense labeling in the control and no signal in the mutant embryos. Occurrence of *copia* transcripts in the gonads is a result consistent with the reported specific expression of a series of retrotransposons in these tissues in *Drosophila* (see reference 18 [although not explicitly mentioned in the text, labeling can also be observed for *copia* in the corresponding figure of reference 18]), and the *lola* dependence of this expression could be a hint of the occurrence of *lola* gene products acting as positive transcriptional regulators of *copia*. However, the in situ immunostaining of the embryos with the anti-Lola-immunopurified antibodies failed to detect *lola* gene product accumulation in the gonads (although a low level of expression of Lola cannot be excluded due to a faint and diffuse labeling which can be detected at the level of the mesodermal cells surrounding the gonads [see also reference 25]), and it cannot, therefore, be concluded that *copia* expression in this tissue is regulated by *lola* gene products through a direct interaction.

DISCUSSION

In the 5′ untranslated domain of the *copia* retrotransposon, we had previously identified a 130-bp sequence involved in the transcriptional regulation of this retrotransposon. We have now precisely identified, within this sequence, a 18-bp target DNA sequence, i.e., 5′-GAAATAGCTTTTTTTTTC-3′, which is footprinted by a factor that we have cloned and characterized. This factor is a transcription factor, since, when introduced into *Drosophila* cells in culture as an expression vector, it enhances the level of expression of *copia*-derived reporter genes by 30-fold. It specifically transactivates, similarly, a minimal heterologous promoter flanked with the targeted sequence alone. This factor, which was isolated by using a cDNA expression library from *D. hydei*, most probably originates from a gene common to both *D. hydei* and *D. melanogaster*. Actually, two functional domains of this factor (namely the zinc finger- and BTB-terminal domains) as well as a third internal domain share very high sequence identity (98, 92, and 96%, respectively) with the corresponding domains of the long product (96.5 kDa) of the recently cloned (25) *D. melanogaster lola* gene (longitudinal lacking gene, first identified by a genetic approach as involved in axon growth and guidance [25, 47]). In

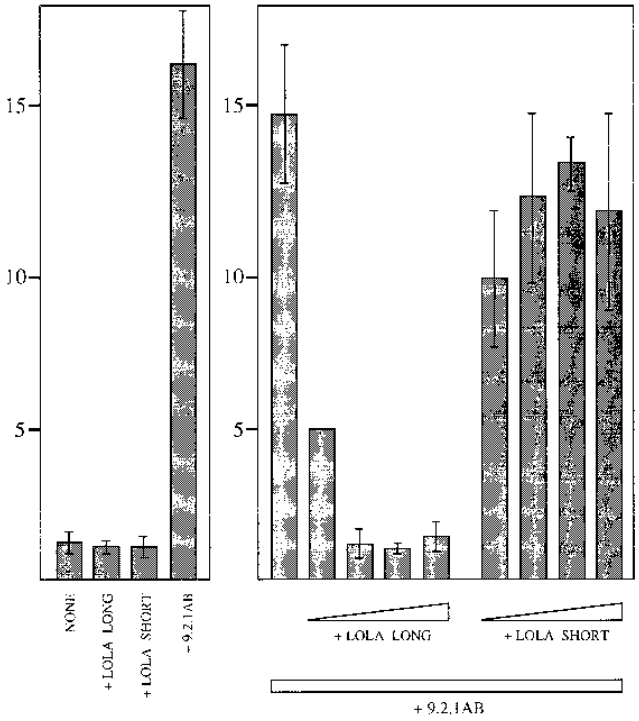

FIG. 9. Assay for Lola effects on *copia* responder plasmids. Cotransfections in S2 cells were performed as described in the legend to Fig. 4, with the *copia* LTR-5′UTR-*lacZ* responder plasmid and expression vectors for the long (+LOLA LONG [pAct5C-lola 4.8]) and the short (+LOLA SHORT [pAct5C-lola 8.13]) forms of Lola, for 9.2.1AB (+9.2.1AB [pAct5C-9.2.1AB]), and a control with no coding sequence (NONE [pAct5C-none]). (Left) cotransfections were with 1 μg of responder plasmid and 1 μg of the indicated expression vectors. (Right) Cotransfections were with 1 μg of responder plasmid, 1 μg of the pAct5C-9.2.1AB expression vector, and increasing amounts of the pAct5C-lola 4.8 or pAct5C-lola 8.13 expression vectors (0.1, 0.3, 0.5, and 1 μg). The expression vectors in the assays were maintained constant (2 μg, final) upon complementation with pAct5C-none, and final DNA was adjusted to 10 μg with the Bluescript plasmid (Stratagene).

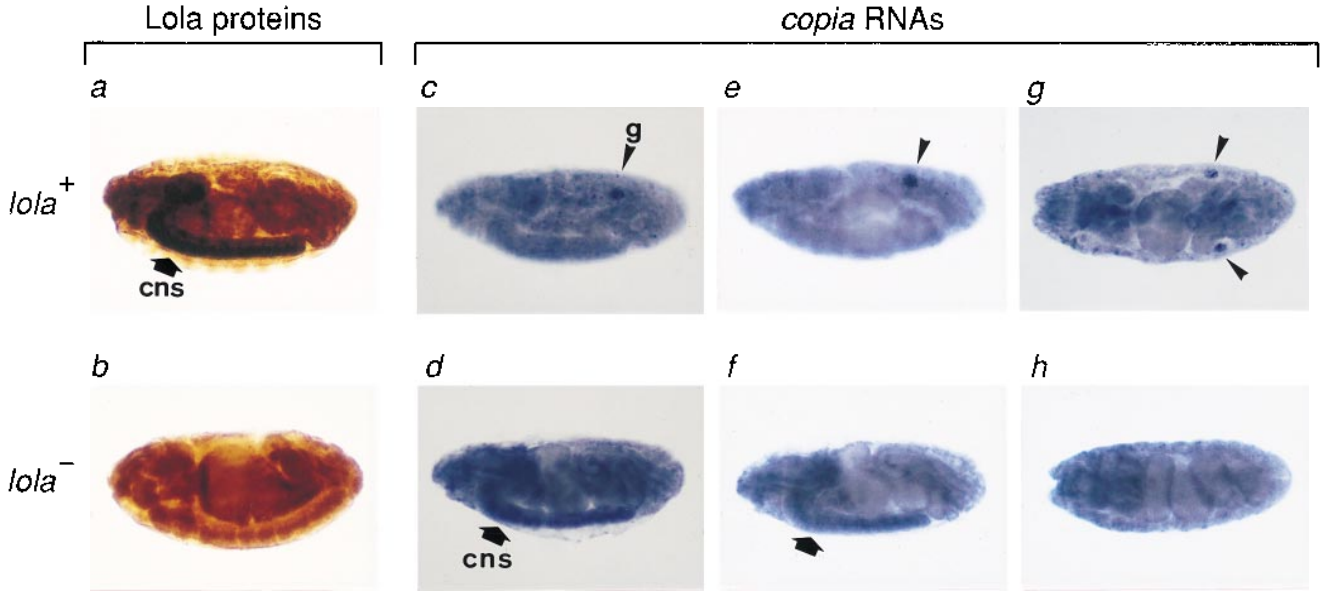

FIG. 10. In situ detection of *copia* RNAs and Lola proteins in *lola* mutant (*lola*$^-$) and wild-type (*lola*$^+$) *D. melanogaster* embryos. Immunostaining (a and b) was performed with α-Lola immunopurified antibodies, and in situ hybridization to *copia* transcripts (c to h) was performed with a digoxigenin-labeled DNA probe. The embryos (stages 15-17) are shown anterior to the left, either in a lateral view with dorsal up (a to f) or in a dorsal view (g and h). Arrows, CNS with the ventral nerve cord and the brain lobes; arrowheads, gonads (g). The embryos in the figure correspond to the 5D2 *lola* allele, but identical results were observed with the 4D4 allele; similarly, identical patterns were obtained for the two *lola*$^+$ control strains (unrelated P insertions originating from the same mutagenesis experiment [see Materials and Methods]). The mutant phenotype for *copia* (i.e., intense labeling of the CNS and absence of *copia* transcript in the gonads) was observed in nearly one-quarter of the progeny from *lola*^{5D2}/+ parents (in a typical experiment, 90 embryos over a total of 369, i.e., 24.4%); a similar ratio was observed for the mutant phenotype for Lola (i.e., absence of immunolabeling of the CNS with the α-Lola antibodies) as previously described (25).

addition, Southern blot analysis of *D. melanogaster* and *D. hydei* genomic DNA with a probe overlapping the internal domain common to both factors revealed, under high stringency hybridization conditions, single-copy genes in both species, with the bands in *D. melanogaster* being unambiguously identified as *lola* associated by using a specific probe for this gene. In addition, gel shift assays using the cloned factor expressed in bacteria resulted in a band of retarded mobility comigrating with the B′ band, which is the specific retarded band common to *D. hydei* and *D. melanogaster* cell line extracts. Finally, one of the *lola* gene products, when expressed in cells in culture, antagonizes the enhancing effect of the cloned factor, and in situ hybridization experiments demonstrate changes in the *copia* expression profile in *D. melanogaster* mutant embryos with strong or null *lola* alleles. Altogether, these data strongly suggest that despite differences in their coding sequences as well as in their activating potencies, the gene for the factor that we have cloned and *lola* are homologous genes that have been conserved, with modifications possibly resulting from the invasion of *D. melanogaster* by *copia*, between the distantly related *D. hydei* and *D. melanogaster* species.

At the structural level, the cloned factor discloses several interesting features. Actually, like the *BR-C*, *ttk*, and *bab* gene products, it possesses a BTB box at its very N-terminal end. This domain is found in an increasing number of proteins belonging to both invertebrates and vertebrates (e.g., see references 9, 19, 40, and 52), and its high amino acid conservation is probably relevant to its functional importance. It has been hypothesized that this domain could be involved in protein-protein interactions and could mediate homo- or heterodimer formation; this was recently demonstrated to be the case in vitro (2, 10) and in vivo (16). The structure of the *copia* regulatory sequence itself, within the 5′UTR, would be consistent with such interactions. Actually, we have previously reported the presence of functional homeoprotein binding sites in the 5′UTR of the *copia* retrotransposon (7), which are located approximately 30 bp upstream of the enhancer sequence and together with it constitute a 130-bp regulatory domain. It is, therefore, tempting to speculate, although direct experimental evidence is still lacking, that protein-protein interactions could take place at the level of these adjacent regulatory sites, resulting in refined regulations for *copia* transcription. Interestingly, Zeng and coworkers have described a very similar organization for the E module of the autoregulatory enhancer of the *Deformed* (*Dfd*) homeogene, with a 120-bp sequence composed of a single Dfd binding site 30 bp upstream of an imperfect inverted repeat sequence (59). The authors have shown that both sequences are required for complete function of the module and suggested that the inverted repeat is a binding site for a putative homeoprotein cofactor. Finally, the BTB domain of the cloned factor could also be involved in the formation of a complex with factors not necessarily binding to DNA. Among these, isoforms of the factor itself lacking the zinc finger DNA binding domain (e.g., the Lola short gene product) would be likely candidates for the formation of such heterodimers.

At its C-terminal end, the cloned 9.2.1AB factor possesses a two-zinc finger domain which is very similar to that of the *lola* gene products and responsible for the DNA binding activity. The Lola long gene product, and not the one from which the zinc finger domain is deleted, competes with 9.2.1AB for its enhancing effect when introduced by transfection into S2 cells, thus rendering likely that both factors actually bind to the same target sequence. An unexpected outcome of the present investigation is that the genes (in addition to *copia*) which are regulated by the *lola* factor (and as such participate in axon growth and guidance in *Drosophila* [25, 47]) could now be identified by using the characterized target DNA sequence.

The internal domains of 9.2.1AB and Lola are the most

divergent elements within these factors, although strong similarities can be detected within subdomains (e.g., 96% identity within the 75-bp sequence shown in Fig. 7). The overall structure of the 9.2.1AB internal domain (sequences of polyglutamine preceded by stretches of serine and threonine residues and presence of acidic domains) renders likely that it corresponds to the activation domain of this transcription factor. In this respect, the amino acid sequence divergence between both factors might be relevant to the differences in their regulation of *copia*, as first evidenced in the S2 cell assay, Lola long acting as an antagonist of the 9.2.1AB activator. Repressing effects of *lola* gene products can further be inferred, in vivo, from in situ hybridization with a *copia* probe and immunostaining with anti-Lola antibodies, carried out in *D. melanogaster* embryos. These experiments have revealed in the CNS, which is the tissue with maximal accumulation of Lola gene products (25), a strong increase in *copia* transcripts in the *lola* mutants, which is consistent with a repressing effect of the *lola* gene products detected in this tissue in wild-type embryos but not in mutant embryos. The localization of the *lola* gene products and *copia* transcripts in the CNS renders very likely the occurrence of a direct regulation of *copia* by an isoform of *lola* with, accordingly, a repressing activity. Such an effect is not unexpected, since negative regulations have been previously documented for a closely related BTB-containing element, Tramtrack, which acts as a transcriptional repressor for several segmentation genes (6, 57). It is also consistent with the general requirement for transposon shutoff, especially in the somatic tissues, that most probably is a biological requisite for elements which, when transcriptionally active, can act as mutagenic agents. An interesting observation finally concerns the occurrence of *copia* transcripts in the gonads of the wild-type *D. melanogaster* embryos, which were not observed in the *lola* mutants. Occurrence of an isoform of Lola with activating effects on *copia* (resembling the cloned 9.2.1AB factor) would account for such effects, but the presence of *lola* gene products could not be demonstrated with the available anti-Lola antibodies. A plausible interpretation for this failure might be that the hypothetical Lola isoform involved in this positive regulation lacks the N-terminal domain recognized by the immunopurified antibodies, but indirect effects of the *lola* mutation are also possible.

Finally, it should be acknowledged that the present investigation leaves unresolved the identification of the *D. hydei*-specific factors, not observed in the *D. melanogaster* cell extracts, which result in the gel shift assays in the B1 and B2 bands, superimposing on the B′ band common to both species. By UV cross-linking experiments (8), it had previously been demonstrated that these two bands correspond to the binding of, respectively, one and then two proteins with apparent molecular masses of 50 kDa, which therefore cannot correspond to the presently cloned 9.2.1AB factor (theoretical molecular mass, 110 kDa [170 to 180 kDa by SDS-PAGE]). However, taking into consideration the numerous transcripts revealed in the present Northern blot analysis, the possibility exists that different factors are produced by alternative splicing from the single *9.2.1AB* gene locus, as similarly observed for the *Broad Complex* (17) and *Tramtrack* (44) genes and also for *lola* (25). In this respect, it should now be tested whether different 9.2.1AB-derived gene products, possibly with different activation potencies, are generated in *D. hydei* cells, and whether some of them have the expected features for generating the B1- and B2-retarded bands in gel shift assays. An interesting corollary would be that modulation of alternative splicing at the *9.2.1AB*/*lola* gene locus, by generating transcription factors with distinct activation domains, has actually been part, in the course of evolution, of an adaptive strategy to limit the extent of activation of *copia* in *D. melanogaster*, when this *Drosophila* species has been invaded by this otherwise highly mutagenic element.

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