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IDENTIFICATION OF A STRONG TRANSCRIPTIONAL ACTIVATOR FOR THE COPIA RETROTRANSPOSON RESPONSIBLE FOR ITS DIFFERENTIAL EXPRESSION IN *DROSOPHILA HYDEI* AND *MELANOGASTER* CELL LINES

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We have characterized the regulatory properties of a 72bp sequence located in the 5' untranslated domain of the *Drosophila* copia retrotransposon, 3' to the left LTR, by transient transfection assays with cell lines derived from either *Drosophila hydei* (DH33 cells) or *Drosophila melanogaster* (Schneider II and Kc cells). Reporter plasmids were constructed which contained the lacZ gene under the control of either the entire copia LTR with 5' untranslated domain, or a minimal heterologous promoter flanked with the identified copia regulatory sequences. Upon transfection into the copia-free DH33 cells, the presence of the 72bp sequence resulted for all reporter plasmids in a 100-700 fold increase in expression level -as well as in reporter gene RNA levels- whereas this sequence had no enhancing effect upon transfection of the same plasmids into the copia-containing Schneider II or Kc cells. Moreover, mobility shift assays with the 72bp enhancer sequence disclosed two specific bands of retarded mobility with whole-cell extracts from DH33 cells, whereas no retarded band could be detected, under identical conditions, with extracts from Schneider II cells. UV crosslinking experiments between the enhancer sequence and DH33 extracts revealed a single protein species -of app. mol. wt. 50kD- for both retarded bands, thus strongly suggesting that they simply correspond to the sequential binding of two identical factor molecules to the enhancer sequence. These data demonstrate that the copia-free *D. hydei* cells express a strong transcriptional activator for the copia element and possible interpretations for the absence of this factor in the copia-containing *D. melanogaster* cells are discussed in terms of a possible "adaptation" of the "host" (*D. melanogaster*) to an otherwise highly mutagenic "parasite" (copia with its transcription factor).

The *Drosophila* transposable element copia is a member of a large class of structurally related genetic elements -the viral-like retrotransposons- which share homologies with the proviral form of the vertebrate retroviruses [reviewed in (1-3)]. This class of elements includes the yeast Ty retrotransposon [reviewed in (4)] and the mammalian IAP sequences [reviewed in (5)], which both have been unambigously demonstrated to transpose in a replicative manner, via an intermediate step involving the reverse transcription of a full-length transcript of these element (6, 7). Copia is a 5.4kb element previously entirely sequenced, which contains two 276bp LTRs, a 145bp 5' untranslated region, and a single ORF with sequence homologies to retroviral integrases, proteases and reverse transcriptases (8, 9). Copia is a widespread element which is found in most species of the *Drosophila* genus, with a few exceptions including *D. hydei* (10). Transposition events are rare, and still little is known about the factors and cellular genes which control their frequency. Actually, a key step in this control -as a consequence of the transposition mechanism of this class of elements-

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should be transcription, as shown for the yeast Ty1 and the mammalian IAP retrotransposons for which induction of transcription at a high level was demonstrated to result in high transposition frequencies (6, 11).

We have recently identified in the copia retrotransposon a sequence which lies outside of the LTR, in the 5' untranslated domain, at the level of which we could demonstrate direct interaction of homeoproteins, resulting in modulations of the level of copia expression (12). We now investigate the properties of a sequence -closely related to enhancer sequences identified in the early promoter of SV40 (8, 13)- which lies adjacent to the homeoprotein responsive element. We demonstrate that this sequence is responsible for high level expression of copia in transient transfection assays using *D. hydei*-derived cells and copia-derived responder plasmids. We further identify, by mobility shift assay and UV cross linking experiments, a 50kD factor, which binds to this enhancer sequence. Interestingly, we demonstrate that this factor is not present in *D. melanogaster*-derived cells, a result which correlates with the measured low level expression of copia in these cells. The presence of a strong activator for copia in cells from a *Drosophila* species devoid of copia elements and its absence in cells from a copia-containing species is discussed in terms of a possible adaptation of the host (*D. melanogaster*) to the "parasitic" element (the copia element) it contains, via the extinction and/or mutation of a transcription factor for an otherwise too mutagenic copia.

MATERIALS AND METHODS

DNA constructions: the copia element used is the "white apricot insertion" [lambda w^a5.9 clone in (14), sequenced in (8)], that we subcloned into the Bluescript vector (Stratagene) as a BanI fragment, leaving only 77bp and 87bp of white DNA respectively 5' and 3' to the copia element (pBScopia). Four oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer: Hom1=(5')CAGTCCATGCCTAATAAAC; Hom2=(5')CACAATTTATTTTCACAATC; Enh1=(5')AAATAGCATTTTTTCACATTC; Enh2=(5')CTGAGAAGGAAATAATTTC; they were used to PCR-amplify from the copia-containing plasmid a 130bp fragment containing both the consensus homeoprotein binding sites and the enhancer sequences (using Hom1 and Enh2), a 58bp fragment containing the homeoprotein binding sites (using Hom1 and Hom2), and a 72bp fragment containing the two dyad symmetry elements of the putative enhancer region (using Enh1 and Enh2).

Construction of the copia LTR-derived responder plasmids: the LTR lacZ responder plasmid was constructed by inserting a 490bp BanI-DdeI fragment from the pBScopia plasmid (containing the entire copia LTR and 5' untranslated sequence) at the unique SalI site of pGEMnlslacZ [which contains a lacZ gene with nuclear location signal (15)], after Klenow treatment of both vector and insert; the polyadenylation sequence from the hsp70 gene [as a BamHI-EcoRI fragment from HZ50 (16)] was then inserted at a unique BamHI site 3' to the nlslacZ gene, after Klenow treatment of both vector and insert. A responder plasmid deleted from the 5'UTR domain (LTR-130 UTR) was similarly constructed by inserting a 360bp BanI-ApaI fragment (LTR alone) from pBScopia at the unique SalI site of a pGEMnlslacZ plasmid containing the polyadenylation sequence above, after Klenow treatment of both vector and insert.

Construction of the HZ50-derived reporter plasmids: the HZ50-derived responder plasmids were constructed by inserting the copia regulatory sequences at the unique NotI site of a plasmid containing a lacZ gene under control of an hsp70 enhancerless promoter and polyadenylation sequence [HZ50 plasmid; (16)]; the inserted sequences were PCR-amplified fragments, either 130bp long (Hom1 and Enh2 primers) or 72bp long (Enh1 and Enh2 primers), which were treated with T4 kinase before ligation with the Klenow-treated vector.

Cells, transfections, ß-galactosidase assays and Northern blot analysis: Schneider II (17) and Kc (18) cells from *D. melanogaster* and DH33 cells (19) from *D. hydei* were grown in Schneider medium (GIBCO) with 10% fetal calf serum (GIBCO) at 25°C. Transfections were performed by the calcium phosphate method (20), with 3µg of reporter plasmid for $2.5x10^6$ cells. ß-galactosidase activity was measured two days post transfection after protein extraction as described in (21), using a spectrophotometric assay with CPRG (Boehringer) as a ß-galactosidase substrate. In standard assays, 10µl of protein extract (0.1 mg/ml) and 1 mM CPRG were used; ß-galactosidase is

expressed as O.D. units per µg of protein extract per minute. For analysis of RNA levels, transfections were performed as above, but with 10^7 cells, and 15µg of responder plasmid. Total cellular RNAs were extracted two days post-transfection and analysed as in (22), upon electrophoresis of 10µg aliquots on 1% agarose-formaldehyde gels and transfer to nylon filters (Hybond N; Amersham) in 0.15M ammonium acetate. Filters were hybridized with a ^{32}P-labelled lacZ probe in Church solution (23) with final wash in 0.1xSSC, 0.1% SDS at 65°C.

Cellular extracts, mobility shift assays and UV crosslinking: whole-cell extracts were prepared according to the procedure in (24), with 10^8 Schneider II or DH33 cells; protein concentrations were measured using a standard Bradford assay, and the extracts were stored at -80°C until use. For the mobility shift assays, the 130bp and 72bp fragments amplified by PCR with respectively the Hom1-Enh2 and Enh1-Enh2 primers were radioactively labelled with $\gamma[^{32}P]$dATP (3000Ci/mmol; Amersham) using T4 polynucleotide kinase (Biolabs); the radiolabelled fragments were then purified on native polyacrylamide gels or on G50 Sepharose columns. Binding reactions were carried out at 20±1°C for 30 minutes in 20µl of a solution containing 20mM Hepes, pH7.9, 100mM KCl, 5mM $MgCl_2$, 0.1mM EDTA, 1mM dithiothreitol, 1µg poly(dI-dC) and 20% glycerol; the samples were then run on native 5% polyacrylamide gels at room temperature for 2h30 at 180V. When indicated, unlabelled double-stranded oligonucleotides were added in competition assays. For UV crosslinking experiments, the 72bp labelled probe was incubated with whole-cell extracts from DH33 cells as above, then irradiated as a liquid drop on a saran wrap for 4 minutes at 254nm, and run on a native polyacrylamide gel as above. The gel was then autoradiographed for 8 hours at 4°C, the DNA-protein complexes were precisely excised, and the acrylamide slices were loaded after a short incubation in Laemmli 2X buffer on a 10% SDS-polyacrylamide gel (5% SDS-polyacrylamide stacking gel). After migration the gel was fixed, dried and autoradiographed.

RESULTS

Characterization of a cell type-specific enhancer in the copia 5' untranslated region. To analyze the role of defined sequences in the copia promoter region, two series of reporter plasmids were used, which contained the lacZ coding sequence as an easily assayable gene (see figure 1): one with the copia LTR plus 5' untranslated region, which then contains the repeated 22bp sequences with dyad symmetry and homology to SV40 enhancer sequences (ENH) and a previously identified homeodomain responsive element (HpRE), and a second series which utilizes a minimal heterologous promoter -the hsp70 promoter deleted for its own enhancer sequences- with elements of the copia untranslated region placed upstream: either a 130bp fragment containing the entire untranslated region, or a 72bp fragment with only the 22bp repeats; for each series, reporter plasmids with deletion of either the 130bp or the 72bp fragment above were also constructed. In a standard assay, 3µg of reporter plasmid DNA were introduced by transfection into either DH33, Schneider II or Kc cells, and ß-galactosidase activity was measured two days post-transfection by a quantitative assay using CPRG (chlorophenol ß-red D galactopyranoside) as a substrate; each transfection experiment was repeated two to four times. Transfection efficiencies in the various cell types were determined in parallel experiments using a lacZ control plasmid containing the entire hsp70 promoter; they were found identical for the Schneider II and DH33 cells (namely 1.0±0.1 for DH33, taking the SII value as unity), but significantly lower with the Kc cells (namely 0.07); results from transfections in Kc cells were therefore corrected for this factor. As illustrated in figure 1B, transfection of reporter plasmids with defined copia sequences disclosed dramatically higher levels of expression in DH33 cells than in either Kc or SII cells: with the extended copia LTR, ß-galactosidase activity was 50-100 fold higher in DH33 cells, and even larger differences were observed (up to 700 fold) with reporters where copia sequences from the untranslated region are placed upstream to a minimal heterologous promoter in the HZ50+130UTR and HZ50+72UTR constructs. The data in figure 1B also demonstrate that the copia

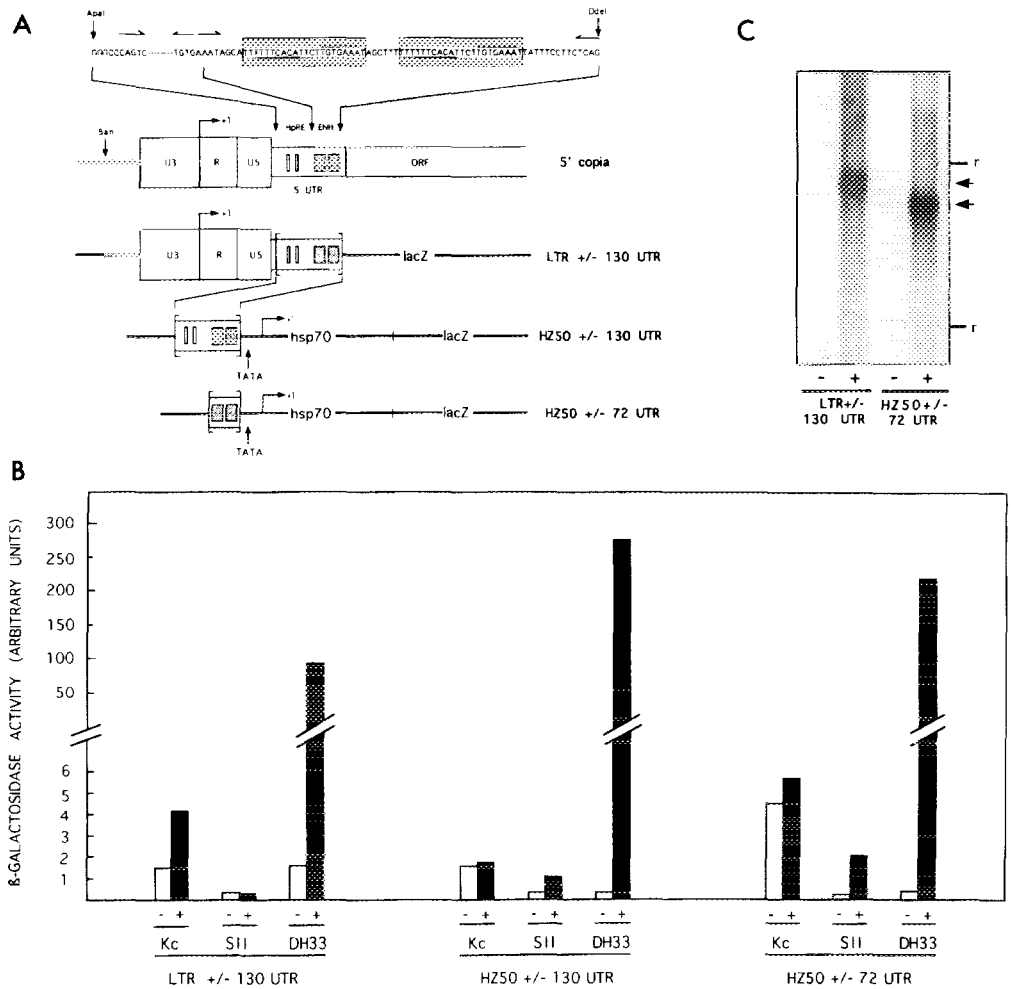

Figure 1. **Characterization of a functional enhancer in the copia 5' untranslated region.**

(A) Schematic representation of the copia LTR with U3-R-U5 organization and RNA start site (+1) and of the 5' untranslated region (5'UTR); the enhancer-like sequence (ENH) containing the 22bp repeats (grey boxes) displaying dyad symmetry (sequence underlined) and the homeoprotein responsive elements (HpRE) are indicated; the 5' ends of the oligonucleotides used for PCR amplification of the 130bp UTR, of the 72bp enhancer-like sequence, and of HpRE are indicated with horizontal arrows. Structure of the "responder" plasmids constructed for the transfection assays: the copia LTR-based responder plasmid containing the lacZ gene for ß-galactosidase with or without the entire 130bp UTR (deletion indicated by the brackets); the HZ50-based responder plasmids containing a "minimal" hsp70 promoter (enhancerless promoter, with TATA box and RNA start site indicated) with or without (deletion indicated by the brackets) the copia 130bp UTR or 72bp UTR, placed 5' to the heterologous promoter.

(B) Activity of the lacZ-based responder plasmids in cell lines from *D. melanogaster* (Kc and SII cells) and *D. hydei* (DH33 cells). Transfections were performed with 3µg of the indicated responder plasmids (see structures in A) either complete (+, dark bars) or deleted (-, open bars) for the 130bp or 72bp copia UTR, and ß-galactosidase activity was assayed two days post-transfection. Transfection efficacies were measured in parallel experiments using a hsp70-lacZ plasmid and were 0.07 for Kc and 1.0 for both SII and DH33. Data in B are the mean of 2-4 independent experiments, after correction for transfection efficacy (for Kc values).

(C) Northern blot analysis of responder plasmid expression. 15µg of the indicated responder plasmids were transfected in 10^7 DH33 cells; each lane corresponds to 10µg of total RNA extracted two days post-transfection; the "responder" transcripts (upon hybridization of the blot with a lacZ radiolabelled probe) and positions of the calf 28S and 18S ribosomal RNA (r) are indicated.

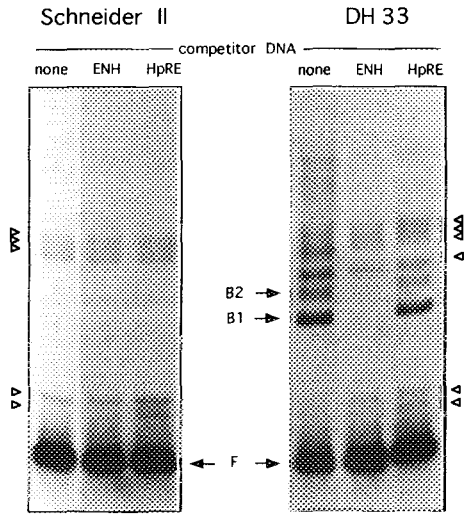

Figure 2. **Mobility shift assay for the interaction of factors from SchneiderII and DH33 whole-cell extracts with the copia 5' UTR.**
A fixed amount (0.2nM) of end-labelled 130bp DNA fragment corresponding to the copia 5' UTR was incubated with 10µg of whole-cell extracts from Schneider II (left) or DH33 (right) cells, and the samples were run on 5% polyacrylamide gels under non denaturating conditions. Specificity of the DNA-protein interactions resulting in the observed retarded bands was assayed upon addition to the incubation buffer of a 50-fold concentration excess of unlabelled DNA fragments (either the ENH sequence or the HpRE sequence) covering the two halves of the copia 5'UTR, or no competitor (none). Non-specific interactions are indicated by open triangles, and the two bands of retarded mobility B1 and B2, which correspond to enhancer-specific interactions with factors found only in DH33 whole-cell extracts, are indicated by arrows; F: free DNA.

5'UTR 72bp fragment is necessary -and not only sufficient- for high level expression in DH33 cells, as its removal totally abolished the cell type-specific effect.

Finally, Northern blot analyses were performed to determine whether the 72bp-mediated increase of reporter plasmid expression in DH33 cells reflected increases in RNA levels or translational effects. LTR-derived and HZ50-derived responder plasmids were introduced by transfection into DH33 cells as above, and RNAs extracted two days later. As illustrated in figure 1C, high RNA levels were observed for both types of reporter plasmids, only when the 72bp ENH sequence is present; a very faint -if any- signal is observed with the control responder plasmids (-UTR), the relative levels of which (at least 50-fold lower, as determined by scanning of the autoradiograms or dot blot analysis - not shown-) are further compatible with -and could then simply account for- the data on ß-galactosidase activities.

Identification and characterization of a cell type-specific factor that binds to the copia enhancer element. It could be hypothesized that the differential behaviour of the copia enhancer elements in DH33 cells (derived from the *D. hydei* species) and in Kc or SII cells (both derived from the *D. melanogaster* species) is due to a specific transcriptional activator, only present in the former cells. We therefore looked for such a factor by a mobility shift assay using either the entire 130bp 5' UTR or the 72bp enhancer sequence as a target. Whole-cell extracts were prepared from DH33 and SII cells, and assayed for DNA binding activity. As illustrated in figure 2, using an end-labelled 130bp DNA fragment, several bands of retarded mobility can be detected with both

extracts; several of them (see open triangles in figure 2) correspond to non-specific interactions as they are not competed out by an excess of unlabelled target DNA (either the HpRE or ENH DNA fragments corresponding respectively to the 5' and 3' halves of the target DNA), but two bands -of higher intensity- could be detected which were only observed with the DH33 whole-cell extract (B1 and B2); moreover, these two bands clearly corresponded to specific interactions with the labelled target DNA, and even more precisely with the 72bp enhancer sequence, as they were competed out by a 50-fold excess of unlabelled 72bp ENH fragment and not by a similar concentration of the adjacent HpRE fragment. These data demonstrate the presence of factors specifically interacting with the 72bp fragment and differentially expressed in the two cell lines, and strongly suggest that these factors are the only ones specifically interacting with the copia 5' untranslated region in the DH33 cell line.

These specific interations were further characterized by varying the concentration of DH33 cellular extract in a mobility shift assay using the 72bp fragment as a reduced target DNA; as illustrated in figure 3A, increasing the concentration of DH33 whole-cell extract resulted in the retardation of the B1 and then of the B2 band, as expected for the sequential binding of two factor molecules to this reduced target; both of them were competed out, again, by an excess of unlabelled 72bp DNA, and were not observed, even at the highest concentration used, with the SII whole-cell extract.

A definite characterization of the DNA binding factor(s) present in the DH33 whole-cell extract and responsible for the retarded B1 and B2 bands was achieved by UV-crosslinking experiments. DH33

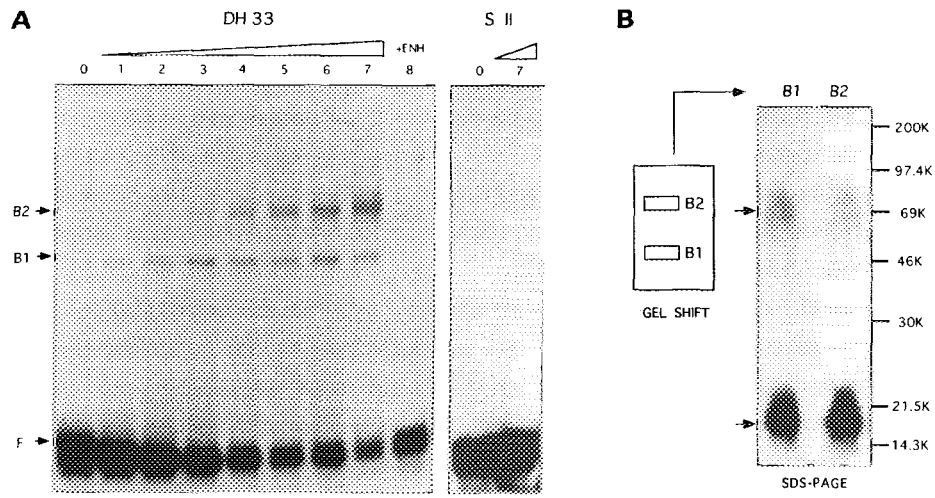

Figure 3. **Characterization of a copia enhancer-specific factor by gel shift assay and U.V. crosslinking.**
(A) left: a fixed amount (0.2nM) of end-labelled 72bp DNA fragment corresponding to the copia enhancer sequence was incubated with increasing concentrations of DH33 whole-cell extracts (4 to 45µg with regular 1.5-fold increase, from lane 1 to 7; lane 8: same conditions as in lane 7, but with a 50-fold excess of unlabelled 72bp ENH fragment); the B1 and B2 bands of retarded mobility are indicated by arrows; right: same conditions, with Schneider II whole-cell extracts.
(B) U.V. crosslinking: incubation between the end-labelled 72bp fragment and the DH33 whole-cell extract was as in (A, lane 4), but the reaction medium was UV-irradiated as indicated in Materials and Methods before non-denaturating polyacrylamide gel electrophoresis (as in A); the B1 and B2 bands were excised from the gel and the acrylamide slices were deposited and run on a 10% SDS-polyacrylamide gel; the marked factor (app.mol.wt. 70kD) and free DNA (app.mol.wt. 20kD) are indicated with arrows, with positions of the molecular weight markers on the right.

whole-cell extracts were incubated with the end-labelled 72bp copia enhancer element, UV-irradiated for crosslinking, and submitted to a gel shift assay as above. The B1 and B2 bands were then cut out of the gel, and each band was submitted to SDS-polyacrylamide gel electrophoresis. As illustrated in figure 3B, for both B1 and B2 a low molecular weight band of app.mol.wt. 20kD can be observed, which corresponds to free labelled DNA, with in addition a single band of app.mol.wt. 70kD, common to both B1 and B2, which should correspond to a factor of app.mol.wt. 50kD before DNA crosslinking. These data strongly suggest that the B1 and B2 retarded bands simply correspond to the sequential binding of two identical factor molecules -of app.mol.wt. 50kD- to the 72bp copia enhancer element, possibly associated in an homodimeric complex (not visible on the SDS-PAGE in figure 3B, because UV crosslinking efficiency is too low to have a statistically significant fraction of the target DNA molecules with two crosslinked factor molecules).

DISCUSSION

The identification of a strong transcriptional activator for the copia retrotransposon in *D. hydei*-derived DH33 cells, not found in *D. melanogaster*-derived cells, settles a debated issue concerning the differential level of expression of the copia element in the two cell types (13, 25), and raises intriguing questions concerning the role of mobile elements and of their regulation in evolutive processes.

A first conclusion is that the previously noticed differential expression of the copia promoter in DH33 versus Schneider II cells can now be very simply accounted for, since we have shown (i) that it relies on the presence of a well-characterized 72bp sequence in the copia 5' untranslated region, which is both necessary and sufficient for high level expression in DH33 cells, and (ii) that this enhancer sequence is targetted by a strong transcriptional activator only present in DH33 whole-cell extracts. The identified sequence -which contains repeats close to sequences also found in the SV40 enhancer (26)- behaves as a classical autonomous enhancer element, placed either downstream or upstream to the transcription start site, or linked to an heterologous minimal promoter. The associated transcription factor is a 50kD molecule, which binds with a stoichiometry of -at least- two molecules per 72bp enhancer DNA sequence.

The presence of a strong activator for the copia element in cells from a *Drosophila* species lacking this element- and conversely its absence in cells from a *Drosophila* species which contains 10-50 copia elements- seems paradoxical, but in fact corresponds to the only possibly viable situation. One can indeed expect that the presence of copia elements in *Drosophila* -such as *D. hydei*- which express a strong activator for this element would be lethal due to excessive transpositions. Actually, a closely related situation is naturally encountered for other mobile elements in P-M and I-R dysgenic crosses, where very high transposition frequencies and lethality are observed when *Drosophila* individuals containing P or I mobile elements are crossed with individuals devoid of the corresponding elements [reviewed in (27-29)]. One can further speculate that the gene for the identified transcriptional activator should be present not only in *D. hydei* but in all *Drosophila* species, and that upon "invasion" of the *Drosophila* genus by copia [possibly by horizontal transmission, as recently strongly suggested for the P and Jockey elements; (30) and references therein, (31)] *Drosophila* have evolved strategies to shut off or severely control this transcription

factor. A very interesting outcome of the present investigation is that one can now clone the corresponding gene from a *D. hydei* cDNA expression library as in (32), and investigate for its presence and level of activity in other *Drosophila* species, as well as for its spatial and temporal pattern of expression in *Drosophila* individuals. This should help in understanding the complex interations which must exist between a "parasitic" element and its host, and the "compromises" that are selected for an evolutionary beneficial solution.

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