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The modifier of position-effect variegation *Suvar(3)7* of *Drosophila*: there are two alternative transcripts and seven scattered zinc fingers, each preceded by a tryptophan box

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ABSTRACT

An increase in the number of copies of the *Drosophila* locus *Suvar(3)7* enhances position-effect variegation, i.e. the inactivation in some cells of genes brought close to heterochromatin by a chromosomal rearrangement. The locus produces two transcripts of 5047 and 4203 nt that differ solely by the length of their 3′ untranslated region. That these transcripts encode the modifier of variegation *Suvar(3)7* is demonstrated by genetic transformation with the corresponding cDNAs. The deduced protein is 1169 amino acids long and contains seven widely spaced zinc fingers. These fingers are each preceded at 11–16 amino acids before the N-terminal cysteine by a tryptophan-containing motif. The transcripts are maternally transmitted, but are also found throughout development. The ubiquitous distribution of transcripts in embryos and the different sequence motifs support our speculation that the locus encodes a chromosomal protein implicated in heterochromatin-mediated DNA silencing.

INTRODUCTION

Chromosomal rearrangements that relocate a euchromatic gene in the vicinity of heterochromatin often result in a variegated, i.e. mosaic, phenotype. The permanent inactivation of the relocated gene in some cells but not others seems to be the consequence of a variable spreading of the heterochromatic conformation into euchromatin, though alternative models have been proposed (see 1–3 for diverse opinions on the matter, and references therein for reviews). A number of dominant mutations, the modifiers of variegation, affect the extent of variegation, namely the proportion of cells in which the relocated gene is inactivated (1). These genes may encode components of the chromosome that play an important role in maintaining an active or inactive state of chromatin. We have previously mapped and cloned the modifier gene *Suvar(3)7* and shown from the deduced protein sequence that it seems to encode a zinc finger protein; genetic transformation with a genomic fragment indicated that an increase in the dose of the gene increases variegation (4). We report here that the locus produces two alternative transcripts and we demonstrate by genetic transformation that both transcripts encode the genetic function. We have also determined the transcript levels and distribution throughout development. We have found that the transcripts are longer at their 5′ end than the sequence we published previously (4) and that the correction of a sequencing error also extends the sequence of the protein at its N-terminus. We now report the complete deduced sequence of the protein encoded by *Suvar(3)7*, revealing not five, but seven scattered putative zinc fingers, each preceded by a tryptophan motif resembling those previously described in front of the homeodomain of proteins encoded by certain homeotic genes. By analogy, this adds to our speculation that the *Suvar(3)7* encoded protein plays a mechanical role in chromosome condensation.

MATERIALS AND METHODS

RNA extraction, poly(A^+) selection

Embryos, larvae, whole adults and dissected ovaries from the Canton S strain were ground in 100 mM Tris–HCl, pH 9.0, 100 mM NaCl, 20 mM EDTA, 1% Sarcosyl-30 (CIBA) and spun for 5 min at 5000 r.p.m. After several extractions of the supernatant with phenol–chloroform–isoamylalcohol (50:50:1) and ethanol precipitation, the pellet was resuspended in diethylpyrocarbonate-treated water (0.02%) and selection of poly(A^+) RNA was achieved on oligo(dT)–cellulose (Pharmacia).

Northern blot analysis

Samples containing 5 μg RNA in Northern buffer (5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 1 mM CH_3COONa, 1 mM EDTA, pH 6.9), 50% formamide, 3.7% formaldehyde, loading buffer (5% glycerol, 0.1 mM EDTA, 0.04% bromophenol blue, 0.04% xylene cyanol) were denatured for 5 min at 55°C and loaded on a 1% agarose gel in Northern buffer containing 2 M formaldehyde. After transfer, the nylon membrane (Hybond-N^+; Amersham) was UV irradiated (Stratalinker; Stratagene) and stained

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for 5 min in 0.04% methylene blue in 0.5 M NaOAc, pH 5.2. After several washes in water, the membrane was prehybridized for at least 1 h at 60°C in 50% formamide, 4 × SSPE, 0.5% SDS, 0.5% non-fat dry milk, 0.5 mg/ml denatured salmon sperm DNA. Hybridization was carried out at the same temperature and buffer conditions with a [^{32}P]CTP-labelled riboprobe, previously denatured for 30 s at 95°C. Washes were for 2 min in 2 × SSPE, 1% SDS at room temperature, twice for 30 min in 1 × SSPE, 0.1% SDS at 60°C and twice for 30 min in 0.1 × SSPE, 0.1% SDS at 60°C.

Transformation vectors

Two transformation vectors were constructed, each containing one of the alternative transcripts. At the 5′-end they both contain the 2304 bp *Hin*dIII fragment spanning 67 bp from the pNB40 plasmid (8), the transcription start site and the first 2237 bp of the transcript. For the 3′-end moiety, fragments defined by the *Hin*dIII site (position 2237) in the transcript and the *Not*I site downstream of the poly(A) tail of each class of cDNA were spliced to the 5′ moiety and the combined fragment introduced into the *Not*I site of the pNHT4 vector (5). Transformation procedures were as previously described (4).

Riboprobe synthesis

The probes were: (A) 1 µg cDNA from the shorter transcript linearized upstream of the cDNA in the polylinker; (B) 1 µg cDNA from the longer transcript digested with *Ava*I (position 4441). The non-coding strand-specific probe was from the same cDNA as was used for probe A but cloned in the opposite orientation. After digestion, the template DNAs were treated for 40 min at 37°C with 20 µg proteinase K (Sigma) in a final volume of 100 µl in 10 mM Tris–HCl, pH 7.8, 5 mM EDTA, 0.5% SDS, extracted twice with phenol–chloroform and ethanol precipitated. For transcription, 5 µl transcription buffer [1 µl 10 mM rATP, 1 µl 10 mM rGTP, 1 µl 10 mM rTTP, 1 µl 1 mM rCTP, 1 µl 0.75 M DTT, 0.5 µl RNAsin, 2.0 µl [^{32}P]rCTP (800 Ci/mmol), 0.5 µl 20 U/µl T7 polymerase] were added to 1 µg DNA in 12.0 µl diethylpyrocarbonate-treated water. Incubation was for 30 min at 37°C. One unit of RNase-free DNAse (Stratagene) was added and incubation pursued for 15 min. The synthesized RNA was precipitated by adding a 1/10 vol 5 M LiCl and 3 vol ethanol. After centrifugation and several rinses with 70% ethanol, the pellet was resuspended in 100 µl water.

Primer extension

Fifty nanograms of the oligonucleotide (5′-GGAGATCACAG-TATACGG-3′) in 20 µl 50 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM spermidine, 1 mM EDTA, pH 8.0, 5 mM DTT, 50 µCi [γ-^{32}P]ATP, 10 U T4 polynucleotide kinase (Biofinex) were incubated for 45 min at 37°C. Then, 50 µl H_2O, 10 µl proteinase K buffer (0.1 M Tris–HCl, pH 7.8, 0.05 M EDTA, 5% SDS) and 20 µl proteinase K (1 mg/ml) were added. After 30 min at room temperature, the labelled primer was extracted with phenol and purified on a Sephadex G25 spin column. Labelled primer (10^5 c.p.m.) was precipitated with ethanol, together with 20 µg poly(A^+) RNA. The pellet was resuspended in 18 µl water and 2 µl 10 × annealing buffer (0.5 M Tris–HCl, pH 8.0, 0.5 M KCl, 80 mM $MgCl_2$) were added. Denaturation was for 5 min at 65°C; the tube was then transferred to 45°C and incubated for 2 h. To this were added 2.5 µl dNTP (10 mM each), 2.5 µl 10 mM DTT, 1.0 µl 8 U/µl AMV reverse transcriptase (Biofinex) and 0.5 µl Rnasin (Boehringer). Incubation was for 90 min at 37°C. The extended product was extracted with phenol–chloroform and ethanol precipitated. The pellet was resuspended in 90% formamide and denatured for 2 min at 95°C before loading on a 10% sequencing gel.

RNA *in situ* hybridization on whole mounts

DIG–DNA probes were labelled according to the DIG–DNA Labeling Kit protocol (Boehringer) using 300 ng of template DNA previously digested with two four-cutters (*Hae*III and *Alu*I) to produce small fragments. Labelling was carried out overnight at 14°C. The DIG–riboprobe was made from the same template used for probe B in the Northern analysis. Five microlitres of transcription buffer, 5 µl RNA DIG labelling mixture (Boehringer), 1 µl 0.75 M DTT, 0.5 µl RNAsin and 0.5 µl 20 U/µl polymerase were added to DNA dissolved in 13.0 µl water. Transcription conditions were as above. The efficiency of labelling was estimated by including a radiolabelled ribonucleotide (diluted 1/100) in the reaction mixture. Complete (100%) incorporation gave a yield of 66 µg of synthesized RNA. To penetrate embryos, the probe had to be partially hydrolysed. the pellet was resuspended in 50 µl 40 mM sodium bicarbonate, 60 mM sodium carbonate, pH 10.2. Incubation was at 60°C for a time determined as follows: time (min) = initial length (kb) – final length (usually 0.2 kb)/0.11 × (initial length × final length). For DNA probes, the protocol is as described (6). For riboprobes, the protocol is an adaptation of that of Harland (7), where prehybridization and hybrization were carried out at 60°C in the same buffer as was used for DNA probes. Hybridization solution contained 2 µg riboprobe denatured for 30 s at 95°C in 400 µl. The washes were done at 60°C: 3 × 20 min in hybridization buffer (50% formamide, 5 × SSC, 50 µg/ml heparin, 0.1% Tween 20, 0.1 mg/ml ssDNA and 0.1 mg/ml tRNA); 20 min in hybridization buffer/PBST (phosphate-buffered saline + 0.1% Tween 20) 1:1; 4 × 20 min in PBST. After hybridization, embryos were treated with RNase in a 30 min incubation at 37°C with 20 µg/ml RNase A and 10 U/ml RNase T1 and again washed for 3 × 20 min at room temperature with PBST. Detection was as described by the manufacturer's protocol with pre-absorbed anti-DIG at a 1:2000 final dilution. Embryos were finally mounted either in 90% glycerol, 10% phosphate-buffered saline or in a permanent medium (DURCUPAN ACM, Fluka) made by mixing 10 ml component A, 10 ml component B, 350 µl component D and 200 µl component C (stored at –20°C). After serial dehydratation in ethanol and 30 s in acetone, dried embryos were dropped into the medium on the slide. The coverslip was added and polymerization induced for 12 h at 60°C.

RESULTS

Suvar(3)7 encodes two alternative transcripts

In order to examine the *Suvar(3)7* transcription unit, Northern blots were first performed with partial cDNA clones as probes (Fig. 1A). Two transcripts of 5.0 and 4.2 kb were detected. The transcripts were hence found to be larger than the previously available cDNAs. We have therefore characterized over 20 new cDNA clones isolated from embryonic stages (data not shown) [the libraries were a gift from M. Goldschmidt-Clermont

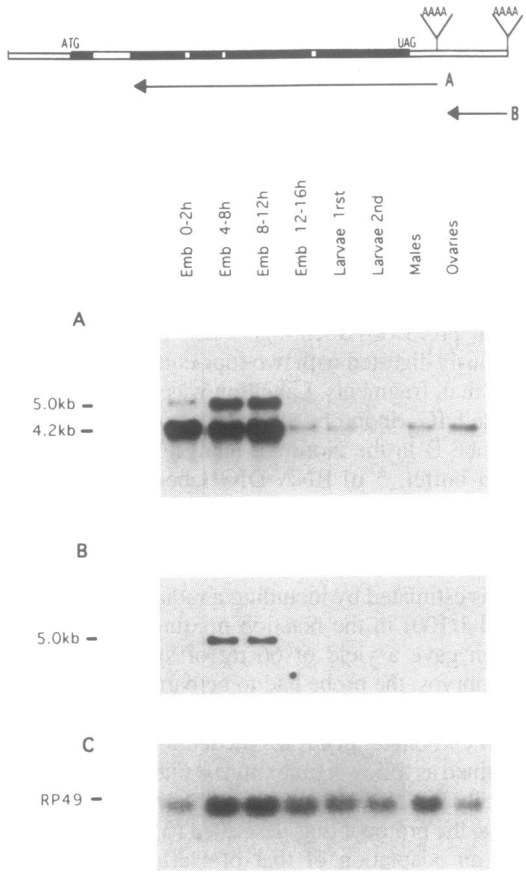

Figure 1. Northern blot analysis. Poly(A+) RNA was prepared from selected developmental stages as indicated. The same membrane was hybridized successively with riboprobes A (**A**) and B (**B**). Probes A and B are delimited above the photograph on a map representing the transcript with the exons in black and the introns in white and the two polyadenylation sites. (**C**) Hybridization of a probe for the ribosomal protein gene rp49 to show that roughly similar amounts of RNA were loaded in each slot. All three panels have been scanned to compare values (not shown). Each panel results from one autoradiograph of one blot. Differences in the backgrounds of the lanes result from cutting and pasting of the photographs of the lanes to reconstitute the course of development stages.

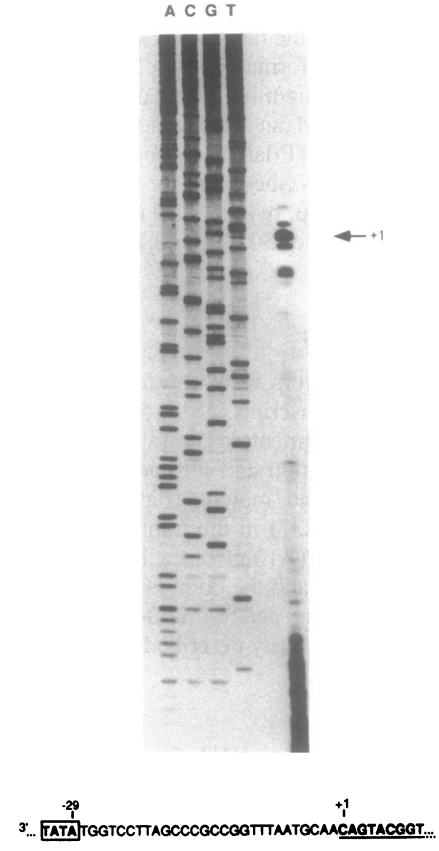

Figure 2. Primer extension analysis. The oligonucleotide primer is complementary to nucleotides 79–96. The sequence shown to the left was determined on the genomic clone pAS (our unpublished work) with the same oligonucleotide used as primer. Note that there is a polymorphism between the cDNA and the genomic sequence of different origin (position 7 is a G in the genomic DNA). The sequence written below is the genomic DNA to show the transcriptional start site (+1) and the TATA box located 29 bp upstream.

(unpublished) and N. Brown (8)]. In addition, to better delineate the transcriptional unit, different genomic fragments spanning the locus were tested for their ability to detect one or the other transcript (Fig. 1B). This analysis provided the first evidence that the two transcripts share the same 5′-end but differ at their 3′-ends. Indeed, fragments at the 3′-end of the locus identify the longer transcript on Northern blots, but not the shorter one (Fig. 1B and data not shown). Sequencing of the cDNA clones determined, first, that the *Suvar(3)7* transcription unit extends further at its 5′-end than we had previously reported and, secondly, that the cDNA clones define two classes of transcripts which differ solely in the length of their 3′-untranslated sequence (see below). The complete sequence of the longer transcript, which comprises 5047 nt [without poly(A)], is not given here, but is accessible in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession number X52187. To confirm that the start of the cDNA sequences indeed represents the start of transcription *in vivo*, we performed the primer extension analysis shown in Figure 2. The major site (+1) is indeed preceded 29 nt upstream on the genomic DNA by a TATA box-like motif. At the 3′-end, the two classes of cDNA clones determine two termination sites that are preceded in the sequence by poly(A) sites at positions 4175 and 5013. The two transcripts hence differ by 844 nt. The two sizes of 5047 and 4203 nt, excluding the poly(A) tails, correspond well to the sizes of 5.0 and 4.2 kb determined on Northern blots (Fig. 1). That the longer transcript on blots actually corresponds to the longer class of cDNA is demonstrated by the use of a 3′-end-specific probe on the blots in Figure 1B. Genomic sequencing (not shown) has led us to map three small introns dispersed over the coding sequence and a larger one close to the 5′-end. The introns are located on the cDNA sequence after positions 927, 1512, 1857 and 2835. We conclude from the data above and restriction analysis (data not shown) that it is very likely that the two classes of transcripts differ solely by the use of an alternative termination site.

Genetic transformation: both cDNAS encode the modifier function

cDNAs corresponding to each class of transcript were cloned downstream of the *hsp70* promoter in the pNHT4 transformation vector (5). Transgenic lines were established for each class of transcript and the presence and integrity of the transgene was

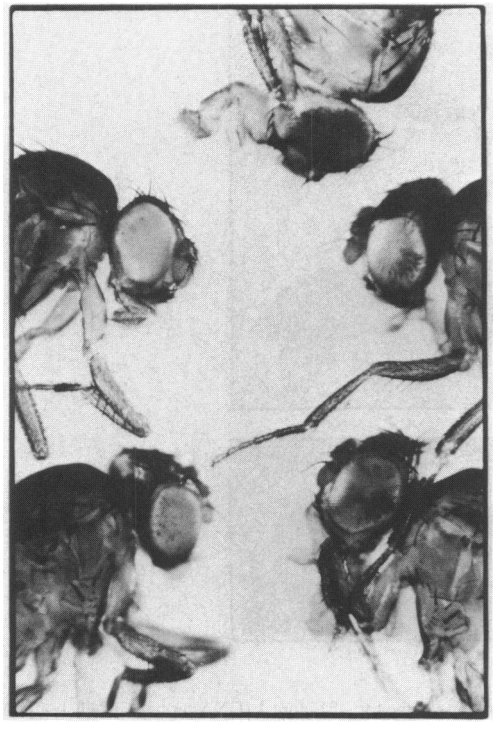

Figure 3. Enchancement of position-effect variegation by genetic transformation with a *Suvar(3)7* cDNA. The fly on top is the control white mottled fly (w^{m4h}). Variegation in this and other flies in the figure was somewhat suppressed by introducing the suppressor of variegation mutation *Suvar(2)101*, so that the effect of two additional copies of *Suvar(3)7* could be distinguished from the effect of one additional copy (see Fig. 4 for details of genotypes in a similar test). The flies on the left side are males and illustrate the effect without heat-shock of one copy (heterozygote, top) or two copies (homozygote, bottom) of the transgene. The two flies on the right side illustrate the same genotypes in females.

controlled by genomic Southern analysis (data not shown). Six independent transgenic lines were crossed to the *white mottled 4h* (w^{m4h}) variegating rearrangement as previously described for the transformation with genomic fragments (4). Figure 3 illustrates the dramatic enhancement of variegation in the resulting progeny, even in the absence of heat shock. Figure 3 also shows that two copies of the transgene enhance variegation more than one and that the effects are stronger in males (only one copy of the variegating rearrangement on the X chromosome) than in females carrying two copies of the rearrangement. These transgenic lines thus provide direct evidence that the transcripts we describe here encode the genetic function of the locus. It is also of interest to note that the phenotypic effects of the large and the small transcript were indistinguishable (data not shown).

Developmental pattern of *Suvar(3)7* transcript levels: a maternal contribution

The developmental Northern blot in Figure 1 reveals two transcripts of 5.0 and 4.2 kb and shows their abundance at selected developmental stages. The smaller transcript is predominant and is found first as a maternal contribution (Fig. 1A, 0–2 h embryos and adult female ovaries). It is detected throughout embryogenesis (4–12 h) and in larvae (2nd instar) and adults (whole males and ovaries). Though at first of maternal origin, the persistent presence of message from late embryos to adults suggests that embryonic and post-embryonic synthesis is also likely. The larger transcript is less abundant than the smaller, but Figure 1A does not show obvious differences in the pattern of expression. A probe specific for the 3′-end of the transcript labels only the larger species (Fig. 1B). The message for the ribosomal protein rp49 was used as a control to compare the different lanes (Fig. 1C).

Tissue distribution of the transcripts: specific pattern in ovaries

The tissue distribution of the transcripts was determined by *in situ* hybridization to whole mounts of embryos and dissected organs. In ovaries, the DNA probe labelling both transcripts (Fig. 4A) confirms the maternal origin of the message inferred from the Northern blots. The message is first detected in the germarium and becomes very intense in the nurse cells of stage 10 oocytes. It is then transferred into the maturing oocyte. The riboprobe specific for the larger transcript (Fig. 4B) shows a strong signal in somatic follicular cells in addition to the nurse cells. That this additional staining was not observed with the DNA probe comprising both transcripts results from a large difference in relative abundance and the lesser sensitivity of DNA probes. The Northern blot (Fig. 1) confirms this large difference in abundance. Figure 4B hence suggests that the signal in somatic tissue is specific for the 5.2 kb transcript.

In pre-blastoderm embryos the RNA is rather uniformally distributed, before being concentrated in the cytoplasm of all cells of the cellular blastoderm. This distribution is found ubiquitously throughout embryogenesis (not shown), but at the end of embryogenesis, the staining is concentrated in the the central nervous system and gut (Fig. 5A). Hybridization with the probe specific for the longer transcript (Fig. 5B) does not show any significant differences from the above, except for the absence of detection in the central nervous system of late embryos.

The complete deduced sequence of the protein: seven zinc fingers each preceded by a tryptophan box

That the cDNA sequence is longer than we had previously determined (4) led us to re-investigate the conceptual translation of the message. This, together with the correction of a sequencing error (introduction of a G at position 52 in the previously published sequence, position 1147 in the present report) allowed us to extend the N-terminus of the protein by 237 amino acids, leading to a 1169 amino acid long protein with a calculated M_r of 131.138×10^3. Figure 6 shows an alignment of the sequences of the seven zinc fingers starting from the upstream tryptophan box. In the lower part of the figure we have schematized the scattered distribution of zinc fingers in the complete protein sequence.

Since we are now confident of having determined the correct start of transcription, we believe that we have also determined the complete protein sequence. Surprisingly, this led us also to discover two new zinc-finger motifs, each with the same characteristics as the five we had described previously (4). Another motif, the so-called tryptophan box (9–13) is found in front of the first cysteine of each of the seven fingers (Fig. 6). The figure shows, in addition, that the number of amino acids separating the tryptophan box from the zinc finger (as determined by the first cysteine) is fairly conserved, with a minimum of 11 and a maximum of 16 amino acids. We also note that the first two tryptophan boxes are themeselves preceded by a small region very rich in tryptophan. The two classes of transcripts do not

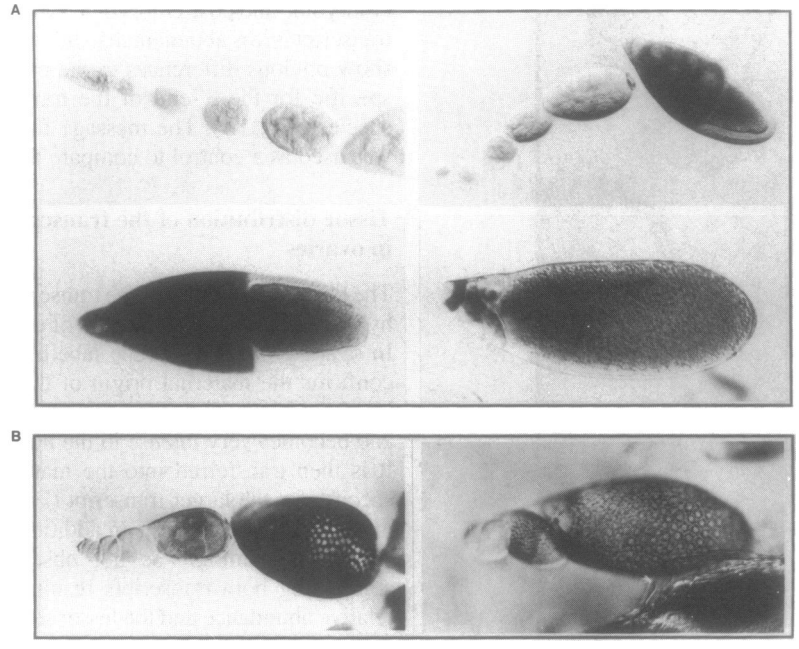

Figure 4. Distribution of the transcripts in adult ovaries. (**A** and **B**) the probes described in the scheme at the top of Figure 1 (DNA probe A labels both transcripts, riboprobe B labels only the large one). Signal in the germarium is weak, but was confirmed in many preparations.

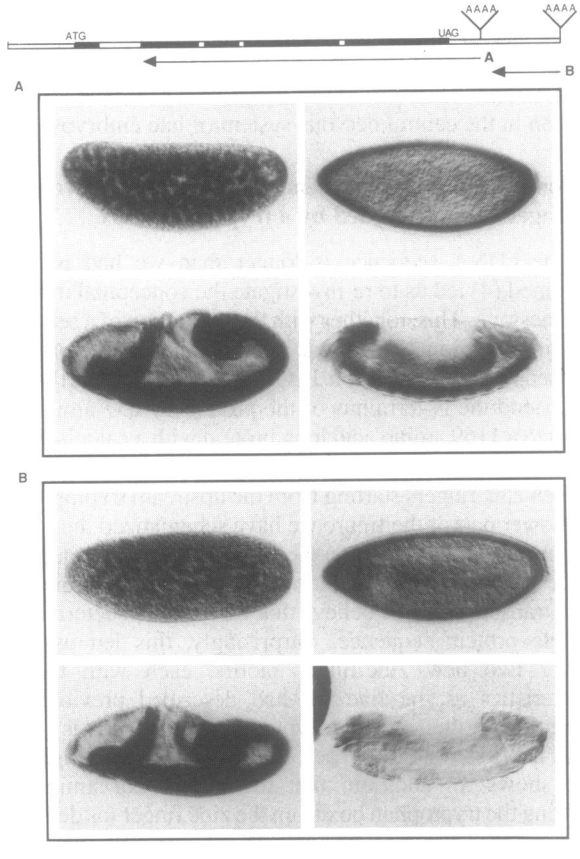

Figure 5. Distribution of the transcripts in whole mount embryos. (**A** and **B**) the probes described in the scheme at the top of Figure 1 (DNA probe A labels both transcripts, riboprobe B labels only the large one).

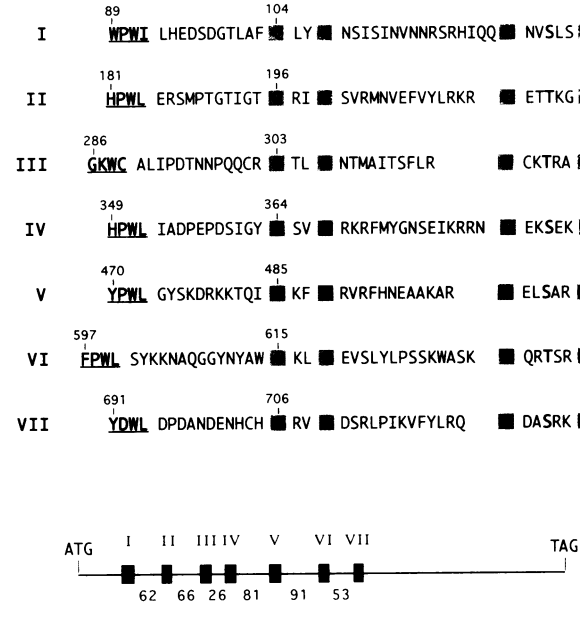

Figure 6. The seven zinc fingers and tryptophan motifs. Roman numbers refer to each of the seven fingers starting from the N-terminus of the protein. The tryptophan boxes are in bold and underlined. The cysteines and histidines characteristic of the zinc fingers are in bold and in a grey square. The amino acids separating the tryptophan box from the corresponding zinc fingers are listed. The potential phosphorylation sites are in bold. each sequence is numbered at the start; the position of the first cysteine of each zinc finger is also numbered. The drawing under the sequences represents the protein sequence with the position of the fingers; the numbers below refer to the spacing of the fingers in amino acids, from the last histidine to the first cysteine.

differ in their open reading frame and should produce the same protein.

DISCUSSION

Alternative termination: two transcripts for one protein

From the evidence presented above, we conclude that the locus produces two transcripts differing in the use of alternative termination sites, but conserving the same protein coding capacity. With regard to the 5′-end of the transcripts, the correspondence between the start of the longest cDNA clones and the result of the primer extension analysis supports our conclusion that we have identified the correct start of transcription. Whether these transcripts each play a specific role is unknown, but we have found that in ovaries the large transcript is detected essentially in follicular cells. This emphasizes the importance of the smaller transcript, which is abundant in nurse cells and transferred into the oocyte. If the product of *Suvar(3)7* serves in the building of heterochromatin, the transcript would be expected to be present in the early embryo, when heterochromatin forms, and at later stages of growth. This is consistent with the developmental levels we see.

Maternal transmission

The high levels of transcript in nurse cells of the ovaries and its presence in oocytes and very early embryos indicate maternal transmission. This is expected for a protein which is a structural component of heterochromatin. Though our developmental analysis is far from being comprehensive, it seems that transcripts are ubiquitous throughout development. This persistence suggests embryonic and post-embryonic expression.

Seven zinc fingers in Suvar(3)7, each preceded by a tryptophan box

The sequencing of additional cDNA clones has allowed us to correct the previously published sequence (4). The predicted protein encoded by *Suvar(3)7* is 1169 amino acids long. This has also led us to the discovery of two additional zinc fingers close to the N-terminus of the protein. These fingers have the same features reported for their previously described brothers, as they are of the C2H2 type, encode a potential phosphorylation site in the middle of the histidine doublet, display an unusually large spacing relative to one another and, last but not least, are each preceded by a tryptophan-containing motif about 10 amino acids upstream of the cysteine doublet. A similar motif (YPWM) was first noted in front of the homeodomain in the product of several homeotic genes in *Drosophila* (9,11) and in vertebrates (10). The tryptophan box in Suvar(3)7 is not well conserved in front of some of the fingers, but it stands out as it precedes the DNA binding motif by a rather similar number of amino acids. Correspondingly, their distant brothers in homeotic gene products precede the DNA binding homeodomain. Though the role of this motif has not been ascertained, it has been determined that it perturbs or even suppresses secondary structure locally in the Antennapedia homeodomain-containing protein and could thus promote protein–protein association in multiprotein complexes with DNA (12; see also 13, a commentary extending the discussion to the yeast α2 homeodomain protein). The role of the tryptophan motif was recently analyzed for this yeast protein (14) and it was concluded that this short and unstructured sequence is responsible for the specific interaction of the homeodomain with a second regulatory domain (MCM1) which permits cooperative binding of the two proteins to an operator. A role in protein–protein interaction by providing flexibility is of great interest for a component believed to promote chromosome condensation.

The function of the Suvar(3)7 protein

Each finger is, at least in the linear sequence, rather far away from its neighbours, a configuration not generally found in zinc finger transcription factors. Moreover, the fact that each finger is preceded by a tryptophan motif (shown in other cases to enhance flexibility, protein–protein interaction and protein–DNA interaction) makes of the *Suvar(3)7* product a potential multifingered gripping tool. We have established that an increase in the dose of *Suvar(3)7* increases the silencing of genes brought close to the highly condensed heterochromatin (4). hence we cannot resist proposing a direct role for the *Suvar(3) 7* gene product in the mechanical compaction of chromatin, resulting in heterochromatin. The fingers are DNA binding motifs, their dispersion in the protein could allow them to make contacts with DNA at a distance, and the tryptophan boxes could provide the flexibility required to pack the bound DNA in a more compact conformation. Both the tryptophans and acidic regions (4) implicate other proteins (other modifers of variegation for example) in this process. Finally, the number of potential phosporylation sites suggests a control of protein function by this modification. Actually, the modifier of variegation *Suvar(3)6* encodes a protein phosphatase (15). In this respect, the potential phosphorylation site in the middle of the histidine doublet of each finger is of particular interest, because it seems unlikely that the finger can maintain an active Zn-dependent conformation with a phosphate group at such a close distance. On the other hand, it has recently been proposed that DNA sequence repeats by themselves induce formation of heterochromatin and variegation of a neighbouring gene, probably by pairing (16). The product of *Suvar(3)7* could be an excellent candidate for mediating orthodox or aberrant DNA–DNA pairing. This hypothesis is now testable. For completeness, we note an alternative model of position-effect variegation (17), in which heterochromatin (and occasionally neighbouring genes) are sequestered in a compartment of the nucleus inaccessible to some transcription factors. How the product of *Suvar(3)7* could mediate this process is more difficult to imagine, but our present knowledge does not exclude this possibility.

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