Mapping the D. melanogaster En1A Enhancer Modules Responsible for Transcription Activation and Long-Distance Enhancer-Promoter Interactions

L. S. Melnikova*, E. A. Pomerantseva, V.V. Molodina, P. G. Georgiev
Institute of Gene Biology, Russian Academy of Sciences, Vavilova str. 34/5, Moscow, 119334, Russia
*E-mail: lsm73@mail.ru
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ABSTRACT
The structure of the new enhancer En1A of the 1A region of the X chromosome of D. melanogaster was investigated. Two distinct regulatory elements were found. The first element is responsible for transcription activation, and the second element provides specific interaction with the promoter of the yellow gene. The findings support the hypothesis of a modular structure for enhancers, including certain sequences that bind transcription activators and special communication elements providing long-distance enhancer-promoter interaction.

KEYWORDS
Drosophila melanogaster, long-distance interactions, transcription activation, enhancer structure, yellow gene.

INTRODUCTION
One of the key properties of enhancers is their ability to specifically activate the transcription of the target gene that, in some cases, covers a distance of tens or even hundreds of kilobase pairs [1]. However, the mechanisms that are at play for maintaining specific long-distance interactions between enhancers and promoters remain elusive. In some cases, the cis-regulatory sequences found within the promoter regions of eukaryotic genes have been known to enable communication between an enhancer and a promoter [2–4]. Collected data [5] suggest that the specificity of some enhancers is due to the presence in them of binding sites for the said transcription factors (TF), which are responsible for transcription activation, and of proteins providing a stable long-distance enhancer-promoter interaction.

The aim of the current study was to investigate the new enhancer En1A found in the intron of the unexplored gene CG3777 located on the X chromosome.

The En1A enhancer was shown to have a modular structure. We found the activation and communication elements in the structure of En1A. The activation element is able to functionally replace the yellow gene body and wings enhancers; i.e., stimulate transcription in the corresponding cuticular structures. The communication element is necessary for the interaction between En1A and the yellow gene promoter and able to provide long-distance GAL4-dependent transcription activation.

EXPERIMENTAL PROCEDURES
All constructs are based on a pCaSpeR3 vector containing the mini-white gene. The plasmid vector pCΔ derived from pCaSpeR3, which contains a deletion of the mini-white gene, has been described previously [6].

For the constructs EcoRI–PstI–Y, PstI–PvuII–Y, and HindIII–y+–Y, the corresponding restriction fragments of the chimeric element from the y+ allele were used. The fragments were inserted upstream of the yellow gene promoter at position -343 bp (hereinafter, including figures, the numeration within the yellow locus is determined relative to the gene transcription initiation site) at the KpnI restriction site.

For (a1–a2)Y construct design, yellow cDNA lacking a bristle intron and an enhancer was used (pCaSpeR3-Yil). A fragment of 362 bp was amplified from the genomic DNA of a y+ fly line using the primers a1 (5′-CTTTTTGCATACACATCCAC-3′) and a2 (5′-GCTGATGGAAGTTGCAGA-3′) and cloned into a vector based on the pBlueScript plasmid between two

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RESULTS AND DISCUSSION

In Drosophila melanogaster, gene yellow is responsible for the pigmentation of cuticular structures: the body, wings, and bristles. The enhancers that control yellow expression in the body and wing cuticle are located on the 5’-end of the gene, whereas the enhancer responsible for expression in bristles is located in the intron [7]. In wild-type flies, the body, wings, and bristles have a dark color.

The allele $y^i$ is often used as a model system in works that study transcriptional regulation in D. melanogaster. The $y^i$ allele has an incorporated retrotransposon, MDG4 (gypsy), between the promoter and enhancer of the body and wings of the yellow gene [8]. As a result, a Su(Hw) insulator comprising MDG4 blocks yellow activation through body and wing enhancers. Thus, the $y^i$ phenotype is characterized by a yellow color of the body and wings, while the bristles are dark-colored.

The superunstable allele $y^{+s}$ (Fig. 1A) was obtained by induction of P-M hybrid dysgenesis in a line containing the $y^i$ mutation [9]. Allele derivatives $y^{+s} - y^{2s}$ and $y^{2s}$ containing a chromosome X region 1A duplication in the pre-promoter region of yellow have also been obtained [9, 10]. The study of the structure of the alleles $y^{+s}$ and $y^{2s}$ made it possible to identify the regulatory element 1A-RE, which activates long-distance yellow expression and is a yellow-specific insulator, within the duplicated fragment comprising the region 1A [10]. In the presented paper, we continued our thorough study of the structure of the $y^{+s}$ allele.

The mutation $y^{+s}$ was a result of the introduction of a chimeric 5.4 kb element at position -69 bp with simultaneous deletion of the yellow sequence between -146 and -70 bp. The chimeric element consists of 1.2 kb P-elements located “tail to tail” and a 3 030 bp sequence trapped between them, which presents a duplication of the region 1A of chromosome X and is located distal to the yellow locus in the genome (Fig. 1A) [9]. This duplication includes a fragment of the unexplored gene CG3777, which is expressed at the same stages of development as the yellow gene.

The body and wings of the flies carrying allele $y^{+s}$ exhibit a dark color close in intensity to the color of wild-type flies. Hence, gene expression is activated in the body and wings in the case when the 1A region from chromosome X is shifted to the yellow gene, despite the fact that the Su(Hw) insulator blocks the corresponding enhancers. We managed to localize a 1748 bp enhancer, which was called enhancer 1A (En1A), in the relocated DNA sequence by using transgenic constructs (Fig. 1B).

First, we tested two restriction fragments which together cover most of the region 1A duplications: EcoRI–PstI of 771 bp and PstI–PvuII of 1748 bp (Fig. 1A). In the transgenic constructs EcoRI–PstI-Y and PstI–PvuII-Y, these fragments were located upstream of the yellow promoter at position -343 bp (Fig. 1B). Both constructs contained no body or wing enhancers. Among the lines carrying the EcoRI–PstI-Y construct, 19 flies out of 20 had an uncolored body and wings. The phenotype of the flies from transgenic PstI–PvuII-Y lines was similar to the wild-type phenotype in 23 out of 31 lines.
obtained, which is proof of the ability of the 1748 bp fragment to functionally replace the body and wing enhancers of the *yellow* gene. Hence, the En1A enhancer is localized within the PstI–PvuII region.

In order to accurately map En1A, two genetic constructs containing distinct PstI–PvuII fragments incorporated at position -343 bp were designed: HindIII–PvuII–Y and (a1–a2)Y (Fig. 1B). The HindIII–PvuII fragment, of 1 511 bp (Fig. 1A), had no enhancer properties: the body and wings of the flies were yellow in all 10 transgenic HindIII-PvuII-Y lines (Fig. 1B). A bio-informatic analysis of the structure of the PstI–PvuII sequence revealed a 362-bp fragment comprising recurring motifs, which, possibly, could serve as binding sites for regulatory proteins. This DNA fragment was amplified by PCR using the primers a1 and a2 and then incorporated upstream of the *yellow* promoter as part of the (a1–a2)Y construct (Fig. 1B). Fragment a1–a2 is surrounded by the Cre recombinase recognition sites (loxP sites), which allows in *vivo* excision of the analyzed element [11]. It should be noted that *yellow* cDNA contained no bristle enhancer in the construct (a1–a2)Y.
We obtained 17 transgenic lines carrying the construct. Despite the absence of a bristle enhancer, the flies of all the lines had the y² phenotype. Excision of the a1–a2 sequence resulted in the disappearance of pigmentation in bristles in 12 out of 15 lines. Thus, the studied 362 bp fragment within the (a1–a2)Y construct interacted with a promoter and stimulated yellow expression in bristles but was incapable of functionally substituting body and wing enhancers.

The obtained results allowed us to suggest that enhancer En1A has a heterogeneous structure. One part of the enhancer, (a1–a2) of 362 bp, named the “communication part” (hereinafter Cm1A), alone stimulates yellow expression only in the bristles. However, it is necessary for the stimulation of yellow expression by the full-length En1A in the body and wings. Another part of the PstI–PvuII sequence of 1,386 bp is capable of inducing a high level of yellow expression in the body and wings only in combination with the communication part (Fig. 1A, B). Full-length En1A of 1,748 bp activates yellow transcription in all cuticular structures. Apparently, the 1,386-bp fragment contains binding sites for yellow transcription activators in the body and wings, but their interaction with the promoter is provided by Cm1A-binding proteins.

To further explore the communication properties of the Cm1A element, we used a model system based on the properties of the yeast activator GAL4. This activator is known to stimulate promoters of various genes in the Drosophila genome [2]. However, GAL4, located at the 3'-end of the gene, is incapable of transcription activation [12]. In the construct YG4(Cm1A), the protein GAL4 binding sites and a potential communicator, Cm1A, surrounded by loxP sites were incorporated at the 3'-end of the yellow gene. In addition, the 5' sequence of yellow containing body and wing enhancers (up to -890 bp) was deleted (Fig. 2). In seven transgenic lines carrying the YG4(Cm1A) construct, the flies had a y² phenotype. Thus, in the absence of GAL4 activation, the Cm1A fragment is incapable of activating the transcription of yellow in the body and wings. Then, we crossed YG4(Cm1A) transgenic lines with a line expressing the GAL4 protein. As a result of GAL4 activation, the body and wings of the flies in all the lines acquired a darker color (Fig. 2). Deletion of Cm1A led to a decrease in yellow expression to its initial level. Therefore, the Cm1A element, indeed, has communication properties. It provides stable long-distance interaction with the GAL4 activator and yellow promoter.

Earlier, we had localized TE at -69 … -100 of yellow, which provides long-distance interaction of body and wing enhancers with the yellow promoter, as well as the heterologous promoter of the gene eve [2]. We hypothesized that the Cm1A communicator functionally

![Fig. 2. Screening of the communication properties of the Cm1A fragment. Results of the phenotypic analysis of the flies in transgenic lines are presented under construct schemes. Designations: y – yellow gene promoter; eve – even skipped gene promoter; black ellipse – Cm1A communicator. The arrow inside an ellipse indicates the transcription direction. Deletion of the yellow gene sequence of -69 to -100 bp (TE) is marked by Δ. Pigmentation of the body and wings is numbered from 5 (dark color, as in the wild-type) to 2 (yellow color corresponding to the phenotype of the y² allele). The designation “+ GAL4” refers to the derivatives obtained after GAL4 activation in transgenic lines of the corresponding genotype. N is the number of lines of flies that acquired a new phenotype after Cm1A deletion or by crossing with the line expressing GAL4. T is the total number of lines examined for each particular construct. For other designations, see Fig. 1.](image-url)
interacts with TE of the yellow gene. In order to test this hypothesis, the constructs eveYG4(Cm1A) and ΔeveYG4(Cm1A) were obtained (Fig. 2). In both constructs, the yellow gene promoter was replaced by a heterologous promoter of the eve gene (-68 ... +130 bp) (Fig. 3). Moreover, the TE sequence of the yellow gene was deleted in the ΔeveYG4(Cm1A) construct (Figs. 2, 3). The results obtained during a phenotypic analysis of five eveYG4(Cm1A) transgenic lines were similar to the results of a YG4(Cm1A) line analysis. As in the previous case, the communicator provided GAL4-dependent transcription activation of yellow. Since the structures of the yellow and eve promoters are different (Fig. 3), one can assume that the core elements of the promoter are not involved in the functional interaction with Cm1A. In six transgenic lines carrying the ΔeveYG4(Cm1A) construct, GAL4 activation did not lead to changes in the initial y phenotype (Fig. 2). Hence, the communicator Cm1A is incapable of supporting long-distance interaction between the transcription activator and the promoter of the gene in the absence of yellow gene TE. Apparently, the proteins binding the communication element Cm1A can interact with the proteins recruited to TE of the yellow gene. Such interaction brings the GAL4 activator and promoter spatially together in the described model system, which enables contact between the activation complex recruited to the GAL4 sequences and the transcriptional complex of the promoter.

CONCLUSION
The presented data allow us to conclude that the new enhancer En1A has a modular structure. In a previous study, we showed that the regulatory system of white also includes elements that do not affect transcription but provide long-distance enhancer–promoter interaction. The pre-promoter region and the eye enhancer of gene white contain binding sites for the Zeste protein. The Zeste protein is not involved in transcription activation but allows the eye enhancer to activate a long-distance promoter through binding to its target sites [5]. The results of the current study support the hypothesis that the regulatory regions of various genes have a modular structure and include activation elements that bind to transcription factors, initiating and providing efficient transcription and communication elements that bind proteins, providing spatial contact between an enhancer and a promoter. The described model systems can be used to study the enhancer structure and identification of the sequences involved in long-distance interactions between the regulatory elements of the genome.

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