10. *Sohn J., Choi E., Kang H., Rhee J., Agaphonov M., Ter-Avanesyan M., Rhee S.* A dominant selection system designed for copy-number-controlled gene integration in Hansenula polymorpha DL-1 // Appl. Microbiol. Biotechnol.- 1999.- vol. 51, P.800–807.
11. *Veenhuis M., Hoogkamer-Te Niet M.C., Middelhoven W.J.* Biogenesis and metabolic significance of microbodies in urate-utilizing yeasts // Antonie Van Leeuwenhoek-1985.-vol. 51, P.33-43.
12. *Li J., Chen Z., Hou L., Fan H., Weng S., Xu C., Ren J., Li B., Chen W.* High-level expression, purification, and characterization of non-tagged *Aspergillus flavus* urate oxidase in *Escherichia coli* // Protein Expr. Purif.- 2006.- vol. 49, P.55-59.
13. *Leplatois P., Douarin B., Loison G.* High-level production of a peroxisomal enzyme: *Aspergillus flaws* uricase accumulates intracellularly and is active in *Saccharomyces cerevisiae* // Gene– 1992.- vol. 122, P.139-145.

Abstract

Strains of *Hansenula polymorpha* overproducing own uricase (UOX) have been constructed. Uricase gene under the control of the strong *H. polymorpha* alcohol oxidase promoter was multicopy integrated to the recipient strain *H. polymorpha* C-105 (*gcr1 catX*). The recombinant producer characterized by a forty-fold increased UOX enzyme activity (up to 3.36 U ml^{-1} in cell-free extract) compared to the initial strain.

Сконструированы штаммы *Hansenula polymorpha* с усиленной экспрессией собственной уриказы. Ген уриказы под контролем сильного промотора гена алкогольоксидазы *H. polymorpha* введён в геном реципиентного штамма *H. polymorpha* C-105 (*gcr1 catX*). Рекомбинантный штамм характеризировался сорокакратным увеличением активности уриказы (3.36 МЕ $мл^{-1}$ в бесклеточных экстрактах) в сравнении с исходным штаммом.

Сконструйовані штами *Hansenula polymorpha* з посиленою експресією власної урікази. Ген урікази під контролем сильного промотора алкогольоксидази *H. polymorpha* було введено в геном реципієнтного штаму *H. polymorpha* C-105 (*gcr1 catX*). Рекомбінантний штам характеризувався підвищенням активності урікази в 40 разів (3.36 МЕ $мл^{-1}$ в безклітинних екстрактах) у порівнянні з вихідним штамом.

OLENKINA O.M.,OLENINA L. V., LAVROV S.A., KLENOV M. S., GVOZDEV V.A.
Department of Animal Molecular Genetics, Institute of Molecular Genetics, Kurchatov sq., 2, Moscow, 123182, Russia; e-mail: olenkina@img.ras.ru

ANALYSIS OF TESTIS - EXPRESSED *STELLATE* GENES PROMOTER REGION IN *D. melanogaster*

The first described case of a natural RNA-mediated silencing found to be necessary for male fertility maintenance was discovered in *Drosophila* (Aravin et al., 2001). In wild-type testes, hyperexpression of tandemly repeated X-linked *Stellate* genes is prevented by homologous, bidirectionally transcribed Y-linked *Suppressor of Stellate* repeats (*Su(Ste)*), and deletion of *Su*(*Ste*) leads to abnormalities of spermatogenesis (Palumbo et al., 1994). In the *Drosophila* germline, selfish genetic elements, such as retrotransposons and repetitive sequences, are suppressed by rasiRNAs (Vagin et al., 2006). However, a little is known about classical transcription regulation of the *Stellate* genes. The expression of the *Stellate* genes is restricted to *D. melanogaster* testes. The transcription start site was determined in (Livak,

1990) by primer extension experiments. Sequence in -30 region upstream transcription start has shown no TATA-element similarity, so the *Stellate* gene promoter appears to be TATA-less but initiator-containing one. As we have shown earlier (Aravin et al., 2001) a 134 bp-long region containing *Stellate* initiator site is sufficient for high-level expression of transgene reporter *LacZ* in testes of cry^1 males. This result provides a strong evidence for a presence of *cis*-regulatory sites within this region. In this article, we present our analysis of the promoter region of the *Stellate* genes.

Materials and methods

Reporter construction design. For PCR amplification, a plasmid template containing 6 full-length *Stellate* genes was used. PCR amplifications of *Stellate* regulatory sequences with primers, generating a duplex with 5' overhangs containing sites for XbaI and BamHI, and ligation in pCaSpeR-β-gal vector opened with XbaI and BamHI, were performed as in Aravin et al., 2001.

Drosophila strains, transformation, and genetic crosses. Flies were reared on standard medium at 25ºC. P-element-mediated germline transformation of $Df(1)w^{67c23(2)}y$ embryos was performed according to standard protocol (Rubin and Spradling, 1982). The number of insertions in genome was estimated by Southern blot. The strain with deletion of the bulk of *Su(Ste)* repeats on the Y chromosome was cry^1Y, described in Palumbo et al., 1994. To produce males carrying the cry^1Y chromosome, $Df(1)\ w^{67c23(2)}y$ females were crosses to $X/cry^1B^sYy^+$ males.

Testis nuclear extract preparation. It was prepared as in (Dignam et al., 1983) with minor modifications.

Electromobility shift assay. The binding probes were terminally [^{32}P]-labeled with T4 polynucleotide kinase. Testis nuclear extract 10-15 micrograms was incubated for 20 min at room temperature with 0.3-0.6 pmol of labeled oligonucleotide. All binding reaction were carried out in 20 mM HEPES pH7.6, 50-60 mM NaCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 1mM DTT, 5% glycerol, 20 ng/μl poly(dI-dC), 0.1 ng/μl heparin in total volume of 15 μl. DNA-protein complexes were resolved by 5% non-denaturing polyacrylamide gel electrophoresis in 0.75xTris-borate-EDTA buffer with 2.5% glycerol at 8ºC.

Results and discussion

Determination of 5' border of proximal promoter region of Stellate genes. General approach taken to determine upstream borders of gene transcription-control regions involves creating a set of 5'-deleted reporter constructs. It was previously shown that *lacZ* reporters driven by 5' *Stellate* fragments carrying the *Ste225-lacZ* and *Ste131-lacZ* constructs were sufficient to provide high-level expression of β-galactosidase in cry^1Y males testes (Aravin et al., 2001). 134 bp 5'-fragment of *Stellate* heterochromatin gene contains 33 bp of 5'-transcribed region from ATG start codon, and promoter–proximal region lying 101 bp upstream of the start site. To determine whether the 134 bp fragment can be account as minimal promoter region of the *Stellate* genes, we constructed two additional 5'-deletion mutant constructs of *Stellate* promoter-proximal region *Ste63-lacZ* and *Ste44-lacZ* with *lacZ*-reporter gene, and established the transgenic fly lines by P-element-mediated germline transformation. Both mutant constructs also contained 33 bp of 5'-transcribed region of *Stellate* gene sequence downstream from the ATG start codon fused with *lacZ* gene and untranscribed 5' upstream fragments of *Stellate* heterochromatin gene of 30 bp or 11 bp, respectively. Analysis of β-galactosidase activity in dissected testes from the cry^1 males revealed that β-galactosidase expression of the *Ste63-lacZ* was significantly weaker than that of the *Ste131-lacZ* (fig.1).

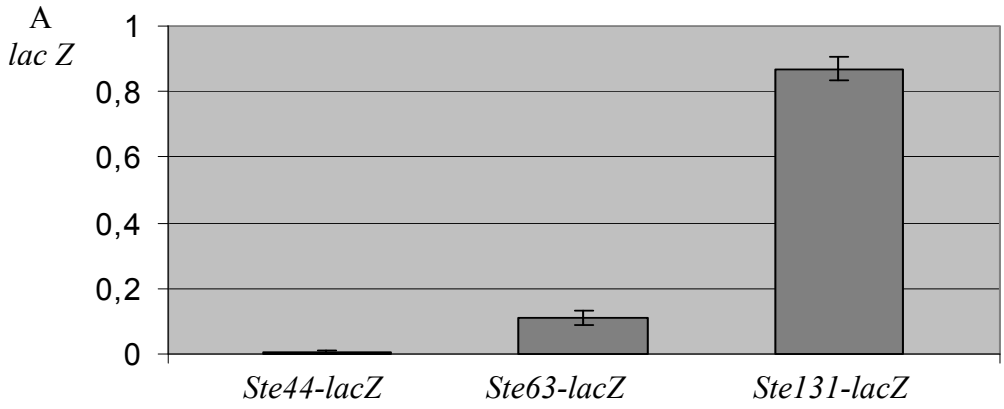

Figure 1. Activity of β-galactosidase expressed in testes of *D. melanogaster* under control of the *Stellate* promoter sequences of different sizes.

The *Ste44-lacZ* construct did not show β-galactosidase activity and did not differ markedly from control fly lines, which were used for P-element transformation. In our experiments, only flies bearing the *Ste131-lacZ* construct allowed high-level expression of the lacZ reporter in testes. As a result, we consider the fragment of 134 bp (-101 bp upstream and +33 bp downstream transcription start site) to be a minimal promoter region of the *Stellate* genes, which is sufficient for their correct testes-specific transcription. We suppose that the short 71 bp fragment (from -101 bp to -30 bp) shared by the *Ste131-lacZ* and the *Ste63-lacZ* constructs contains a *cis*-acting protein-binding control element, or a few elements, which regulate *Stellate* gene transcription in testes.

Identification of cis-acting elements within Stellate promoter. To solve the question which *cis*-acting elements for testes-specific transcription are present in *Stellate* promoter, electromobility shift assay was performed. We used four 5'-radiolabeled oligonucleotides in 27-37 bp encompassing the minimal promoter of 134 bp and named Ste1, Ste2, Ste3 and Ste4 as probes. We observed three specific DNA-protein complexes formed with Ste1, Ste2, and Ste4 oligonucleotides, respectively, after their incubation with testis nuclear extract (fig.2). In all of these cases, 50-fold excess of unlabeled specific oligonucleotide was able to compete for binding, whereas nonspecific was not. The signal intensity of binding complexes decreased reproducibly in next array: Ste2 > Ste4 > Ste1. Within Ste2 we found palindrome hexanucleotide sequence CACGTG. Sequence CA*NN*TG is well known as transcription control element named E-box (Atchley et al., 1997; Ledent and Vervoort, 2001). We aligned Ste1 and Ste4 oligonucleotides using Ste2 as a template and found that both of them contained degenerated E-box sequences: CATCTG and CAAGTG, respectively.

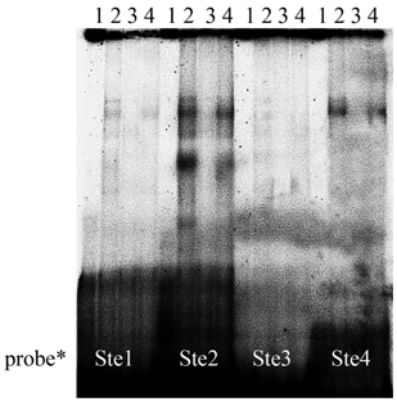

Figure 2. Electromobility shift assay.1- without extract; 2, 3, 4- with testis nuclear extract; 3- in presence of 50-fold excess of unlabeled specific oligonucleotide; 4- in presence of 50-fold excess of unlabeled nonspecific oligonucleotide.

To verify if this testes-specific protein factor binds just E-box in the Ste2 sequence, we performed gel shift assay with mutated Ste2 oligonucleotide, Ste2mut, where E-box CACGTG sequence was replaced by GGCTAT. Using Ste2mut as radiolabeled probe with testis nuclear extract abolished the gel shift (data are not shown). These observations suggest that the gel shift complex of Ste2 oligonucleotide is determined by the E-box motif. We also have noted that all three complexes run shifted probes in gel nearly with the same mobility. To confirm that all three oligonucleotides Ste1, Ste2 and Ste4 bind the same factor, we designed cross-competition assay. We assumed that the unlabeled Ste2 oligonucleotide containing the perfect E-box would compete with all E-box-containing oligonucleotides for the E-box binding factor, whereas Ste2mut would not. Actually, we observed that the 70-fold molar excess of unlabeled Ste2 competed effectively for complex formation with all three radiolabeled oligonucleotides, however the same excess of unlabeled Ste2mut did not affect the binding (fig. 3). Therefore, we determined three *cis*-acting motifs within minimal *Stellate* promoter to be E-boxes and detected the single protein factor from testis nuclear extract interacting with them *in vitro* in our experiments.

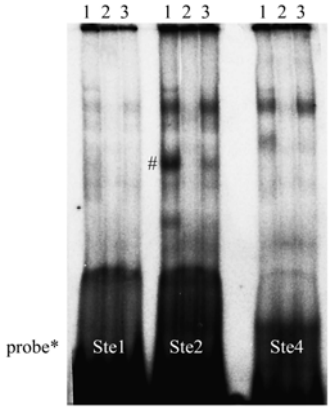

Figure 3. Cross-competition shift assay. Testis nuclear extract was incubated with oligonucleotide probes. 1- without competitors; 2- in presence of 70-fold excess of unlabeled Ste2 oligonucleotide; 3- in presence of 70-fold excess of unlabeled Ste2mut oligonucleotide. #-unspecific binding.

Discussion. The most of genes are transcribed during the organism life cycle according to the environmental conditions and in certain cell types and organs. The changing set of transcription factors provides control of place, time, and level of transcription for every particular gene. The composition of transcription factors binding sites (*cis*-regulatory regions) near a gene is the major determinants of its expression. The binding sites comprise a small part of nucleotides within promoter region. This fraction ranges from 10-20% within well-studied regulatory regions (Wray et al., 2003). Some genes analyzed to date, expressing only during *Drosophila* spermatogenesis, have extremely short regulatory regions located near basal promoter (Santel, 2000; Blumer et al., 2002). Here we determined a fragment of 134 bp as a minimal promoter region of the *Stellate* genes, which is sufficient for their correct testes-specific transcription. We identified three *cis*-regulatory sites within this minimal *Stellate* promoter *in vitro*. These *cis*-regulatory sites are known as E-boxes. The perfect E-box CACGTG is located from -47 bp to -42 bp upstream transcription start site of the *Stellate* gene. It resides within the promoter fragment of 71 bp which has been shown above to be responsible for the high-level expression of the reporter gene. E-box regulatory sites have been identified in a lot of promoter and enhancer elements. The E-box motif is known as cognate recognition sequence for basic helix-loop-helix (bHLH) superfamily of transcriptional regulatory proteins that are found in organisms ranging from yeast to humans.

According to our data, we can expect that the protein which binds to the E-boxes in our experiments also belongs to the bHLH superfamily.

Conclusions

We determined fragment of 134 bp (-101 bp upstream and +33 bp downstream transcription start site) to be minimal promoter region of the testis-expressed *Stellate* genes in *D. melanogaster*. We also identified three *cis*-regulatory sites within it to be E-boxes and showed that all of them interacted with the same DNA-binding factor from testis nuclear extract.

References

1. *Aravin A.A. et al.* Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the D. melanogaster germline//Curr.Biol.-2001.-v.11(13).-P.1017-1027.
2. *Palumbo G et al.* Genetic analysis of Stellate elements of Drosophila melanogaster//Genetics.-1994.-v.138.-P.1181-1197.
3. *Vagin V.V. et al.* A distinct small RNA pathway silences selfish genetic elements in the germline//Science.-2000.-v.313(5785).-P.320-324.
4. *Livak K.* Detailed structure of the Drosophila melanogaster stellate genes and their transcripts//Genetics.-1990.-v. 124(2).-P.303-316.
5. *Rubin G. and Spradling A.* Genetic transformation of Drosophila with transposable element vectors//Science.-1982.-v.218.-P.348-353.
6. *Dignam J.D. et al.* Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei//Nucleic Acid Res.-1983.-v.11.-P.1475-1489.
7. *Atchley W. and Fitch W.* A natural classification of the basic helix-loop-helix class of transcription factors//Proc. Natl. Acad. Sci. U S A.-1997.-v.94(10).-P.5172-5176.
8. *Ledent V. and Vervoort M.* The basic helix-loop-helix protein family: comparative genomics and phylogenetic analysis//Genome Res.-2001.-v.11(5).-P.754-770.
9. *Wray GA et al.* The evolution of transcriptional regulation in eukaryotes//Mol. Biol. Evol.-2003.-v 20(9).-P.1377-1419.
10. *Santel A et al.* The initiator element of the Drosophila β2tubulin gene core promoter contributes to gene expression in vivo but not required for male germline specific expression//Nucleic Acid Res.-2000.-v.28.-P.1439-1446.
11. *Blumer N et al.* A new translational repression element and unusual transcriptional control regulate expression of don juan during Drosophila spermatogenesis//Mech. Dev.-2002.-v.110.-P.97-112.

Abstract

Here we present our analysis of a promoter-proximal region of *Stellate* genes expressed in *D. melanogaster* germline. We determined the minimal core promoter of *Stellate* genes using the series of deletion transgene constructs. We analyzed this region by gel shift assay and revealed three E-box sites which bound to the protein factor from testis nuclear extract.

Здесь мы представляем наш анализ промоторной области генов *Stellate*, экспрессирующихся в герминальных клетках *D.melanogaster*. Мы определили минимальный коровый промотор с помощью делеционных трансгенных конструкций. Мы проанализировали его с помощью гель-шифта и выявили три Е-бокса, связывающихся с белковым фактором из ядерного экстракта семенников.

Тут ми представляємо наш аналіз промоторної ділянки генів *Stellate*, що експресується в гермінальних клітинах *D.melanogaster*. Ми визначили мінімальний коровий промотор за допомогою делеціонних трансгенних конструкцій. Ми проаналізували його за допомогою гель-шифту та виявили три ділянки так званих Е-боксів, що зв'язуються з білковим фактором з ядерного екстракту семенників.