Russian Journal of Developmental Biology, Vol. 33, No. 2, 2002, pp. 70–74. Translated from Ontogenez, Vol. 33, No. 2, 2002, pp. 95–99.
Original Russian Text Copyright © 2002 by Trunova, Dubatolova, Omel'yanchuk.

CELL DIFFERENTIATION AND PROLIFERATION

A Study of Expression of Cell Cycle Genes in *Drosophila* Using an Enhancer Trap and Radioautography

S. A. Trunova, T. D. Dubatolova, and L. V. Omel'yanchuk

Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences, pr. Akad. Lavrent'eva 10, Novosibirsk, 630090 Russia

Received May 3, 2000; in final form, February 28, 2001

Abstract—The phase of expression of genes *CycB, CycE*, and *chb* were determined in the cell cycle of neuroblasts of *D. melanogaster* 3rd instar larvae using the previously described radioautographic method and software. *CycB* was expressed at G_2 phase and upon transition from G_2 phase to M phase, while *CycE* was expressed at the end of G_1 phase and upon transition from G_1 phase to S phase. The phase of expression of the centrosome-associated protein chb was determined more precisely in G_2 phase. The mean life span of reporter β-galactosidase in neuroblasts was 4 h. The existence of more than one peak of expression of the gene in question in the cell cycle is discussed.

Key words: cell cycle, expression, cyclins, centrosome gene, *Drosophila*.

Expression of *Drosophila* zygotic genes controls the course of both cell cycle and parallel ontogenetic events (Gatti and Baker, 1989). It is now evident that the cell cycle in postembryonic development of *Drosophila* represents not only the regular transformations of cell structures, but also an orderly sequence of transcription of certain genes, which is studied using the so-called mitotic-wave method in larval eye imaginal discs (Wolff and Ready, 1993). While recording the number of cell layers separating the bean of expression of a given gene in the first mitotic wave from that of label incorporation (S phase of cell cycle) or from the morphogenetic groove (middle of G_1 phase of cell cycle), the phase and length of expression of this gene in the cell cycle is determined (Richardson *et al.*, 1995; Finley *et al.*, 1996). For this purpose, it is essential that the gene in question be expressed in the eye imaginal disc. We discovered that the expression of the insertion alleles of the *CycB* gene is detected by means of reporter β-galactosidase in the nerve ganglia but is absent in imaginal discs (Trunova *et al.*, 1998a). For such cases of tissue-specific expression of a reporter gene, the method has already been developed; it can be used to study any organs and combines visualization of gene transcription in the cells with the help of the enhancer trap reporter gene and radioautographic detection of radioactive label incorporation (Trunova *et al.*, 1998b).

The goal of this work is to compare the results of the study of the transcription of the *CycE* gene using the mitotic-wave method with the data obtained by our method (*CycE*) and determination of the phase of expression of the genes *CycB* and chb^{v40} in the cell cycle.

MATERIALS AND METHODS

Preparations were made for the radioautographic study of the stage of expression of reporter β-galactosidase vector *P*[*lArB*] in the cell cycle as described earlier (Trunova *et al.*, 1998b). Nerve ganglia of heterozygous larvae at the middle of the 3rd instar were isolated and labeled by ^{3}H-thymidine for 15 min; they were then incubated at pH7.8 for 10 min in a dissociating solution containing 3 mM sodium tetraphenyl borate, 50 mM sucrose, 140 mM NaCl, and 5 mM Na_2HPO_4. Thereafter, the ganglia were crushed on a slide using a coverslip, frozen, and postfixed by 0.75% glutaraldehyde in a 0.1 M sodium-cacodylate buffer for 20 min. The preparations were washed from the fixative by phosphate buffer and stained for β-galactosidase overnight. Photoemulsion was then applied onto the dried preparations for detection of ^{3}H-thymidine; the time of exposure was 14 days. A developed D-19 was used for detection. The stained and labeled cells were counted visually.

The experimental curves shown in Fig. 1 were obtained as a result of 5–16 independent measurements at each point in time; 95% confidence intervals of the mean values are shown in the graphs.

The following lines of *D. melanogaster* were used:

(1) Line chb^{v40} (*y w*; *P*[*lArB*]/*TM*6 *Ubx; y*$^+$) containing an insertion of *P[lArB]* (enhancer trap) in the region 78D of chromosome 3. Phenotypically, this mutation is expressed as a defect of centrosome disjunction in meiosis leading to polyploidy (Omel'yanchuk and Volkova, 1996).

(2) Line $CycB^{2g}$ (*y w*; *P*[*lArB*]/*CyO; y*$^+$) containing an insertion of *P[lArB]* in the region 58F–59A; this mutation is characterized by full male and female ste-

rility and disturbed M–A transition in mitosis and meiosis (Trunova *et al.*, 1998a).

(3) Insertion mutation $CycE^{P1396}$ (*P1396 y w*; *P*{*ry=PZ*} *cn*/*CyO*; *ry*) kindly provided by the Bloomington Stock Center; it was rebalanced (*y w*; *P*{*ry=PZ*} *cn*/*CyO*; y^+).

RESULTS

Figure 1 presents the graphs illustrating changes in the phase index φ in cells of the nerve ganglia in the chb^{v40}, $CycB^{2g}$, and $CycE^{P1396}$ larvae from the middle of 3rd instar. The index φ is the ratio of the number of cells positively stained for β-galactosidase and, at the same time, incorporated the radioactive label, to the total number of cells stained for β-galactosidase as a function of the time of incubation after pulse labeling. The experimental curves obtained using this method contain information about the phase of expression of the studied gene carrying insertion *P[lArB]*. The curves plotted for the genes with different phases and lengths of expression in the cell cycle differed not only in the position of the peaks, but also in the shape and half-width of these peaks. A special software was developed to extract the information from the experimental data as completely as possible.

The cells that incorporated the label are designated in the computer model as segment *S*, which corresponds to the period of DNA synthesis (Fig. 2). After the label incorporation, segment *S* moves along the circumference and, starting from a certain moment, crosses segment *X* (whose position and length vary). Segment *X* corresponds to the cells that express reporter β-galactosidase and the length of circumference to the period *T*-cell cycle. At the moment of the segments' intersection, the visually recorded parameter φ is calculated as the ratio of the length of *S* and *X* intersection to the length of segment *X*. Theoretical values of the parameters of the expression phase are selected in such a way as to minimize the mean square deviation of the theoretical curve from the experimental one.

The thus-obtained theoretical curve approximating the data on the expression of the gene $CycE^{P1396}$ is given in Fig. 1a. It can be seen that the height of the peaks and the steepness of the descents and ascents of the theoretical curve correspond fairly well to the experimental points in the time period after 6 h.

Figure 3 shows the data on the beginning of the phase of expression of gene *CycE* and length of the phase in cell cycle as calculated in accordance with the optimized theoretical curve.

It was already shown using the mitotic-wave method that the expression of this gene began in the eye imaginal disc cells at G_1 phase (Finley *et al.*, 1996), which corresponds fairly well with the peak of expression we observed in the nerve ganglion cells in atime interval of 7 to 12 h (Fig. 1a). At the same time, according to the data for the eye imaginal disc, the expression of *CycE*

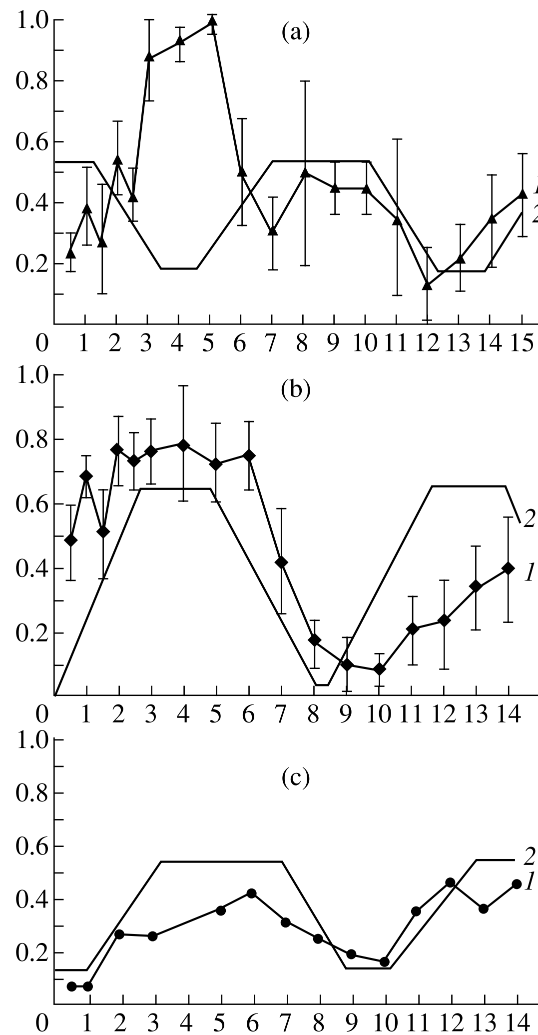

Fig. 1. Curves of changes in the phase index φ of the nerve ganglion cells in 3rd instar larvae of $CycE^{P1396}$ (a), $CycB^{2g}$ (b), and chb^{v40} (c) *D. melanogaster* lines. Abscissa: time of cell incubation after ^{3}H-thymidine pulse incorporation, h; ordinate: index φ. (1 and 2) experimental and theoretical curves, respectively.

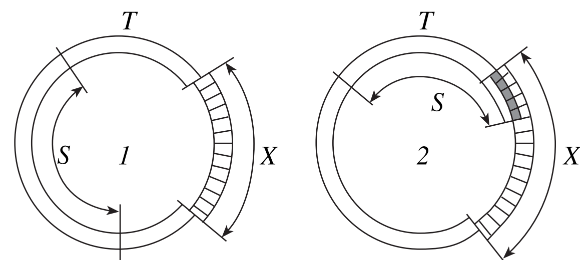

Fig. 2. Schematic diagram illustrating the method of determination of the length and phase of gene expression, on which the computer analysis is based. (*1*) termination of cell labeling by ^{3}H-thymidine, (*2*) moment of counting of a registered parameter. *T*, length of cell cycle; *S*, synthetic phase; *X*, period of expression of the studied gene.

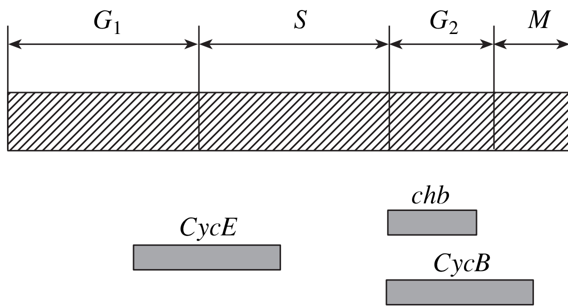

Fig. 3. Theoretical approximation of the data for genes *CycE, CycB*, and *chb*. The scheme reflects the length of cell cycle stages (▨) and the phase of the expression of reporter β-galactosidase (▬) in the cell cycle, except for the 4-hour period related to β-galactosidase degradation.

is terminated in the first third of *S* phase, while our data suggest that it lasts much longer. The source controversy is quite clear: when using the mitotic-wave method, expression is visualized by *in situ* hybridization of the labeled probe onto the gene intracellular mRNA, while our method takes into account the reporter β-galactosidase synthesized as a result of transcription of the reporter gene, which can exist in the cells far longer than the corresponding mRNA. Taking into account that the expression of *CycE* is terminated in the first third of *S* phase (Finley *et al*., 1996), the timing of the β-galactosidase degradation can be calculated (4 h); this allows the correction of the results obtained by our method.

A significant scattering of the experimental data (Figs. 1a, 1b) was observed within the first two hours after the label incorporation. According to the available data, this scattering is reproducible and deserves special consideration. It may well be that the formation of intracellular ^{3}H-thymidine pool serves as a source of scattering, which leads to an increased number of labeled nuclei not related to the cell cycle course. However, the estimates of the life span of the pool of the labeled precursor in the cytoplasm markedly differ: according to some authors, this effect is present in the mouse fibroblasts within 3–4 h after pulse labeling (Cleaver and Holford, 1965), while others reported that the main amount of ^{3}H-thymidine was incorporated in the DNA within 20–30 min after incubation termination (Epifanova *et al*., 1977).

Figure 1b shows an experimental curve for the parameter in question in heterozygotes for an insertion mutation of gene $CycB^{2g}$. As in the preceding case, the optimal theoretical curve differs from the experimental curve within the first 3 h after label incorporation. Expression of gene *CycB* starts at G_2 phase (Fig. 3). While subtracting the time of β-galactosidase degradation from the expression peak width, one can evaluate the length of *CycB* expression in the cell cycle as 1.5 h, i.e., the *CycB* transcription should be terminated at metaphase. It is known that the functioning of *CycB* is related to the metaphase–anaphase transition. For example, removal of the destruction block from the coding area of gene *CycB* blocked the exit of cells from mitosis (Gallant and Nigg, 1992). The presence of kinetochores that were not attached to the spindle or not related to the poles (Gorbsky, 1995) or disturbed polymerization of microtubules (Hunt *et al*., 1992) inhibited the degradation of *CycB*. In addition, studies of intracellular *CycB* localization showed that this protein was associated with centrosomes at the pro- and metaphase, and this association disappeared upon transition to anaphase (Debec and Montmory, 1992). Thus, the available data about the function of the product of this gene correspond fairly well to our results concerning its transcription at G_2 phase, i.e., just before the spindle formation in the cell. We have no data on the determination of the phase of *CycB* expression by the mitotic-wave method.

Use of the software that interprets the experimental curves facilitates visualization of complicating “parasitic” effects. Therefore, the earlier data on expression of the centrosome gene chb^{v40} (Trunova *et al*., 1998b) characterized, unlike cyclins, by a low level of expression can be made more precise. The phases of its expression (Fedorova *et al*., 1997) corresponding to the theoretical curve (Figs. 1c and 3) agree with our previous results concerning its expression in the last third of *S* phase of the cell cycle. This means that the centrosomes in the somatic cells are duplicated later, i.e., at G_2 phase. Indeed, it is known that, in yeasts, the duplication of the centers of microtubule organization, analogs of centrosomes, is timed to G_2 phase. Termination of the centrosome duplication in the cells of vertebrates also occurs during this phase (Murray and Hunt, 1993).

DISCUSSION

Comparison of the results on determination of the phase of *CycE* gene expression in the eye imaginal disc by the mitotic-wave method and by our method based on pulse labeling and visualization of expression of the reporter gene β-galactosidase allowed estimation of the duration of β-galactosidase degradation in *Drosophila* cells (4 h) and the duration during which the amount of label incorporated in chromatin increases at the expense of the cytoplasmic pool of DNA precursors (2 h). Taking into account these specific features, it was shown that transcription of the components of the cell oscillator *CycB* is timed to G_2 phase and G_2–M transition, which precedes directly the period of the cell cycle, where the action of the protein product of the gene in question is focused. This result agrees quite well with the known data on the expression of gene *CycB* in the cell cycle of mammals (Lees and Harlow, 1995).

Comparison of the results concerning the expression of genes *CycE, CycB*, and our earlier data for the centrosome gene *chb* (Trunova *et al*., 1998b) convincingly

shows that the method we chose is capable of detecting the phases of gene expression in the cell cycle.

The problem of the number of peaks of gene expression during one cell cycle deserves special consideration. When there is more than one phase of expression, the mitotic-wave method cannot reveal this type of gene expression. For the control experiment, the *CycE* gene was chosen based on the published data on transcription of this gene and functioning of its protein product. However, the experimental curve for *CycE* significantly differed from the theoretical curve (Fig. 1a). The second peak of expression in the interval from 2 to 6 h (Fig. 1a) was timed to the end of phase G_2, phase M, and the beginning of phase G_1. Unfortunately, we are not yet able to determine more precisely the time, since M-phase in *Drosophila* neuroblasts lasts 0.5 h (Trunova *et al*., 1998b), which is comparable to the method error.

Transcription of *CycE* at G_2 phase in the nerve ganglion cells seems quite likely. During the postblastodermal mitoses, *CycE* is constantly transcribed at S, G_2, and M phases during 14–16 cycles of embryogenesis in epidermal cells, whose cell cycle comprised only G_2, S, and M phases (Richardson *et al*., 1993; Jones *et al*., 2000). Thus, the period of transcription of the *Drosophila* gene *CycE* is stage- and tissue-specific.

Since in the cases of *CycE, CycB*, and *chb*, insertions of enhancer traps were applied for studying the expression, this means that a weak promoter of P-element used as an enhancer-sensitive element has no specificity with respect to the phase of expression. At the same time, these data suggest that the specificity of gene expression with respect to the cell cycle phase is provided by enhancer areas of these genes, since the transposons we used are activated by enhancer zones of the genes, under whose control they are incorporated into genomic DNA.

As a result of the findings of the Berkley and European *Drosophila* Genome Projects (BDGP and EDGP) in 2000, the genome of *D. melanogaster* was markedly saturated with insertion mutations and enhancer traps, sites of insertion were localized (project EDGP-sequence tagged sites, EDGP-STS), full genomic sequence was obtained, and collections of cDNA from different tissues were obtained. The functions of many genes identified within the framework of EDGP-STS screening remain unclear and identification of the phase of expression may become an important characteristic for description of the gene activity. For the method that we developed to be applied, it is necessary that, in the cells of imaginal discs or nerve ganglia of the 3rd instar larvae, such a product of a given gene be transcribed under the enhancer of which the P-element carrying the β-galactosidase gene would be incorporated. On the other hand, it is possible to apply the mitotic-wave method only to the genes transcribed in the eye imaginal disc at a certain developmental stage. Therefore, our method has certain advantages for the solution of tasks directed at the search for enhancer areas specific with respect to the phase of their activity in the cell cycle.

ACKNOWLEDGMENTS

The authors would like to thank Prof. D.P. Furman for his valuable comments.

This study was supported by the Russian Foundation for Basic Research, project no. 99-04-49730.

REFERENCES

Cleaver, J.E. and Holford, R.M., Investigations into the Incorporation of [^{3}H] Thymidine into DNA in L-Strain Cells and the Formation of a Pool of Phosphorylated Derivatives during Pulse Labeling, *Biochim. Biophys. Acta*, 1965, vol. 103, no. 4, pp. 654–671.

Debec, A. and Montmory, C., Cyclin *B* Is Associated with Centrosomes in *Drosophila* Mitotic Cells, *Biol. Cell*., 1992, vol. 75, pp.121–126.

Epifanova, O.I., Terskhikh, V.V., and Zakharov, A.F., Formation of an Intracellular Pool of Labeled DNA Precursors, *Radioavtografiya* (Radioautography), Moscow: Vyssh. Shkola, 1977, pp. 60–62.

Fedorova, S.A., Chubykin, V.L., Gusachenko, A.M., and Omel'yanchuk, L.V., Mutation *chromosome bows (chb^{v40})* inducing a Division Spindle Defect, *Genetika*, 1997, vol. 33, no. 11, pp. 1502–1509.

Finley, B.L., Thomas, B.J., Zipursky, S.L., and Brent, R., Isolation of *Drosophila* Cyclin *D*, a Protein Expressed in the Morphogenetic Furrow before Entry into *S* Phase, *Proc. Natl. Acad. Sci. USA*, 1996, vol. 93, pp. 3011–3015.

Gallant, D. and Nigg, E.A., Cyclin *B2* Undergoes Cell Cycle Dependent Nuclear Translocation and, when Expressed as a Non-Destructible Mutant, Causes Mitotic Arrest in HeLa Cells, *J. Cell. Biol*., 1992, vol. 117, pp. 213–224.

Gatti, M. and Baker, B.S., Genes Controlling Essential Cell-Cycle Function in *Drosophila melanogaster*, *Genes Devel*., 1989, vol. 3, no. 4, pp. 433–453.

Gorbsky, G.J., Kinetochores, Microtubules and the Metaphase Checkpoint, *Trends Cell Biol*., 1995, vol. 5, pp. 143–148.

Hunt, T., Luca, F.C., and Ruderman, J.V., The Requirements for Protein Synthesis and Degradation, and the Control of Destruction of Cyclin *A* and Cyclin *B* in the Meiotic and Mitotic Cell-Cycles of the Calm Embryo, *J. Cell Sci*., 1992, vol. 116, pp. 707–724.

Jones, L., Richardson, H., and Saint, R., Tissue-Specific Regulation of Cyclin E Transcription during *Drosophila melanogaster* Embryogenesis, *Development*, 2000, vol. 127, pp. 4619–4630.

Lees, E.M. and Harlow, E.D., Cancer and Cell Cycle, *Cell Cycle Control*, Hutchison, C. and Glover, D.. Eds., New York: Oxford Univ., 1995, pp. 228– 263.

Murray, A. and Hunt, T., *The Cell Cycle*, New York: Oxford Univ., 1993, pp. 121–122.

Omel'yanchuk, L.V. and Volkova, E.I., Pattern of Expression of Cell Division Genes in Tissues of *Drosophila melanogaster*, *Dokl. Ross. Akad. Nauk*, 1996, vol. 347, no. 2, pp. 284–286.

Richardson, H. E., O'Keefe, L.V., Reed, S.I., *et al.*, A *Drosophila* G1-Specific Cyclin *E* Homolog Exhibits Different Modes of Expression during Embryogenesis, *Development*, 1993, vol. 119, pp. 673–690.

Richardson, H., O'Keefe, L.V., Marty, T., and Saint, B., Ectopic Cyclin *E* Expression Induces Premature Entry into *S* Phase and Disrupts Pattern Formation in the *Drosophila* Eye Imaginal Disc, *Development*, 1995, vol. 121, pp. 3371–3379.

Trunova, S.A., Gur'ev, V.P., Lebedeva, L.I., *et al.*, Tissue Specificity of Cyclin *B* Expression, *Dokl. Ross. Akad. Nauk*, 1998a, vol. 361, no. 6, pp. 839–842.

Trunova, S.A., Dubatolova, T.D., and Omel'yanchuk, L.V., Determination of the Phase of Expression of Gene chb^{v40} in Cell Cycle of *Drosophila melanogaster*, *Ontogenez*, 1998b, vol. 29, no. 5, pp. 342–346.

Wolff, T. and Ready, D.F., Pattern for Mutation in the *Drosophila* Retina, *The Development of Drosophila melanogaster*, New York: Cold Spring Harb. Lab., 1993, pp. 1277–1325.