Requirement of *Rbp9* in the maintenance of *Drosophila* germline sexual identity

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Received 5 September 1999; received in revised form 12 December 1999

Edited by Shmuel Shaltiel

Abstract ***Drosophila*** **germline sex determination is controlled by a group of genes expressed at early stages of oogenesis (*ovo*, *otu*, *bam*, and *Sxl*, etc.). Mutations in these genes cause not only sex transformation of female germ cells, but also ovarian tumors. Although mutations at the *Rbp9* locus also cause an ovarian tumor phenotype, *Rbp9* has been shown to function during later developmental stages than do other ovarian tumor-causing genes. To test whether *Rbp9* is also required for germline sex determination, we examined the sex transformation process of female germ cells in *Rbp9* mutant flies. The detection of *Sxl* male transcripts and other male germline markers in *Rbp9* mutant ovaries revealed that the *Rbp9* mutation caused a partial germline sex transformation. Therefore, sex determination signals that persist throughout oogenesis appear to be required for proper maintenance of germline sexual identity.**

Key words: *Rbp9*; Ovarian tumor; Germline sex transformation; Maintenance of sexual identity; *Drosophila*

1. Introduction

While sex determination of the *Drosophila* soma has been studied extensively, sex determination of the germline is poorly understood. Genes that have been shown to be involved in germline sex determination include *ovo*, *ovarian tumor* (*otu*), *sans-fille* (*snf*), *bag-of-marbles* (*bam*), and *Sex-lethal* (*Sxl*) [1–6]. Mutations in these genes yield similar phenotypes, such as an ovarian tumor phenotype. *ovo*, *otu*, and *snf* act upstream of *Sxl* in the germline sex determination process by causing *Sxl* to produce female-specific transcripts [7]. As the SXL protein is synthesized only from female-specific *Sxl* transcripts [8], no SXL protein is detected when mutations in *ovo*, *otu*, or *snf* occur [9].

In wild type flies, SXL protein can be detected in the cytoplasm of oogonial cells of the third instar larval gonads [9]. This cytoplasmic expression persists until the cystocytes undergo their first mitotic division. As the cystocytes undergo the second mitotic division, SXL protein loses its cytoplasmic localization pattern. In *Drosophila* strains carrying mutations in *Sxl* that cause an ovarian tumor phenotype, SXL protein remains in the cytoplasm during cystocyte differentiation [9]. Therefore, cystocyte differentiation in these mutants is arrested prior to the stage when the level of cytoplasmic SXL protein decreases (i.e. prior to transition from the 2-cell to the 4-cell cyst).

bam is also required quite early in cystocyte differentiation [10]. BAM protein begins to be expressed as soon as the cystoblast is produced from the stem cell. If BAM protein is not expressed in the cystoblast, daughter cells from the germ cell division are maintained as stem cells, rather than as cystoblasts. Because mutations in both *Sxl* and *bam* arrest germ cells prior to completion of the first or second mitotic divisions of cystocytes and sexual transformation is already detected in these mutants [4], SXL and BAM proteins appear to be required prior to the 2- or 4-cell stage for proper germline sex determination.

We showed previously that mutations in *Rbp9* cause an ovarian tumor phenotype [11]. Although expression of the other ovarian tumor genes is required prior to the 2–4-cell stages of germ cell development, *Rbp9* only begins to be expressed at the end of the fourth mitotic division. Because the ovarian tumor phenotype is shared in mutated versions of genes involved in germline sex determination, we sought to determine whether *Rbp9* is required to maintain the sexual identity of germ cells during cystocyte differentiation. To this end, we examined whether sexual transformation of germ cells occurs in *Rbp9* mutant flies and found that male-specific *Sxl* transcripts and other male-specific markers are expressed in *Rbp9* mutant ovaries. These results suggest that even though female germ cells acquire sexual identity at the beginning of oogenesis, germ cells at the 16-cell stage still require certain signals to maintain their sexual identity. *Rbp9* appears to provide at least a part of the signal required for the maintenance of female identity in female germ cells.

2. Materials and methods

2.1. Fly strains

Rbp9 alleles used in this experiment are described in Kim-Ha et al. [11]. *LacZ* reporter-expressing fly strains are described in Gönczy et al. [12].

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Sex-specific, poly(A)$^+$ RNA was prepared from wild type and *Rbp9* mutant flies and reverse transcribed with MMLV reverse transcriptase (Promega, Madison, WI) and oligo(dT) to generate complementary (c) DNAs [13]. In order to remove the primer and unincorporated nucleotides, the reaction was subjected to centrifugation for 30 min at 5000×*g* through a Centricon-10 filter. The cDNAs were then amplified by PCR (cycle: 94°C, 55°C, 72°C; 1 min each for 25 cycles) in the presence of trace amounts of [^{32}P]dATP using the *Sxl*-specific primers described in Oliver et al. [2]. The amplified products were separated on 2% agarose gels and visualized with a phosphorImager (Molecular Dynamics, Sunnyvale, CA).

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PII: S0014-5793(99)01745-7

2.3. Generation of flies carrying a male-specific lacZ enhancer trap with an Rbp9 allele

To generate *Rbp9* mutant alleles in *lacZ* enhancer trap-containing *Drosophila* lines, we performed the genetic crosses diagrammed in Fig. 1. Homozygous $Rbp9^{\Delta 1}$ males were crossed with both of the homozygous enhancer trap lines *lacZ* 542 and *lacZ* 590 [12], which are described collectively as P[*w*+; *lacZ*] (P). P[*w*+; *lacZ*]/$Rbp9^{\Delta 1}$ transheterozygous females were selected from this mating and crossed with heterozygous $Rbp9^{\Delta 1}$ male flies (F1). As enhancer trap lines were marked with the *w*+ gene, red-eyed progenies with *CyO* balancer were selected. Some fraction of these flies would be the recombinants that carry both the $Rbp9^{\Delta 1}$ and the enhancer trap. Therefore, these flies were crossed with heterozygous $Rbp9^{\Delta 1}$ females (F2), and red-eyed flies without the *CyO* balancer were selected. Flies carrying recombinant chromosomes showed female sterility, and stocks were generated from the vial that carried the recombinant chromosomes. Flies from these stocks were used for further analysis.

2.4. Whole mount in situ hybridization

Ovaries from 3–10-day-old females were dissected in 1×phosphate-buffered saline (PBS) and fixed for 20 min with 4% paraformaldehyde. In situ hybridization was performed following the protocol described in Kim-Ha et al. [14]. Digoxigenin-labeled antisense RNA probes were made from the male-specific exon and used for the in situ hybridization assay. As wild type and $Rbp9^{P[2690]}$ ovaries can be distinguished easily by morphology, they were all processed in the same test tube.

2.5. Staining for β-galactosidase expression in ovaries

Ovaries were dissected and fixed as described above. After rinsing once with 1×PBS, ovaries were incubated at 37°C for 12–16 h in a staining solution containing 10 mM sodium phosphate (pH 7.2), 150 mM sodium chloride, 1 mM magnesium chloride, 3.1 mM each of ferro- and ferricyanide, 0.3% Triton X-100, and 0.2% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The ovaries were washed once with 1×PBS, dehydrated in an ethanol series, and mounted in Gary's Magic Mountant as described in Kim-Ha et al. [14].

3. Results

3.1. Male-specific Sxl transcripts are expressed in Rbp9 mutants

Because a tumorous ovarian phenotype is observed when germline sex determination fails to occur [2], we examined in *Rbp9* mutants the sex-specific pattern of *Sxl* pre-mRNA splicing (with respect to the presence of the male-specific *Sxl* exon). Because distinct *Rbp9* alleles show differing degrees of oogenesis defects [11], we used three *Rbp9* alleles, $Rbp9^{P[2567]}$, $Rbp9^{P[2775]}$ and $Rbp9^{P[2690]}$, which display ovarian phenotypes ranging from nearly wild type to tumorous, respectively. Using RT-PCR and a set of primers complementary to the *Sxl* male-specific exon and the neighboring (5′) common exon (that is, an exon found in *Sxl* transcripts from both male and female flies; Fig. 2A), we amplified male-specific *Sxl* transcripts from wild type and *Rbp9* mutant flies. The male-specific *Sxl* transcript was not observed in wild type or $Rbp9^{P[2567]}$ females (Fig. 2B and data not shown). However, $Rbp9^{P[2775]}$ females had a small amount and $Rbp9^{P[2690]}$ homozygote females had a higher amount of male-specific *Sxl* transcript (Fig. 2B). RT-PCRs with *Sxl* primers complementary to the two common exons that flank the male-specific exon resulted in amplification of the corresponding *Sxl* transcripts from both sexes of *Rbp9* mutant and wild type flies. The identities of the RT-PCR amplified products were confirmed by Southern hybridization with a *Sxl*-specific probe (data not shown).

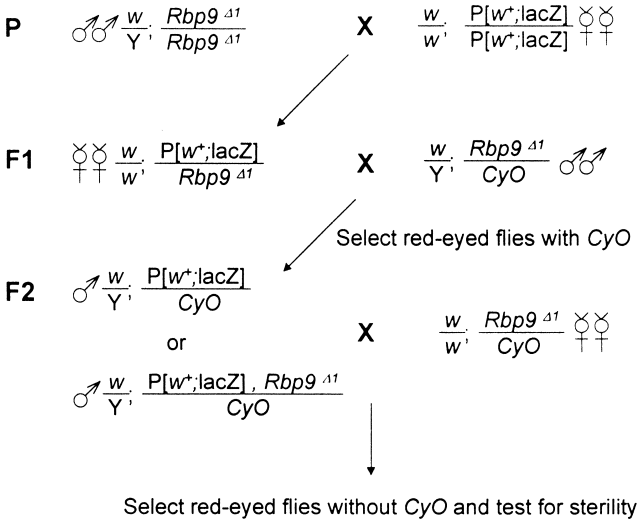

Fig. 1. Genetic scheme to generate *lacZ* reporter-carrying *Rbp9* mutant strains. For a detailed description of strains and crosses, see Section 2. Enhancer trap lines *lacZ* 590 and *lacZ* 542 used in this experiment are abbreviated collectively as P[*w*+; *lacZ*].

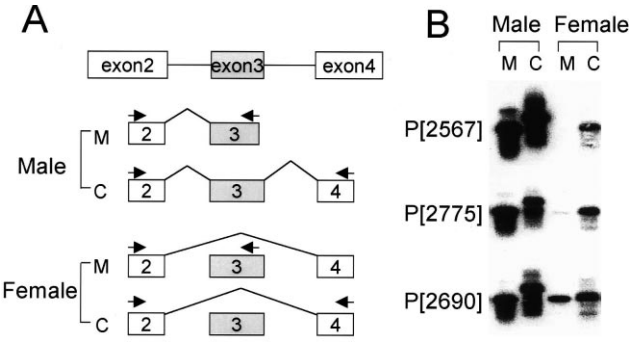

Fig. 2. Allele-specific defect of *Rbp9* mutants in germline sex determination. A: The regions of *Sxl* exons that show different splicing patterns in male and female flies are indicated. The male-specific exon (exon 3) is shaded. The locations of the primers (arrows) used for PCR and the predicted RT-PCT products are indicated. B: Sex-specific RNA prepared from flies carrying the indicated *Rbp9* alleles was analyzed for sex-specific *Sxl* mRNA splicing by RT-PCR with a set of primers that are (i) complementary to the male-specific exon and to the neighboring 5′ common exon (M); or (ii) complementary to the two common exons flanking the male-specific exon (C). The films were over-exposed to show the presence of the male-specific *Sxl* transcript in $Rbp9^{P[2775]}$ females. No male-specific *Sxl* transcript was detected in $Rbp9^{P[2567]}$ females even after a prolonged exposure.

The sexual identities of the cells in *Rbp9* mutant ovaries were assessed further using in situ hybridization with male exon-specific probes labeled with digoxigenin. As the presence of male-specific transcripts was most pronounced in ovaries from $Rbp9^{P[2690]}$ homozygous females in the RT-PCR analysis, we used this line for in situ hybridization. Male-specific transcripts were observed in a mottled pattern throughout the ovaries from $Rbp9^{P[2690]}$ homozygous females (Fig. 3A), whereas no hybridization signal was detected in wild type ovaries (Fig. 3B). Taken together, the results from the RT-PCR and in situ hybridization experiments indicate that as the oogenesis defect becomes more severe, germline cells express more male-specific *Sxl* transcripts.

3.2. Expression of male-specific markers in Rbp9 mutants

Expression of male-specific *Sxl* transcripts in *Rbp9* mutant

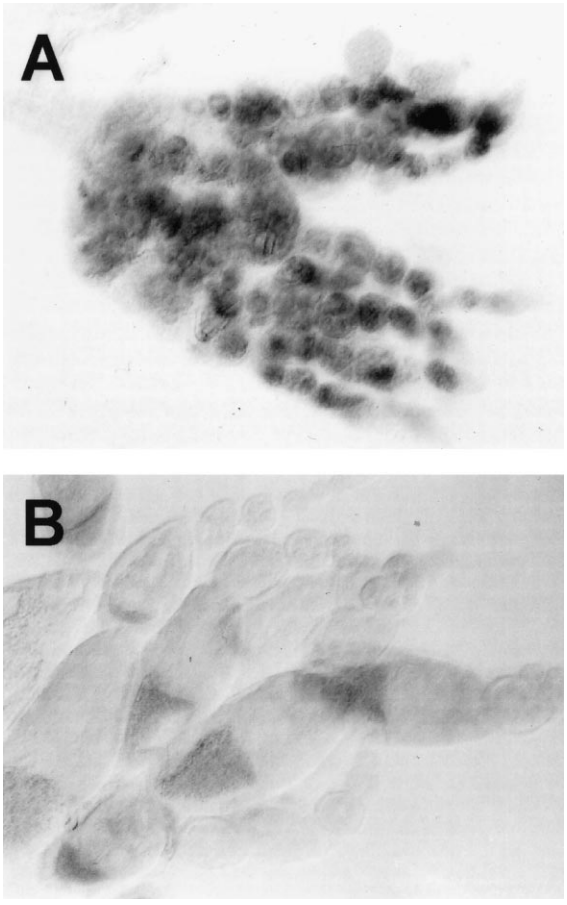

Fig. 3. Expression of male-specific *Sxl* transcripts as assayed by in situ hybridization with a digoxigenin-labeled male exon-specific probe. The hybridization signal appears as darkly staining material. A: Male-specific transcripts are observed in a mottled pattern throughout the $Rbp9^{P[2690]}$ ovaries. B: No hybridization signal is detected in wild type ovaries.

flies suggests that the sexual identity of the female germ cells in the *Rbp9* mutants is perturbed. To investigate further the possibility that germline sex is disrupted in *Rbp9* mutants, we tested whether other male-specific markers are expressed in *Rbp9* mutants. As shown above, females with the most severe *Rbp9* mutant phenotype produced more male-specific *Sxl* transcripts than did less severely affected *Rbp9* mutants; thus we expected that sexual identity in flies carrying a severely affected *Rbp9* mutant allele would be perturbed to a greater extent. Because strain $Rbp9^{\Delta 1}$ displays a tumorous ovarian phenotype (as does $Rbp9^{P[2690]}$) and was shown to be a true protein null allele [11], we used $Rbp9^{\Delta 1}$ in the following experiments.

For male-specific markers, two spermatogenesis-specific enhancer traps, *lacZ* 542 and *lacZ* 590 [12,4] were used to test the expression of these markers in *Rbp9* mutants (Fig. 1 and see Section 2). Homozygous $Rbp9^{\Delta 1}$ females carrying either *lacZ* 542 or *lacZ* 590 were tested for β-galactosidase (β-gal) expression in germ cells. Although β-gal was not expressed in the ovaries of $Rbp9^{\Delta 1}$ heterozygotes carrying either *lacZ* 542 or *lacZ* 590 (Fig. 4A and data not shown), β-gal activity was detected in nearly all egg chambers of the female germ cells of $Rbp9^{\Delta 1}$ homozygotes carrying *lacZ* 590 (Fig. 4B). In addition, we observed β-gal expression in pseudonurse cells from $Rbp9^{\Delta 1}$ homozygotes, as reported for other ovarian tumors [4]. Cells that expressed β-gal activity also were detected in $Rbp9^{\Delta 1}$ homozygotes carrying *lacZ* 542, although the β-gal signals were much weaker and were detected less frequently (Fig. 4C). To confirm that the β-gal activity we observed did not result from a non-specific activity of dying cells, we tested for β-gal expression in $Rbp9^{\Delta 1}$ homozygotes that did not carry the *lacZ* reporter, and did not detect any β-gal expression in the germ cells of these flies (Fig. 4D). Therefore, the expression of spermatogenesis-specific markers in the female germ cells of $Rbp9^{\Delta 1}$ demonstrates that the *Rbp9* is required during the cystocyte differentiation stage for the maintenance of germline sexual identity.

4. Discussion

Although several genes involved in germline sex determination in *Drosophila melanogaster* have been identified, the precise function of each gene product and their interactions are not fully understood. Once germ cells acquire their position in the midgut rudiment, germ cells committed to the female lineage require *ovo* for their survival during the embryo and larva stages [1,15,16]. Temperature shift analysis using a heat-inducible *otu* gene construct revealed that *otu* is required during pupal and adult oogenesis [16]. The activity of *otu* seems to be required in the germline for several stages during oogenesis, as different alleles show abnormal germ cells arrested at different stages of oogenesis [17]. The main function of *otu* regarding germline sex determination is its interaction with somatic signals [18,19] and its regulatory function in *Sxl* expression together with *ovo*. The function of *Sxl* in germ cell development is quite unusual when compared to its role in somatic sex determination. *Sxl* is well known to function as a master splicing regulator in the somatic sex determination pathway (see [20] for a review). However, in germ cell development, *Sxl* is distributed in the cytoplasm during the oogonia proliferation stage and acts downstream of *otu* and *ovo* [9]. With respect to the molecular function of these gene products in germ cell development, many questions remain unanswered. For example, we still do not know what role the cytoplasmic SXL protein plays in germ cell development or how *otu* and *ovo* regulate *Sxl* expression.

As reported previously [11], genetic studies have shown that *Rbp9* has more than one function during oogenesis; it is not only required for cystocyte differentiation, but is also involved in oocyte determination and positioning. In this study, we observed an additional developmental defect in *Rbp9* mutants; that is, germline sex determination is perturbed. This observed defect in the germline sex determination process was limited to *Rbp9* mutants with an abnormal cystocyte proliferation phenotype ($Rbp9^{P[2690]}$ and $Rbp9^{P[2775]}$, which are oncogenic alleles) and was not detected in *Rbp9* mutants with defects only in oocyte determination and positioning ($Rbp9^{P[2567]}$, which is a differentiated allele). These results further support the idea that *Rbp9* controls at least two developmental processes in germ cells. These include (i) a *Sxl*-dependent germline sex determination function, and (ii) a *Sxl*-independent function required for cystocyte differentiation. We hypothesize that the varying levels of activity characteristic of each individual *Rbp9* mutant allele are responsible for the degree and type of defects observed in ovary development. Oncogenic *Rbp9* alleles appear to be defective in both functions, whereas the differentiated *Rbp9* alleles are defective only in the *Sxl*-independent function of *Rbp9*.

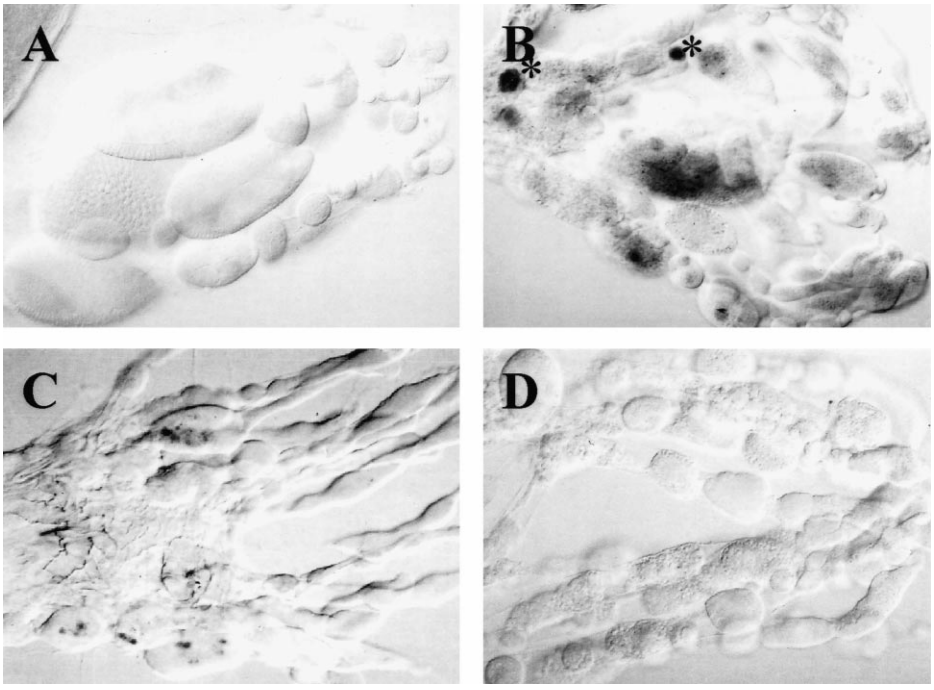

Fig. 4. Expression of male germline-specific markers in *Rbp9* mutant flies. Ovaries were stained for β-gal activity. The anterior end of the ovaries is oriented at the right side of each panel. A: Female germ cells bearing the *lacZ* 590 enhancer trap and $Rbp9^{\Delta 1}$ allele as heterozygotes show no β-gal activity. B and C: β-Gal activity can be detected in ovaries from flies carrying homozygous $Rbp9^{\Delta 1}$ alleles with the *lacZ* 590 (B) or *lacZ* 542 (C) enhancer traps. Most of the egg chambers in B contain cells expressing β-gal activity. Pseudonurse cells expressing β-gal activity are marked with asterisks. D: No β-gal activity is detected in germ cells from flies carrying homozygous $Rbp9^{\Delta 1}$ alleles without the *lacZ* enhancer trap.

Our finding that male germ cell markers are expressed in *Rbp9* mutant female germ cells provides evidence that sexual identity has to be maintained even after completion of the fourth mitotic division of cystocytes to produce 16-cell clusters. With respect to the production of male-specific *Sxl* transcripts in *Rbp9* mutants, we do not know whether *Rbp9* controls *Sxl* splicing directly or indirectly. Because RBP9 protein is localized in the cytoplasm of 16-cell stage cystocytes, it may regulate certain signal(s) required for the proper maintenance of *Sxl* splicing in the female mode. We reported previously that RBP9 protein binds to *bam* transcripts and down regulates BAM protein expression [11]. Although perturbation of sexual identities has also been observed in *bam* ovaries [4,9], cystocyte differentiation is arrested at much earlier stages in *bam* ovaries than in *Rbp9* ovaries [10,11]. The mechanism for the sexual perturbation in *bam* ovaries has not been reported. As the *bam* gene appears to function in multiple steps during oogenesis [11], *bam* may be involved in both the initial stages and later stages of germline sex determination. However, another possibility is that later stages of germline sex determination are not mediated by *bam*, but by other signals that are also regulated by *Rbp9*. Failure to properly maintain female-specific *Sxl* transcription may cause the sexual transformation we observed in *Rbp9* mutant flies. Our findings are significant in that they allow us to divide the germ cell sex determination process into at least two steps, commitment and maintenance. Whether *Rbp9* is involved in this process directly or indirectly will be revealed in further studies.

Acknowledgements: We are grateful to S. DiNardo for providing the *lacZ* reporter strains. This work was supported by grants from SBRI (B-98-023) to J.K.-H. and Korea Science and Engineering Foundation, Republic of Korea (98-0403-04-01-5) to Y.-J.K. and J.K.-H.

References

[1] Oliver, B., Perrimon, N. and Mahowald, A.P. (1987) Genes Dev. 1, 913–923.
[2] Pauli, D., Oliver, B. and Mahowald, A.P. (1993) Development 119, 123–134.
[3] Steinmann-Zwicky, M. (1988) EMBO J. 7, 3889–3898.
[4] Wei, G., Oliver, B., Pauli, D. and Mahowald, A.P. (1994) Dev. Biol. 161, 318–320.
[5] Marsh, J.L. and Wieschaus, E. (1978) Nature 272, 249–251.
[6] Schüpbach, T. (1985) Genetics 109, 529–548.
[7] Oliver, B., Kim, Y.-J. and Baker, B.S. (1993) Development 119, 897–908.
[8] Bell, L.R., Maine, E.M., Schedl, P. and Cline, T.W. (1988) Cell 55, 1037–1046.
[9] Bopp, D., Horabin, J.I., Lersch, R.A., Cline, T.W. and Schedl, P. (1993) Development 118, 797–812.
[10] McKearin, D. and Ohlstein, B. (1995) Development 121, 2937–2947.
[11] Kim-Ha, J., Kim, J. and Kim, Y.-J. (1999) Mol. Cell. Biol. 19, 2505–2514.
[12] Gönczy, P., Viswanathan, S. and DiNardo, S. (1992) Development 114, 89–98.
[13] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[14] Kim-Ha, J., Smith, J.L. and Macdonald, P.M. (1991) Cell 66, 23–35.
[15] Oliver, B., Pauli, D. and Mahowald, A.P. (1990) Genetics 125, 535–550.
[16] Rodesch, C., Geyer, P.K., Patton, J.S., Bae, E. and Nagoshi, R.N. (1995) Genetics 141, 191–202.
[17] King, R.C., Mohler, D., Riley, S.F., Storto, P.D. and Nicolazzo, P.S. (1986) Dev. Genet. 7, 1–20.
[18] Nagoshi, R.N., Patton, S.J., Bae, E. and Geyer, P.K. (1995) Development 121, 579–587.
[19] Hinson, S. and Nagoshi, R.N. (1999) Development 126, 861–871.
[20] Baker, B.S. (1989) Nature 340, 521–524.