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Isolation and Characterization of the Ribosomal Protein 46 Gene in *Drosophila melanogaster*

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A cDNA clone coding for ribosomal protein 46 (rp46) which is a component of 60S ribosomal large subunit has been identified from *Drosophila melanogaster*. A cDNA clone encoding *S. cerevisiae* rp46 was used as a probe to screen a *Drosophila* larvae cDNA library. The DNA sequence analysis revealed that the cDNA coding for *Drosophila* rp46 contains a complete reading frame of 153 nucleotides coding for 51 amino acids. The deduced amino acid sequence showed 71-75% homology with those of other eukaryotic organisms. Northern blot analysis showed that about 1-kb rp46 transcripts are abundant throughout fly development. Whole mount embryonic mRNA *in situ* hybridization also showed no preferential distribution of the transcripts to any specific region. The chromosomal *in situ* hybridization revealed that the identified gene is localized at position 60C on the right arm of the second polytene chromosome with a possibility of single copy.

Eukaryotic ribosomes are complex organelles consisting of 4 molecules of RNA and 70-80 proteins. Ribosome biosynthesis is an excellent example of a process involving the coordinate expression of different sets of genes. The coordinate synthesis of the many protein and RNA molecules must involve a complex series of regulatory mechanisms (reviewed in Wool, 1979; Nomura et al., 1984). The synthesis of most ribosomal proteins changes in concert with the physiological or developmental state of the cells or organisms. Under these conditions of varying demand, it is still unclear how the different genes are coordinately up or down regulated. Evidence suggests that regulation of the ribosomal protein synthesis occurs at a variety of levels of gene expression, including transcription, RNA processing, translation and protein turnover in bacteria as well as in higher organisms (Pierandrei-Amaldi et al., 1982; Al et al., 1985; Kay and Jacobs-Lorena, 1985).

A number of ribosomal protein genes have already been identified from various eukaryotes. They show remarkable conservation of DNA and amino acid sequences among homologous proteins, suggesting that the coordinate control of expression requires common features of evolutionary constraints on the structures and rules of ribosomal proteins.

Researchers have long been interested in the coordinate regulation of ribosome biosynthesis in *Drosophila melanogaster* because of the availability of sufficient quantities of animals at known developmental stages and also because of great opportunities for genetic and molecular approaches. The genes for *Drosophila* ribosomal proteins have also been extensively studied (Kongsuwan et al., 1985; Qian et al., 1987; Wigboldus, 1987; Rafti et al., 1988, 1989; Armes and Fried, 1995). However, a considerable number of *Drosophila* ribosomal protein genes have not yet been isolated.

In the present study, we identified and characterized *Drosophila melanogaster* ribosomal protein 46 (rp46), a component of the 60S ribosomal large subunit, in order to provide more evidence of coordinate regulation of ribosome biosynthesis. The *Drosophila rp46* gene was isolated from screening of the *Drosophila* larvae cDNA library constructed in this study by using the *S. cerevisea rp46* cDNA as a probe. We report here the DNA and amino acid sequences, and developmental and spatial expression patterns of the cloned gene. We also investigated the polytene chromosomal localization of the gene.

Materials and Methods

cDNA library construction and screening

Construction of a cDNA library: A *Drosophila melanogaster* larvae cDNA library was constructed using a library synthesis kit (Clontech). Poly(A^+) RNA was purified from 4-day-old larvae of *Drosophila* Canton-S wild type, and was used for cDNA synthesis. The cDNAs were size-fractionated using a fractionation column, and then inserted into the pGAD10 plasmid

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vector. The recombinant plasmids were introduced into the *E. coli* XL2-Blue (MRF') strain.

cDNA library screening: The constructed cDNA library was screened for the isolation of *Drosophila rp46* cDNA using digoxigenin (DIG)-labeled *S. cerevisae rp46* cDNA fragment as a probe (Leer et al., 1985). The cDNA library screening was performed by the method of hybridization to nitrocellulose filters containing replicas of bacterial colonies (Ford et al., 1989).

DNA sequencing

The nucleotide sequence was determined by the dideoxynucleotide chain termination method of Sanger et al. (1977). Double stranded recombinant plasmid DNAs were purified by a plasmid purification system (Promega), and then used for sequencing reaction using the pGAD forward and reverse primers (forward, 5'-TACCACTACAATGGATG-3'; reverse, 5'-ATTGAGA-TGGTGCACGAT-3').

Northern blot analysis

Total RNA was isolated from animals at different stages using a single step method of acid guanidiniun thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Each 10 µg of RNA sample was electrophoresed under the denaturing condition in 1.2% agarose gels containing 2.2 M formaldehyde. The gels were blotted onto nylon membrane filters, which were then used for the hybridization reaction with the isolated *rp46* cDNA as a probe. The hybridization reaction and signal detection were carried out by a DIG-labeling and detection kit (Boehringer Mannheim) and supplier's recommendations.

Whole mount embryonic in situ hybridization

Embryos were collected from well-fed flies in yeast-coated egg collecting medium (25% apple juice, 2.5% sucrose, 2.25% agar and 0.017% methyl D-hydroxy-benzoate). The collected embryos were dechorionated in 3% sodium hypochlorite and washed thoroughly in distilled water. After washing twice in PBST (PBS containing 0.3% Triton X-100), the embryos were transferred into fresh microtubes containing 350 µl of devitellinizing buffer (6.17% formaldehyde, 16.7 mM KH_2PO_4/K_2HPO_4, pH 6.8, 75 mM KCl, 25 mM NaCl, 3.3 mM $MgCl_2 \cdot 6H_2O$) and 700 µl of n-heptane, and then incubated for 15 min with gentle agitating. After washing twice in PBST for 10 min, the embryos were treated with proteinase K (40 µg/ml) for 5 min. The hybridization reaction was carried out using the DIG-labeling and detection kit mentioned above. The *rp46* cDNA was used as a probe in the hybridization reaction.

In situ hybridization to polytene chromosomes

The salivary glands were dissected from late third instar larvae and fixed in 45% acetic acid for 5 min. After they were dropped onto a gelatin-coated slide glass, a cover glass was used to spread the polytene chromosomes. Immediately after freezing at -70℃ for 10 min, the cover glass was removed with a razor blade. Slides were dehydrated with 75% ethanol for 10 min, dried and stored in a dry place. Before hybridization, the chromosomes were denatured in 0.07 M NaOH solution at room temperature for 3 min. The DIG-labeled *rp46* cDNA fragment was also used in hybridization reactions. When signals were detected, slides were counter-stained with Giemsa.

Results and Discussion

Construction of a Drosophila larvae pGAD10 cDNA library

For the construction of a *Drosophila* larvae cDNA library, poly(A^+) RNA was purified from 4-day-old larvae of Canton-S wild type. The poly(A^+) RNA was then used for the first-stranded cDNA synthesis by two separate priming methods, oligo(dT) priming and random priming. These reactions were pooled prior to second-stranded cDNA synthesis. The cDNA fragments longer than 0.3 kb were collected, and then attached to *Eco*RI linkers. Thereafter, they were inserted into the polycloning site of the pGAD10 plasmid. The *E. coli* XL2-Blue (MRF') strain was used as the host cell of transformation of recombinant plasmids.

When the constructed library was examined for quality, the number of independent colonies was approximately 9×10^5 and insert sizes ranged from 0.3 to 3 kb. The independent colony number of this library is somewhat low compared with general commercially available cDNA libraries, but is still valuable for further studies such as library screening. The cDNA library was amplified once onto the LB-agar plates (135 mm), and then stored as 1 ml aliquots of bacterial culture in 25% glycerol at -70℃.

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GAATTCGCGGCCGCGTCGACCGCCGGTCGGCACGGCTCGTTGAAAAGATTGGAC    54
GAAATGGCTGCACACAAGTCGTTCAGAATAAAGCAGAAGCTGGCTAAGAAGCTG   108
    M  A  A  H  K  S  F  R  I  K  Q  K  L  A  K  K  L     17

AAGCAGAACAGATCCGTTCCCCAATGGGTTCGCCTAGCTACTGGCAACACTATT   162
 K  Q  N  R  S  V  P  Q  L  V  R  L  R  T  G  N  T  I     35

CGTTACAACGCTAAGCGCCGTCACTGGAGGCGTACCAAGTTGAAGCTGTAAGCT   216
 R  Y  N  A  K  R  R  H  W  R  R  T  K  L  K  L  *        51

CGCCAATTTTGTGGAATGCTGAAGATCTTTTCGAGAATATGAAATAATAAATTG   270
CTCTACAATTTGCGGATTAAAAAGAAAAAAAAAAAAAAAAAAAAAGTCGACGCG   324
GCCGCGAATTC                                               355
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Fig. 1. Nucleotide sequence of *Drosophila rp46* cDNA and its deduced amino acid sequence. The deduced amino acid sequence is indicated below the nucleotide sequence as a single latter. The TAA stop codon at the end of the open reading frame is marked with an asterisk. The putative polyadenylation signal and poly(A) tail sequences are underlined. The regions of dotted underlines are linker sequences. The nucleotide sequence reported in this paper has been deposited in the GenBank nucleotide sequence database with the accession number AF012422.

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Dro.rp46  MAAHKSFRIKQKLAKKLKQNRSVPQLVRLRTGNTIRYNAKRRHWRRTKLKL
S.rp46    ---Q--------M--AK----PL--WI----N----------N-----MNI
Klu.L46   ---K---I-------AKN---PL--WF--K-N----------------VC
C.L39     -S-L-KSF--R-----Q----PM--W--MK----MK---------------
Hum.L39   -SS--T----RF----Q----PI--WI-MK---K----S----------G-
Rat L39   -SS--T----RF----Q----PI--WI-MK---K----S----------G-
          :...:...::...::._:::_::._:.:.:...:::.:::::...
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Fig. 2. Alignment of the amino acid sequence of *Drosophila* rp46 with those of other eukaryotic ribosomal proteins. The *Drosophila* rp46 amino acid sequence reported in this study (Dro.rp46) was aligned with those of evolutionary counterparts from other organisms, such as *S. cerevisae* rp46 (S.rp46), *Kluyveromyces* rp46 (Klu.rp46), *C. elegans* L39 (C.L39), human L39 (Hum.L39) and rat L39. A summary of the analysis is given in the lower line with the following categories: (:), perfect match; (.), conservative match; (-), no match.

Analysis of DNA and amino acid sequences

Because the yeast *rp46* gene is known to be a well conserved gene with homologues in other species (Lin et al., 1984; Leer et al., 1985; Bergkamp-Steffens et al., 1992; Wilson et al., 1994; Chan et al., 1995; Otsuka et al., 1996), we used a yeast *rp46* cDNA as a probe for the isolation of a *Drosophila* homologue. From the screening of the pGAD10 plasmid cDNA library which was constructed in this study (approximately 1.2×10^6 colonies), a positive clone containing a cDNA of 0.35 kb long was isolated.

The nucleotide and deduced amino acid sequences of the isolated cDNA are shown in Fig. 1. The cDNA turned out to be a *rp46* cDNA containing an complete reading frame of 153 nucleotides coding for 51 amino acids. The sequence of the 3'-nontranslated region contains the polyadenylation signal (AATAAA) at positions 262 to 267 and a poly(A) tail at position 22 nucleotides downstream from the polyadenylation signal. The molecular weight calculated from the deduced amino acid sequence was approximately 7,000. *Drosophila* rp46 is a protein containing basic amino acids at a very high rate, such as 9 lysine, 9 arginine

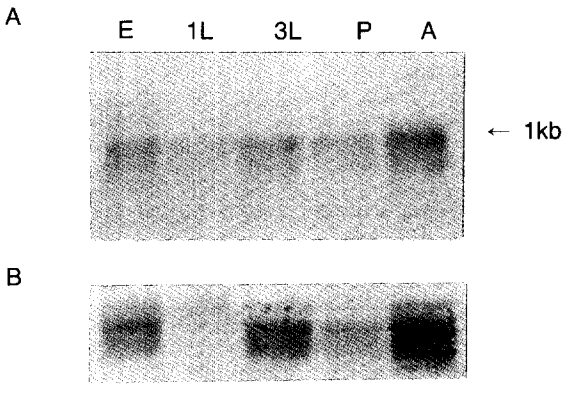

Fig. 3. Northern blot analysis of the *rp46* gene. Total RNAs were isolated from animals at different developmental stages and then separated on denaturing 1.2% agarose gels containing 2.2 M formaldehyde (10 μg/lane). The gels were used for the Northern blot analysis using the DIG-labeled *rp46* cDNA fragment as a probe. A, Expression patterns of the *Drosophila rp46* gene during development (E, embryos; 1L, 1st instar larvae; 3L, 3rd instar larvae; P, pupae; A, adults). B, Control. Expression of the *Drosophila rp49* gene (O'Connell and Rosbash, 1984) was examined as a control.

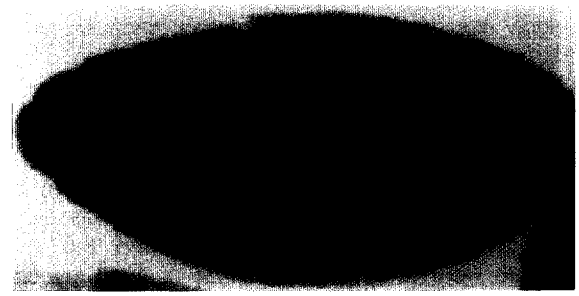

Fig. 4. Whole mount embryonic mRNA *in situ* hybridization. Embryos were collected from well-fed Oregon-R flies, and dechorionated in 3% sodium hypochlorite. The hybridization reaction was carried out using the DIG-labeled *rp46* cDNA as a probe. The figure shows an early-staged embryo orientated with the anterior to the left and the posterior to the right.

and 2 histidine residues, similar to other homologues (Lin et al., 1984; Leer et al., 1985). It contained no acidic amino acids.

Comparison of homology

The deduced amino acid sequence of *Drosophila* rp46 showed a striking homology with ribosomal proteins from other eukaryotic organisms (Fig. 2). *Drosophila* rp46 has an overall identity in 75%, 75%, 73%, 71% and 71% of its amino acid sequences with *S. cerevisae* rp46 (Leer et al., 1985), *Kluyveromyces* rp46 (Bergkamp-Steffens et al., 1992), *C. elegans* L39 (Wilson et al., 1994), human L39 (Otsuka et al., 1996) and rat L39 (Lin et al., 1984; Chan et al., 1995), respectively. At the nucleotide sequence level, the coding region of the *Drosophila rp46* cDNA shares approximately 72% homology with that of the *S. cerevisae rp46* cDNA (Leer et al., 1985). This result implies that *Drosophila* rp46 is obviously the evolutionary counterpart of L39 and rp46 proteins from other eukaryotes.

The remarkable homology at the nucleotide and amino acid levels hints at the function and evolution of

Fig. 5. Polytene chromosomal *in situ* hybridization. Salivary gland polytene chromosome squashes from the Oregon-R strain were hybridized with the DIG-labeled *rp46* cDNA. A single band at 60C on the right arm of the second chromosome is indicated with an arrow.

ribosomal proteins. It is suggested that the *rp46* gene is highly conserved during evolution because of its fundamental role in the structure and function of the ribosome.

Expression of the Drosophila rp46 gene

The relative concentration of the transcripts from the *Drosophila rp46* gene during development was determined by Northern blot analysis. When the *rp46* transcripts were compared with those of the *Drosophila rp49* gene as a control, it showed that about 1 kb *rp46* transcripts were abundant throughout fly development (Fig. 3). This is similar to previous reports that the transcripts of some *Drosophila* ribosomal proteins show no quantitative differences during development (O'Connell and Rosbash, 1984; Rafti et al., 1988; Armes and Fried, 1995). The result of little variation in the *rp46* mRNA level during development suggests that regulation of the gene expression does not occur at the transcriptional level.

Whole mount embryonic mRNA *in situ* hybridization was performed to investigate the spatial expression patterns of the *rp46* gene during the embryonic stage. The staining patterns throughout embryogenesis showed no preferential distribution of the transcript for any specific region (Fig. 4). The broad expression through the whole embryo implies that the *rp46* gene has no tissue specificity during embryogenesis.

Polytene chromosomal localization

To determine the polytene chromosomal localization and copy number of the *rp46* gene, polytene chromosomal *in situ* hybridization was carried out. The salivary glands were dissected from Oregon-R wild type flies.

As shown in Fig. 5, *in situ* hybridization to polytene chromosomes gave a single hybridization band at 60C of the second chromosome right arm. The appearance of a single hybridization band implies that the *Drosophila pr46* gene exists as a single copy in the fly genome. However, the possibility of tandem repeats of the gene cannot be ruled out.

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