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CHROMOSOMAL LOCATION BY FLUORESCENCE *IN SITU* HYBRIDIZATION OF THE 28S RIBOSOMAL RNA GENE OF THE EASTERN OYSTER

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ABSTRACT The physical location of the 28S ribosomal RNA gene (28S rDNA) was localized to the short arm of chromosome number 2 of the eastern oyster (*Crassostrea virginica*) by fluorescence *in situ* hybridization. The existence of a single locus of the 28S rDNA in the genome of the eastern oyster was concluded based on the findings from metaphase chromosomes prepared from gill, mantle, and embryos and from meiotic chromosome bivalents prepared from ripe gonad tissue. The region of the chromosome occupied by the gene was found to be GC-rich, and the location of probe DNA used to identify the 28S gene corresponded exactly with the location of the nucleolus organizer region. The transcriptional activity of the gene did not vary among different cell types but changed with different phases of mitosis. This study is the first report of physical mapping of a specific gene in mollusks and provides techniques for detection of integration of foreign DNA in the oyster genome.

KEY WORDS: fluorescence *in situ* hybridization, 28S rDNA, chromosome, *Crassostrea virginica*

INTRODUCTION

The ribosomal RNA genes are a group of DNA sequences that produce structural rRNA to support protein synthesis. For example, the 28S ribosomal RNA gene (28S rDNA) encodes a major component of the large subunit of the eukaryotic ribosome. The nucleotide sequences of these genes are polymorphic, which has enabled taxonomic investigation of organisms with ambiguous phylogenetic relationships (Littlewood 1994).

In addition, 28S rDNA is useful for the initiation of physical genome mapping, because the gene exists in multiple copies, which improves the ease of detection. There are various methods for verifying the chromosomal location of 28S rDNA. Fluorescence *in situ* hybridization (FISH) (Pendas et al. 1993) or *in situ* polymerase chain reaction (Zhang et al. 1997) can be used for direct assignment of gene location. The 28S rDNA loci can be revealed indirectly by chromomycin A3 staining to target GC-rich regions (Amemiya and Gold 1987). Moreover, the 28S RNA genes are associated with the nucleolus organizer regions (NOR) (Long and Dawid 1980). By use of silver staining, which targets protein components associated with RNA synthesis (Howell and Black 1980), the active 28S rDNA loci can be detected.

The eastern oyster has been harvested commercially in the United States for hundreds of years. An organized system of oyster leases has been maintained in Louisiana for over 100 years, and harvest in Louisiana was valued at more than $50 million in 1997 (Louisiana Summary of Agricultural and Natural Resources 1997). Overall, genetic improvement in this species has been hampered by the absence of basic genetic information. Study of the physical location of DNA sequences in oysters is still preliminary, and reports are restricted to nonspecific DNA elements derived from the mammalian genome (Guo and Allen 1997) or oyster genome (Clabby et al. 1996). The procedures for physical mapping of specific genes must be developed and validated in this species.

The chromosomal location of the 28S rDNA in the eastern oyster has been preliminarily investigated using FISH techniques (Zhang et al. 1999a; Xu et al. 1999). Our goal was to use the 28S rDNA as a first step to verify techniques for physical mapping of genes in oysters. Specific objectives of the present study were to: 1) localize the 28S rDNA on eastern oyster chromosomes by fluorescence *in situ* hybridization; 2) compare these sites of the 28S rDNA to those detected by silver staining and chromomycin A3 staining; and 3) evaluate the transcriptional activity of the 28S rDNA in different cell types.

MATERIALS AND METHODS

Probe Construction

Nuclear DNA was isolated from hemolymph collected from adult oysters ($n = 5$) using a QIAamp blood kit (Qiagen Inc., Chatsworth, CA). Probe DNA was synthesized by polymerase chain reaction (PCR). A pair of primers was designed to target the oyster 28S rDNA gene based on a published sequence (Littlewood 1994) and was synthesized by the Gene Probes and Expression Systems Laboratory, Louisiana State University, Baton Rouge. The primer sequences were (5′ to 3′): GCTAAATACTTCCCG-AGTCCGATAGC and GCACCTTCCTCCAGCTCTTCTGAC. Conditions for PCR were initial denaturation at 94 °C for 2 min; 35 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min, with a final elongation step at 72 °C for 7 min. The PCR products were labeled by inclusion of biotin-16-dUTP in the reaction mixture. Labeling was visualized by agarose electrophoresis, in which the shift of band size was detected because of incorporation of label into the PCR products.

Chromosome Preparation

Ten ripe oysters (five males and five females) were used in this study. Chromosomes were prepared from gonad tissues, embryos at 5 h after fertilization (Paniagua-Chavez et al. 1998) and gill tissues with methods reported elsewhere (Zhang et al. 1999b). For obtaining bivalent chromosomes, the gonad tissue was not treated with colchicine, but a prolonged hypotonic treatment in a 1% sodium citrate solution (~5–7 h) was used to promote separation of

chromosomes. Chromosomes were prepared using standard air-drying methods (Zhang et al. 1999b).

Fluorescence In Situ *Hybridization*

Chromosomes were prepared on two-well, *Teflon*-coated slides (Cel-Line Associates, Inc., Newfield, NJ) and digested with RNase A (100 μg/ml) in 2-x SSC buffer at 37 °C for 1 h. After dehydration with a series of ethanol concentrations (75%, 85%, 95%, and 100%), chromosomal DNA was denatured in 70% formamide at 70 °C for 5 min. The slides were chilled immediately in 70% ethanol at −20 °C, and were dipped in 100% ethanol and dried in a laminar-flow hood.

The hybridization mixture was composed of biotin-labeled probe (1.2 ng/ml), 50% deionized formamide, 2-x SSC buffer, 10-x Denhardt's solution, 0.1% sonicated salmon sperm DNA, and 0.1% sodium dodecyl sulfate (Chen, 1994). The mixture was heated at 95 °C for 10 min and chilled immediately on ice. Twenty μl of the mixture was applied to each well of slides prewarmed to 37 °C, and coverslips were sealed using clear nail polish. The slides were incubated in a wet box at 37 °C for 24 to 36 h. Hybridization was detected with avidin-fluorescein isothiocyanate (FITC) (Zhang et al. 1997). Slides were counterstained with propidium iodide (PI) (0.5 μg/ml) prepared in an antifading medium (100 mg *p*-phenylenediamine in 100 ml glycerol, pH 7.0).

Identification of NOR-Bearing Chromosomes by Silver or CMA Staining

Slides were treated with 50% acetic acid for 20 min to remove background materials on embryonic and gonadal chromosomes. To determine the relationship between the 28S rDNA and NOR, chromosomes were stained with chromomycin A3 (CMA) made in a modified McIlvaine's buffer (Amemiya and Gold 1987). After the images of chromosomes were recorded, slides were rinsed gently with 2-x SSC and left in the buffer for 20 min. The slides were dehydrated through a series of ethanol concentrations (70%, 80%, 95%, and 100%) and stained with a one-step silver staining procedure (Howell and Black 1980). Slides were covered with a solution of 33% silver nitrate and 0.7% gelatin and incubated for 8 to 10 min at 50 °C.

Image Analysis and Map Construction

Fluorescent images of chromosomes stained with CMA or FITC and PI were examined under a fluorescence microscope (Microphot-SA, Nikon Inc., Garden City, NY) equipped with filters for FITC and CMA (excitation wavelengths of 480 nm) and PI (excitation wavelength of 535 nm). Fluorescent images of nuclei and chromosomes were photographed using Kodak Ektachrome (400 ASA) color slide film. The negatives were scanned into a computer with a slide scanner (SprintScan 35, Polaroid scanner model CS-2770, Needham Heights, MA) for analysis. For silver staining, chromosomal images were captured and analyzed by a computer-based image analysis technique (Zhang and Tiersch 1998). Individual chromosomes were identified based on a previously developed karyotype (Zhang et al. 1999b). Relative length (RL) and centromeric index (CI) of each chromosome were calculated using the following formulae:

$$\text{RL}\ (\%) = (\text{length of the chromosome pair/total complement length}) \times 100$$

$$\text{CI}\ (\%) = (\text{length of short arm/total length of the chromosome}) \times 100$$

The location of the 28S rDNA gene was analyzed by microdensitometry, and a map of the chromosome bearing the 28S rDNA was constructed using Microsoft PowerPoint (Office 97 version).

RESULTS

The location and activity of the 28S rDNA in the genome of the eastern oyster was investigated using four cell sources (Table 1). Two chromosomes from embryo cells were found to hybridize with the 28S rDNA probe (Fig. 1a). The RL of one chromosome was 6.14 ± 0.12, and the CI was 42.3 ± 1.8; whereas, the other chromosome had a RL of 5.90 ± 0.10 and a CI of 36.5 ± 2.1 (n = 10 spreads). Two chromosomes from gill cells (Fig. 1b) were found to hybridize with the 28S rDNA (Fig. 1b & 1c). The same region identified by the 28S rDNA probe was found to stain intensely with chromomycin A3, indicating the presence of GC-rich regions (Fig. 2a), and the location of the 28S rDNA was found to be the same as that of the NOR, as indicated by silver staining (Fig. 2b).

One of 10 bivalent chromosomes prepared from gonad tissue was found to hybridize with the 28S rDNA probe (Fig. 3a). Two hybridization signals sometimes appeared on the same bivalent corresponding to the presence of homologous chromosomes that were in the process of separating (Fig. 3b). The chromosome bivalent was consistently stained with CMA at diakinesis (Fig. 3c) and pachytene stages (Fig. 3d) and was stained positively with silver nitrate for the NOR at pachytene stage (Fig. 3e). However, in most cases (30 out of 50 spreads), this NOR site was not detectable by silver staining of diakinesis and pachytene chromosomes.

Upon more detailed examination of the chromosome in question (number 2, from embryo), the location of the 28S rDNA, as measured by microdensitometry, was identified at the telomeric regions corresponding to the site of maximal hybridization intensity of the 28S rDNA probe (Fig. 4).

DISCUSSION

In this study, the 28S rDNA of the eastern oyster was found to be localized to the telomeric region of the short arm of chromosome number 2 by fluorescence *in situ* hybridization. Although the two 28S rDNA-bearing chromosomes from embryo cells were different in size and centromeric index, a single chromosomal location of 28S rDNA was found using meiotic chromosome bivalents. The area of the gene was found to be GC-rich and to

TABLE 1.

Characterization of the 28S ribosomal RNA gene on chromosomes of the eastern oyster by different techniques.[a]

Tissue Type	Phase of Cell Division	AgNOR Staining	CMA Staining	FISH
Embryo	Prophase	Two, NP	Not studied	Not studied
	Prometaphase	Two, NP	Two, NP	Two, NP
	Early metaphase	Two, NP	Not studied	Not studied
	Metaphase	One	Two, P, AS	Two, P
Gill	Metaphase	One	Not studied	Two, P
Mantle	Metaphase	One	Not studied	Two, P
Gonad	Pachytene	One bivalent	One bivalent	One bivalent
	Diakinesis	One bivalent	One bivalent	One bivalent

[a] *Abbreviations:* AgNOR, nucleolus organizer regions stained by silver nitrate; CMA, chromomycin A3; FISH, fluorescence *in situ* hybridization; NP, not pairable; P, pairable, and AS, asymmetric staining or different staining intensity between the two chromosomes.

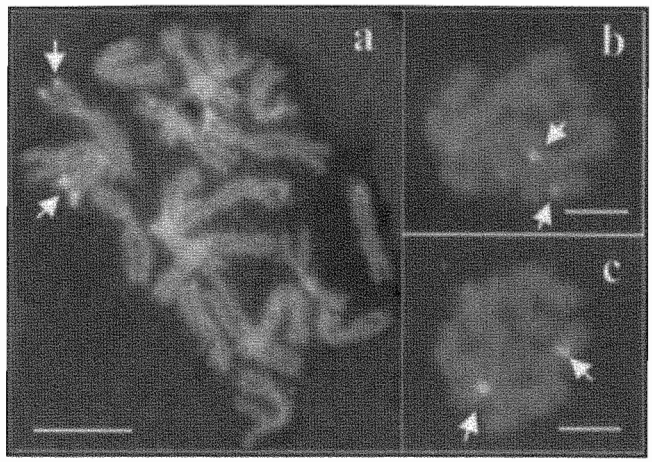

Figure 1. Localization of the 28S ribosomal RNA gene (28S rDNA) of the eastern oyster by fluorescence *in situ* hybridization. Metaphase chromosomes were prepared from cells of (a) embryos and (b) and (c) gill. Arrowheads point to the location of 28S rDNA; bars = 10 μm.

match exactly with the location of nucleolus organizer regions (NOR).

These results indicate that there is a single NOR-bearing chromosome pair in this species. In a previous study, two NOR-bearing chromosomes with measurable differences in size and centromeric index were found in preparations of embryo cells of the eastern oyster by use of silver staining (our unpublished data). Also, the number of the NOR sites was found to be variable among different phases of the cell cycle. Asymmetric features have been observed between homologous chromosomes prepared from embryo cells (Zhang et al. 1999b). Therefore, it was worthwhile to investigate chromosomes prepared from other tissue types for clarification of the NOR sites in embryo cells and to develop methods for physical genome mapping in this valuable species. In the present study, analysis of the location of the 28S rDNA on gonadal chromosomes and the transcriptional activity of the 28S rDNA among different tissues indicates that the two NOR-bearing chromosomes observed in embryo cells are homologous and pair during meiosis.

Variation in chromosome morphology among closely related oyster species has been used to investigate evolutionary relationships (Thiriot-Quievreux and Insua 1992). However, our studies demonstrate that variation in morphology of homologous chromosomes, such as in the pair of NOR-bearing chromosomes, can exist in the eastern oyster, especially in chromosomes obtained from cells of embryos. Centromeric position can change when pericentric inversions involve different lengths of the chromosomal segments on each side of the centromere. This mechanism has been proposed to explain most of the chromosomal variation within oysters of the genus *Crassostrea* (Landron De Guevara et al. 1996) and could be used to interpret the difference of centromeric index between the two homologous 28S rDNA-bearing chromosomes found in this study, although it is likely not appropriate.

The small difference we observed in the homologs of chromosomes would be at the limit of detection when using basic procedures (Longwell and Stiles 1996). In catfish chromosomes, we have reported that a computer-assisted image system can routinely identify a size difference of ~0.2% in relative length, which is equivalent to ~0.1 μm under the microscope (Zhang and Tiersch 1998). The size difference we observed between the two 28S rDNA-bearing chromosomes of the eastern oyster was ~0.4% in prophase, prometaphase, and early metaphase of embryo chromosomes, but the difference was reduced in late metaphase and was not distinguishable in highly contracted somatic chromosome spreads. Explanations for the size differences between chromosomes include differential activity of the homologs, which may be useful for studying early development and gene expression in oysters.

The results of the present study provide valuable information for physical genome mapping in mollusks. Probe DNA was synthesized by polymerase chain reaction and required only DNA sequence information from two primer regions, eliminating time and labor-intensive cloning and screening procedures. Primers prepared for physical mapping of oyster genes could be derived in some cases from mammalian species because of evolutionary conservatism, which is especially beneficial in such species as the eastern oyster that lack DNA sequence information.

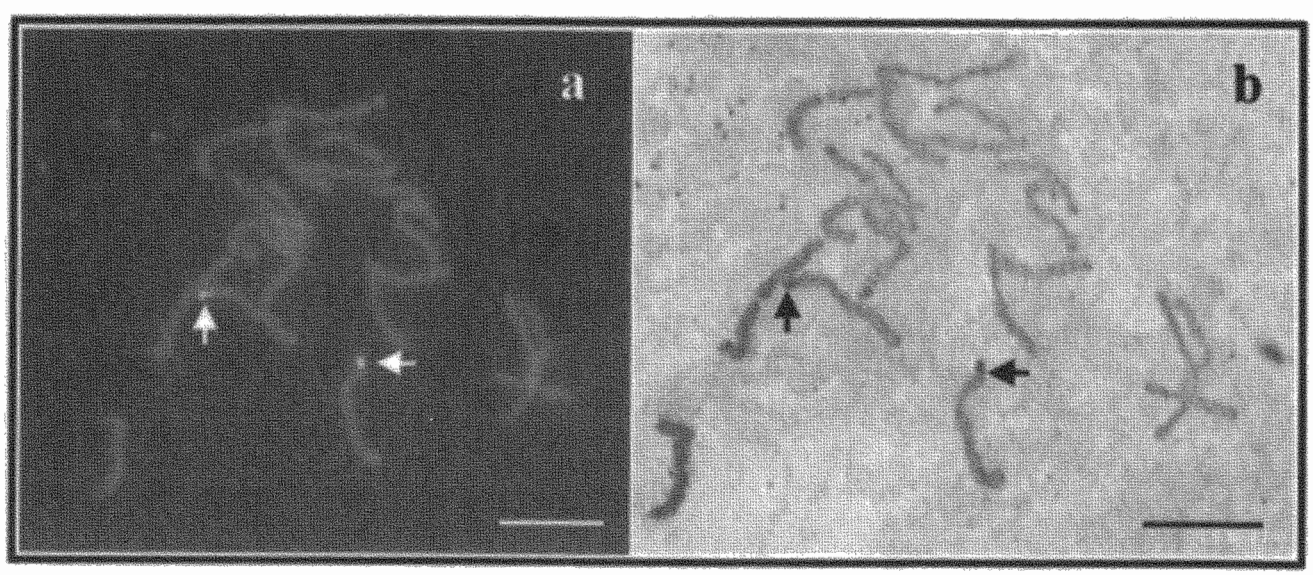

Figure 2. Relationship between the 28S rDNA locus and the nucleolus organizer region of the eastern oyster. The same chromosome spread prepared from embryonic cells was subjected to chromomycin A3 staining (a), followed by staining with silver nitrate (b). Arrowheads indicate location of 28S rDNA and NOR; bars = 10 μm.

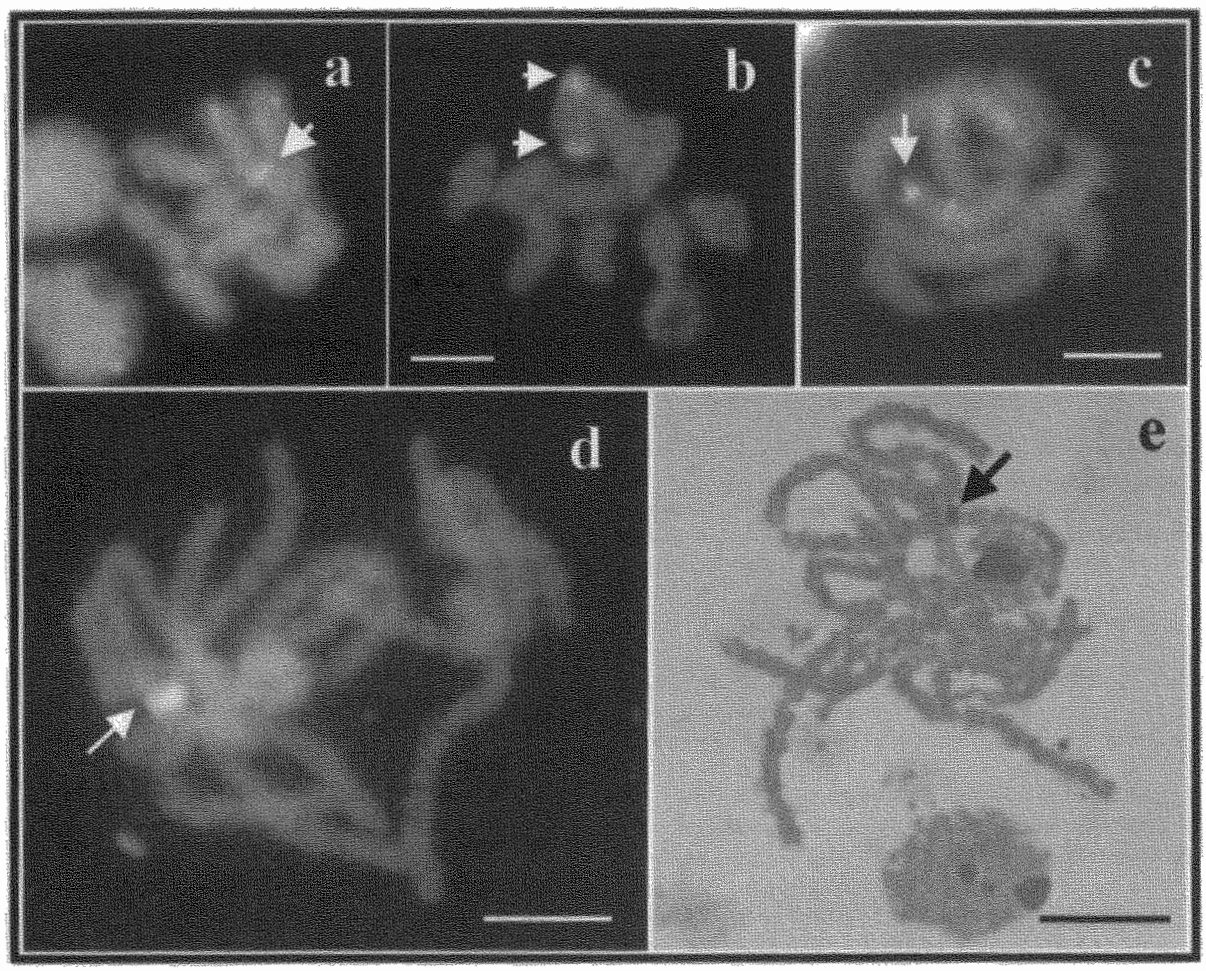

Figure 3. Location and activity of the 28S rDNA on meiotic chromosomes of the eastern oyster. Diakinesis (a, b, and c) and pachytene chromosome bivalents (d and e) were prepared from ripe gonad tissue. Fluorescence *in situ* hybridization (a and b), chromomycin A3 staining (c and d) and silver staining techniques (e) were used to analyze the chromosomal location of this gene (arrowheads); bars = 10 μm.

The 28S rDNA, because of its association with the NOR, can be informative for verifying such specific hybridization techniques as FISH and *in situ* PCR and provides an internal positive control for mapping studies. This is useful for such economically important species as the eastern oyster, which are poorly characterized at the chromosome level. Indeed, given the intrinsic difficulties of cytogenetic analysis in oysters, the association between NOR and 28S rDNA may be the only gene-level marker available at present to test physical mapping techniques, although the associations of such other genetic markers as microsatellite loci may prove beneficial in this regard (e.g., McGoldrick 1997). The techniques developed in this study could be applied for identification of chromosomal integration of genetic material foreign to the genome of the eastern oyster, such as that in transgenic studies (Zhang et al. 1998).

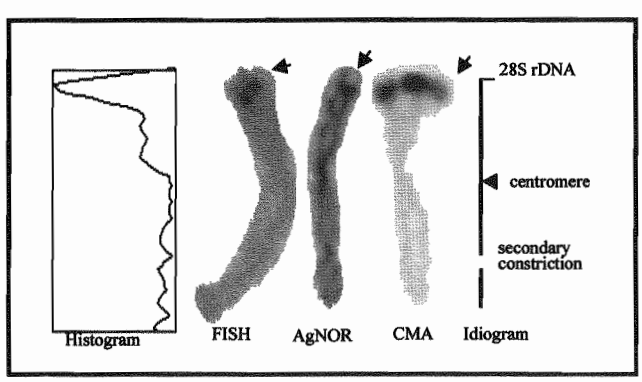

Figure 4. Analysis of the chromosomal location of the 28S rDNA of the eastern oyster by microdensitometry. Arrowheads indicate location of 28S rDNA, revealed by fluorescence *in situ* hybridization (FISH), silver staining (AgNOR) and chromomycin A3 staining (CMA).

Localization of the 28S rDNA represents the first report of physical mapping of a gene in mollusks. More importantly, results of this study provide methodology for examining the validity of hybridization techniques. This is especially important in physical mapping of species without pre-existing genetic information, such as mollusks. Large-scale mapping studies in oysters await improvements of techniques such as probe labeling to increase detection efficiency.

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