08. GENE PROBES AND MAPPING

8.1 Introduction

The advent of In situ hybridization (ISH) techniques in the late 1960's opened a new horizon in the field of Molecular cytogenetics and DNA diagnostics. Application of these technologies can help in the detection of target nucleic acid sequences or genes by using DNA probes. A DNA probe is a specific sequence of a single stranded DNA, which can single out and bind with the specific region of complementary DNA. Fluorescent in situ hybridization (FISH) which is a variation of the ISH technique is used to determine the chromosomal locus of a gene and in physical mapping of particular gene sequences on the chromosomes (Bucioni-Nardelli and Amaldi, 1970; Gall and Pardue 1969a; John et al. 1969; Lodish et al. 1999).

The sensitivity and efficiency of these techniques depend on several variables like probe construction and hybridization conditions, the type and efficiency of probe labelling, the tissue or cell suspension preparation and finally on the method used for signal detection (Chevalier et al. 1997).

Traditional In-situ hybridization involved radioactive probes (Gall and Pardue, 1969; John et al. 1969), which are still widely applied for ISH because of their high sensitivity and the amplificatory effect of autoradiography. But number factors such as health hazards, reduced stability of radioactively labeled probes, and speed of visualization as well as the extensive development of immunogold cytochemistry, which allows a much more precise location of antigen sites in a tissue section compared to the autoradiographic technique, have stimulated interest in the development of non-radioactive probes (Van der Ploeg et al. 1986; Baumann 1985; Forster et al. 1985).

Non-radioactive probes based on the fluorochrome-labeled DNA or RNA (Baumann 1985) was not widely used because of its relatively low sensitivity. Intensive research on immunohistochemical detection of chemically modified nucleic acids, such as acetylaminofluorene (Landegent et al. 1984; Tchen et
al. 1984), dinitrophenyl (Dnp) groups as a hapten (Shroyer and Nakane 1983), and mercurated probes and sulfhydrylhapten ligands (Hopman et al. 1986), were proved very successful in the initial period. Significant improvement was achieved in 1974 with the application of the biotin-avidin system (Heitzmann and Richards, 1974) to detect antigens at the electron microscope level. It was a significant improvement over the immunocytochemical methods. Synthesis of biotin-labeled d-UTP, led to the construction of biotinylated nucleic acids (Brigati et al. 1983; Hutchison et al. 1982; Singer and Ward 1982; Langer et al. 1981; Forster et al. 1985) and opened important new prospects in non-radioactive in situ Hybridization at both the light and the electron microscopic levels. Since living tissue synthesize biotin, which is associated with carboxylases and is involved in number of important biochemical reactions in the kidneys and liver (Kirkeby et al. 1993; Varma et al. 1994). In some cases especially in histology studies, this endogenous biotin can lead to false-positive results. Due to this fact research in the identification of alternative led to the isolation and characterization of digoxigenin, a steroid isolated from digitalis plants (Digitalis purpurea or Digitalis lanata). Since the blossoms and leaves of these plants are the only natural sources of this steroid no binding of the anti-digoxigenin antibody occurs in other biological material, so it was proposed as an alternative to biotin for labeling of hybridizing probe.

8.1.1 The Digoxigenin (DIG)

In the present study Digoxigenin (DIG), DIG labeled nucleotides are used for the labeling reactions. Digoxigenin is a steroid molecule, isolated from digitalis plants (Digitalis purpurea and Digitalis lanata). Since the flowers and the leaves are the only natural sources of this heptane, the anti-DIG antibody does not bind to other biological material. Digoxigenin is linked to the C-5 position of uridine nucleotides via a spacer arm containing eleven carbon atoms. The DIG labeled nucleotides may be incorporated, at a defined density, into nucleic acid probes by DNA polymerases like E. coli DNA Polymerase I, T4 DNA Polymerase, T7 DNA Polymerase, Reverse Transcriptase, and Taq DNA Polymerase) as well as RNA Polymerases (SP6, T3, or T7 RNA Polymerase), and Terminal Transferase. DIG label
may be added by random primed labeling, nick translation, PCR, 3'-end labeling/tailing, or \textit{in vitro} transcription.

\textbf{Structural formula}

\begin{center}
\begin{tikzpicture}
\end{tikzpicture}
\end{center}

\section*{8.1.2. Nucleic Acid labeling}

A lot of work has been published on the DNA labeling techniques like nick translation (Forster et al. 1985; Liehr et al. 1995), and DOP-PCR (Liehr et al. 2002) other sophisticated labeling procedures have also been published (Samiotaki et al. 1997; Adarichev et al. 1998; Tanke et al. 1998; Wiegant et al. 1999; Henegariu et al. 2000; Nimmakayalu et al. 2000)

Fluorescent \textit{in situ} hybridization (FISH) techniques is very useful to assign the position of the gene on the chromosome. In this technique a specific DNA sequence is labeled with a fluorescent molecule and this allows specific nucleic acid sequences to be detected in interphase and metaphase chromosomes. Incorporation of the DIG labeled nucleotides in the DNA sequence is possible by many methods, but three method that is random primed labeling method, Nick translation method and DOP-PCR method. Presently other sophisticated labeling procedures like probes prepared from 4-aminooxybutylamine (Adarichev et al. 1998) multicolour FISH (Samiotaki et al. 1997), use of platinum coproporphyrin and delayed luminescence imaging to extend the number of targets (Tanke et al. 1998), FISH based on a monofunctional reaction of cisplatin derivatives with guanine moieties, custom fluorescent-nucleotide...
synthesis as an alternative method for nucleic acid labeling (Henegariu et al. 2000), simple method for preparation of fluor/hapten-labeled dUTP (Nimmakayalu et al. 2000) have also been used according to the requirement of the investigation.

Random primed labeling (Feinberg and Vogelstein, 1984) is the most widely used method for generating homogeneously labeled DNA probes. It produces sensitive probes that can detect single-copy genes even in complex targets (such as human and plant genomic DNA). In random primed labeling, template DNA is first linearized and denatured. Then, Klenow polymerase incorporates labeled dUTP into the template as multiple locations that are determined by the binding of a random hexamer primer mixture.

Degenerative oligonucleotide priming PCR (DOP PCR), which is often used to label complex DNA probes (Liehr et al. 2002), the DNA fragments may be up to 4-5 kb long. Long DNA fragments will not penetrate the proteic structure surrounding the DNA target and will result only in background hybridization. To shorten the DNA, we usually perform a partial DNAse digestion. The labeling method is normally used and is efficient for the large molecules.

8.1.3. Nick translation

This method of nucleic acid labeling uses the simultaneous activity of two enzymes first one being DNase I, which in the presence of Mg++ ions becomes a single stranded endonuclease and creates random nicks in the two strands of any DNA molecule. The second enzyme E. coli polymerase I, which through it’s 5'-3' exonuclease activity removes nucleotides “in front” of itself, while the 5'-3' polymerase activity adds nucleotides to all the available 3' ends created by the DNase (Fig. 1b, red bars). This exonuclease/polymerase activity, moves (or “translates”) any single stranded nick in the 5'-3' direction. When nicks on opposite strands meet, the DNA molecule breaks.

Number of molecules are presently used for the labeling of the dUTPs very common among them are DIG-dUTP, biotin dUTP or fluorescein, coumarin, CY3, rhodamine and texas red. In the present study, DIG labeled nucleotides is incorporated by using nick translation method of DNA labeling, since
this method is very efficient for the DNA sequence of 200 to 500 bps and the 18S ribosomal gene
which is used for the amplification and labeling is about 459 bp.

Molecular cytogenetic is a relatively new field of studies, where molecular techniques are applied to
resolve the molecular details of the chromosomes. In situ hybridization technique was found to be a
boon in the chromosome research, were the hybridization of a nucleic acid probe to a chromosome
spread allows localization of specific chromosome region. Fluorescent in situ Hybridization (FISH) is
applied to the conventional cytogenetic preparations on the microslides. This technology utilizing
fluorescently labeled DNA probes to physical map gene sequences on the interphase and metaphase
chromosomes. The technique is having immence importance in physical mapping of genes and to repeti-
tive sequences that occur at several places on a chromosome. FISH can also be used to do chromo-
some painting to make a comparison between two species or varieties by using DNA from entire
chromosomes or even the entire genome of one species or variety as a probe on the other,thus chromo-
sonal abnormalities can be identified and evolutionary relations can be deduced (Liehrand Claussen
2002a; Liehrand Claussen 2002b). In medical science, this technique is frequently used for the detec-
tion of numerous chromosome associated pathological conditions. Apart from these this technique is
widely used in the identification of the microorganisms and in the field of microbial ecology.

The advantage of FISH over other techniques like somatic cell hybridization, contiguous clones and
STS (STS gene mapping) for gene mapping is that it is not dependent on recombination and thus can be
used in chromosome regions where recombination is a rare event, such as the centromere. FISH is
efficient in mapping single copy gene and alphoid sequences to the repetative sequences. DNA frag-
ment as small as 200 bp can be, efficiently be mapped on the chromosomes.

8.1.4. 18S ribosomal RNA gene

In the present study, 18S gene in Perna viridis (Goswami, 2005), was reamplified and characterized
and was used for the construction of the DNA probe for the hybridization reaction. This gene is se-
lected to localize the position of this gene on the chromosome and to map the position of gene by using rDNA FISH and NOR data.

18S ribosome RNA gene is a part of the major ribosomal RNA repeated unit, which comprises of 28S, apart from 18S ribosomal RNA gene, and are present in large copy numbers. Available literature data report on the localization of major (18S-28S rDNA) and minor (5S rDNA) ribosomal clusters on different chromosome pairs as the most frequent configuration in vertebrates (Lucchini et al. 1993; Suzuki et al. 1996; Liu and Fredga, 1999). Among invertebrates, the 18S-28S and 5S rDNAs were found in the same chromosome in the nematode Meloidogyne arenaria (Vahidi et al. 1991) and in many species of calanoid copepods so far analyzed.

The genes encoding 18s and 28s ribosomal RNA (rDNA) are present in numerous copies in eukaryotes and are found clustered at chromosomal sites, which are known as nucleolar organizer regions (NORs). Special interest has been paid to the NORs, because they can provide valuable information about basic chromosomal organization and gene function. Moreover, NOR site variations could be used as taxonomic and systematic characters to infer phylogenetic hypotheses of species relationships (Amemiya and Gold, 1990). The silver nitrate staining only stains those NORs which were transcriptionally active in the last cell cycle, because the silver binds to a complex of acidic protein associated with the nucleolus and nascent pre-RNA. The rDNA, corresponding to NORs, can be located on chromosomes by using in fluorescent situ hybridization (ISH) with an rDNA probe labeled with the fluorescence molecule. Molecular cytogenetic techniques have been applied to number of invertebrates (Cross et al. 2005)

Homologous or heterologous ribosomal sequences (rDNA FISH) proved to be essential for conclusively mapping major (18S–28S) ribosomal clusters, otherwise unidentifiable after silver and/or chromomycin A₃ staining, in the karyotype of several vertebrate and invertebrate species (Sánchez et al. 1995, Pendás et al. 1993, Lorite et al. 1997, Colomba et al. 2000 and Libertini et al. 2000).

Molecular cytogenetic studies have been carried out in number of vertebrate and invertebrate species belonging to various phyla (Appels, 1980; Lorite et al. 1997; Libertini et al. 2000; Vitturi et al.)

Molecular cytogenetic studies in the phylum mollusca is limited to very few species (Lopez-Pinon et al. 2005; Vitturi, et al. 2005; Insua et al. 2001; 2006). Fluorescent in situ hybridization has been carried out in some species like Nucella lapillus, Fasciolaria lignaria, Oxynoe olivacea, Melarhaphe neritoides in the class gastropoda (Pascoe et al. 1996; Vitturi et al. 2000a; 2000b; Colomba et al. 2002), Pectin maximus, Mimachlamys varia, Mytilus edulis, Mytilus galloprovincialis (Insua and Mendez, 1998; Insua et al. 2001; 2006). In these studies repetitive probes like 18S-28S rDNA (Pascoe et al. 1996; Vitturi et al. 2000a; Insua et al. 2006), 5S rDNA (Insua et al. 2001; 2006), (TTAGGG), and (GATA), (Vitturi et al. 2000a; 2000b;) are mainly used for fluorescent in situ hybridization.

In the present study the 18S ribosomal RNA gene is reamplified (Goswami, 2005), probe is developed using Digoxigenin – dUTP (Roche) and the distribution of the major ribosomal gene units is localized by in situ hybridization of 18S gene.

Materials and methods are described in chapter 2 section VI.

8.2. Results

8.2.1. Genomic DNA extraction

Genomic DNA was extracted from the gill and gonadal tissues of the Perna viridis. DNA samples were ran on 0.8 % agarose microgel along with the DNA molecular weight marker, this allowed the visualization of the intense bands under UV gel documentation system and image was captured with the mounted camera system. The qualitative analysis was done using Spectronic Genesis 2 spectrophotometer and the results of the genomic extraction data are tabulated in the table 44. Spectrophotometric analysis gave an indication to the amount and the quality of genomic DNA. The DNA yield in all the samples ranged from 1.22 µg/ml in gill tissue of animal 1 to 4.8 µg/ml gill tissue of animal 2. Spectrophotometric analysis at O.D. 260 / O.D.280, the ratio of the genomic DNA ranged between 1.806 to 1.957 indicating recovery of more than 1100 ng per microliters in all the DNA samples and
was sufficient to carry out PCR successfully. The sample A3 (Gonad of animal 1) was selected due to the good ratio and minimum protein contamination for further experiments involving amplification of gene.

From Left to Right

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Ratio</th>
<th>DNA Conc.(µg / L)</th>
<th>Protein conc.(mg / L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>1.848</td>
<td>3.691</td>
<td>7.02</td>
</tr>
<tr>
<td>A2</td>
<td>1.957</td>
<td>2.003</td>
<td>2.076</td>
</tr>
<tr>
<td>A3</td>
<td>1.867</td>
<td>1.221</td>
<td>1.017</td>
</tr>
<tr>
<td>A4</td>
<td>1.806</td>
<td>4.813</td>
<td>2.412</td>
</tr>
</tbody>
</table>

**Figure 28.** Photograph of Agarose gel showing Genomic DNA bands

Lane 1: 5 KB DNA Marker
Lane 2: A1 Genomic DNA extracted from gonad of animal 1
Lane 3: A2 Genomic DNA extracted from gonad of animal 2
Lane 3: A3 Genomic DNA extracted from gill of animal 1
Lane 4: A4 Genomic DNA extracted from gill of animal 2

**8.2.2. 18S ribosomal gene amplification and sequencing**

Agarose gel (Figure 29) shows thick bands of amplified gene and the molecular weight was determined to be less than 0.5 kb. The amplified product was sequenced. In the present study the amplified product...
of 459 bp was characterized (Goswami, 2005).

**Figure 29.** Photograph of 1% Agarose gel showing amplified gene bands along with the pGEM marker.

From Right to Left
Lane 1 pGEM marker
Lane 2 Replicate 1
Lane 3 Replicate 2

**18S gene sequence**

**ORIGIN**

1 ttgattccttgaaagccccctgtttgcaaaacctcgtactttacctggtgaaaccgcga

61 atggetcattaaatcagttatggttcttaagatcgtacactctacttggaataactgtgg

121 taattctagaatcatacagctccaattacccactccgacggattcgtgatatttcagaggtttcgg

181 tagaacaagaccagggctccggacggcgcaggttgaccgtttcaaatgtgagttctgctgtaactgtggat

241 gatggtgatgccgcgccggcgacgtatccttcacaggtttcagatctggccctactctgc

301 ggtacgtgatatgctaccaaatggtattctgacgggaatgcagtggttggcatggctacggcgcaggtttcgg

361 agaggagacagtccagacccgctaccacatcggagggagcagaacgacgacgtttcagatggctacggtttcgg

421 acctccgcaaggaggaggtgtgagaaaaaaattacacaacggtttcgg

Molecular Cytogenetic Studies in *Perna viridis* (Bivalvia:Mollusca) from Goa, West Coast of India.
8.2.3. 18S ribosomal RNA gene probe and molecular weight determination

18S ribosomal RNA gene probe constructed by using Digoxigenin—dUTP nick translation mix (Roche). The molecular weight determination of the probe was determined by loading the probe on 1% agarose gel along with pUC 19 DNA digest. Molecular weight comparison showed that the molecular weight of the probe was between 300 bp to 500 bp.

**Figure 30.** 1% agarose gel showing Digoxigenin labeled 18S gene probe

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8.2.4. Fluorescent *in situ* hybridization of 18S ribosomal RNA gene

As many as, 50 metaphase plates prepared from 18 animals were examined to evaluate the presence of signal of 18S ribosomal gene probes on the chromosomes. Figure 31 A shows four signals in the nucleus, whereas figure 31B and 31C shows metaphase plates with 4 and 8 signals respectively. The discriminated signals showed greenish yellow colors on the red propidium iodide-counterstained nuclei and chromosomes. The fluorescent signals of all the detected loci were strong and ranged from 2 to maximum of eight 18S ribosomal gene sites were located at the sub-terminal positions of short arms of three chromosomes (1M/7sM/11sM) and long arm of one chromosomes (3 M). The frequency of the distribution of the rDNA FISH signal varied from 4 % with 8 signals, 10 % with 5 signals and most of the metaphases with 4 (64 %) and 2 (22 %) signals.
FISH - showing 4 signals in metaphase plate

FISH - showing 8 signals in metaphase plate
Fig 32. A

FISH - Metaphase spread

Fig 32. B

FISH - Karyogram
8.2.5. Physical mapping of 18S gene.

Morphometric measurements were taken in as many as 25 cell, indicated six signals on the short arms of chromosome pairs 1, 7 and 11 (1p, 7p and 11p) and two signals were localized on the long arm of the chromosome pair 3 (3q). Based on the method developed by Levan and coworkers (1964), karyogram was prepared as shown in figure 32B.

8.3. Discussion

Uniformity was observed in rDNA location (NORs) and in Ag-NOR specific staining pattern in the chromosomes of Perna viridis. The results of the fluorescence in situ hybridization were consistent with the results of silver staining. The silver impregnation observations in the larvae and the adults in all the populations studied, have also exhibited maximum of eight NOR’s and most common frequency being four in all the three populations. Measurements of the metaphase chromosomes with eight signals indicated chromosome pairs 1, 3, 7 and 1, which is also similar to the NOR studies. NOR data and the 18S rDNA data suggest that the ribosomal genes are distributed on four chromosome pairs.

Based on silver impregnation data, ribosomal gene clusters vary considerably in the pelecypods (Insua and Thiriot-Quievreux, 1991; Thiriot-Quitvreux and Insua, 1992; Martinez-Exposit et al. 1994; Martinez-Lage et al. 1997; Insua et al. 2001)

The number of rDNA fluorescence in situ hybridization signals correlates with the number of number of ribosomal units it contains (Appels et al. 1980; Wachtl et al. 1986; Leitch and Heslop-Harrison, 1992). It is generally established that the in the invertebrate species belonging to different phyla, the rDNA units are repeated from 80 to 600 times per diploid genome (Long and Dawid, 1980). But in the phylum mollusca a low to very high rDNA units are reported in different groups (Insua et al. 2006; Vitturi, et al. 2000; 2005).

Survey of different groups of animals in the molusca, reveal a high degree of variation in the distribution of ribosomal genes. In pectinidae, the distribution of the 18S-28S rDNA varies from two in Pectin...
maximus and Mimachlamys varia to four in Hinnites distortus (Lopez-Pinon et al. 2005; Insua et al. 2006). In opisthobranchia, Oxynoe olivacea, 18S gene probe was localized on the single chromosome and in the some pulmonata species such as Cantareus aspersus and C. mazzullii as many as ten 18S rDNA signals were localized (Vitturi, et al. 2005).

In Mytilidae, two species belonging to genus Mytilus that is Mytilus edulis and M. galloprovincialis, chromosomes carrying 18S-28S rDNA were identified by fluorescence in situ hybridization. The signals correspond to two different submetacentric-subtelocentric pairs (Insua et al. 2001), but the nucleolus organizing regions was found to be four in the Mytilus edulis and two in Mytilus galloprovincialis. The distribution of 18S-28S rDNA, on the metaphase plates prepared from different populations of Mytilus galloprovincialis showed two to three signals (Insua and Mendez, 1998), whereas the NOR impregnation data suggest two two NOR regions (Martinez-Exposit et al. 1994). This clearly shows that the even in Mytilus galloprovincialis, the major unit of the ribosomal RNA gene unit is distributed on more than two chromosomes.

In some bivalves, Crassostrea angulat, high NOR and rDNA polymorphisms in both the size and/or the number occurred intra-individually, inter-individually and inter-populationally and comparative studies showed that the some NORs were transcriptionally inactive (Cross et al. 2003). Transcriptional activity of the ribosomal genes is influenced by the prevailing environmental conditions (Morgan et al. 2005).

Fluorescence in situ hybridization this study suggests a high copy number of 18S rDNA in the diploid genome as against the general admittance in invertebrates. 18S rDNA-FISH and NOR data suggests, though 18S gene is present in high copy number, frequent transcriptionally active genes are present only on two chromosome pairs.

If general account, which is established in the invertebrate species belonging to different phyla of having low copy number of ribosomal genes (Long and Dawid, 1980), it appears that there are at least few DNA sequences which are homologus to the 18S ribosomal gene sequence.

Molecular Cytogenetic Studies in Perna viridis (Bivalvia:Mollusca) from Goa, West Coast of India.
8.4. Conclusion

The fluorescent in situ hybridization data corresponds to the NOR impregnation data. Both data confirmed that in *Perna viridis* the major repeated units of ribosomal RNA genes are distributed on the four chromosomal pairs. The fluorescent signals were detected and the position of the 18S gene was mapped on the chromosomal pairs 1, 3, 7 and 11.