Chromosomal Localization of the Islet Neogenesis Associated Protein (INGAP) Gene in Syrian Hamster by Tyramide Signal Amplification-Fluorescence in Situ Hybridization (TSA-FISH)

Sallie A. Smith
Old Dominion University

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CHROMOSOMAL LOCALIZATION OF THE ISLET NEOGENESIS ASSOCIATED PROTEIN (INGAP) GENE IN SYRIAN HAMSTER BY TYRAMIDE SIGNAL AMPLIFICATION-FLUORESCENCE IN SITU HYBRIDIZATION (TSA-FISH)

by

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A Thesis Submitted to the Faculty of Old Dominion University in Partial Fulfillment of the Requirement for the Degree of

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ABSTRACT

CHROMOSOMAL LOCALIZATION OF THE ISLET NEOGENESIS ASSOCIATED PROTEIN (INGAP) GENE IN SYRIAN HAMSTER BY TYRAMIDE SIGNAL AMPLIFICATION-FLUORESCENCE IN SITU HYBRIDIZATION (TSA-FISH)

Sallie A. Smith
Old Dominion University, 2005
Director: Dr. Christopher Osgood

Diabetes mellitus is a group of conditions characterized by hyperglycemia due to an inability to produce or properly utilize insulin. The majority of cases fall into two categories, Type 1 and Type 2. Type 1 results from the autoimmune destruction of pancreatic β-cells of the islets. The beta cells are the exclusive source of insulin and the patient becomes entirely dependent on exogenous insulin to survive. Patients with Type 2 are distinguished by insulin resistance, a condition that develops due to the inability of the body to effectively use the insulin being produced. The β-cells gradually lose their ability to produce sufficient amounts of insulin due to the increased demand. A viable approach to alleviating this reliance is with regeneration of the β-cell mass. The islet neogenesis associated protein (INGAP) gene is a member of the Reg family of genes and has been shown to induce islet regeneration and reverse diabetes in STZ-rendered diabetic hamsters. Regulation of INGAP expression may represent a method of ameliorating diabetes through the growth of endogenous islet cells. The chromosomal localization of INGAP will provide information for a complete analysis of the spatial relationships involved in this gene family and how this relates to the regulation of expression. Using a tyramide amplification system along with fluorescence in situ hybridization, the INGAP gene was localized to chromosome eleven in the Syrian hamster.
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CHAPTER I

INTRODUCTION

Diabetes Mellitus

Diabetes mellitus refers to a group of diseases in which high blood glucose levels are present due to either an inability to produce insulin or an inability to correctly utilize insulin. The first known reference to diabetes is believed to have been in the Ebers Papyrus of Egypt, approximately 1550 B.C., in which a remedy for excessive urination is mentioned. In 1922, Banting and Best, at the University of Toronto, successfully isolated insulin from a dog pancreas. Soon thereafter, insulin was made available to diabetics through its purification from pigs and cows (Sanders, 2002; History of Diabetes Timeline, 2005). Though the primary structure of insulin is highly conserved across species, allergic reactions are still possible. With the advent of genetic engineering, human insulin was made accessible to diabetics without the aggravation from an exogenous source. The availability of insulin to diabetics turned the disease into a manageable series of complications rather than an outright death sentence (endocrineweb.com; The Pancreas: Introduction and Index).

The complications associated with diabetes are wide-ranging and chronic. Many organs of the body are deleteriously affected by poorly managed high blood glucose levels, including; the eyes, kidneys, heart, blood vessels, and nerves (American Diabetes Association (ADA), Diagnosis and classification of diabetes mellitus, 2004). People with

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This thesis is modeled after the journal Cytology and Genome Research
diabetes are at greater risk for high blood pressure, stroke, blindness, kidney failure and diseases of the nervous system. According to the World Health Organization (WHO), nearly 50% of all deaths related to diabetes are from heart disease (WHO, Diabetes mellitus, 2002).

In the U.S. alone, there are 18.2 million people with diabetes, 5.2 million of which are, as of yet, undiagnosed. It is estimated that there are an additional 41 million more with a condition known as pre-diabetes. A person is said to have pre-diabetes if their blood glucose levels are higher than normal, but not yet at the levels required to be diagnosed with the disease (ADA). It is estimated that by the year 2025, nearly 300 million people worldwide will be afflicted by the disease. It is striking to note that in 1985 that number was 30 million, a ten-fold increase in 40 years. According to the WHO, the estimated number of deaths worldwide due to diabetes is greatly underestimated and may be nearly 4 million annually (WHO, Diabetes: the cost of diabetes, 2002).

Not only is there a physical and emotional toll upon individuals with diabetes, but there is also an economic component. Health care costs for someone living with diabetes in the U.S. are four times that of someone without the disease. As of 2002, this amounts to just over $13,000 per capita according to the American Diabetes Association (National Diabetes Fact Sheet, ADA, 2002). The direct and indirect cost to the nation is around $132 billion annually. That works out to be one out of every ten dollars spent on health care (Direct and Indirect Costs of Diabetes, ADA, 2002).
Pancreas and Insulin

Insulin is produced exclusively in the β-cells of the pancreas, which is a glandular organ made up of exocrine and endocrine cells. The exocrine cells make up the bulk of the pancreas and produce enzymes involved in digestion, such as trypsin and chymotrypsin. The endocrine cells account for 5% of the pancreas tissue and consist of clusters of cells called the islets of Langerhans. The majority of cells that make up the islets are beta cells, which produce and secrete insulin into the blood. Several other cell types are present in the islets and produce hormones such as glucagon (alpha cells) and somatostatin (gamma cells). After a meal, insulin is secreted in response to the elevated levels of blood glucose. Insulin then stimulates uptake of glucose by the liver, which in turn stores that glucose in the form of glycogen. Muscle and adipose tissue are also stimulated by insulin to use the excess blood glucose to produce glycogen and triacylglycerols, respectively. Insulin also has a stimulatory effect on protein synthesis while it inhibits protein degradation. Above all, insulin is a regulator of metabolism; it promotes the storage of essential fuels (endocrineweb.com).

Types of Diabetes

There are many different forms of diabetes mellitus. The majority of cases, however, fall into two main categories. Type 1 diabetes (previously known as insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes) accounts for between 5-10% of all cases. Type 1 diabetes develops as a result of the autoimmune destruction of the beta cells of the pancreas. This form of the disease is most often diagnosed in childhood at an average age of fourteen, but can occur at any time. Though the etiology
of the disease is unclear, there appears to be both a genetic susceptibility and an
environmental component in Type 1 (ADA, Diagnosis and classification of diabetes
mellitus, 2004; National Diabetes Fact Sheet, ADA, 2002).

The second major classification of diabetes is Type 2 (previously known as non-
insulin dependent diabetes mellitus (NIDDM) or adult-onset diabetes mellitus) which
accounts for 90% to 95% of all cases. This form of the disease usually begins with
insulin resistance, an inability of fat, muscle and liver tissue to properly utilize the insulin
being produced. Insulin secretion is also seen in patients with Type 2, however less
severe than those with Type 1. Eventually, patients can become insulin dependent due to
the beta cells inability to produce sufficient amounts. Risk factors for developing Type 2
diabetes include obesity, prior gestational diabetes, race or ethnicity, genetic
predisposition and age (ADA, Diagnosis and classification of diabetes mellitus, 2004;
National Diabetes Fact Sheet, ADA, 2002). An alarming new trend is the emergence of
large numbers of children diagnosed with Type 2 diabetes, previously thought to be
primarily an adult disease. In a great number of cases this is due to the rising obesity rate
in young children. The National Center for Health Statistics reports that as of 2002, 16%
of children in the United States between the ages of six and nineteen are overweight.
They also find that another 15% are at risk of becoming overweight. (National Center for
Health Statistics, 2002).

Other forms of diabetes include genetic defects of the beta cells, which results in
impaired insulin secretion and are inherited in an autosomally dominant fashion. They
are referred to as maturity onset diabetes of the young (MODY). Six genetic loci have
been identified with the most common being in a hepatic transcription factor on
chromosome 12. Other forms include genetic defects in insulin action (mutations in insulin receptors), diseases of the exocrine pancreas and gestational diabetes. Anything that affects insulin structure, secretion, and insulin receptors can also result in some form of the disease (ADA, Diagnosis and classification of diabetes mellitus, 2004).

**Symptoms of Diabetes**

Symptoms resulting from the hyperglycemia of impaired insulin production and/or action include excessive urination and thirst along with unexplained weight loss. Because the body does not process the glucose that is present in the blood, frequent hunger can result. Other symptoms can include blurred vision, fatigue, tingling in the feet, poor wound healing and dry or itchy skin. Not all of these symptoms will present themselves, and often with Type 2, the development of hyperglycemia occurs over an extended period of time with symptoms going unnoticed. Because the person is unaware of the presence of disease, complications may proceed unobserved until the disease reaches a critical level. (ADA, Diagnosis and classification of diabetes mellitus, 2004; endocrineweb.com)

**Current Research**

Current research into curing or managing diabetes follows many paths including islet transplantation, pancreas transplantation, stem cells, and gene therapy. All of these therapies hope to either replace the lost β-cell mass or the ability of the existing tissue to produce sufficient quantities of insulin to maintain normal glucose levels. Difficulties, however, arise in all forms of treatment ranging from: the shortage of available donor
tissue and need for immunosuppressive drugs in transplantation, to the problems in delivery and regulation of the gene of interest to the diseased cell in gene therapy (Harlan, 2004). Another promising approach is islet regeneration, which has the potential of ultimately allowing the diabetic patient to produce endogenous insulin.

**Islet Neogenesis Associated Protein**

The ability of islets to regenerate has been recognized since the early 1900’s when it was observed after complete pancreatic duct obstruction (within Vinik et al., 1997). In 1983, Rosenberg et al. developed a model to study islet neogenesis in the adult Syrian hamster without many of the difficulties found in other models, including widespread pancreatitis, autoimmune destruction and tissue atrophy, or the need for additional chemical agents. They found that cellophane wrapping (CW) of the head of the pancreas resulted in partial obstruction of the pancreatic duct and led to the formation of new islets (Rosenberg et al., 1983). They also demonstrated that the islets appeared to come from precursor cells in the ducts as opposed to mitosis of β-cells already present in the islets, and that the increased islet mass did not compromise the ability to maintain normal insulin output and blood glucose levels. In further studies, cellophane wrapping was performed on hamsters that had been rendered diabetic with the β-cell toxin, streptozotocin (STZ) to determine if the newly produced insulin was capable of producing a biologic response. They observed normal blood glucose and insulin levels in 50% of the diabetic animals after seven weeks in comparison to 12% of the control group. After determination that the previously described results were due to local factors within the pancreas, the group isolated an extract of the wrapped pancreas, which they
termed Ilotropin. The next step was to determine if Ilotropin was capable of reversing diabetes in hamsters. After six weeks of treatment, the diabetes was reversed or stabilized 60% of the time compared to 10% in control animals (Vinik et al., 1997; Rosenberg et al., 1998). Further studies were conducted to determine the genes expressed in the wrapped pancreas as opposed to the normal pancreas. Using mRNA differential display, a cDNA clone (RD-19-2) was found that was unique to the cellophane wrapped pancreas (Rafaeloff et al., 1996). This clone was used to scan a cDNA library of the hamster wherein a novel gene was identified and named islet neogenesis associated protein (INGAP). To determine the expression pattern of INGAP in CW pancreas in contrast to normal control animals, northern blot analysis was performed. The control showed no expression in duodenum spleen, stomach, lung, liver, heart, or skeletal muscle. In the CW animals, INGAP was found in the duodenum and pancreas. Within the pancreas, INGAP was found only in acinar (exocrine) cells and not in islets. They went on to perform \([^3H]Tdr\) incorporation studies and found that duct epithelium and an epithelial cell line (ARIP) showed a 2.4-fold increase in incorporation while mature human islets and cells from a hamster insulinoma tumor cell line (HIT-T15) showed none (Rafaeloff et al., 1997). More recently INGAP was found to be expressed in islets and exocrine cells in normal hamsters (Flores et al., 2003). The authors attribute the discrepancy to the difference in sensitivity in the different techniques used. Rafaeloff et al. concluded that the evidence described above suggests that INGAP plays a role in islet neogenesis (Rafaeloff et al., 1997). In 2000, Wang et al. showed that a portion of the INGAP protein, INGAP 104-118, increased the \(\beta\)-cell mass, insulin content, and reversed diabetes in three out of four hamster that had been rendered diabetic with STZ
(Wang et al., 2000). These findings were later confirmed with the reversal of the diabetic state in all animals, an increased expression of pancreatic duodenal homeobox-1 (PDX-1), a transcription factor, in duct cells and islet regeneration with a reduction of insulitis in the newly formed islets (Rosenberg et al., 2004). Gagliardino et al. concluded that PDX-1/INGAP-positive cells (as opposed to PDX-1 cells that showed no concomitant expression of INGAP) denote a stem cells population that may be activated in islet neogenesis (Gagliardino et al., 2003).

INGAP is a 175 amino acid protein that is a member of the Reg family of genes (Table 1) and shows significant homology to mouse Reg IIIδ in particular (77% identity) (Abe et al, 2000; Taylor-Fishwick et al., 2003a). Reg genes are involved in tissue injury, inflammation, islet neogenesis and carcinogenesis. Reg I was the first gene of this family to be isolated and was found in a regenerating islet cDNA library of 90% depancreatized rats. The gene was found to encode a secretory protein that stimulates the growth of β-cells. All of the Reg-related family members exhibit the same genomic structure of six exons with five introns spanning a 3 kb region, suggesting a common ancestral gene followed by duplication to generate the paralogous family of Reg genes. There are four subgroups in the Reg gene family and both Reg IIIδ and INGAP fall into group III. It has been hypothesized that the two may be orthologs (Abe et al, 2000; Zhang et al., 2003). The expression patterns of both are complex. Two differing reports show Reg IIIδ being expressed either in the pancreas only (Abe et al., 2000), or in the pancreas, stomach, duodenum and skeletal muscle (Sasahara et al., 2000). The latter
Table 1. Known regenerating gene family members.

<table>
<thead>
<tr>
<th>Type I</th>
<th>Type II</th>
<th>Type III</th>
<th>Type IV</th>
<th>Chromosomal Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Reg I</td>
<td>PAP III</td>
<td>Reg IV</td>
<td>4,2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PAP/Peptide 23</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reg III/PAP II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Reg I</td>
<td>Reg II</td>
<td>Reg III alpha</td>
<td>Reg IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reg III beta</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Reg III gamma</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reg III delta</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>REG I alpha</td>
<td>HIP/PAP</td>
<td>Reg IV</td>
<td>2,1</td>
</tr>
<tr>
<td></td>
<td>REG I beta</td>
<td>REG III/PAP IB</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RS (pseudogene)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hamster</td>
<td></td>
<td>INGAP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td></td>
<td>PTP</td>
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The four subgroups and chromosomal location are shown. In each species the gene residing on a separate chromosome from the other family members is the *Reg IV* gene: 2, 3, and 1 for rat, mouse, and human, respectively.

study is of a mouse protein named islet neogenesis associated protein related protein (INGAPrP), which is identical to the cDNA of mouse *Reg IIIδ*. As stated previously, there is discrepancy as to the expression pattern of INGAP. Recent evidence, however, suggests the two (INGAP and *Reg IIIδ*) may be differentially regulated (Taylor-Fishwick et al., 2003). More evidence is needed to determine if the two are, in fact, distinct genes or orthologs being expressed differently in different animals.

A gene family is defined as a group of genes, descended from an ancestral gene, showing similarity in function and DNA sequence homology. Clustered gene families are thought to arise from tandem duplication of a genomic region, segmental duplication, or via unequal crossover events during meiosis. These repeats may then undergo
diversification through mutations resulting in genes with novel functions or pseudogenes that have lost all function (divergent evolution). Conversely, the repeats may retain the function of the original gene as in histone and rRNA gene families. This is referred to as concerted evolution. Gene duplication can also arise through retrotransposons. These are mobile elements in the genome that arise by way of an RNA intermediate. An RNA polymerase transcribes the RNA intermediate, which is then converted back into double stranded DNA by reverse transcriptase. These retrotransposons can move throughout the genome, but are often non-functional. Interspersed duplicates can also arise through rearrangements following tandem duplication events producing fully functional genes (Hurles, 2004; Twyman, 1998). It has been hypothesized that the Reg family of genes in human arose from three gene duplications followed by diversification (Nata et al., 2004).

The chromosomal localization of INGAP in relation to other Reg members is fundamental to a full understanding of its regulation. Though none of the Reg family members have been mapped in hamster, several have been mapped in mouse, human, and rat. The Reg genes that have been mapped in mouse reside in a contiguous region of chromosome six, with the exception of Reg IV, which is located on chromosome three (Abe et al., 2000; National Center for Biotechnology Information). In human, the results are similar with four of the five known Reg genes being mapped to chromosome two and the fifth (Reg IV) mapping to chromosome one (Nata et al., 2004). In rat, three Reg genes have been mapped to chromosome four and one (Reg IV) to chromosome two.

The regeneration of fully functioning islet cells represents a unique approach to the restoration of lost β-cell mass in both Type 1 and Type 2 diabetes. A comprehensive understanding of all of the factors involved in the regulation of islet neogenesis is
required to allow for manipulation of that process in the treatment of diabetes.

Regulation of gene expression is an extremely complex process and occurs at many levels including changes in methylation states, nuclear positioning, and transcription regulatory elements. There is also recent evidence of genes located on separate chromosomes interacting during regulation of expression (Spilianakis et al., 2005).

Very little is yet known about the regulation of gene expression in the Reg family. To thoroughly understand and possibly control the expression of INGAP the spatial relationship between the INGAP gene and other Reg family members is essential. As INGAP is a member of the Reg gene family, it should co-localize to the same chromosomal region as other family members. The goal of this study is to cytologically localize the INGAP gene in the hamster using a tyramide signal amplification system in conjunction with fluorescence-in-situ hybridization.
CHAPTER II
MATERIALS AND METHODS

Metaphase Spread Preparation

Metaphase spreads were prepared using a Syrian hamster cell line (HIT-T15: ATCC® CRL-1777) provided by the Strelitz Diabetes Institute (Norfolk, VA). Cells were grown in VitaCell® F-12K media supplemented with 10% horse serum and 2.5% fetal bovine serum. Prior to metaphase spread preparation, the cells were resuspended in 10 mls fresh media and grown overnight. Four hundred microliters of colcemid (10 μg/ml; Invitrogen) was added to the flask which was incubated at 37°C for 5 minutes then washed with Hanks Buffered Saline Solution (HBSS). After removal of wash, 1 ml of trypsin was added and the cells incubated for 10 minutes at 37°C. Four milliliters of media was added and the cells collected in a 15-ml tube. The cells were centrifuged at 1000 rpm for 5 minutes. The supernatant was removed and the cells resuspended in 0.075 M KCl (pre-warmed) for 15 minutes at 37°C. The cells were again centrifuged at 1000 rpm for 5 minutes and resuspended in a small amount of supernatant (1-2 mls). Fixative (3:1 methanol/glacial acetic acid) was added dropwise while flicking the tube. The cells were centrifuged and fixed two more times in the same manner as described above. After the final spin, the cells were resuspended in a small amount of fixative (3-5 mls) and stored at -20°C until further use. Slides for the spreads were first cleaned with 70% ethanol. The slides were held over a steam bath for 2-3 seconds before a drop of cells was dropped from a height of 2-3 inches. At the point when the cells first began to contract, a drop of fixative was added. The slides were stored in ethanol at -20°C for at
least 2 days prior to use.

**Polymerase Chain Reaction (PCR)**

*Amplification of DNA probe fragment*

A 1.5 kb portion of the *INGAP* genomic region was generously provided by the Strelitz Diabetes Institute in a pCR2.1 vector. The probe was then amplified with polymerase chain reaction (PCR). The PCR reaction mixture was prepared by combining 5 μl 10X buffer (Eppendorf MasterTaq Kit), 1μl 25mM Mg²⁺, 10 μl 5X Enhancer (Eppendorf MasterTaq Kit), 4 μl 2.5 mM dNTPs, 1 μl (approx. 50pmol) M13 forward/reverse primers (M13F 5'-AGGTTTTCCAGTCACGAC-3', M13R 5'CACACAGGAAACAGCTATGAC-3'), 1 μl pCR2.1 vector template (355ng), 1 μl Taq polymerase and molecular grade water to bring the final volume to 50 μl. A negative control was prepared with the same components as above with the substitution of 1 μl water for DNA template. The reaction mixture was incubated at 94°C for 2 minutes followed by 30 cycles of the following program: 94°C for 1 minute (denature), 60°C for 1 minute (anneal) and 72°C for 2 minutes (extend). After the final cycle the reaction was extended at 72°C for 10 minutes and then held at 4°C. An OD₂₆₀ reading was taken of the product.

**Cleaning of probe for labeling**

The DNA probe was cleaned to remove all by-products of the PCR amplification in order to achieve the best result in the subsequent labeling reaction. A Quantum Prep® PCR Kleen Spin Columns Kit (BIO-RAD Laboratories) was used following the
manufacturer's instructions.

Labeling of probe

The probe was labeled in a PCR reaction utilizing the same conditions as described above with substitution of 65% of the dTTP with biotin-16-dUTP (Roche). To confirm labeling, the probe was visualized on an agarose gel. The restricted migration through the agarose gel of the labeled probe due to the biotin addition provides evidence of labeling. Both labeled and unlabeled probe were run on a 1% agarose gel with 1X Tris-borate EDTA (TBE) buffer at 90 volts for 1 hour. The gel was then stained in ethidium bromide for 15 minutes and visualized with UV light.

Restriction Enzyme Digestion

Effective hybridization of the probe to the target requires that the probe be, on average, 500 bp in size. Digestion with restriction enzymes following the labeling procedure should yield probe fragments of adequate size. Two enzymes, SspI and BgIII (Invitrogen) were used, based on analysis of enzyme cutting sites and the INGAP sequence being used. The restriction digest was prepared by mixing the following: 4 μg labeled probe DNA, 2 μl 10X restriction enzyme buffer and molecular grade water to bring the volume up to 20 μl. To this, 1μl of SspI (10U/μl) was added and incubated in a 37°C waterbath overnight. The next day, 1μl BgIII (10U/μl) and 2.2 μl buffer were added and the reaction mixture incubated at 37°C for 1-2 hours. To visualize the result, 1 μg of the digested, labeled probe DNA was run on a 2% agarose gel in 1X TBE at 90 V
or 1 hour. The gel was stained in ethidium bromide for 15 minutes and viewed with UV light.

**Probe Hybridization Mix**

The probe was prepared by combining 500 ng labeled probe, 20 μg salmon sperm (10 mg/ml; Sigma) and 25 μg Mouse Hybloc DNA (1 mg/ml; Applied Genetics Laboratories, Inc.) in a 1.5 ml tube. To precipitate the DNA, 1/10 volume of 3 M sodium acetate and 2 volumes of ice-cold 100% ethanol were added and then vortexed. The tube was placed in −70°C for 1 hour and then centrifuged at 13,000 rpm for 5 minutes. The supernatant was removed and the DNA pellet washed with 70% ethanol. The tube was centrifuged at 13,000 rpm for 5 minutes, the ethanol removed and the DNA pellet dried in a speed-vac. The pellet was then dissolved in 10 μl hybridization buffer consisting of 50% formamide, 2X Standard Saline Citrate (SSC) and 10% dextran sulfate. The probe was then stored at 4°C.

**Slide Pretreatment**

The metaphase spreads were pretreated to minimize endogenous peroxidase activity and therefore reduce background. Slides were washed with 0.3% hydrogen peroxide (H₂O₂) in 100% methanol at room temperature for 10 minutes and then rinsed with 1X phosphate-buffered saline (PBS) for 5 minutes at room temperature. To remove excess cytoplasm and ensure strong hybridization, the slides were placed in a pre-warmed (37°C for 15 minutes) solution consisting of 0.01N HCl and 0.005% pepsin (10% w/v stock) for 2 minutes at room temperature and then rinsed in 1X PBS for 5 minutes at
room temperature. The slides were then fixed with a 1% formaldehyde/1X PBS/50mM MgCl₂ solution for 2 minutes at room temperature and rinsed in 1X PBS for 5 minutes at room temperature.

**Tyramide Signal Amplification-Fluorescence in situ Hybridization (TSA-FISH)**

The tyramide signal amplification system utilizes the enzyme horseradish peroxidase (HRP) to deposit labeled tyramide at the site of the probe (Fig. 1). Tyramide is a phenolic compound that is converted by the HRP into a highly reactive intermediate. It is this short-lived intermediate that binds covalently to electron rich portions of adjacent proteins, usually with tyrosine sidechains. The deposition of labeled, reactive tyramide occurs at the site of the bound HRP enzyme (HRP-streptavidin bound to biotin label of probe) resulting in little diffusion of the signal. Multiple tyramide molecules are
Streptavidin-fluorescein

Biotin-labeled probe annealed with denatured target DNA

Horseradish peroxidase (HRP)-streptavidin

Tyramide-biotin-XX
(XX=14-atom spacer)

Tyramide-biotin-XX derivative (activated)

Tyramide-biotin-XX conjugated to streptavidin-fluorescein

Fig. 1. Tyramide signal amplification (TSA). (A) TSA reaction. (B) Reaction components.
rapidly deposited with a subsequent amplification of the signal. With the indirect process used for this study, the tyramide was labeled with biotin-XX (XX = 14-atom spacer) that required detection through the use of streptavidin conjugated to a fluorescent molecule (fluorescein) (Perkin Elmer, 2005).

Immediately following pretreatment, the slides were dehydrated in an ethanol series, 70%, 85% and 100% for 2 minutes each at room temperature. The slides were then denatured in 70% Formamide/2X SSC at 70°C for 1.5 minutes and washed in an ice-cold ethanol series (as above). The probe was denatured at 70°C for 4 minutes and then immediately put in ice. The probe was added to the quickly air-dried slides and covered with a coverslip. Slides were sealed with rubber cement and incubated in a lightproof box at 37°C overnight. The TSA-FISH was carried out using an amplification kit from Molecular Probes (Lot: 67C2-1, #21). The slides were washed in 1X PBS for 5 minutes at room temperature. A second wash was performed in 0.1X SSC for 5 minutes at room temperature. To the slide, 100μl 1% blocking solution was added and covered with a plastic coverslip. The slide was incubated in a moist chamber at room temperature for 30 minutes. To this, 100 μl streptavidin-horseradish peroxidase (1:50 in 1% blocking solution) was added and again covered and incubated in the moist chamber at room temperature for 30 minutes. The slide was then washed three times in 1X PBS at 37°C for 5 minutes. The tyramide solution was prepared by adding stock tyramide (resuspended in dimethyl sulfoxide-DMSO) to 0.0015% H2O2/Amplification buffer (1:50). This was added to the slide, a plastic coverslip applied and incubated at room temperature for 10 minutes. The slide was again washed in 1X PBS as described above. Avidin-fluorescein isothiocyanate (FITC) (50ul of a 1:200 dilution in dH2O) was added
and the slide incubated in a moist chamber at 37°C for 30 minutes. The slide was again washed in 1X PBS as above and then counterstained with 10ul DAPI/vectashield (Vector Laboratories) coverslip applied. The slides were visualized on a Zeiss Axiovert fluorescent microscope equipped with a cooled CCD camera.
CHAPTER III
RESULTS AND DISCUSSION

Cloning of INGAP probe fragment

The Strelitz Diabetes Institute, Norfolk, VA provided a portion of the Islet Neogenesis Associated Protein (INGAP) gene to be used as a probe in this investigation. Previous to this study, a 6 kb portion of Syrian hamster DNA corresponding to the INGAP promoter and genomic regions was cloned and sequenced (Taylor-Fishwick, 2003). A 1.5 kb portion (2_1 L/R, Taylor-Fishwick, 2003) was PCR amplified and sequenced by the Institute before being provided for this study in a pCR2.1 vector. Fig. 2 shows the subsequent PCR amplification of that fragment for this study. The M13 primers used to amplify the probe from the vector added approximately 150 bp to the probe fragment resulting in the increased size seen. The other bands of higher molecular weight may be due to genomic DNA or vector DNA.

Fig. 2. INGAP probe amplification. Lane 1: 1 kb ladder, lane 2: INGAP probe fragment, lane 3: INGAP fragment.
Labeling and Restriction Digest of INGAP Probe

Label verification

PCR was used in this study to label the probe fragment. Because of this, determination of the efficacy of the labeling process was required. The added biotin molecule is large enough to inhibit movement of the labeled probe through the agarose gel; thereby, the retarded migration of the labeled fragment provides evidence that the probe was successfully labeled (Fig. 3).

![Image of agarose gel with bands at 3.054 kb, 2.036 kb, 1.636 kb, and 1.018 kb]

Fig. 3. Probe label verification. Lane 1: 1 kb ladder, lane 2: probe labeled with biotin-16-dUTP, lane 3: unlabeled probe.

Restriction digest of labeled probe

Prior to hybridization, the labeled probe was digested with restriction enzymes to produce fragments of an optimal size for FISH analysis. Ideally, the fragments should be an average size of 500 bp. A search was first performed to determine which restriction enzymes would digest the probe into adequate fragment sizes (RestrictionMapper. Output, 2003). Two enzymes, BglII (A/GATCT) and SspI (AAT/ATT) were found to have recognition sequences within the probe that should result in fragments averaging
360 bp. The enzyme SspI, however, cuts at an AT junction, which was problematic in that the probe was labeled with biotin-16-dUTP. If a thymine in the enzyme recognition sequence was replaced with the labeled dUTP, the enzyme may not cut the DNA in that region with 100% efficiency. In addition, any labeled dUTP near the recognition sequences could be bulky enough to inhibit the enzyme. Due to these issues, the resulting fragments were other than expected, however the major portion of the resulting fragments were between 100 and 1000 bp as can be seen in Fig. 4.

Fig. 4. Restriction enzyme digest of labeled probe. (A) Expected digestion map of probe fragment by restriction enzymes SspI and BglII with expected fragment sizes shown in parenthesis. (B) Agarose gel of probe digest. Lane 1: 1 kb ladder, lane 2: probe digest, lane 3: probe digest.
INGAP Localization

Probes used in FISH analysis are routinely on the order of 40 kb or greater. The larger the probe being used, the larger the fluorescent signal will be. The small size of the probe (1.5 kb) in this study made necessary the use of an amplification system. An amplification system effectively increases the size of the probe to allow for better visualization. Tyramide amplification systems have been used to map DNA probes less than 1 kb in size (Schriml et al., 1999). Schriml et al mapped two PCR-amplified and labeled DNA probes that were 855 bp and 608 bp. Physical mapping was confirmed with a probe 319 bp in size. The results for this smaller probe, however, were not sufficient to accurately map with the tyramide system. High background is an inherent problem associated with the tyramide amplification system. Because of this, all parameters must be optimized to allow for a good signal to background ratio (Table 2). Another difficulty in working with small probes comes with the hybridization step. To obtain two signals on two chromatids of the same chromosome, they must both denature and hybridize to the probe. The smaller the probe the less chance there is of this encounter. The spatial position of the gene to be mapped is also a factor. The target DNA location, within or towards the outside of the chromatin structure, will make a difference as to whether or not the probe can readily hybridize.

An inability to acquire consistent results with regard to the metaphase chromosome spreads along with the problems inherent with the tyramide system made localization difficult. Problems arose in obtaining uniform metaphase spreads in terms of number of chromosomes detected, length, and banding of those chromosomes. The Syrian hamster chromosome complement is 44. None of the metaphase spreads prepared
contained this number. There were generally between 70 and 85 chromosomes. This in itself is not inconsistent with good results as this is a common problem with cell lines; however, the difficulties with chromosome length and banding did make localization troublesome. As far as the length is concerned, it is important to obtain consistent spreads with similar chromosome contraction patterns in order to localize the gene to a specific chromosome and band on that chromosome. If several chromosomes normally demonstrate similar banding patterns and/or size and the metaphase chromosome lengths vary from spread to spread, correct identification is made difficult. Chromosome lengths were inconsistent from spread to spread on a single slide, as well as from experiment to experiment. For this reason, only a few spreads were found to have distinguishable chromosomes with banding sufficiently clear to enable accurate identification.

**Table 2.** Experimental parameters of TSA-FISH technique.

<table>
<thead>
<tr>
<th>Date</th>
<th>Hybloc</th>
<th>Salmon Sperm</th>
<th>Spreads Prepared</th>
<th>HCl conc.</th>
<th>Tyramide</th>
<th>Strep-HRP</th>
<th>Tyramide Reaction</th>
<th>Probe Denature</th>
</tr>
</thead>
<tbody>
<tr>
<td>11/18/2004</td>
<td>50X</td>
<td>none</td>
<td>11/15/2003</td>
<td>0.30%</td>
<td>1 to 50</td>
<td>1 to 100</td>
<td>5, 7, 10 minutes</td>
<td>4 minutes</td>
</tr>
<tr>
<td>1/5/2004</td>
<td>50X</td>
<td>10ug/slide</td>
<td>12/29/2003</td>
<td>3%</td>
<td>1 to 50</td>
<td>1 to 100</td>
<td>5 minutes</td>
<td>4 minutes</td>
</tr>
<tr>
<td>1/12/2004</td>
<td>75 X</td>
<td>none</td>
<td>12/29/2003</td>
<td>0.30%</td>
<td>1 to 50</td>
<td>1 to 100</td>
<td>5 minutes</td>
<td>4 minutes</td>
</tr>
<tr>
<td>1/20/2004</td>
<td>75 X</td>
<td>10 ug/slide</td>
<td>1/13/2004</td>
<td>0.30%</td>
<td>1 to 50</td>
<td>1 to 100</td>
<td>5 minutes</td>
<td>4 minutes</td>
</tr>
<tr>
<td>1/27/2004</td>
<td>75 X</td>
<td>10 ug/slide</td>
<td>1/13/2004</td>
<td>0.30%</td>
<td>1 to 50</td>
<td>1 to 100</td>
<td>6 minutes</td>
<td>4 minutes</td>
</tr>
<tr>
<td>2/17/2004</td>
<td>75 X</td>
<td>10 ug/slide</td>
<td>1/13/2004</td>
<td>0.03%</td>
<td>1 to 50</td>
<td>1 to 100</td>
<td>10 minutes</td>
<td>2 minutes</td>
</tr>
<tr>
<td>2/26/2004</td>
<td>50 X</td>
<td>20 ug/slide</td>
<td>1/13/2004</td>
<td>0.03%</td>
<td>1 to 50</td>
<td>1 to 50</td>
<td>10 minutes</td>
<td>4 minutes</td>
</tr>
<tr>
<td>3/22/2004</td>
<td>50 X</td>
<td>10 ug/slide</td>
<td>3/9/2004</td>
<td>0.03%</td>
<td>1 to 50</td>
<td>1 to 50</td>
<td>10 minutes</td>
<td>4 minutes</td>
</tr>
</tbody>
</table>
The *INGAP* gene was tentatively localized to chromosome 11. Figure 5A shows two, clearly distinguishable, doublets with the DAPI banding pattern seen in 5B. Ten spreads were found with two fluorescent signals (doublets) on one or both sister chromatids and of these, only two showed doublets on both chromatids. The chromosomes on these spreads were too compact to permit banding that would allow identification. Two spreads had one doublet signal. One metaphase spread was found to have single spots at homologous sites on two separate chromosomes and one spread had a single spot. Other spreads did show possible doublet signals; however, the background to signal ratio was too high for any absolute identification. As is illustrated in Fig. 6A, the chromosome hybridizing to the probe was identifiable as number 11 due to the size, banding pattern and centromere location with this occurring on all spreads where there were discernable signals and adequate DAPI banding. It can also be shown that the signal resides above the second dark band of the q arm by a small margin which puts the chromosomal localization at 11qa.
Fig. 5. Localization of the INGAP gene by TSA-FISH. (A) Metaphase chromosome showing doublet. (B) DAPI banding of chromosome for identification. (C) Ideogram of Syrian hamster chromosome 11 (Li et al., 1982).

Fig. 6. Metaphase spread of HIT-T15 cells. (A) Two single signals on separate sister chromatids. (B) DAPI banding of the same spread showing the two chromosomes are identical in banding, size and centromere location.
CHAPTER IV

CONCLUSION

Taylor et al. discuss the regulation of INGAP as an approach to the growth of endogenous islets and therefore a possible treatment for diabetes (Taylor-Fishwick et al., 2003a). Using new methodologies currently available it is possible to analyze gene expression in relation to chromosomal location. Differential gene locus mapping (DIGMAP) is a computational tool that allows the researcher to evaluate expression of genes which are located contiguously on a chromosome (Yi et al., 2005). Another group has used another recently developed technique, chromosome conformation capture (3C), to study interchromosomal interactions of genes. (Spilianakis et al., 2005).

These are just a few of the many new tools that are enabling researchers to further clarify the complex regulation of gene expression. One of the first steps is to determine the chromosomal location of the gene, or genes, of interest. Cloning and chromosomal localization of INGAP along with localization of the other Reg family members will allow for a comprehensive understanding of the control of regulation in this multigene family. Using tyramide signal amplification along with fluorescence in situ hybridization, INGAP has been tentatively localized to the Syrian hamster chromosome position 11qa in this study. Further cloning and mapping of the other member of the Reg family in Syrian hamster will enable researchers to work toward the ultimate goal of controlling the expression of INGAP as a means of addressing the lack or loss of β-cell mass in diabetics.
REFERENCES


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