

CHROMOSOMAL LOCALIZATION OF A PROINSULIN TRANSGENE INSERTED
WITH A TRANSPOSON-BASED VECTOR INTO JAPANESE QUAIL,
COTURNIX COTURNIX

A Dissertation

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This dissertation is dedicated in honor of my family,
Molly and Kelsey McNally, who always encouraged and
supported me in each endeavor.

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Abstract

The overall goals of this research were to develop a reproducible method of detecting stable DNA insertion into Japanese quail and provide a method for gene location on avian chromosomes. This research resulted in the development of a different method of obtaining chromosome spreads in Japanese quail, the establishment of primed *in situ* hybridization as a method for the chromosomal gene detection in birds, development of Teflon-coated coverslip slides to facilitate laser microdissection of 0.5 μm samples, and chromosomal identification of proinsulin transgene insertions by laser microdissection and nucleotide sequence from G_2 Japanese quail. The 28S rDNA was found on a macrochromosome and a microchromosome pair by primed *in situ* hybridization, fluorescent *in situ* hybridization, and silver staining. Teflon-coated coverslip slides were created to facilitate laser microdissection of avian chromosomes for DNA amplification and nucleotide sequencing. Transgenic G_2 Japanese quail produced in Dr. Richard Cooper's laboratory were identified by laser microdissection and found to have 2-5 chromosomal insertions of the proinsulin transgene.

Chapter 1: Overview

Introduction

Advances in molecular medicine have been based upon the development of tools needed to identify genes responsible for many genetic disorders and gene therapy procedures that may allow restoration of normal function. Gene therapy may provide a method of treatment and prevention of genetic and infectious diseases. In gene therapy, a DNA fragment is inserted into a host genome with a vector (Kuhnel et al. 2004) either to inactivate a gene or to provide additional genetic potential. To confirm successful gene therapy, gene detection methods are used to determine that the specific nucleotide sequence is incorporated into the host genome in a stable manner (Yamashita et al. 2001). Limitations in DNA vectors used to deliver a desired gene and the methods used to detect the DNA incorporation into the host genome have restricted the development of gene therapy. It is a goal of the present work is to develop a reproducible method of gene detection that can identify a single copy gene with nucleotide specificity.

As a result of technologies developed in the human genome project, the application of primed *in situ* labeling techniques (PRINS) (Werner et al. 1997a; Krejci & Koch 1998), and the use of fluorescent *in situ* hybridization (FISH) (Suzuki et al. 1999), advances have been made in determining the sequence and location of naturally occurring genes in a variety of species (human, Kallioniemi et al. 1992; pig, Rogel-Gaillard et al. 1997; chicken, Suzuki et al. 1999; oyster, Zhang et al. 1999). Although these advances have resulted in the knowledge of gene sequences that

cause some genetic diseases (ex. sickle cell anemia (Ingram 1959)), researchers have not employed PRINS or used laser microdissection for isolating single chromosomes for DNA sequencing to examine existing methods of transgene delivery. Currently, FISH (Gussoni et al. 1996), polymerase chain reaction (PCR) (Yamashita et al. 2001), *in situ* PCR (Catzavelos et al. 1998), and Southern blots (Liu et al. 2001) are used to identify possible transgenic cells or animals. These techniques, however, only determine the presence of a gene in the cell, not DNA incorporation into the host's chromosome, nor are they able to determine in which chromosome the transgene is incorporated. More specific techniques are required in order to advance this field of research.

Gene Delivery Systems

The gene delivery systems used today to create transgenic animals are retroviruses (Orwig et al. 2002), adenoviruses (Sato et al. 1998), microinjection of linear DNA (Sang & Perry 1989), nuclear transfer (Wilmut et al. 1997), and transposon-based vectors (Sherman et al. 1998) and *Agrobacterium* to create transgenic plants (Florack et al. 1995).

VIRAL VECTORS

Although retroviruses can be constructed so that most of the native viral genes are absent while containing large DNA inserts up to 7kb (Miller et al. 1988), retroviruses can only infect dividing cells which would have reduced application for adults (Blau & Springer 1995). Despite the benefits of adenoviruses, which have the ability to infect nondividing cells (Acsadi et al.

1995; Knowles et al. 1995), they do not incorporate the desired DNA into the host chromosome and they may have the ability to produce infectious virus (Blau & Springer 1995). Additionally, the use of adenoviruses as vectors for gene therapy has resulted in little or no success (Knowles et al. 1995; Kajiwara et al. 1997) and has been associated with problems (Byrnes et al. 1995) including cytotoxic immune response to virally infected cells (Yang et al. 1994) and the death of one individual (Stolberg 1999; Beardsley 2000).

MICROINJECTION AND NUCLEAR TRANSFER

Microinjection of linear DNA into the pronuclei of fertilized oocytes has resulted in up to 5% of mammals which integrate the transgene DNA into their genome (Eyestone 1994). In avians, linear DNA was microinjected into the cytoplasm of a fertilized chicken ovum. Although the expression of the reporter gene was detected, no evidence of chromosomal integration of the injected DNA was found which indicated that the linear DNA persisted without integration (Sang & Perry 1989). Nuclear transfer involves the genetic manipulation of DNA in donor cells, cells cultured in a serum-free system and tested for the transgene, which is electric transfused to a DNA free egg and transplanted into a surrogate mother (Ikumi et al. 2003). While animals produced by nuclear transfer are not mosaics, the main problems of nuclear transfer are spontaneous abortion and the incidence of prenatal mortality (Schnieke et al. 1997).

TRANSPOSON-BASED VECTORS

Because of the limitations of viral vectors, such as pathogenicity and production expense, nonviral vectors are becoming more popular due to their low costs and lack of specific host's immune response. Nonviral vectors, such as transposon-based vectors (Zhang et al. 1998) or yeast artificial chromosomes (Takahashi et al. 2000), may be introduced into a cell by one of two methods: 1) naked DNA delivery by a physical method, such as electroporation and (2) delivery mediated by a chemical carrier, such as a lipid. Transposons, mobile DNA elements, are generally characterized as either transposons, which transpose directly into the genomic DNA as DNA (Kleckner et al. 1975), or retrotransposons, which transpose into the genomic DNA through an RNA intermediate and reverse transcriptase (Haynes & Jelinek 1981; Moran et al. 1999). While the transposons are common in bacteria, for example Tn10 (Foster et al. 1981), in nature, the retrotransposons, like Alu and P elements, are often found in mammals (Haynes & Jelinek 1981) and other eukaryotes (Laski et al. 1986). A vector based on transposable elements has been applied in both bacteria and eukaryotes to verify whether a cloned DNA fragment contains the whole functional gene of interest (Rubin & Spradling 1983). Bacterial transposons are also used to create eukaryote mutants to interrupt a gene sequence with transposon carrying an identifiable tag, ex. *lacZ*, and test for a specific mutation (Amariglio & Rechavi 1993).

A transposon-based vector that will force DNA incorporation into a recipient chromosome has excellent potential for DNA

delivery for gene therapy applications in humans and animals (Yant et al. 2000). In this vector, the transposase (an enzyme responsible for excising the transposon from the plasmid vector and inserting it into the recipient DNA) is under control of a eukaryotic promoter upstream of the transposon. Having the transposase outside of the insertion sequences allows insertion of the transposon with subsequent degradation of the delivery vector and hence the source of the transposase; the result is a stable insertion incapable of undergoing any further transposition. This vector has been designed with a multiple cloning site between the insertion sequences to allow easy insertion of a desired gene (Cooper, 1998; Cooper & Enright, 1999).

To date, transgenic catfish, koi, oysters, and mice have been successfully made using this transposon-based vector and, in both mice and catfish, the gene has been shown (by PCR and *in situ* PCR) to transfer to the F1 generation (Zhang et al. 1998). Long term detection in sperm and heritability to G₁ and G₂ generations indicates stable incorporation, but the number of gene insertions and specific chromosomes carrying the gene(s) have not been identified. In order to use this vector for gene therapy, it must be demonstrated which chromosomes are carrying the transgene and the number of copies present. This information will eventually allow specific insertion sites to be identified and a determination made on whether or not a detrimental effect has been induced through gene inactivation or activation of an oncogene. In addition, this gene must be passed from one generation to the next.

Transgene Detection Methods

There are several methods of gene detection, including fluorescence *in situ* hybridization (FISH), PCR, and *in situ* PCR, currently available; each of these has limited application in detecting genes. Another method of gene detection, primed *in situ* hybridization, has been shown to distinguish between centromeric nucleotide sequences that have only a few base pairs different by using an oligonucleotide with a different base on the 3' end. To date, transgene insertions have not been identified with primed *in situ* hybridization. Laser microdissection is often used to separate cancerous cells or tissue from normal tissue (Paterson et al. 2003; Schneider-Stock et al. 2003) and to isolate individual chromosomes (Schermelleh et al. 1999) for genetic analysis. To date, laser microdissection has not been used to isolate tissue or chromosomes for verifying transgene incorporation into any animal or plant. Its potential to isolate each chromosome from a single nucleus of a potential transgenic animal for further sequence analysis may, however, provide analysis beyond current techniques. This might, for example, include the ability to determine the sequence of each transgene copy and identify the chromosome in which the transgene was inserted.

FLUORESCENT *IN SITU* HYBRIDIZATION

The fluorescent *in situ* hybridization (FISH) procedure, currently the most common method of chromosomal location used in gene therapy, hybridizes a large DNA probe (> 4 kb) (Herrick &

Bensimon 1999). The DNA probe has an incorporated labeled-nucleotide and is complementary to the target gene on the chromosome (Azzalin et al. 1997). Using a fluorescein or FITC labeled-nucleotide, the probe may be viewed directly with a fluorescent microscope (Simon et al. 1997). Alternatively, a biotin or digoxigenin labeled-nucleotide is incorporated into the probe and a fluorescent-labeled avidin or antibody detects the biotin or digoxigenin (Lichter et al. 1990; Chevalier et al. 1997). Although this technique is popular, it has several disadvantages which include: 1) FISH can only be used to detect a large gene sequence (Herrick & Bensimon 1999); 2) FISH requires multiple copies of a DNA sequence for detection in a light microscope (Nouvo 1992); and 3) FISH lacks the specificity to distinguish among sequences with high homology because of the size probe required (Koch et al., 1989; Gosden et al. 1991; Gosden & Lawson 1994; Pellestor et al., 1996).

Fluorescent *in situ* hybridization has been used to identify chromosomal insertion of transgenes. Beta-glucuronidase, in wheat (Perret et al. 2003) and green fluorescent protein in dwarf goats (Keffer et al. 2001) are examples of transgenes identified with FISH. Because gene therapy targets are generally a small (<5kb), single copy genes, and may only have one nucleotide different from the host's original copy (as would be the case with sickle cell anemia), the FISH technique would not provide reliable confirmation of gene therapy with the transgene having one nucleotide change.

POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) amplifies a DNA fragment with >30 cycles of denaturing DNA, annealing specific oligonucleotide primers, and elongating target DNA (Saiki et al. 1988). After the DNA fragment is amplified, PCR products are separated by agarose gel electrophoresis (Brito et al. 2003). The PCR technique allows examination of several samples a day with a high degree of nucleotide specificity (Orita et al. 1989). Polymerase chain reaction cannot distinguish among integrated and free plasmid/or unincorporated vector DNA (Page et al. 1995) nor can copy number of a gene be established, or can single base substitutions or deletions be easily identified.

IN SITU POLYMERASE CHAIN REACTION

In situ PCR combines aspects of FISH and PCR techniques. A PCR reaction, using free nucleotides including one fluorescently labeled nucleotide, is conducted on immobilized cells on a glass slide for >30 cycles (Zhang et al. 1998). A fluorescent-labeled antibody binds to the labeled nucleotide to allow for visualization of gene location. Considerable caution must be used in interpreting results of *in situ* PCR because amplified products can diffuse out of a cell into adjacent cells, which may not contain the target sequence (Komminoth et al. 1992; Sallstrom et al. 1993; Teo & Shaunak 1995a). As a result, a second amplification of these products may occur in adjacent cells, resulting in a false positive. Additionally, *in situ* PCR results have often been difficult to reproduce (Teo & Shaunak 1995b).

PRIMED *IN SITU* HYBRIDIZATION

Primed *in situ* hybridization (PRINS) uses oligonucleotide primers to amplify a DNA sequence on eukaryotic chromosomes immobilized onto a glass slide (Koch et al. 1989; Gosden et al. 1991; Gosden & Lawson 1994; Hindkjaer et al. 1994). The PRINS technique requires one cycle of DNA denaturing, oligonucleotide primer annealing, and elongation with a fluorescently labeled nucleotide. A fluorescent-labeled antibody binds to the labeled nucleotide and is detected using a fluorescent microscope (Pellestor et al. 1995a; Yan et al. 2001). Because only one cycle of denaturing, annealing, and elongating is used, amplified products cannot diffuse into an adjacent cell and be amplified again, eliminating one of the problems of *in situ* PCR. The small size of the oligonucleotide, <30 nucleotides, does not hinder hybridization and locates DNA sequences regardless of size (Kadandale et al. 2000). Primed *in situ* hybridization has been used to detect aneuploidy in human sperm and cancer cells (Coignet et al. 1996; Werner et al. 1997b), to titer virus (Claudio et al. 2001), and for identification of inverted terminal repeats (Reiter et al. 1999). Additionally, PRINS has been shown to distinguish between alphoid centromeric sequences of human chromosomes 13 and 21, which share 99.7% homology (Pellestor et al. 1994; Pellestor et al. 1995a). Primed *in situ* hybridization has only been utilized in mammals (human; Gosden & Lawson 1995; Pellestor et al. 1995a; Pellestor et al. 1995b; Pellestor et al. 1996; cattle, sheep, horse, and reindeer; Gu & Hindkjaer 1996; pig, Rogel-Gaillard et al. 1997) and plants (rye, garden pea, and field bean;

Kubalaková et al., 1997; barley; Abbo et al., 1993). By using PRINS to locate a gene, it is possible to determine chromosome incorporation and the copy number of a gene. In chapter 2, a new application of PRINS to locate genes inserted by a transposon vector (Cooper 1998) for the purpose of gene therapy is examined. One advantages of using PRINS in gene therapy includes the ability to locate a variety of genes regardless of size and to be able to distinguish among alleles.

Laser Microdissection

Laser-assisted microdissection techniques have been used extensively to evaluate DNA mutations in malignant and nonmalignant cells in a variety of tumors (Manning et al. 2002), minute tissue areas (Ling et al. 2001), single cells (Ponten et al. 1997; Sokolova et al. 2003), and chromosomes (Schermelleh et al. 1999). The areas of interest are microdissected and isolated from the surrounding tissue, cell, or adjacent chromosomes. Subsequent PCR amplification has yielded invaluable gene sequence and gene expression information on the biological behavior of tumors (Garay et al. 2004). A growing number of studies have applied laser-assisted microdissection techniques to the analysis of gene expression in complex tissues (Neira et al. 2001). In most cases, the approach has been limited to the use of frozen sections, because of the difficulty and unreliability of isolating high-quality DNA or RNA from formalin-fixed or paraffin-embedded tissues (Keohavong et al. 2004).

While laser microdissection techniques offer an unparalleled opportunity to study cell biology, there are a number of

technical concerns. The manufacturers (Artcurus, Leica, and PALM) utilize different technologies for the microdissection and subsequent sample collection. The Artcurus system uses a low-power infrared laser to attach the target tissue or cell to a capsule with a transparent ethylene vinyl acetate film (Godstein et al. 1999). The Artcurus system requires strict dehydration of the sample and removal of the tissue from the ethylene vinyl acetate before subsequent sample processing for gene expression assays (Willenberg et al. 2002; Kondapalli et al. 2003). The Leica system uses a pulsed UV-laser to cut around the material and gravity to collect the sample and has a sample size limit of 4-5 μm (Schutze & Lahr 1998; Koelble 2000). Both the Leica system (Burbach et al. 2003; van Dijk et al. 2003) and the PALM system (Fellenberg et al. 2004) may use the PEN, polyethylene naphthalene, membrane slides to facilitate the collection of microdissected tissue or cells. The PALM system, however, uses a UV-laser as an optical knife to cut the targeted tissue or cells and an additional laser pulse to catapult the sample into a microfuge cap (Schutze et al. 1998; Schneider-Stock et al. 2002). Although each laser microdissection microscope has its own advantages and disadvantages, the critical aspect of an instrument for the studies on Japanese quail reported in chapters 3 & 4 is the ability to microdissect and collect 0.5 μm Japanese microchromosomes.

Laser pressure catapulting (LPC), available on the PALM system, catapults the dissected material directly into the cap of a standard 0.5 ml microfuge tube without any mechanical contact.

This enables the rapid collection of specimens 0.5 μm up to several hundreds of micrometers in diameter without contamination from adjacent areas (Schermelleh et al. 1999). Although a variety of tissue protocols for DNA and mRNA identification and amplification are increasingly available (Paterson et al. 2003), chromosome protocols have focused on chromosome painting (Kubickova et al. 2002). Other applications including its potential use for successful gene therapy detection have not been reported. Isolation by laser microdissection allows the identification of a transgene on a single chromosome by agarose gel electrophoresis and nucleotide sequencing. With improvements, laser microdissection may be an extremely useful tool to apply to transgenics and genomics. Although laser microdissection can isolate small sections of tissue and mammalian chromosomes, laser microdissection has not been used to study gene insertion in transgenic species. Genes used for gene therapy using a transposon vector are often single copy genes, may contain single nucleotide changes, and may be of small size. The properties make such genes difficult to identify *in situ* using other techniques.

Avian Karyotypes

Avian karyotypes are generally characterized by a small number of macrochromosomes, a large number of microchromosomes, and sex chromosomes Z and W. The chicken, *Gallus gallus*, and Japanese quail, *Coturnix coturnix*, karyotypes contain eight macrochromosome pairs, Z and W sex chromosomes (with the female being ZW (Saitoh et al. 1993; Ogawa et al. 1995), and 30 microchromosome pairs

(Ryttman & Tegelstrom 1981; Rodiouov 1998). The distinction between macrochromosomes and microchromosomes is somewhat arbitrary (Stock & Bunch 1982), but in chicken the macrochromosomes are numbered 1-8 based on size and the microchromosomes are not assigned numbers because that the distinguishing centromeres and telomeres are difficult to establish (Ladjali-Mohammedi et al. 1999). Due to the high number of microchromosomes in avian karyotypes, obtaining clearly defined preparations, without chromosome overlaps, and constant staining patterns is difficult (Krishan 1962; Ponce de Leon et al. 1992).

Although karyotypes of Galliform birds have been studied with both modern techniques such as FISH (Habermann et al. 2001), macrochromosome chromosome paints (Guillier-Gencik et al. 1999), and chromosome banding techniques (Stock & Bunch 1982; Ladjali-Mohammedi et al. 1999), the avian karyotypes are still largely undefined because the microchromosomes are not distinguishable from one another. The microchromosomes of avian species appear to be gene-rich (McQueen et al. 1996) containing at least 50% of the genes, while only accounting for 23% of the total DNA (Habermann et al. 2001) and are both mitotically and meiotically stable (Bloom 1981). The only similarity the avian microchromosomes share with supernumerary chromosomes, which are small chromosomes $\leq 1 \mu\text{m}$ that contain little or no coding gene information, are not mitotically or meiotically stable (Foresti et al. 1989; Fenocchio & Bertollo 1990), is their small size $\leq 1\mu\text{m}$.

My objectives of this research are to 1) identify a native Japanese quail gene with primed in situ hybridization and laser

microdissection and PCR; 2) identify interchromosomal insertion of a stable of a transgene (proinsulin) inserted with a transposon vector in Japanese quail, *Coturnix coturnix*; 3) to determine the number of chromosomal insertions; and 4) to determine the nucleotide sequence of each transgene copy. The gene detection methods (PRINS and laser microdissection with PCR) could provide a tool currently lacking in avian genetics, for gene location. The ability to confirm specific nucleotide sequence of an inserted gene interchromosomally also provides a tool to confirm both interchromosomal insertion and nucleotide sequence. Additionally, laser microdissection, subsequent PCR, and sequencing provide chromosomal location independently of native genes and nucleotide sequence with high certainty.

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Chapter 2: Physical Mapping of the 28S Ribosomal RNA Gene in Japanese Quail, *Coturnix coturnix*, Using Primed *in situ* Hybridization

Introduction

Genetic maps are the basic tools for identifying genes of importance, and are increasingly being used for agricultural species. For bovine, porcine, and sheep, genetic maps contain 1000–2000 microsatellite markers, but only 10% of genetic markers are coding genes (Andersson et al. 1996; Rohrer et al. 1996; O'Brien et al. 1999). Comparisons between genomes can be based on molecular cytogenetic approaches, nucleotide sequence, or chromosomal location of homologous genes in different species (Chowdhary et al. 1998). Because partial nucleotide sequences can be inferred among related organisms, comparative gene mapping can be used to combine genetic information from related species. Most comparative studies have focused on mammals primarily mouse and human genomes (O'Brien et al. 1993; Carver and Stubbs 1997; Parker et al. 2001; Li et al. 2002), however, recent comparisons between chicken (Jones et al. 1997; Pitel et al. 1998) and mammals reveal a high degree of conservation of genome organization and nucleotide sequence. In poultry, genome mapping efforts have concentrated on the chicken (*Gallus gallus*), which has 235 gene-based markers (Cheng et al. 1995; Groenen et al. 2000), but little has been done on other avian species. For example, current turkey and Japanese quail genome maps are based primarily on microsatellite sequences (Huang et al. 1999; Kayang et al. 2000), while in other Galliformes, such as Guinea fowl and pigeon, genome mapping has been limited to a few studies using

comparative mapping through sequence tags (Smith et al. 2001). Because of the moderate homology between chicken and human nucleotide sequence (Suchyta et al. 2001), the construction of genetic maps for other avian species could be easier through the use of comparative genome mapping by taking advantage of sequences for the human genome. The transfer of mapping information from "gene rich" species, with completely sequenced genomes, to "gene poor" species, with partially or non-sequenced genomes, will be viable, if the quantity and quality of the data on the comparative organization of the two genomes are available (Andersson et al. 1996). Detailed comparative information at the chromosomal level and gene level has been emphasized in the search for candidate loci governing traits of biological and economic importance in farm animals.

Traditionally, genes are located *in situ* through fluorescent *in situ* hybridization (FISH) (Azzalin et al. 1997). With FISH, a DNA probe (> 4 kb) that has an incorporated labeled-nucleotide is hybridized to complementary DNA on a chromosome and is detected with a fluorescent-labeled antibody (Lichter et al. 1990; Herrick & Bensimon 1999). The major limitations of FISH are that it reliably detect large sequences, nucleotide sequences of at least 2kb, (Fransz et al. 1996; Herrick & Bensimon 1999) or shorter DNA sequences if they are tandemly arranged with multiple copies (Werner et al. 1997a). Because fluorescent *in situ* hybridization does not distinguish among sequences with high homology (Gosden & Lawson 1995; Pellestor et al., 1996), it is not suitable for comparative genome mapping because the evolution of nucleotide

sequences from species to species could result in many differences. In this work, to overcome the limitations of probe size and copy number, primed *in situ* hybridization (PRINS) was used to locate the 28S ribosomal RNA (rRNA) gene on Japanese quail chromosomes.

Primed *in situ* hybridization is a hybrid technique based on FISH and *in situ* hybridization (Koch et al. 1989). Primed *in situ* hybridization uses short DNA oligonucleotide primers (~20bp) to amplify the target DNA with one cycle of DNA to localize a target DNA sequence by amplifying the target with one cycle of DNA denaturing, oligonucleotide primer annealing, and elongation (Gosden & Lawson 1994; Hindkjaer et al. 1994). A labeled nucleotide is incorporated, during the elongation phase, and detected with a fluorescent-labeled antibody (Pellestor et al. 1995; Yan et al. 2001). The small size of the oligonucleotide, 20-30 nucleotides locates target DNA sequences without a lower limit on the length of target DNA (Kadandale et al. 2000).

Primed *in situ* hybridization has been used to detect aneuploidy in human sperm and cancer cells (Coignet et al. 1996; Werner et al. 1997b), centromeric repeated sequences on chaffinch chromosomes (Saifitdinova et al. 2001), and for identification of inverted terminal repeats (Go et al. 2000). Additionally, PRINS has been shown to distinguish between alphoid centromeric sequences of human chromosomes 13 and 21, which share 99.7% homology (Pellestor et al. 1994; Pellestor et al. 1995). Through the use of PRINS, genetic maps may be created in sequence-poor species, i.e. Japanese quail, by taking advantage of nucleotide

sequences in more defined species, such as human or chicken. The data obtained in this research demonstrates that Japanese quail have two pairs of NORs (1 macrochromosome pair and 1 microchromosome pair) in contrast to the previously reported 1 microchromosome pair (Schmid et al. 1989) and PRINS provides a reliable and sensitive method for detection of a low-copy sequence on avian chromosomes using oligonucleotides derived from the chicken 28S rDNA nucleotide sequence.

Materials and Methods

Metaphase Chromosomes

Metaphase chromosomes were obtained from Japanese quail using one of two methods, tissue disassociation or feather pulp cell culture.

TISSUE DISASSOCIATION

Japanese quail embryos (4d) were harvested from eggs, placed in 0.05% colchicine for 45 min, followed by a hypotonic solution of dH₂O for 50 min, and fixed in a methanol: acetic acid solution (3:1) for 3h. Tissue was disassociated with 60% acetic acid and cells were placed in a methanol solution at -20°C (Stock et al. 1972; McNally et al. 2000).

FEATHER PULP

Feather pulp was removed from the shafts of 5 flight feathers from the same bird and cultured at 40°C in RPMI 1640 (Hyclone, Logan, UT) with 10% fetal calf serum (Hyclone), 5% chicken serum (Sigma, St. Louis, MO), and antibiotic/antimycotic (10,000 units/ml penicillin G, 10 mg/ml streptomycin sulfate and

25 µg/ml amphotericin B) (Sigma) (Bloom 1981; Van Tuinen & Valentine 1982; Tiersch et al. 1991). Colchicine (0.05%) was added to the media for 30 min. Cells were harvested and pelleted (200 g). A hypotonic solution of 0.075M KCl was added for 15 min and the cells were pelleted (200 g). Cells were fixed in methanol: acetic acid solution (3:1) for 3 h.

Fluorescent *in situ* Hybridization

Fluorescent *in situ* hybridization was conducted using metaphase chromosomes (see above). The 28S rRNA gene was located as a control with the FISH technique. The FISH procedure was based on methods used by Azzalin et al. (1997) and Trask (1991). Internal controls included: 1) no probe added, 2) no antibody added, 3) non-labeled nucleotides, and 4) silver staining.

PROBE CONSTRUCTION

Japanese quail genomic DNA was obtained from feather pulp purified with a QIAamp Tissue Kit (Qiagen, Inc, Valencia, CA). DNA concentration was estimated using a GeneQuant RNA/DNA calculator (Pharmacia, Inc, Piscataway, NJ). The 28S gene was amplified by PCR with primers 28S-F (5'GTGCGGTAACGCAAGCGATC 3') and 28S-R (5'CGCGAGATTTACACCCTCTC3') with the inclusion of a digoxigenin-dUTP. The PCR product was sequenced (Gene Probes and Expression Laboratories, Louisiana State University School of Veterinary Medicine) and compared with chicken (*Gallus gallus*, GENBANK accession #AH001604) 28S rDNA sequence using the BLAST program (GENBANK). The PCR product was purified with a Zymoclean column (Zymogen, Zymo Research, Orange, CA) and served as a FISH probe.

PRE-DENATURE TREATMENT

Metaphase chromosomes were dropped onto two-well slides (Erie Scientific, Portsmouth, NH) and allowed to air dry. Slides were dehydrated in 70% ethanol for 1 h and treated in 2 x saline sodium citrate (SSC) for 10 minutes. Slides were then dehydrated in an ice-cold ethanol series (70%, 90%, 100%) for 1 min each. A pre-denature solution (2 parts water: 1 part 20 x SSC: 7 parts formamide) was added to each well and incubated at 90°C for 5 min.

IN SITU HYBRIDIZATION

A 4x hybridization solution containing (ddH₂O, 20 x SSC, fish DNA (Sigma), 10% dextrose sulfate, formamide, and probe DNA) was heated at 94°C for 5 min and 25µl was added to each well. Wells were sealed with rubber cement to prevent evaporation, and the slides incubated overnight at 37°C.

Primed *in situ* Hybridization

SLIDE PREPARATION

Metaphase chromosomes prepared with either technique (listed above) were dropped onto two-well slides (Erie Scientific). Slides were air dried and incubated for two days at room temperature or heated on a thermocycler at 37°C for 2 h. Slides were dehydrated with a series of ethanol washes (70%, 90%, 100%) for 3 min each. Chromosomal DNA was denatured with 70% formamide in 2 x SSC for 2 min at 72°C. Slides were dehydrated with 70% and 100% ethanol for 5 min each, excess ethanol removed, and the slides air-dried.

PRIMED *IN SITU* HYBRIDIZATION

Primed *in situ* hybridization was conducted with a modified procedure based on methods used by Pellestor et al. (1995). The PRINS reaction solution (10mM nucleotides, 1mM DIG-dUTP, glycerol, Taq polymerase, Taq buffer, and primer) was heated to 60°C in a water bath for 5 min. Slides were pre-warmed on a thermocycler at 55°C for 10 min, and the PRINS solution was added (25µl) to each well. The PRINS cycle consisted of 56°C (annealing temperature) for 15 min and 72°C, elongation temperature, for 30 min. After one cycle, slides were immediately removed and placed into stop buffer (500mM NaCl, 50 mM EDTA) at 60°C for 3 min, and washed with washing solution (Tween 20 in 4 x SSC).

Detection for FISH and PRINS

Slides were washed with a solution containing formamide and 20x SSC for 10 min. DNA was blocked with 3mg/ml (Blocking Reagent, Sigma) in water for 20-30 min at 37°C. Anti-dioxigenin-Fab fragment labeled with fluorescein (10µg/ml blocking solution) was applied to each well for 1 h (except to the no antibody control wells). Unbound antibody was removed by washing with 2 x SSC and Tween 20. Chromosomes were counterstained with 1µg/ml propidium iodide. Vectashield (Vector Laboratories, Burlingame, CA) was added to prevent bleaching. Slides were sealed with nail polish, and coverslips added.

Image Capture

Chromosomes were viewed with a Zeiss Axioplan microscope with a 100x objective and a triple-band pass fluorescent filter (Chroma, Battleboro, VT). Images were captured with a RT slider camera (Diagnostic Instruments, Sterling Heights, MI) containing a 2.5x video coupler (Diagnostic Instruments). Images were viewed with Adobe Photoshop 6.0 (Adobe).

Silver Staining

Metaphase chromosome spreads were dropped onto slides and incubated at 40°C for 2 d. Silver staining was conducted using a modified protocol of Bloom and Bacon (1985) in low light conditions. Briefly, slides were treated with a 50% silver nitrate in Walpole's buffer (Humason 1997) at 60°C for 17-20 min. Slides were then treated in the AS solution and 3% formalin for 10 sec-3 min. Slides were mounted in Permount (Fisher Scientific, Fair Lawn, NJ), and a coverslip was added.

Results

The tissue disassociation method for preparing chromosome spreads resulted in more spreads per embryo than the feather pulp cell culture and less chromosome overlap. Also, tissue disassociation resulted in chromosome spreads quicker than the feather pulp method because cell culture was not required for tissue disassociation.

Primed *in situ* hybridization of 28S rDNA in the Japanese quail showed positive hybridization signals on 2 pairs of chromosomes (1 macrochromosome pair and 1 microchromosome pair) (Figure 1). The control preparations (no antibody, no labeled

nucleotide, and no primer) did not contain positive hybridization signals (Figure 2). Fluorescent *in situ* hybridization showed four positive hybridization signals (Figure 3) and zero positive signals in the control preparations (no antibody or no probe) (Figure 4). The FISH and PRINS methods showed similar intensities for the localization of the 28S rDNA, which was expected because the 28S is a highly conserved nucleotide sequence. Silver staining showed between 2 and 4 active nucleolar organizer regions (NOR)s in a chromosome spread (3 NOR locations shown in Figure 5). The localization of the 28S rDNA with PRINS and silver staining indicates that the 28S rDNA was mapped to the location of the NOR in Japanese quail (Figure 5).

Discussion

The PRINS technique was useful in locating the 28S rDNA, which was confirmed by silver staining. In this study, the 28S rDNA was located on two pairs of chromosomes (1 macrochromosome and 1 microchromosome pair) with both the PRINS technique and the FISH technique. Primed *in situ* hybridization has not been previously documented to localize specific genes in avian species and therefore silver staining NOR proteins as an internal positive control was necessary. The NOR proteins have been associated with the 28S rDNA and the 18S rDNA, but only transcriptionally active NORs have been shown to stain with silver (Miller & Beatty 1969; Bloom & Goodpasture 1976).

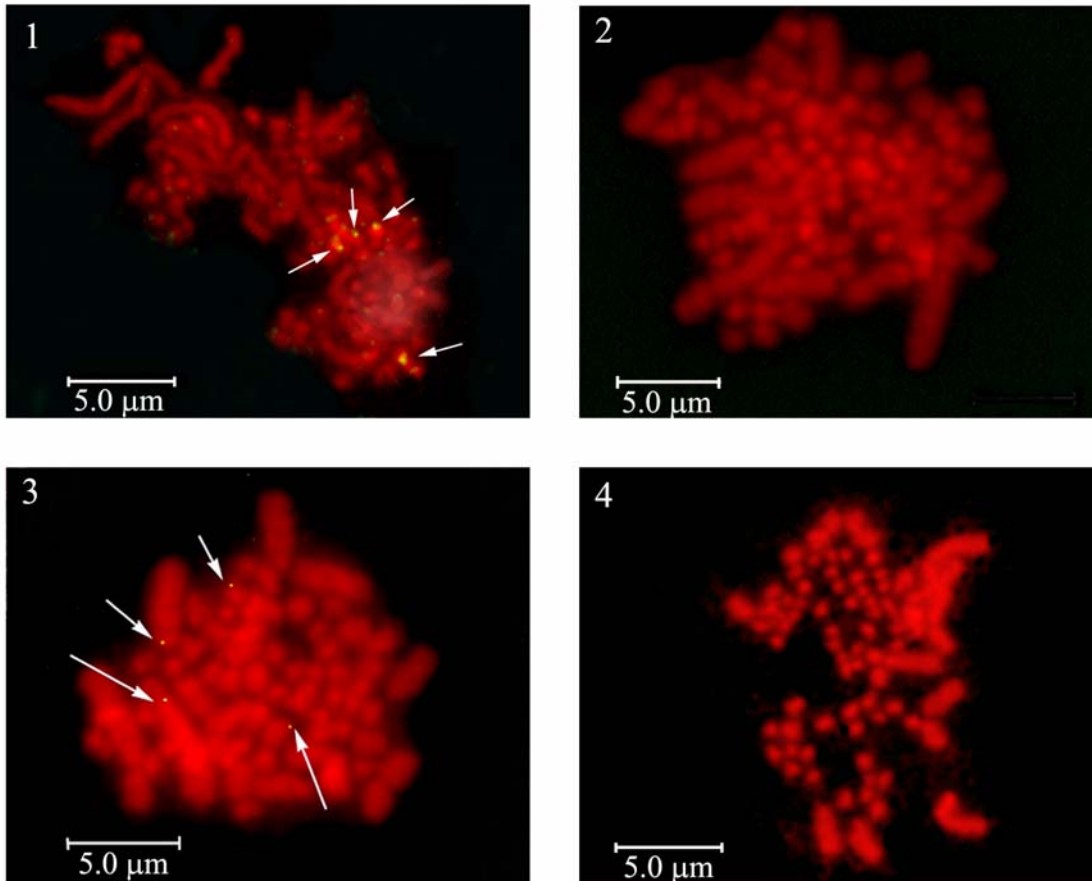


Figure 1: 28S rDNA localized with PRINS on Japanese quail chromosomes; arrows identify localized gene on one microchromosome pair and one macrochromosome pair

Figure 2: PRINS negative control (no primers) for 28S rDNA on Japanese quail chromosomes

Figure 3: 28S rDNA localized with FISH on Japanese quail chromosomes; arrows identify localized gene on one microchromosome pair and one macrochromosome pair

Figure 4: FISH negative control (no probe) for 28S rDNA on Japanese quail chromosome

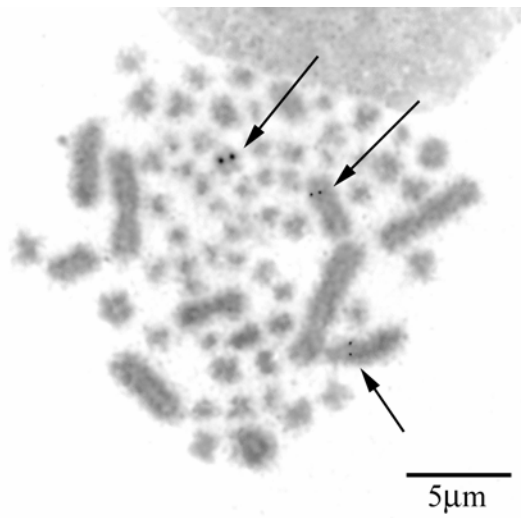


Figure 5: Silver staining of the NOR in Japanese quail; arrows indicate positive staining

In a previous work of Schmid et al. (1989), the Japanese quail NOR genes, 18S and 28S rDNA, were localized to chromosome pair 18 (a microchromosome pair) through silver staining and an additional band was localized with Distamycin A/ mithramycin in chromosome pair 4 (a macrochromosome pair). The band on pair 4 was discounted because it did not show a positive signal with silver staining. Schmid et al. (1989) used the Goodpasture and Bloom technique that was developed for mammals instead of the modified technique for avian species in Bloom and Bacon (1985). Because silver staining only stains active NORs, our results varied by obtaining 1, 2, 3, and 4 positively stained NORs in different chromosome spreads. Using the modified technique, we were able to stain both pairs of NOR bearing chromosomes (1 microchromosome pair and 1 macrochromosome pair). The difference in the number of NORs obtained from the work of Schmid et al. (1989) (1 pair microchromosome) and our results (2 pairs: 1 macrochromosome and 1 microchromosome) can be explained by the difference in the technique used to identify the NORs.

Localization of the 28S rDNA with PRINS, FISH, and silver staining indicates that the 28S rDNA was mapped to the location of the two pairs of NORs in Japanese quail. Because one pair of NORs had previously been reported in Japanese quail (Schmid et al. 1989), it was necessary to confirm the two pairs of NORs obtained using avian silver staining through PRINS and FISH. No difference between PRINS and FISH was expected or occurred because the 28S rDNA is a conserved nucleotide sequence among

closely related species. The benefits of PRINS, in comparison to FISH, lie in its ability to discriminate single nucleotide changes and its lack of requiring a long nucleotide sequence (Werner et al. 1997a, 1997b). It was important to determine plausibility of using a nucleotide sequence from "gene rich" species to localize the same gene in a "gene poor" species, Japanese quail. Primed *in situ* hybridization has been shown to be useful in anchoring alphoid sequences (Saifitdinova et al. 2001). Identification of 28S rDNA with PRINS suggests that PRINS may aid current efforts to develop high-density chicken and turkey genomic maps and may be useful for studying other avian species. While differences in gene size may hinder gene localization using FISH (Werner et al. 1997b), PRINS does not have the same restraint of gene size. For avian genetics to continue to advance, it is important for a method to be developed which can take advantage of the nucleotide sequences of "gene rich" species.

Avian karyotypes, in general, have a similar chromosome number, sex chromosome composition, and size distribution of macro- and microchromosomes (Stock et al. 1982; Bloom & Bacon 1985; Saitoh et al. 1993; Shibusawa et al. 2001). Additionally, chromosomal banding patterns of many avian species are similar (Ladjali et al. 1995; Rodionov 1996; Shaw et al. 1989; Suzuki et al. 1999). Although nucleotide sequences have been shown to be similar for some genes among species in the order Galliformes, the nucleotide sequence is not identical and gene size differences have been reported in among chicken, Japanese quail,

turkey, and guinea fowl (Levin et al. 1995; Pang et al. 1999; Pimentel-Smith et al. 2001; Smith et al. 2001). Primed *in situ* hybridization would be a better technique to localize unsequenced genes in Japanese quail, turkey, or guinea fowl than FISH because PRINS would have the ability to localize a gene on two species in which the gene size and sequence are different.

As DNA sequencing and genome mapping continue in avians, chicken sequences could be utilized to facilitate the identification of economically important genes in less-well studied poultry species through the use of PRINS for comparative genome analysis. Comparative genome mapping of genes and genomes among avian species would aid in poultry breeding, understanding the evolution of birds, and emphasize the degree of homology in both gene sequence and gene arrangement in Galliforms. For future investigations focused on genome analysis in birds, PRINS may be used to obtain required information on chromosome rearrangements and gene location.

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Chapter 3: Laser Pressure Catapulting of Japanese Quail Macrochromosomes and Microchromosomes with Newly Developed Teflon-Coated Coverslip Slides

Introduction

Laser technology and laser microdissection is increasingly used to identify DNA and mRNA nucleotide sequences. Although laser microdissection is generally used to isolate specific cells from fixed, sectioned material, it has been effective in the isolation of living cells for re-culture and isolation of individual mammalian chromosomes for chromosome paints (DNA probes specific to sites scattered along the length of the chromosome which are fluorescently labeled) (Makinen et al. 1998; Stich et al. 2003). Different approaches are used for the laser microdissection. With the Artcurus system, a thermoplastic (ethylene vinyl acetate; E.V.A.) film is applied above the target tissue (Willenberg et al. 2002). This system uses a low-power infrared laser to melt the area immediately above the target tissue to the thermoplastic cap with heat causing the thermoplastic to become glue-like and stick the target tissue area to the thermoplastic film (Godstein et al. 1999). The Artcurus system requires specific sample preparation and subsequent removal of the embedded tissue from the E.V.A. prior to sample processing for gene expression assays or DNA amplification (Emmert-Buck et al. 1996; Willenberg et al. 2002; Kondapalli et al. 2003). Although this method is useful for whole single cells or large tissue targets >10 μm , it is not useful for chromosome dissection due to the small size of most animal

chromosomes, <10 μm (Godstein et al. 1999). The Leica system uses a UV-laser to cut around the target tissue and thereby isolate the material and gravity to collect the sample into a microfuge cap and has a lower size limit of 4-5 μm (Schutze & Lahr 1998; Koelble 2000). Both the Leica system (Burbach et al. 2003) and the Position Ablative Laser Microbeam (PALM) system (Fellenberg et al. 2004) may use the polyethylene naphthalene, PEN, membrane slides in the microdissection of tissue or cells. The PALM system uses a UV-laser to cut the sample and an additional laser pulse focused beneath the sample to catapult it into a microfuge cap (Schutze et al. 1998; Schneider-Stock et al. 2002). Although the PEN membrane slides may be used to keep the tissue sample intact while using the PALM microscope, the PALM approach removes the requirement of PEN membrane slides. Although each has its own advantages and disadvantages, the critical aspects of the approach for the purpose of microdissecting Japanese microchromosomes are a minimal cut diameter as small as 0.5 μm and minimal diameter of sample collected. Laser pressure catapulting (LPC), available on the PALM system, catapults the dissected material directly into the cap of a sample tube without any mechanical contact. This enables the rapid procurement of a homogeneous specimen from 0.5 μm up to several hundred micrometers in diameter without intrusion into the adjacent area (Schermelleh et al. 1999).

Using a PALM microscope, individual mammalian chromosomes have been microdissected from PEN membrane slides for

construction of fluorescent probes to be used as chromosome paints (Schermelleh et al. 1999), but multiple copies of the same chromosome are required to construct chromosome paints (Kubickova et al. 2002). To date, individual chromosome isolation by laser microdissection has only resulted in the construction of chromosome paints. While the PEN membrane allows the whole chromosome or tissue section to be cut and catapulted as a single piece, it requires that closely adjacent chromosomes be ablated in order to collect a single chromosome. The current laser technology allows for microdissection of 0.5 μm and is limited by the PEN membrane slides that do not allow microdissection of less than 1 μm thus requiring closely adjacent chromosomes to be ablated. In order to microdissect individual Japanese quail chromosomes from a single chromosome spread, the small size and high number of Japanese quail microchromosomes (0.5 μm , 78 respectively) (Ryttman & Tegelstrom 1981), required a new method for coating slides. The goals of the investigation presented here were to: (i) develop microscope slides that would enable the laser capture of a single 0.5 μm microchromosome; (ii) compare quality of chromosome spreads on PEN membrane slides and Teflon-coated coverslip slides; (iii) demonstrate the feasibility of laser pressure catapulting for removing single avian chromosomes; (iii) amplify the B-actin gene by polymerase chain reaction, PCR, from a single avian chromosome; and (iv) obtain the nucleotide sequence of the B-actin gene from a single chromosome.

Materials and Methods

Chromosome Preparation

Japanese quail embryos (4d) were harvested from eggs, placed in 0.05% colchicine for 45 min, followed by a hypotonic solution of dH₂O for 50 min, and fixed in a methanol: acetic acid solution (3:1) for 3h. Tissue was disassociated with 60% acetic acid and cells were placed in a methanol solution at -20°C (Stock et al. 1972; McNally et al. 2000). The suspension was dropped onto coverslips with the PEN membrane or the Teflon-coated coverslip slides (described below) and stained with Wright's stain.

Teflon-Coated Coverslip Slide Construction

For the construction of Teflon-coated coverslip (TCCS) slides, a 22 x 60 coverslip (no. 1.5) was attached at the ends only to a normal 3" x 1" slide with rubber cement for stability only. The coverslip was initially washed with 90% ethanol for 10 min followed by a treatment with 50% acetic acid for 5 min. Coverslips were then coated with a thin coat of Teflon (Fluoroglide CP, Electron Microscopy Sciences, Hatfield, PA), obtained from the clear liquid phase. The Teflon coat was buffed with microscope lens paper to create a monolayer of Teflon. The coverslip was treated with two washes of 0.05 N HCl for 1 h each. Coverslip slides were allowed to air dry and then treated with 0.02 N HCl for 10 min, and dH₂O for 10 min. The slides were dehydrated with an ethanol series of 70%, 80%, and 90% for 5 min each and allowed to air dry. The Teflon-coverslip slide was removed from the normal 3" x 1" slide prior to use on the laser microscope. The TCCS slides at this point only consists of the

coverslip without the normal slide which was used to ensure that the coverslip did not break before use on the laser microscope. Because the chromosome samples are dropped onto the coverslip part of a TCCS slide, the coverslip, in essence, acts as a microscope slide instead of its general purpose, therefore further mention of TCCS slides is understood to not include the normal microscope slide and to only include the coverslip with its coating as the slide.

Laser Pressure Catapulting and Microdissection

Cell nuclei, macro-, and microchromosomes were visualized for both types of slides. Individual chromosomes or cell nuclei were marked and catapulted by the LPC function into a drop of water (2.5 μ l) placed on a lid of 0.5 ml microfuge tube. One problem with the PEN membrane slides is the requirement of ablating adjacent chromosomes or tissue, therefore each chromosome in a single chromosome spread was catapulted to ensure the validity of the TCCS slides; PCR was not conducted on these samples.

Polymerase Chain Reaction

Polymerase chain reaction was performed on one microchromosome, one macrochromosome, one nucleus, and three nuclei each in its own 0.5 ml microfuge tube. The PCR reaction mixture used by Sokolova et al. 2003 was modified as listed below. The first PCR reaction mixture included a total volume of 20 μ l: 10 μ l buffer E (FailSafe, Epicenter, Madison, Wisconsin), 1 μ l of each of 5 mM primers (Table 1), 0.5 μ l FailSafe enzyme

(Epicenter), 5 μ l water and 2.5 μ l of water containing the chromosome. Primers targeted specifically to Japanese quail B-actin used for PCR analyses are summarized in Table 1. The first reaction mixture was placed into the cap of the microfuge tube. Once all components were added, the microfuge tube was vortexed and centrifuged. The PCR was conducted in a MJ Research thermocycler 100 with a heated lid which contacted the tops of the microfuge tubes to hold the caps in place. The first PCR reaction included an initial denaturation of 98°C for 7 min followed by 10 cycles (98°C for 1.5 min, 57°C for 1.5 min, 72°C, 1.5min) and 25 cycles (98°C for 1 min, 57°C for 30s, 72°C for 1.5 min) and an additional elongation at 72°C for 5 min. The second PCR reaction mixture included a total volume of 50 μ l, 20 μ l from the first reaction, and additional buffer, primers (10mM) and enzyme. The second PCR cycle parameters were similar to the first cycles, but were slightly modified to be 35 cycles (98°C for 1 min, 57°C for 30s, 72°C for 1.5 min). The PCR sample was run on an agarose gel with the entire 50 μ l loaded into the lane. The DNA was extracted from the gel and purified on a Zymo column (Orange, CA). For nucleotide sequencing, the purified DNA was PCR amplified a third time and using the same parameters as the second PCR cycles.

DNA Analysis and Sequencing

The positive bands were extracted with a gel extraction kit (Zymo) to obtain pure DNA samples. The nucleotide sequences of the DNA samples were obtained using a Big Dye kit (Applied

Biosystems, Foster City, CA) and the corresponding forward or reverse primer. The PCR product was vacuum dried and sequencing was performed by GeneLab (School of Veterinary Medicine, Louisiana State University) on an Applied Biosystems 377 DNA sequencer. After direct sequencing of the amplicons, the resultant sequences were aligned with the B-actin sequence of chicken, *Gallus gallus* (Genbank #NM_205518) using Vector NTI.

Results

Chromosome spreads that were dropped onto the PEN membrane coverslips clumped. It was not possible to catapult each chromosome from a single spread without destroying neighboring chromosomes (Figure 6). The cell nuclei dropped onto the PEN membrane were obtained individually using the cutting and catapulting function of the PALM microscope. The chromosome spreads dropped onto the TCCS slides separated nicely allowing single cell nuclei, macro- and microchromosomes (Figure 7) to be catapulted individually into the microfuge caps. Once a target cell was found (Figure 7A.1, 7B.1, 7C.1), a target was identified and marked with a red dot (Figure 7A.2, 7B.2, 7C.2). A cut is made with the laser which is marked with a blue triangle (Figure 7). The target is catapulted (Figure 7A.3, 7B.3, 7C.3) into a microfuge cap. The laser energy was set so that a hole would remain after the material was catapulted to aid in chromosome identification after gel electrophoresis. To test TCCS slides for the ability to microdissect every chromosome individually, each chromosome from a single spread was catapulted (Figure 8), however these chromosomes were not used for PCR or sequencing.

The actin gene was amplified from a cell nucleus, single macrochromosome, and single microchromosome in figure 7. After gel electrophoresis (Figure 9) and nucleotide sequencing, the resulting sequence, corresponding to regions amplified by Actin 3' - Actin 5' primer pair, showed 80% homology to B-actin of chicken, *Gallus gallus*, Genbank #NM_205518 (Figure 10).

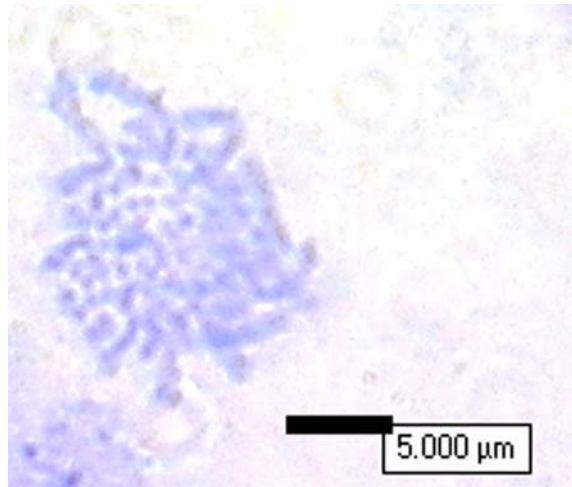


Figure 6: Japanese quail chromosomes dropped onto PEN membrane slides

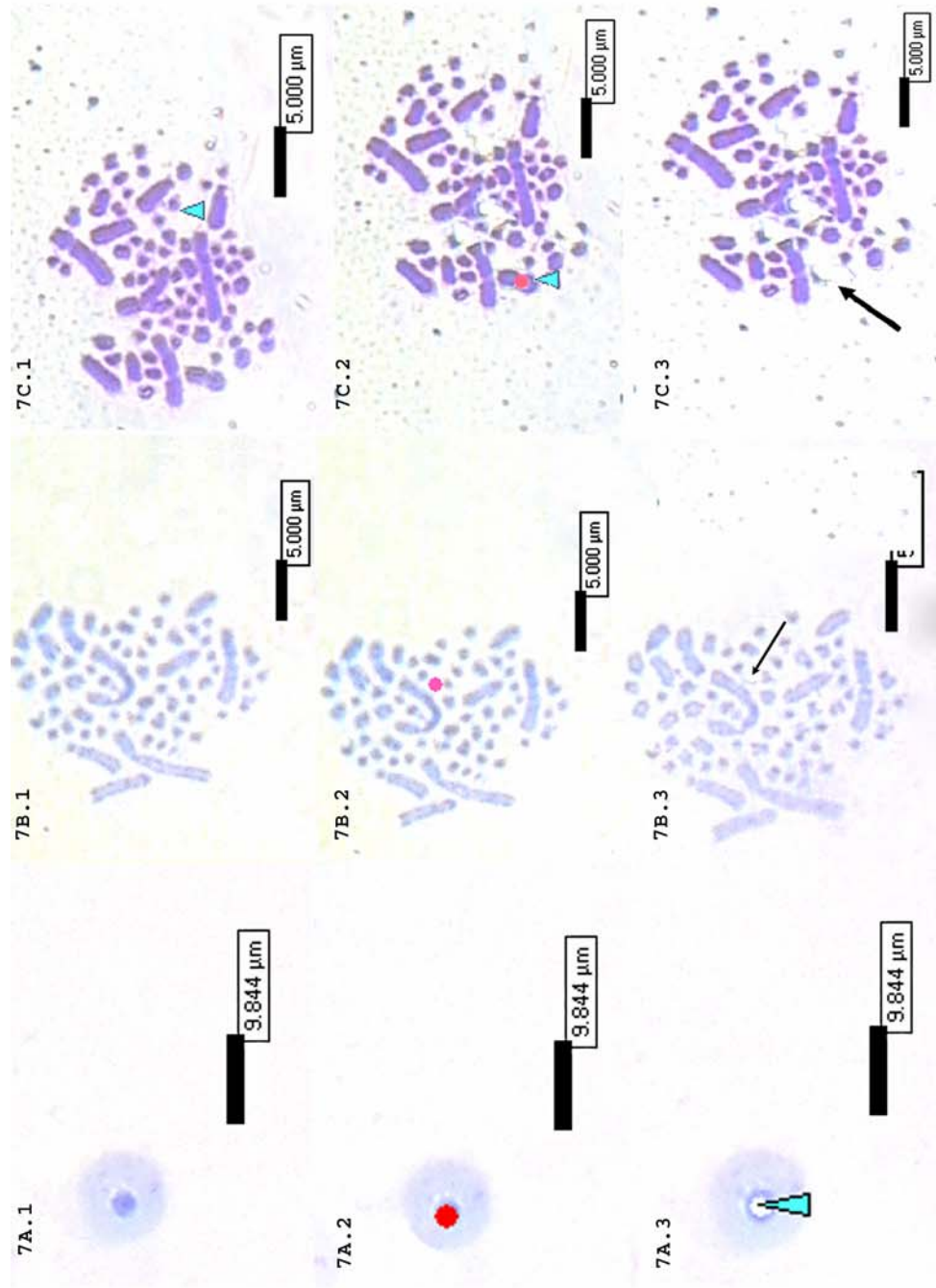


Figure 7: Japanese quail chromosomes dropped on to new slides for laser microdissection

A: single cell; B: chromosome spread; C: chromosome spread

1: Before identification; 2: Identification of material to be microdissected; 3: After microdissection

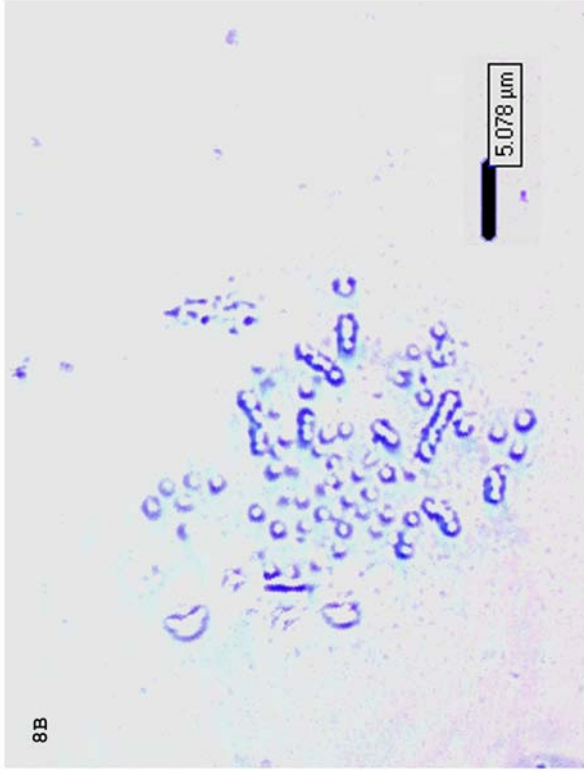
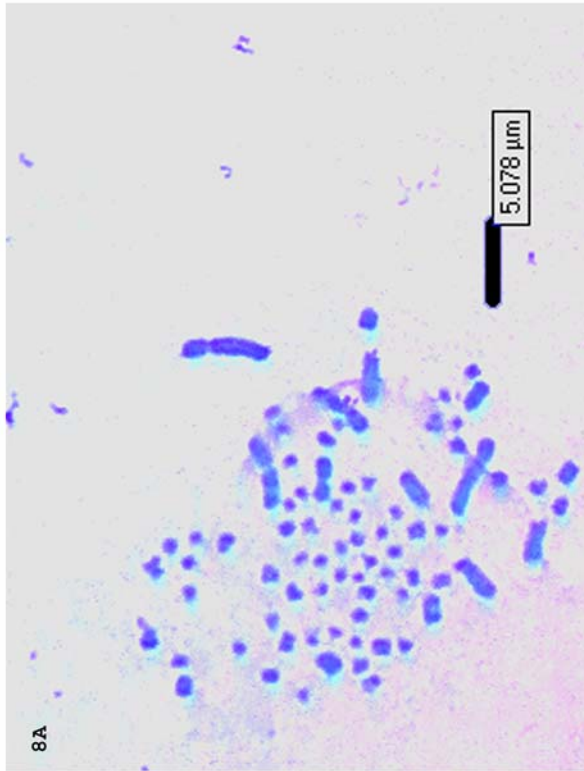


Figure 8: Japanese quail chromosomes dropped onto new slides for laser microdissection
8A Japanese quail chromosomes before microdissection
8B Japanese quail chromosomes microdissected

Table 1: Primers used for amplification of B-Actin from Japanese quail

Primer	Sequence 5'-3'	Expected Size	Obtained Size
Actin 5'	actggtactcactatccaag	750bp	730bp
Actin 3'	cagcatgtatatgcactactggagc		

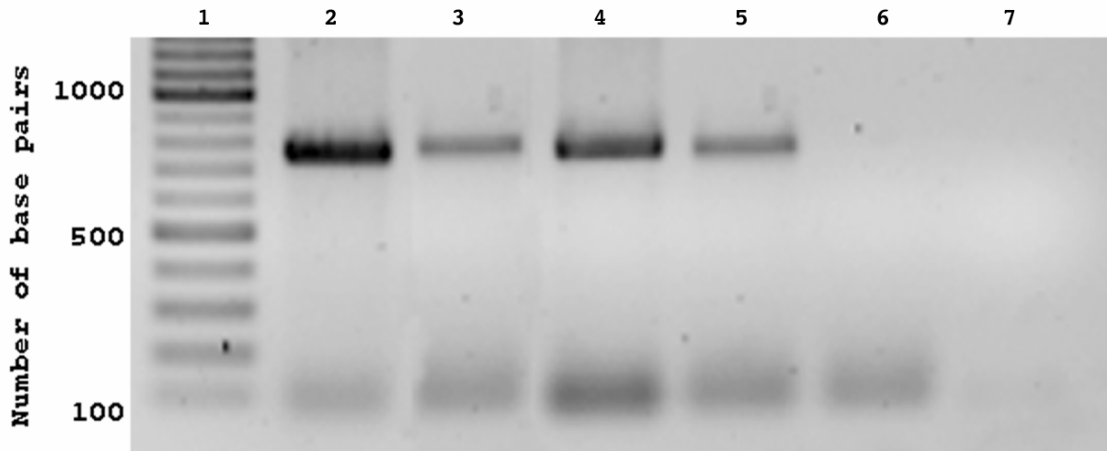


Figure 9: Gel electrophoresis of B-actin fragments obtained from different chromosomes or a cell nucleus and amplified with the Actin3', Actin 5' primer pair **1** -- 100-base DNA mass ladder; **2** - positive DNA; **3** - 1 macrochromosome; **4** - 1 microchromosome; **5** - 1 nucleus; **6** --negative DNA control (first PCR cycle; **7** - negative DNA control (no primers)

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1
AG ATCTGGCACC ACACCTTCTA CAATGAGTTG CGTGTGCTC CAGAGGAGCA
AG ATCTGGCACC ACACCTTCTA CAATGAGCTG AGAGTAGCCC CTGAGGAGCA
AG ATCTGGCACC ACACNTTCTA CAATGAGNTG NGNGTNGCNC CNGAGGAGCA
53
CCCCGTCCTG CTCACTGAGG CCCCCTGAA CCCTAAAGCC AACCGGAGGA AGATGACCCA
CCCTGTGCTG CTCACAGAGG CTCCCCTGAA CCCCAAAGCC AACAGAGAGA AGATGACACA
CCCNGTNCTG CTCACNGAGG CNCCNCTGAA CCCNAAAGCC AACNGNGAGA AGATGACNCA
113
GATCATGTTT GAGACCTTCA ACACTCCAGC TATGTACGTG GCCATCCAGG CTGTGTTGTC
GATCATGTTT GAGACCTTCA ACACCCAGC CATGTATGTA GCCATCCAGG CTGTGCTGTC
GATCATGTTT GAGACCTTCA ACACNCCAGC NATGTANGTN GCCATCCAGG CTGTGNTGTC
173
TCTTTATGCC TCCGGTCGCA CCACTGGCAT TGTCATGGAC TCTGGGGATG GTGTCACCCA
CCTGTATGCC TCTGGTCGTA CCACTGGTAT TGTCATGGAC TCTGGTGATG GTGTTACCCA
NCTNTATGCC TCNNGTCGNA CCACTGGNAT TGTNATGGAC TCTGGNGATG GTGTNACCCA
233
CACGGTGCCC ATCTATGAGG GCTATGCTCT GCCCATGCC ATCCTGCGTC TGGACTTGGC
CACTGTGCCC ATCTATGAAG GCTACGCCCT CCCCATGCC ATCCTCGTC TGGATCTGGC
CACNGTGCCC ATCTATGANG GCTANGCNCT NCCCCATGCC ATCCTNCGTC TGGANNTGGC
293
CGGCCGTGAC CTGACTGATT ACCTCATGAA GATCCTGACA GAGAGAGGCT ATAGCTTCAC
TGGCCGTGAC CTGACGGACT ACCTCATGAA GATCCTGACA GAGAGAGGCT ACAGCTTCAC
NGGCCGTGAC CTGACNGANT ACCTCATGAA GATCCTGACA GAGAGAGGCT ANAGCTTCAC
353
CACCACGGCT GAGAGGGAGA TCGTCCGTGA TATCAAAGGAG AAGCTGTGCT ATGTCGCTCT
CACCACAGCC GAGAGAGAAA TTGTGCGTGA CATCAAAGGAG AAGCTGTGCT ACGTCGCACT
CACCACNGCN GAGAGNGANA TNGTNCGTGA NATCAAAGGAG AAGCTGTGCT ANGTGCGNCT
413
AGACTTTGAG CAGGAGATGG CAACAGCTGC CTCATCTTCT TCCCTGGAGA AAAGCTATGA
GGATTTGAG CAGGAGATGG CCACAGCTGC CTCATGCTCT TCCCTGGAGA AGAGCTATGA
NGANTTNGAG CAGGAGATGG CNACAGCTGC CTCNNNTTCT TCCCTGGAGA ANANCTATGA
473
GCTGCCGAT GGGCAGGTGA TCACCATTGG GAACGAGCGC TTCCGTTGCC TCGAGGGCAT
ACTCCCTGAT GGTACGGTCA TCACCATTGG CAATGAGAGG TTCAGGTGCC CCGAGGCCCT
NCTNCCNGAT GGCACGGTNA TCACCATTGG NAANGAGNGN TTCMNGTGCC NCGAGGNCNT
533
ATTCAAAGCCT TCTTTCTTAA GCATGGAGTC CTGCCGTATF CACAAAAACA CCTTCAACTC
CTTCCAGCCA TCTTTCTTGG GTATGGAGTC CTGTGGTATC CATGAAACTA CCTTCAACTC
NTTCNAAGCCN TCTTTCTTNN GNATGGAGTC CTGNGGTATN CANNAAAANNA CCTTCAACTC
593
CATCATGAAG TGGCAGCTGG CTATCAGGAA GGATCTGTAT GCCAACACTT GTGCTGTCTG
CATCATGAAG TGTGATGTGG ATATCCGTA AATCCTGTAT GCCAACACAT GTGCTGTCTG
CATCATGAAG TGNGANGTGG NTATCNGNAA GGATCTGTAT GCCAACACNT GTGCTGTCTG
653
GGGTACCAC CATGTACCCT GGCATNNCTN ACAGGATGCA NAAAGGAGAT CACAAGCCTT
GTGGTACCAC AATGTACCCT GGCATTGCTG ACAGGATGCA GAAAGGAGAT CACAGCCCTG
GNGGTACCAC NATGTACCCT GGCATNNCTN ACAGGATGCA NAAAGGAGAT CACANNCTN

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Figure 10: Nucleotide sequencing of B-Actin from DNA of a single Japanese quail chromosome amplified by PCR with Actin 5' in red, *G. gallus* mRNA nucleotide sequence in blue, consensus sequence in black, unmatched bases in green

Discussion

Laser microdissection and laser pressure catapulting have primarily been used to isolate specific tissues or single cells (Stoehr et al. 2003; Stich et al. 2003) for DNA or mRNA analysis. Single avian microchromosomes (~ 1 μ m) have not, to my knowledge, been isolated with laser microdissection. Because avian karyotypes, in most species, have a large number of microchromosomes and few macrochromosomes, they differ drastically from mammalian karyotypes (Saitoh et al. 1993; Andreozzi et al. 2001). In addition to the chromosome size difference, avian species typically have chromosome numbers exceeding 70 (Stock et al. 1982). Because of the high chromosome number and small size of the microchromosomes, chromosome spreading is crucial to microdissecting single avian chromosomes. With the PEN membrane, isolation of individual chromosomes becomes increasingly difficult with smaller sizes or higher chromosome numbers because the chromosomes attach to the PEN membrane and clump. The TCCS slides are unique in that their use has resulted in separated avian chromosomes and allowed each chromosome to be isolated for further analysis.

Chromosome isolation by laser microdissection has not identified specific genes, such as B-actin, on chromosomes. Through the use of thermostable sequenase (Thermosequenase) polymerases (Kubickova et al. 2002) and PEN membrane slides, laser microdissection has become a widely used tool to isolate chromosomes. To date, chromosome isolation has resulted exclusively in chromosome paints for a variety of mammal species

(Makinen et al. 1998; Schermelleh et al. 1999). These chromosome paints, fluorescent-labeled DNA probes specific for sites scattered along the length of the chromosome, are used to identify chromosomes that have similar size and shape using hybridization (Kasai et al. 2003). With the exception of Guan et al. 1993, researchers, generally, have found it necessary to dissect multiple copies of a target to produce quality paint probes. Chromosome overlap can make it difficult to dissect an entire mammalian chromosome and insure that all of the starting DNA for probe construction or PCR is from only one chromosome (Christian et al. 1999). Because of the general lack of morphological differences among avian microchromosomes, obtaining multiple copies of the same chromosome for PCR is nearly impossible. The DNA from the initial single chromosome was PCR amplified 70 cycles to overcome the lack of initial starting material. Additionally, chromosome overlapping increases with either larger chromosome size or high chromosome number making it more difficult to catapult all avian chromosomes from a single spread. The TCCS slides facilitated chromosome spreading with the high avian chromosome number and allowed each chromosome to be catapulted from a single spread.

Chromosome paints have been created with flow cytometry and traditional microdissection for macrochromosomes (Guillier-Gencik et al. 1999) and one microchromosome pair (Griffin et al. 1999) in chicken. Multiple copies of each macrochromosome were identified and microdissected with a 1- μ m glass needle (Guillier-Gencik et al. 1999), but using a micromanipulator and glass

needle components from single cells may extremely difficult or impossible to remove without tissue contamination with this method (Whesell et al. 1992). By using a glass needle and micromanipulator to remove only one chromosome which has been successfully transferred into a microfuge tube, the risk of DNA contamination is high and may require a large amount of DNA (up to 10 copies of the single chromosome) to construct the chromosome paint. Traditional microdissection with a 1- μ m glass needle was used to construct chromosome paints for the macrochromosomes in chicken, but these were created from incomplete chromosome spreads (Guillier-Gencik et al. 1999) and may not have been possible due to chromosome overlaps if complete chromosome spreads were used. The restrictions in using traditional microdissection for isolation of microchromosomes includes the 1- μ m glass needle diameter, potential DNA contamination, and lack of chromosome spreading.

Laser microdissection with the TCCS slides provided a method for individual isolation of each microchromosome in a "noncontact" manner. Laser pressure catapulting of all individual chromosomes from the PEN membrane was prevented because the smaller chromosome size and higher chromosome number of Japanese quail. The TCCS slides are unique in that their use has resulted in separated avian chromosomes and allowed individual chromosomes to be isolated for further analysis. These TCCS slides greatly simplify laser microdissection of avian microchromosomes and cell nuclei.

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Chapter 4: Chromosomal Localization of a Proinsulin Transgene in Japanese Quail by Laser Pressure Catapulting

Introduction

Transgenic technology introduces foreign genes into a host genome in a stable manner. Therapeutic proteins are currently produced with the aid of recombinant DNA technology in microbes, animal cell cultures, and transgenic animals. Microbial systems have inherent limitations: microbes cannot carry out the translational modification reactions, such as glycosylation or signal peptide cleavage (Buckholz & Gleeson 1991), required for full biological activity of many proteins in higher eukaryotes. Many of these disadvantages, especially the lack of post-translational modifications, can be overcome with large-scale animal cell culture for the production of proteins of pharmaceutical interest. Because the requirements of a production facility, cell culture media, and personnel, it is cost prohibitive to produce many proteins in this manner. In cell culture, virally transfected animal cells may produce recombinant proteins containing the required post-translational modifications as a native protein (Datar et al. 1993).

Transgenic technology has produced a large variety of mammals (mice, rats, pigs, (Hammer et al. 1985)) & rabbits (Taylor & Fan 1997), which can serve as valuable experimental models for human disease, and as bioreactors for the production of foreign proteins such as human growth hormone, Hammer et al. 1986) and human superoxide dismutase (Stromqvist et al. 1997). Most efforts to provide recombinant proteins have focused on creating

transgenic mammals (rabbits Lipinski et al. 2003) pigs, sheep (Hammer et al. 1985), goats, or dairy cattle (Chan et al. 1998)) or transgenic mammary tissue with the recombinant protein secreted in the milk. Although mammary cells naturally secrete N- or O-glycosylated proteins, recombinant proteins produced in milk are glycosylated, but not necessarily in the correct manner (Houdebine 2000).

The goal of transgenic technology is stable incorporation of a gene into a host genome. Transgenic animals are currently created by using: 1) injection of linear DNA into the nucleus of one-cell embryos (Gordon et al. 1980); 2) injection of transgenic stem cells into blastocysts (Capecchi 1989); 3) somatic cell transfer or nuclear transfer (Schnieke et al. 1997); 4) insertion of DNA with vectors either retroviral (Kuhnel et al. 2004) or transposon-based (Koprek et al. 2001). In chickens, transgenic chimeric intermediates, created by blastodermal injection of Barred Plymouth Rock cells into Dwarf White Leghorn embryos, were the first transgenic avians (Pettite et al. 1990), but incorporation of that transgene into the germ-line was rare. To overcome this problem, the recipient embryo was γ -irradiated prior to injection of the blastodermal cells (Fraser et al. 1993). To date, transgenic chickens have been created by 1) microinjection of a replication-defective retrovirus vector into chicken embryo blastoderms (Bosselman et al. 1989); 2) embryonic stem cell microinjection (Etches et al. 1999); and 3) Mariner transposon (Verrinder-Gibbins 1998). The creation of transgenic Japanese quail was attempted through the use of avian leucosis

virus, a retrovirus, but transgenic progeny were only produced by viremic females and no germline transmission from males was observed (Salter et al. 1999). To date, an efficient method of gene delivery in avian species is not readily available.

To confirm successful gene incorporation, gene detection methods are used to determine that the DNA is inserted into the host genome in a stable manner (Yamashita et al. 2001). One method of determining transgene incorporation is by conventional backcross breeding which would allow confirmation of stable incorporation of the transgene through the progeny. Using conventional backcross, the number of chromosomal insertions of the transgene would not be known. Additionally, the analysis of conventional backcross requires significant numbers of animals bearing well-defined gene markers, which are not currently present in Japanese quail and are only marginally available in chicken (Groenen et al. 2000) to cover the entire genome. It is not uncommon to take months to conclusively identify the chromosomes carrying a transgene and even longer if several chromosomes have integrated the transgene (Silver 1995).

Southern blot analysis of genomic DNA isolated from potentially transgenic animals or plants shows that a transgene is integrated into the genome of F_1 animals (Lu et al. 2002; Perret et al. 2003), but Southern blots do not allow for each individual transgene copy to be sequenced. Fluorescent in situ hybridization (FISH) is often used to identify transgene insertions into specific chromosomes of mammals or plants such as mice (Matsui et al. 2002) and hexaploid oat (Perret et al. 2003).

Transgene loci ranging in length from 10 kb (Wang et al. 1995) to 17 kb (Ambros et al. 1986) have been localized on plant chromosomes using FISH and a smaller 2.7 kb probe has been used to localize a transgene insert by FISH in plants (Fransz et al. 1996). Detecting single copy, low copy, or short nucleotide sequence transgene inserts by FISH can also be difficult (Moscone et al. 1996). For incompletely sequenced genomes, such as chicken or Japanese quail, the FISH probe may bind to native sequences instead of the transgene sequence as a result of unknown homologies among native sequences and the transgene. Because current DNA vectors used to deliver a desired gene may be inefficient and methods used to detect DNA incorporation into the host genome do not result in both transgene location and nucleotide sequence, transgenic animals created as protein bioreactors, currently, have limited application.

Laser microdissection is often used to isolate specific cells from fixed, sectioned material, is effective in isolating of living cells for re-cultivation, and is used for the isolation of individual mammalian chromosomes for chromosome paints (Makinen et al. 1998; Stich et al. 2003). The PALM system uses a UV-laser to cut a sample and an additional laser pulse to catapult the sample into a microfuge tube cap (Schutze et al. 1998; Schneider-Stock et al. 2002). Laser pressure catapulting (LPC) catapults the dissected material directly into the cap of a sample tube without any mechanical contact. This enables the rapid collection of a homogeneous specimen from 0.5 μm up to

several hundred micrometers in diameter without contamination from adjacent areas (Schermelleh et al. 1999).

The goals of this investigation are to: (i) use laser microdissection to isolate each chromosome from a potentially transgenic F₂ Japanese quail chromosome spread; (ii) determine the number of intrachromosomal insertions of the proinsulin transgene; (iii) PCR amplify the proinsulin transgene from individual chromosomes; and (iv) obtain the nucleotide sequence of each positive copy and compare to the original proinsulin sequence in the transposon-based vector.

Materials and Methods

Construction of Transgenic Japanese Quail

Transgenic Japanese quail were constructed in the laboratory of Dr. Richard Cooper. In that laboratory, a transposon vector containing proinsulin was used to transfect male Japanese quail. Briefly, male Japanese quail were transfected using proinsulin in plasmid pTnMod. Plasmid DNA was complexed with Superfect[®] (Qiagen, Inc., Valencia, CA) transfecting reagent at a 1:3 (w/v) ratio of DNA to Superfect[®]. DNA was administered by injection into the testes. Control chicks received plasmid only. Birds were held for two weeks to allow any unincorporated DNA to be cleared before testing for the proinsulin transgene by PCR. Positive birds, G₀, were mated to normal females and offspring were tested for the proinsulin transgene by PCR. The positive G₁ offspring were mated to produce G₂ embryos for DNA and chromosome preparation.

Chromosome Preparation

Japanese quail embryos (4d) were harvested from G₂ eggs and normal Japanese quail eggs, placed in 0.05% colchicine for 45 min, followed by hypotonic solution of dH₂O for 50 min, and fixed in a methanol: acetic acid solution (3:1) for 3h. Tissue was disassociated with 60% acetic acid and cells were placed in a methanol solution at -20°C (Stock et al. 1972; McNally et al. 2000). The suspension was dropped onto TCCS (listed below) and stained with Wright's stain.

Teflon-Coated Coverslip Slide Construction

For the construction of Teflon-coated coverslip (TCCS) slides, a 22 x 60 coverslip (no. 1.5) was attached at the ends only to a normal 3" x 1" slide with rubber cement for stability only. The coverslip was initially washed with 90% ethanol for 10 min followed by a treatment with 50% acetic acid for 5 min. Coverslips were then coated with a thin coat of Teflon (Fluoroglide CP, Electron Microscopy Sciences, Hatfield, PA), obtained from the clear liquid phase. The Teflon coat was buffed with microscope lens paper to create a monolayer of Teflon. The coverslip was treated with two washes of 0.05 N HCl for 1 h each. Coverslip slides were allowed to air dry and then treated with 0.02 N HCl for 10 min, and dH₂O for 10 min. The slides were dehydrated with an ethanol series of 70%, 80%, and 90% for 5 min each and allowed to air dry. The Teflon-coverslip slide was removed from the normal 3" x 1" slide prior to use on the laser microscope. The TCCS slides at this point only consists of the coverslip without the normal slide which was used to ensure that

the coverslip did not break before use on the laser microscope. Because the chromosome samples are dropped onto the coverslip part of a TCCS slide, the coverslip, in essences, acts as a microscope slide instead of its general purpose, therefore further mention of TCCS slides is understood to not include the normal microscope slide and to only include the coverslip with its coating as the slide.

Laser Pressure Catapulting and Microdissection

Microdissection was carried out using the Position Ablative Laser Microbeam (PALM) system, which consists of a pulsed, low-energy 337 nm nitrogen laser coupled into an inverted microscope (Axiovert 200, Carl Zeiss, Göttingen, Germany). The individual chromosomes were localized and marked under the microscope and catapulted into the cap of 0.5 ml PCR reaction tubes (Carl Zeiss) without circumscribing individual chromosomes. Photographs were taken after laser pressure catapulting of each chromosome to identify positive chromosomes after gel electrophoresis of each PCR product. Because microchromosomes are unnumbered, all chromosomes were assigned a number based on the order they were catapulted for subsequent PCR amplification and gel electrophoresis (see below). Immediately before catapulting, the caps were coated with 2 μ l of Pinpoint DNA extraction buffer (Epicenter, Madison, Wisconsin). After catapulting the chromosome of interest, the cap was put on the corresponding tube and centrifuged for 1 min. The sample was heated at 55°C for 45 min and placed at 4°C until the PCR reaction mixture was added.

Polymerase Chain Reaction

Polymerase chain reaction was performed on each individual chromosome in one chromosome spread from the G₂ embryo in the original microfuge tube into which it was collected. The controls for the experiment included normal Japanese quail DNA, normal individual cell nuclei catapulted in the same manner as individual chromosomes, whole chromosome spreads from normal Japanese quail, individual cell nuclei microdissected from undividing cells of the G₂ chromosome preparation, and G₂ embryo DNA. The PCR reaction mixture (Sokolova et al. 2003) was modified as listed below to amplify DNA. The modified PCR reaction mixture included an initial PCR reaction mixture and a secondary PCR reaction mixture. These modifications for the initial PCR reaction mixture included a total volume of 20 µl: 10 µl buffer G (FailSafe, Epicenter), 1 µl of each of 5 mM primers, 0.5 µl diluted FailSafe enzyme (Buffer G, dH₂O, FailSafe enzyme 1:1:1), 5 µl water and 2.5 µl of water containing the chromosome. Primers targeted specifically to the proinsulin transgene and chicken Chpkci, a single copy gene found on the Z chromosome of chicken, were used for PCR analyses are summarized in Table 2. The initial reaction mixture was placed into the cap of the microfuge tube. Once all components were added, the microfuge tube was vortexed and centrifuged.

The PCR was conducted in a MJ Research Thermocycler 100 with a heated lid which contacted the tops of the microfuge tubes to hold the caps in place and eliminates the need for oil. The

initial PCR reaction included an initial denaturation of 98°C for 5 min followed by 45 cycles (98°C for 1.5 min, 60°C for 1.5 min, 72°C, 1.5min) and an additional elongation of 72°C for 5 min. The second PCR reaction mixture included a total volume of 50 µl, 20 µl from the first reaction, and additional buffer, primers (10mM) and enzyme. The second PCR cycle parameters were similar to the first cycles, but were slightly modified to be 45 cycles (98°C for 1 min, 60°C for 30s, 72°C for 1.5 min). The PCR sample was run on an agarose gel with the entire 50 µl loaded into the lane. The DNA was extracted from the gel and purified on a Zymo column. For nucleotide sequencing, purified DNA was PCR amplified a third time using the same parameters as the second PCR procedure. To construct a composite gel, the extracted DNA was re-amplified with proinsulin primers for the positive bands from individual chromosome, single cell nucleus catapulted from the same G₂ preparation, genomic DNA of a full sibling G₂ embryo, single cell nucleus from a normal Japanese quail embryo, and genomic DNA created from normal Japanese quail was run on gel electrophoresis along with the Chpkci DNA controls (Figure 13).

DNA Sequencing and Analysis

Positive bands were extracted with a gel extraction kit (Zymo, Orange, CA) to obtain pure DNA samples. Nucleotide sequences of the DNA samples were obtained using a Big Dye kit (Applied Biosystems, Foster City, CA) and the corresponding forward or reverse primer. Each reaction was vacuum dried and sequencing was performed by Gene Probes and Expression Laboratory

(School of Veterinary Medicine, Louisiana State University) on an Applied Biosystems 377 DNA sequencer. After direct sequencing of the amplicons, the resultant sequences for the 330bp fragments were aligned with the original proinsulin sequence from the plasmid and the resultant sequences of the 1000bp fragment were aligned with the Chicken Wpkci (AB026678) gene because only the mRNA gene has been sequenced in Japanese quail.

Results

Each individual chromosome was catapulted with the PALM laser microscope into separate microfuge tubes from a single chromosome spread prepared from a potentially transgenic G₂ Japanese quail embryo (Figure 11). The laser energy was set so that a hole would remain after the material was catapulted to aid in identification of chromosomes that contain positive proinsulin electrophoresis bands. The blue ring does not contain remnant DNA, but was the result of light diffraction. Each positive chromosome, as observed by gel electrophoresis of PCR product from each chromosome, was identified on the original photograph by arrows (Figure 11A). The 10th (Figure 11B), 25th (Figure 11C), 58th (Figure 11D), 75th (Figure 11E), and 77th (Figure 11F) chromosome catapulted were positive for the human proinsulin transgene.

A single cell nucleus was catapulted in the same fashion from undivided cells in the chromosome preparation created from normal Japanese quail embryos and from the same G₂ Japanese quail chromosome preparation served as controls (Figure 12). The blue triangle in Figure 12A and 12 B identifies the location of the

laser cut. The dark blue edges of the laser cut were a result of light diffraction after the laser cut and were not the result of uncatapulted DNA.

The gel electrophoresis of the PCR products resulted in identification of 5 positive bands (Figure 13) and thus 5 positive chromosomes (Figure 11). After positive proinsulin bands were cut out and reamplified from each of the 5 positive proinsulin bands of the G₂ Japanese quail along with DNA from the sibling embryo, reamplified samples were run on an agarose gel (Figure 13). The agarose gel (Figure 13) resulted in positive proinsulin bands for a single nucleus from the same preparation, genomic G₂ DNA, and the 5 positive chromosomes 10, 25, 58, 75, and 77. No bands were observed for the normal Japanese quail single nucleus and normal Japanese quail DNA amplified with proinsulin primers. The normal Japanese quail DNA control was amplified with Chpkci primers and resulted in a positive band (Figure 13).

The bands from each positive chromosome were sequenced and resulted in nearly identical sequence to human proinsulin portion of the original vector, the only difference being a single nucleotide error in the EninspyA2 primer (Figure 14). Although it is difficult to identify homologous microchromosomes, the single positive macrochromosome demonstrates incorporation of the transgene in a nonhomologous manner (Figure 11).

Table 2: Primers used for amplification of the proinsulin transgene and Chpkci control gene from G₂ Japanese quail chromosomes microdissected by the PALM microscope
 *Gallus gallus Wpkci-8 gene Genbank number AB026678

Primer	Sequence 5'-3'	Expected Size	Obtained Size
Enttag-3	cctgctggatgacgatgaca	330bp	330bp
EntinspyA2	caggcgctggtctagagca		
ChpkciE1F	gtcgccatgtctgacgagatc	1088bp*	
WpkciI1R	gtgagatatcatggaacgcaagg		

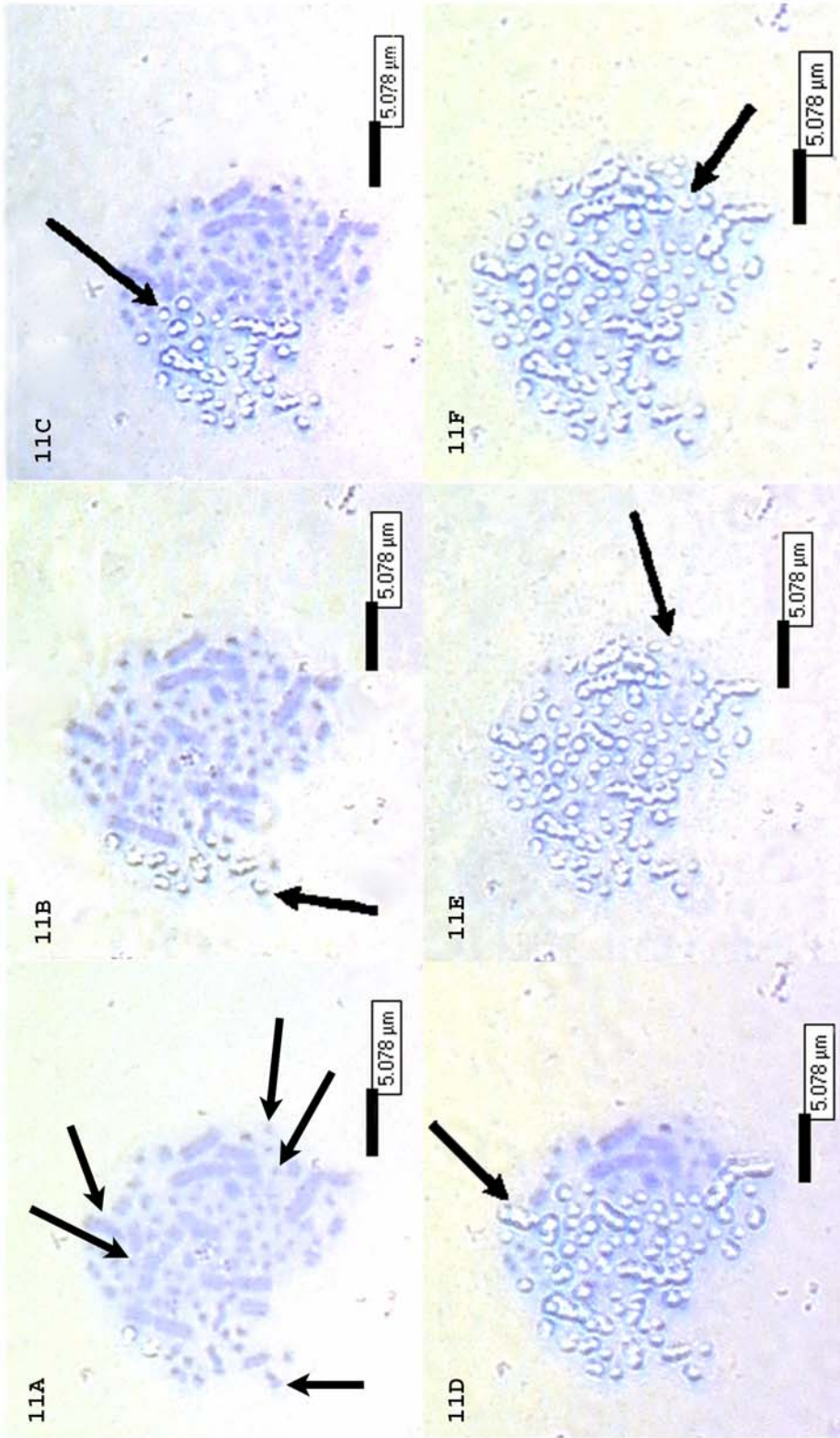


Figure 11: G_2 Japanese quail chromosomes laser microdissected with the PALM microscope
 arrows indicate location of chromosomes with the proinsulin transgene inserted
 11A - positive chromosomes identified; 11B - positive microchromosome;
 11C - positive microchromosome; 11D - positive macrochromosome;
 11E positive microchromosome; 11F - positive microchromosome

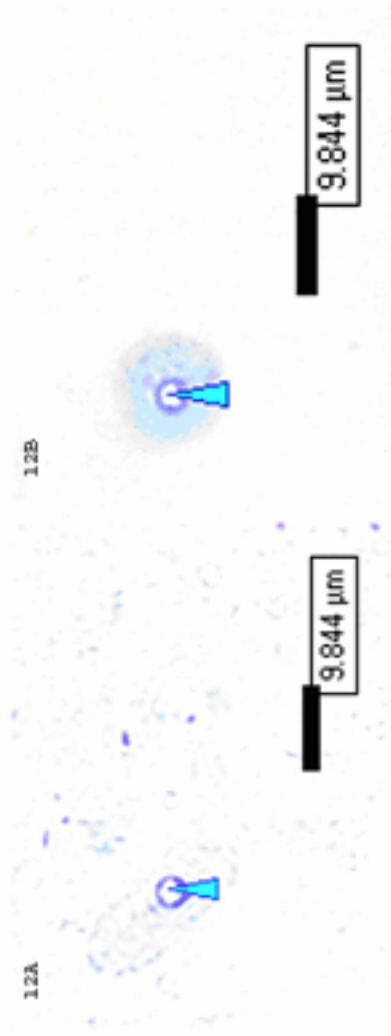


Figure 12: Single cell nucleus microdissected from normal preparation and G₂ Japanese quail preparations
12A - Normal Japanese quail nucleus microdissected,
12B - G₂ Japanese quail nucleus microdissected

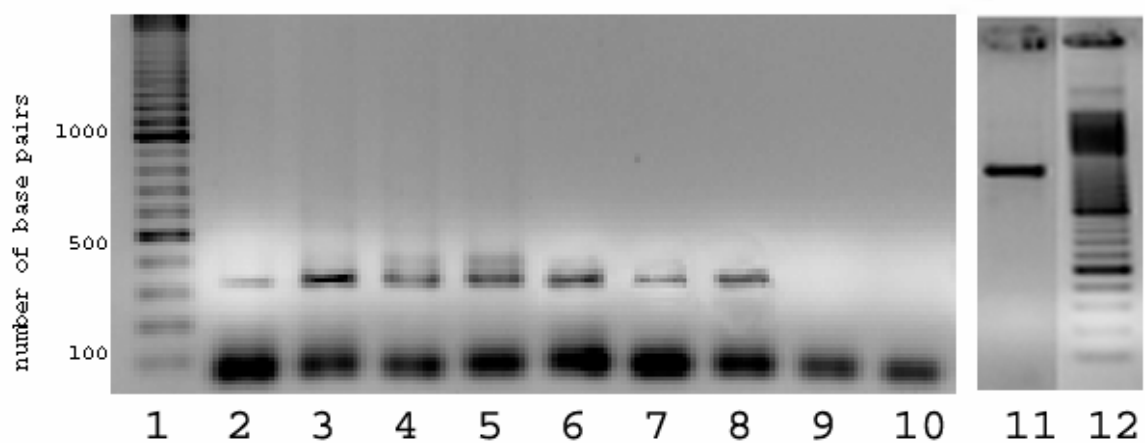


Figure 13: Composite gel electrophoresis of Proinsulin positive bands 1- 100 bp size marker; 2 - single nucleus isolated from same chromosome preparation; 3 - genomic DNA from sibling embryo; 4 - 10; 5 - 25; 6 - 58; 7 - 75; 8 - 77; 9 - single nucleus isolated from normal chromosome preparation; 10 - genomic DNA from normal bird; 11 Chpkci control; 12 100 bp size marker

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1
.....GTGCG GCTCACACCT GGTGGAAGCT CTCTACCTAG TGTGCGGGGA
CACCTGTGCG GCTCACACCT GGTGGAAGCT CTCTACCTAG TGTGCGGGGA
-----GTGCG GCTCACACCT GGTGGAAGCT CTCTACCTAG TGTGCGGGGA

51
ACGAGGCTTC TTCTACACAC CCAAGACCCG CCGGGAGGCA GAGGACCTGC
ACGAGGCTTC TTCTACACAC CCAAGACCCG CCGGGAGGCA GAGGACCTGC
ACGAGGCTTC TTCTACACAC CCAAGACCCG CCGGGAGGCA GAGGACCTGC

101
AGGTGGGGCA GGTGGAGCTG GCGGGGGGCC CTGGTGCAGG CAGCCTGCAG
AGGTGGGGCA GGTGGAGCTG GCGGGGGGCC CTGGTGCAGG CAGCCTGCAG
AGGTGGGGCA GGTGGAGCTG GCGGGGGGCC CTGGTGCAGG CAGCCTGCAG

151
CCCTTGGCCC TGGAGGGGTC CCTGCAGAAG CGTGGCATTG TGGAACAATG
CCCTTGGCCC TGGAGGGGTC CCTGCAGAAG CGTGGCATTG TGGAACAATG
CCCTTGGCCC TGGAGGGGTC CCTGCAGAAG CGTGGCATTG TGGAACAATG

201
CTGTACCAGC ATCTGCTCCC TCTACCAGCT GGAGAACTAC TGCAACTAGG
CTGTACCAGC ATCTGCTCCC TCTACCAGCT GGAGAACTAC TGCAACTAGG
CTGTACCAGC ATCTGCTCCC TCTACCAGCT GGAGAACTAC TGCAACTAGG

251
GCGCCTAAAAG GGC GAATTAT CGCGGCTGCT CTAGACCAGG C.....
GCGCCTAAAAG GGC GAATTAT CGCGGCGCT CTAGACCAGG CGCCTGGATC
GCGCCTAAAAG GGC GAATTAT CGCGGCGCT CTAGACCAGG C-----

```

Figure 14: Nucleotide sequence of proinsulin from a single positive chromosome isolated with the PALM microscope and amplified by PCR in red, original vector TnMod with proinsulin and Enttag bp#7460-7756 in blue, consensus sequence in black, all unmatched bases in green

Discussion

This study was intended to identify integration of a human proinsulin transgene into the chromosomal DNA of Japanese quail with the transgene passed to offspring and determine the potential of laser microdissection in identifying intrachromosomal transgene insertions with nucleotide sequence from each insertion point. Japanese quail transfected with a transposon based vector containing the proinsulin transgene were previously identified through PCR and positive birds mated. Resulting offspring were tested for the proinsulin transgene through PCR. Because a FISH probe may not differentiate between the native proinsulin Japanese quail nucleotide sequence and the human proinsulin located in the transposon vector, a FISH probe was not used to initially probe the G_2 chromosome spread due to potential of false positives. Because transgene insertions have not, to my knowledge, been identified with laser microdissection in any species, G_2 Japanese quail were used for this study to ensure each cell would contain the transgene. The G_2 Japanese quail were shown to contain 2-5 chromosomes with the transgene insertion through laser microdissection, PCR, and nucleotide sequencing. Prior to this study, transgenic Japanese quail have not been successfully produced (Salter et al. 1999).

In previous transgenic avian studies, transgenic birds have been shown to have stable incorporation into the genome with PCR or Southern blots (Mozdziak et al. 2003; Rapp et al. 2003). Although both PCR and Southern blots may confirm the presence of the transgene, actual chromosomal incorporation of the transgene

can not be confirmed because both PCR and Southern blots use genomic DNA (Tolmachova et al. 1999). Chromosomal inserted DNA cannot be positively distinguished from circular original vector DNA by Southern blots or PCR. In the present study, each of the resulting PCR bands contained sequencing matching the proinsulin sequence from the original vector, thus confirming that the inserted DNA was not rearranged prior to its chromosomal insertion and that each copy contained the correct sequence.

Currently, the most widely accepted method for confirming transgene in corporation is fluorescent in situ hybridization, FISH (Kulnane et al. 2002). Fluorescent *in situ* hybridization lacks capacity to distinguish among sequences with high homology (Gosden & Lawson 1994; Pellestor et al., 1996) as may be the case with transgenes and native hosts genes. Because the chicken Proinsulin mRNA, Genbank number X58993, shows some homology (80%) with the proinsulin transgene in the transposon-based vector and could potentially give false positives with the FISH technique, nucleotide sequencing of each positive chromosome was essential. Additionally, the general lack of nucleotide sequence information available for Japanese quail, specifically the single copy Chpkci gene, resulted in the construction of primers based on the chicken sequence. Although nucleotide sequence differences occur between chicken and Japanese quail, the Chpkci primers amplified a portion of the Chpkci gene in Japanese quail and similar nucleotide sequences. The Japanese quail genome is not sequenced and chromosome paints do not exist for all of the microchromosomes and some of the macrochromosomes which made it

essential to microdissect each chromosome from an individual chromosome spread.

In my study, the proinsulin transgene was found to insert more often in microchromosomes of Japanese quail. Because the microchromosomes contain the majority of the coding genes in avians (Smity et al 2000), the proinsulin transgene is less likely to be silenced by heterochromatin. Although transgenes inserted into plants are more likely to be silenced if found in higher copy number (Assaad et al. 1993), high copy number has not been found to silence transgenes in transgenic mammals.

This study has confirmed the transgenic status of the G_2 Japanese quail, 2-5 chromosomal insertions in G_2 Japanese quail, and the nucleotide sequence of each transgene insertion. This study has also introduced laser microdissection with subsequent analysis as a method of choice for confirming chromosomal insertion of a transgene and for obtaining nucleotide sequence of the inserted transgene. Because many transgenes are closely related to the host native gene (as is the case with proinsulin), laser microdissection and subsequent analysis may result in details required for approval of transgenic protein production.

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Chapter 5: Summary

The overall goals of this research were to develop a reproducible method of detecting stable DNA insertion into an eukaryotic genome (using Japanese quail as a model species) and provide a method for determining which chromosomes carry a particular nucleotide sequence. A series of techniques were developed or modified to facilitate this study, including chromosome preparation from Japanese quail embryos, culture of feather pulp cells, primed *in situ* hybridization, Teflon-coated coverslip slides, and polymerase chain reaction and nucleotide sequencing from a single chromosome. This research resulted in different methods of obtaining chromosome spreads in Japanese quail, the establishment of primed *in situ* hybridization as a method for chromosomal gene detection in birds, development of Teflon-coated coverslip slides to facilitate laser microdissection of 0.5 μm samples, and chromosomal identification of proinsulin transgene insertions obtained by laser microdissection and nucleotide sequence from G₂ Japanese quail.

Two methods for obtaining chromosome spreads were utilized in this study. A tissue disassociation method for preparing chromosome spreads resulted in more spreads per embryo than the feather pulp cell culture and was less time consuming. Because tissue disassociation is a lethal chromosome procedure, feather pulp cell culture was developed for future use in transgenic identification to prevent transgenic bird mortality.

Primed *in situ* hybridization, PRINS, was tested as a potential technique for transgene identification by identifying the 28S rDNA in the Japanese quail. Because PRINS has not been used to identify a gene on avian chromosomes, fluorescent *in situ* hybridization, FISH, was used as a technique control. Both PRINS and FISH showed positive hybridization signals on 2 pairs of chromosomes (1 macrochromosome pair and 1 microchromosome pair). Silver staining for nucleolar organizer regions, NOR, was also used as a control technique for PRINS as it localizes active NORs. The silver staining resulted in localizing between 2 and 4 active NORs in a chromosome spread. The localization of the 28S rDNA with PRINS and silver staining indicates that the 28S rDNA was mapped to the location of the NOR in Japanese quail.

The small size and large number of Japanese quail microchromosomes, 0.5 μm , presented some difficulties for isolating each individual Japanese quail chromosome with laser microdissection. Although current laser microdissection technology has the ability to microdissect 0.5 μm chromosome, the polyethylene naphthalene, PEN, membrane slides used for laser microdissection prevented the microdissection of target chromosomes 0.5 μm . Cell nuclei were microdissected from the PEN membrane, but chromosome spreads on PEN membrane coverslips clumped and were not successfully catapulted. Teflon-coated coverslip slides, TCCS, were developed to facilitate the microdissection of small sized tissue. Japanese quail

chromosome spreads dropped onto TCCS slides were not clumped; single cell nuclei, macro-, and microchromosomes were individually catapulted into PCR caps. The actin gene was amplified from cell nuclei, single macrochromosome, single microchromosome and sequenced. The nucleotide sequence from the single chromosomes or cell nuclei showed 80% homology to B-actin of chicken, *Gallus gallus*.

A portion of a single copy control gene, Chpkci located on the Z chromosome and Wpkci located on the W chromosome, was identified and sequenced for Japanese quail as a technique control for single copy genes. The ChpkciE1-F and WpkciI1-R primers were used as DNA controls for the proinsulin transgenic experiments because of unknown embryo sex. The ChpkciE1-F and WpkciI1-R primers were used in the PCR reaction of single cell nucleus from the G₂ Japanese quail and single cell nucleus from normal Japanese quail as DNA controls and nucleotide sequence was similar to *Gallus gallus*.

Potential proinsulin transgenic G₂ Japanese quail chromosome preparations were prepared from embryos and dropped onto TCCS slides. Each individual chromosome was catapulted with the PALM laser microscope into separate microfuge tubes from a single chromosome spread and proinsulin primers were used in PCR amplification. The proinsulin transgene was inserted into 5 chromosomes, 1 macrochromosome and 4 microchromosomes as determined by gel electrophoresis. Each band from a positive chromosome was sequenced and resulted in nearly identical sequence to the original vector, the only

difference being a single nucleotide error in the EninspyA2 primer.

To my knowledge, this is the first report of the use of Teflon-coated coverslip slides to overcome the obstacle of small tissue size which has hampered potential use of laser microdissection. Additionally, this is the first report of using laser microdissection for transgenic animal identification. Although this study demonstrated the usefulness of laser microdissection which showed chromosomal insertions of the proinsulin transgene and nucleotide sequence of each chromosomal insertion, future studies should determine adjacent nucleotide sequences to the inserted transgene. Laser microdissection also has the potential to solve the chromosome identity problem in avian genetics by creating chromosomal paints for each chromosome pair in the chicken or Japanese quail.

Vita

Lacey Renn McNally from Memphis, Tennessee, is the daughter of Molly McNally. Lacey's brother, Kelsey, graduated from the School of Veterinary Medicine, Louisiana State University, with his master's degree in December of 2003. Lacey graduated from Houston High School, in Germantown, Tennessee, in 1996. Lacey went on to the University of Memphis where she was the President of the University of Memphis Honors Program and played bassoon the University's Wind Ensemble. She was a graduate assistant for Dr. Melvin Beck and taught the genetics lab for several semesters. She graduated with a Bachelor of Science degree in biology after two years and continued her education at the University of Memphis where she obtained her master's in biology the summer of 2000. She began her doctoral program at Louisiana State University School of Veterinary Medicine in the fall of 2000. Her major research interest was identifying genes in avian chromosomes through *in situ* hybridization and laser microdissection.