COMPARATIVE MAPPING: HOMOLOGY WITHIN THE ORDER PERISSODACTYLA OF FOUR GENES LOCATED ON EQUUS CABALLUS CHROMOSOME 20

Christine Marie Mains
University of Kentucky, christinemains@yahoo.com

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ABSTRACT OF THESIS

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Since changes in chromosome morphology contribute to the knowledge of evolution as well as to chromosome dynamics, this study looks specifically at one chromosome compared in twelve different species of Perissodactyls: Equus caballus (ECA), E. przewalskii (EPR), Equus africanus somaliensis (EAF), E. asinus (EAS), E. hemionus onager (EHO), E. h. kulan (EHK), E. h. kian (EKI), E. zebra hartmannae (EZH), E. grevyi (EGR), E. burchelli (EBU), Tapirus indicus (TIN), and Rhinoceros unicornis (RUN). While chromosome morphology studies have been done in some of the extant equids, none have followed the evolution of this chromosome, homologous to Equus caballus chromosome 20 (ECA20), which contains the major histocompatibility complex (MHC).

The gene order on the chromosome arm homologous to human chromosome six in most Equidae is reversed with respect to the centromere in comparison to humans. Multicolor fluorescence in situ hybridization was used to show that four probes from ECA20 hybridized to ECA20 (control), SWA5, EAS8, EHO16, EHK14, EKI16, EZH10, EGR11, EBU13, TIN4, and one of RUN12, 14, 15, or 22. The order for the four genes in the horses, zebras, and rhinoceros were as follows: cen-EDN1-MHC-ITPR3-MUT. Hybridization to the ass and tapir chromosomes displayed a possible neocentromere formation. It is apparent the chromosome has gone through several morphological changes while undergoing speciation in the Equidae, yet the overall gene order is conserved.

KEYWORDS: Chromosome Mapping, FISH, Equus caballus, Equidae, Major Histocompatibility Complex

Christine Marie Mains
8/26/04

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Christine Marie Mains

Teri L. Lear
Director of Thesis

Thomas Chambers
Director of Graduate Studies

8/26/04
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COMPARATIVE MAPPING: HOMOLOGY WITHIN THE ORDER PERISSODACTYLA
OF FOUR GENES LOCATED ON EQUUS CABALLUS CHROMOSOME 20

THESIS

A thesis submitted in partial fulfillment of the
Requirements for the degree of Master of Science in the
College of Agriculture at the University of Kentucky

Christine Marie Mains
Lexington, Kentucky

Director: Dr. Teri Lear
Lexington, Kentucky
2004

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DEDICATION

I dedicate this thesis to my parents, Trish and Edward Mains, without whom, I could not have succeeded.
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Chapter 1: Thesis Introduction

I. A Brief History

The history of our knowledge of chromosomes and inheritance is summarized briefly in figure 1.1 below (reviewed in: McFeely 1990, Sumner 2003, and Lewis 1995). In 1847, Torok first observed the process of mitosis, or the replication of “essential information” in the nucleus of a cell in preparation for replication. Virchow first looked at this “essential information” in the nucleus in 1857, though he did not know what he was looking at, nor did these objects have a name. Two years later, Charles Darwin published *The Origin of the Species*, the leading book on the process of evolution. In 1866, a monk named Gregor Mendel published a paper “Experiments in Plant Hybridization”, reviewing the methods of inheritance in pea plants. In 1882, Flemming stained chromosomes using Perkin’s stain, which contained aniline and potassium dichromate. Weismann (1887) developed his theory of inheritance, stating that the nuclear substances of the egg and the sperm are equal and join together during sexual reproduction to produce variability by passing half of the information from each parent to their offspring. The term “chromosome” was not actually coined until 1888 by Waldeyer who derived it from “chroma” meaning color, and “soma” meaning body. Three men separately referenced Mendel’s paper in their own research in 1900, refreshing the scientific community’s mind: Hugo de Vries, Carl Correns, and Tschemak-Seyenegg (Dunn 1965). In 1903 Sutton and Boveri associated chromosome behavior in cells with the conclusions of Mendel’s “Experiments in Plant Hybridization”, nearly forty years later. Thomas Hunt Morgan was a skeptic of this theory until 1910 when he showed Mendelian inheritance in the recessive white eye color in *Drosophila melanogaster* (Morgan 1910). Three years later one of his students, Sturtevant, demonstrated that genes occur linearly on a chromosome. The Nobel Prize in Physiology or Medicine was awarded to Morgan in 1933 for these breakthrough discoveries made in genetics. In 1952 Hsu found that using a hypotonic solution on cells prior to fixing them to a slide spread the chromosomes for easy counting. T.C. Hsu and Kurt Benirschke published the *Atlas of Mammalian Chromosomes* in 1967, detailing the karyotypes of over 400 mammalian species. Caspersson and Zech developed a banding method in 1970 using
quinacrine mustard, which allowed for the identification of all human chromosomes. One year later, Seabright developed G-banding using Giemsa stain, a technique still practiced thirty-four years later. Bauman first practiced fluorescent in situ hybridization (FISH) in 1980; this process will be described in more detail in the following text. Three years later, one of the most widely used molecular techniques was invented, polymerase chain reaction (PCR), by Kary Banks Mullis.

![Timeline of selected genetic events](http://bacpac.chori.org/equine241.htm)

**Figure 1.1:** Timeline of selected genetic events

II. **Fluorescence in situ hybridization (FISH)**

FISH is a process utilizing fluorescent labeling of DNA to visualize the location of a gene on a metaphase chromosome. FISH probes can be made using a variety of source DNA. This source DNA ranges in size depending upon the plasmid or vector used. For example, inserts of genomic DNA 171 kilobase pairs (Kb) in size are inserted into a bacterial artificial chromosome (BAC) and replicated in the host (http://bacpac.chori.org/equine241.htm). These bacterial clones, collectively called a library, can be scanned for a gene or part of a gene contained in their insert by PCR or radioactivity, grown, and isolated of their DNA. This bacterial chromosome, and more importantly the insert, can be labeled radioactively or by fluorescence, called a “probe”. The probe is then denatured, and applied to metaphase chromosomes that have been isolated and prepared from lymphocytes or fibroblasts of the animal being studied and
applied to glass slides. In turn, the fluorescent DNA probe hybridized to the metaphase chromosome of interest can be visualized using a fluorescent microscope equipped with different filters that distinguish between wavelengths of emitted light producing fluorescent colors. (Summary in Levsky & Singer 2003.) Figure 1.2 below shows a schematic of the process.

**Figure 1.2:** Schematic of the FISH experimental process

FISH has been employed for simple mapping projects, the simplest of which is visibly showing the location of a gene on a chromosome. These techniques can be expanded to look at the relative locations of one gene to another on the same chromosome or chromosome arm. Furthermore, the genes of one species can be hybridized to the chromosomes of a closely related species to look at the homology or similarity between the animal genomes. The expansion of this principle across several species can aid in looking at the process of evolution using parsimony and can add to current knowledge of chromosome evolution. Not only genes have been labeled; repetitive elements of telomeres, centromeres, and other heterochromatic regions have also been visualized using FISH (Levsky & Singer 2003). Diseases caused by microdeletions of DNA can be diagnosed using FISH, such as Cri-du-chat and Prader-Willi/Angelman Syndromes (Nussbaum 2001).
III. Perissodactyla

The order Perissodactyla is made up of three families: Equidae (horses, zebras, true asses, and Asiatic asses), Tapiridae (tapirs), and Rhinocerotidae (rhinoceroses). Each family is characterized by a number of species, distinguished via multiple characteristics, including: toe number, dental formula, location, mitochondrial DNA, and body size and shape.

The Equidae have been characterized as ten species: domestic horse (*Equus caballus*) 2n=64, Przewalski’s horse (*E. przewalskii*) 2n=66, donkey (*E. asinus*) 2n=62, Somali Wild Ass (*E. africanus somaliensis*) 2n=62(63, 64), kulan (*E. hemionus kulan*) 2n=54(55), onager (*E. h. onager*) 2n=56(55), kiang (*E. kiang*) 2n=52(51), Hartmann’s or mountain zebra (*E. zebra hartmannae*) 2n=32, Burchell’s zebra (*E. burchelli*) 2n=44(45), and Grevy’s zebra (*E. grevyi*) 2n=46. The numbers in parenthesis indicate polymorphisms in the population. Figure 1.3 shows how these species are related based upon their mitochondrial DNA as described by Oakenfull (2000).

The species of Equidae have a rapidly evolving karyotype (Bush, et al. 1977), and therefore have rearrangement in their respective genomes, which will be discussed further in Chapter 3. Evolution of the Equidae has been previously studied using physical means such as morphological postcranial characteristics (Harris & Porter 1980), cranial and dental structure (Eisenmann 1974), and cranial characteristics (Bennet 1980). Mitochondrial DNA (George & Ryder 1986), protein studies (Henry, et al. 1991, Kaminski 1979, Clegg 1974, Flint 1990, and Lowenstein & Ryder 1985), and comparative mapping (as reviewed by Forozan and co-workers 1997) are a few molecular ways to standardize the evolution of these animals.
Figure 1.3: A simplified phylogenetic tree of the family Equidae based on mitochondrial DNA (Oakenfull 2000).

Tapiridae have been characterized as four species: *Tapirus terrestris* (South American tapir) 2n=80, *T. pinchaque* (mountain tapir) 2n=76, *T. bairdii* (Baird's tapir) 2n=80, and *T. indicus* (Malayan tapir) 2n=52.

Rhinocerotidae have been characterized as five species in four genera: *Dicerorhinus sumatrensis* (Sumatran rhinoceros) 2n=82, *Rhinoceros sondaicus* (Javan rhinoceros) 2n=unknown, *R. unicornis* (Indian rhinoceros) 2n=82, *Diceros bicornis* (Black rhinoceros) 2n=84, and *Ceratotherium simum* (White rhinoceros) 2n=82.

IV. Comparative mapping in Equidae

Using a heterologous probe (DNA from one species hybridized to the chromosomes of another species) in the process of FISH allows for the comparison of genes among species, and is termed “comparative mapping”. In this type of experiment, gene order can be compared between species while the chromosomes may have gone through changes. The genomes can be compared and the evolution of the animal or animals from a common ancestor can be deduced by the utilization of parsimony. In this study, four syntenic genes of the horse were compared to the order found within genomes of eleven other Perissodactyla species, including nine within the family Equidae. Similar studies have been done comparing gene order from twenty horse chromosomes to donkey chromosomes (Raudsepp, et al. 2001); selected genes
from all horse chromosomes except ECA27 and Y to human chromosomes (Milenkovic, et al. 2002); two genes from horse to kulan, kiang, onager, Burchell’s zebra, and Somali wild ass chromosomes to map a fission-fusion event (Myka, et al. 2003); and one gene from every chromosome arm of the horse except 11q, 12p, and 27q to Przewalski’s horse chromosomes (Myka, et al. 2003). Other comparative mapping studies have been done in these species using whole chromosome paints. Raudsepp and Chawdhary compared horse metacentric autosomes, sub-metacentric autosomes, and sex chromosomes to those in the donkey (1999) as well as horse chromosomes 9, 12, 15, 16, 19, and 20 (2001). They also compared horse chromosome 3 with donkey and human chromosomes (1999). Yang (2004) created a complete set of painting probes for the domestic horse and obtained a set available for human to compare entire horse, donkey, mule, and human genomes. Yang and co-workers (2003) also used cross-species chromosome painting to reveal genome-wide chromosomal rearrangements between horse, Grevy’s zebra, and Burchell’s zebra, as well as between horse and Przewalski’s horse, showing that ECA20 was conserved as an arm through all the species studied. Richard and co-workers (2001) used human chromosome painting probes to deduce that HSA6/ECA20 is syntenic with mountain zebra (EZH) chromosomes 4, 7, and 10. Chaudhary and co-workers (1998) compared human chromosomes 2, 5, 6, 16, and 19 with pig and horse chromosomes. Trifonov and co-workers (2003) also showed homology between Burchell’s zebra and White and Black Rhinoceros chromosomes.

V. Major Histocompatibility Complex

The Major Histocompatibility Complex (MHC) was chosen as a subject for comparative mapping based on the extensive information available about MHC genes and knowledge about the structure of the complex in many species. The basic role of the MHC is thought to provide an immune response to a wide variety of infectious agents. The MHC is divided up into three groups of genes called class I, class II, and class III. Class I genes produce proteins that participate in the presentation of antigen to cytotoxic T cells (CD8+). Class II genes produce proteins necessary for antigen presentation to helper T cells (CD4+). Between the class I and class II genes of
humans are a set of genes not related to class I or class II genes, but conserved in the MHC of most species, called class III. Class III genes have a wide variety of functions. The MHC has been mapped in several species, and primarily the order of class I, II, and III clusters has been preserved, but there are a few exceptions to that rule. The cattle class II genes have been found to consist of two regions on chromosome 23 (Band, et al. 1998). The Swine MHC spans the centromere (Smith, et al. 1995). The class I genes are on a different chromosome from class II genes, and there appears to be a duplication of class I genes on at least one other chromosome in Rainbow trout (Phillips, et al. 2003). Class III genes of the chicken are located outside the class I and class II genes (Kaufman, et al. 1999). Recently, a contig map was supplied for the horse MHC, showing the equine MHC to be organized in the same manner as in humans and in mice, but reversed with respect to the centromere (Gustafson, et al. 2003). (See figure 2.1.)

VI. MHC mapped in Equidae

The MHC has been mapped in the domestic horse to chromosome 20 (Ansari et al. 1988) but not in the other members of the Equidae. The purpose of this project is to investigate the extent to which the region surrounding MHC has been conserved among the Equidae. Based on the previous studies mentioned here, we can anticipate which chromosome shares homology with ECA20 in a few of the extant species. It can be deduced that MHC will occur on chromosome 18 in the Przewalski’s horse (EPR18), because the MUT gene was FISH mapped to this chromosome location (Myka, et al. 2003). We anticipate MHC hybridization to donkey chromosome 8 (EAS8) based on previous mapping of ECA20 genes (GNL1, HSPA4, and OR10C1) by Raudsepp and co-workers (2001). Yang and co-workers (2004) used a horse chromosome paint to ECA20, which further confirms this homology, as well as contributes to the understanding of the karyotypic evolution among the Equidae. Yang and co-workers (2003) describe how chromosome specific painting probes were used to compare horse, Hartmann’s zebra, and Burchell’s zebra. This paper shows homology between ECA20, EBU13, and EZH10. Richard and co-workers (2001) also compared human chromosomes to those of Hartmann’s zebra using human chromosome specific painting
probes, showing homology of HSA6 to EZH10, 7, and 4. Comparative mapping studies for the MHC region of ECA20 have not been done in kiang, kulan, onager, or Grevy's zebra.

VII. Chromosome morphology

The evolution of a species is often associated with chromosome rearrangement. The mechanisms for these rearrangements are active areas of research. Many chromosomes have “weak” points called break-points or fragile sites. These sites have been studied in the horse and results indicate fragile sites in each of chromosomes 1, 4, 8, 11, 16, 17, 23, and X (Ronne 1992). No break point has been found within the horse MHC region. A study in cattle showed two fragile sites in chromosome 23 where the MHC is located (Rodriguez, et al. 2002). One of these fragile sites could have been responsible for the evolution of two MHC regions in cattle. A study showed that a breakpoint occurs in human chromosome 6p22 (Tchinda, et al. 2004), the MHC is located on chromosome 6p21.3, directly adjacent to the breakpoint.

Chromosomes also evolve by fission-fusion events. According to Imai and co-workers (2001) fission events are the major contributors to speciation. Figures 1.4 and 1.5 show the difference between chromosome fusion and fission.

![Figure 1.4](image)

**Figure 1.4:** Two acrocentric chromosomes line up via the centromere and fuse creating a metacentric chromosome during a fusion event.
Figure 1.5: One metacentric chromosome splits at the centromere and one of the arms forms a new centromere to form two acrocentric chromosomes during a fission event.

This process causes an overall change in chromosome number. Fusion events cause the diploid chromosome number to decrease, while fission events increase it.

Another mechanism for altering chromosome morphology is the establishment of a neocentromere, or formation/replacement of one centromere in the chromosome for a new centromere. Centromeres are necessary for attachment to the spindle fibers for proper chromosome segregation during mitosis and meiosis. The composition of centromeres remains unknown. Centromeres may contain a highly repetitive element such as in budding yeast (Saccharomyces cerevisiae) (Clarke 1998). However, studies have shown that a repetitive element is not essential for centromere formation (Choo 1997; Barry, et al. 1999; Williams, et al. 1998; and Fuchs, et al. 1998). Centromeres may be determined by methylation blocking the activity of the minor satellite DNA (Mitchell, et al. 1996). The conditions necessary for neocentromere formation remain unknown.

Chromosomes may also go through internal changes such as pericentric or paracentric inversions, when an internal portion of the chromosome breaks from the ends and reattaches to the opposite ends. Translocations may also occur, when two different chromosomes exchange arms or portions of arms. Some chromosomes may even form rings by fusing at the ends or at breakpoints (Nussbaum 2001).
VIII. Objectives

This project had three main objectives:

Objective 1: To discover if the chromosome location and chromosome morphology of the MHC class I and class II genes among Equus africanus somaliensis, E. burchelli, E. grevyi, and E. kiang are conserved relative to that of the horse.

Objective 2: To discover if gene order was conserved throughout the chromosomes possessing MHC among all of the extant Equidae.

Objective 3: To determine the map position and if the morphology of the region surrounding the MHC genes are conserved among rhinoceroses and tapirs, the other representatives of the Perissodactyla.
Chapter 2: Determination of MHC class I and II location in Equus burchelli, E. grevyi, E. kiang, and E. africanus somaliensis

I. Introduction

The Major Histocompatibility Complex (MHC) has been extensively studied among animal species. The primary function of the MHC is to bind to a wide variety of antigens. Genes within the complex encode proteins involved in a wide variety of immune responses (Trowsdale 2001). The genes that compose the MHC are clustered into three groups, designated as class I, II, and III. Class I and II genes code for proteins that present antigen sections to T-cells. Class III genes are those genes that fall into neither class I or class II. In most species, the gene classes are located together on the same chromosome; however, the class order may be rearranged. For example, the arrangement of the classes in humans, when starting at the centromere and moving distally is class II, class III, and class I. In mice and rats it is similar except for a set of class I genes inserted between the centromere and the class II genes (Helou, et al. 1998). In cattle, the order is reversed with respect to humans, and the class II genes are separated into two distinct groups (Band, et al. 1998). Class III genes are on the outside of the class I and class II genes in chicken (Kaufman, et al. 1999). The gene classes of bony fish like rainbow trout, are on different chromosomes as well as both class I and class II regions are split into two groups, resulting in four chromosomes containing parts of the MHC, or “MH” since the complex is too divided to be a complex (Phillips, et al. 2003, Quiniou, et al. 2003). (See figure 2.1)
Figure 2.1: A visual representation of the MHC in several animals: Black dots represent the centromere. Human (Complete sequence and gene map of a human major histocompatibility complex. The MHC sequencing consortium 1999), mouse/rat (Helou, et al. 1998), cattle (Relative MHC class III location is unknown.) (Skow, et al. 1996), chicken (ABCB2A is associated with MHC class I.) (Kaufman, et al. 1999), pig (Smith, et al. 1995), rainbow trout (Phillips, et al. 2003), and horse (Gustafson, et al. 2003). The diagram is not to scale for the relative sizes of the chromosomes or the sizes or locations of the MHC class regions. The MHC has already been mapped to domestic horse chromosome 20 (Ansari, et al. 1988, Mäkinen, et al. 1989) but not explored in the other Equidae species. In order to infer the gene location in these species, four MHC BAC clones were obtained and FISH mapped to metaphase chromosomes. One or two animals were chosen from each phylogenetic group: *Equus burchelli* (EBU) and *E. grevyi* (EGR) from the zebras, *E. kiang* (EKI) from the Asiatic asses, and *E. africanus somaliensis* (EAF) from the true...
asses. This chapter focuses on the location of those genes relative to one another in the genomes of these four Equidae species.

II. Materials and Methods

A. Chromosome Preparation

Metaphase chromosome spreads were prepared by the Center for Reproduction of Endangered Species (CRES) at the San Diego Zoo. Animals referenced by their studbook numbers were used for the following study: Equus. burchelli (EBU) #12781, E. kiang (EKI) #13264, E. africanus somaliensis (EAF) #11061, and E. grevyi (EGR) #unknown. Chromosomes were prepared as previously described (Kingswood, et al. 1998) from fibroblast cell cultures from solid tissue. On a sterile glass plate, a tissue biopsy was minced into tiny fragments with sterile forceps and scalpel. The biopsy was not allowed to dry; it was kept moist with media. The tissue was placed into two T25 culture flasks. The flasks were placed on a warming tray at 37° C until the fragments had adhered. Media (5 cc) was added to each flask. Flasks were incubated in an open system 6% CO2 incubator. The cultures were given fresh media twice a week. The media was removed and the cells were rinsed with 4 cc Earle's balanced salt solution (EBSS). EBSS was removed and 4 cc ATP was added and swirled around flask to coat. All but 1 cc ATP was removed. The flasks were placed on a warming tray set at 37° C until cells detached. Media was added (10 cc) to the flask and swirled around to coat the cells in media. The cells were transferred to a T75 culture flask and the primary cells were re-fed in media. Both cultures were incubated. The flasks were checked for confluency for harvesting. If mitotic cells were abundant, 50 µl colcemid was added for up to 120 min. If few mitoses were evident, 50 µl colcemid was added and the flasks were incubated for 4-6 hours. The culture was transferred to a 15 ml centrifuge tube. EBSS (3 mL) was added to the flask to rinse media. The rinse was poured into the centrifuge tube. Trypsin/EDTA (1:250) solution was added to detach the cells from the flask. Trypsin solution was poured into the centrifuge tube leaving a tiny amount still in flask. EBSS (3 mL) was added, and poured into the centrifuge tube. The cells were centrifuged 10 min at 120 x g. The supernatant was removed, leaving approximately 0.5 cc. The pellet was gently resuspended. Warmed (37°C) 0.075 M KCl
(7 mL) was added to the cells and mixed gently. The cells were incubated in a 37°C water bath for 6-8 min. Three drops of cold fixative was added to the hypotonic solution while the tube was gently agitated. The solution was centrifuged at 120 x g for 10 min. All but 1 cc of the supernatant was removed and the cells were resuspended. The suspension was pulled into the pasteur pipette, and approximately 6 cc of fixative was added to the tube. The pipette with the cells was placed in the fixative and released in a slow steady stream. The cells were resuspended with pipette thoroughly and incubated at 4°C for min. The fixative wash was repeated at least two more times. After the last rinse, depending on the size of pellet, approximately 0.5-1 cc of fixative was left to make a cloudy suspension.

The slides were immersed into methanol for cleaning. The slides were swirled in water until a sheet of water formed on the frosted side. Two to three drops of cell suspension were dropped across the horizontal axis of the slides and 2-4 drops of fix were immediately placed across the slides on top of the cells. The edges and back of the slides were dried. The slides finished drying according to the relative humidity.

**B. Competitor DNA Preparation**

Competitor DNA was made to anneal to repetitive elements in the probe mix, which might interfere with hybridization of the probe to its counterpart in the genome. Fibroblast cells were grown up from a stock stored on liquid nitrogen. All of the methods involving cell culture were done using sterile technique. The cells were quickly thawed in a 37°C water bath and transferred to a 10 mL sterile tube. The first mL of cell media (see Appendix 2) was added to cells at a rate of one drop per minute, then 3 mL were added slowly. The cells were added to T25 cell culture flasks (BD Biosciences) and grown overnight at 37°C. When confluency was reached, the media was removed and the cells were rinsed with 4 mL of 0.8% sodium citrate. A 4 mL quantity of 1:4 trypsin: 0.8% sodium citrate was added to the flasks to dislodge the cells from the flasks and were rinsed with MEMα and transferred to 15 mL conical centrifuge tubes. They were centrifuged at 120 x g for 8 min. The supernatant was removed and discarded. The cells were resuspended in 3 mL media and transferred to T150 cell culture flasks and grown at 37°C until confluent. The media was removed and 5 mL of 0.8% sodium
citrate was added to the cells. A 5 mL quantity of 1:4 trypsin: 0.8% sodium citrate was added to the flasks to dislodge the cells from the flasks and were rinsed with MEMα and transferred to 15 mL conical centrifuge tubes. The supernatant was removed and discarded. The cells were resuspended in 10 mL media and transferred to two T150 cell culture flasks. Media was added to 30 mL, and the cells were grown at 37°C until confluent. The same procedure was followed to split the cells into 4 T150 cell culture flasks. When the cells reached confluency, they were rinsed, collected, and centrifuged as before. The pellets were combined and resuspended in 10 mL of 55°C SE-buffer and 100 µL of proteinase K was added in a brown Oakridge tube. The cells were incubated at 55°C overnight. Prewarmed (55°C) 6 M sodium chloride (NaCl) was added (2.5 mL) along with 12.5 mL CHIASM. Cells were centrifuged at 15,000 x g for 20 min at 4°C. Supernatant was removed to a clean brown Oakridge tube, 6 M NaCl and CHIASM was added, and the pellet was centrifuged as above. The supernatant was removed to a 50 mL centrifuge tube and 18 mL of 90-100% ethanol (EtOH) was added. DNA was placed at 4°C overnight. The DNA was centrifuged at 20,000 x g for 10 min at 25°C and the EtOH was removed. The DNA was transferred to a 1.5 mL twist-cap tube and washed in 70% EtOH. The DNA was centrifuged at 15,000 x g for 10 min at 4°C. The EtOH was removed and the pellet was dried in a vacuum savant for 30 min. The DNA was resuspended in 800 mL TE and rotated over the weekend. The DNA was run on a .4% agarose gel with ethidium bromide (EtBr) in a ratio of 1 µL EtBr: 40 µL 1x TBE for visualization with the IS-1000 Digital Imaging System (Alpha Innotech Corporation), and an OD reading were used to determine the quantity and quality of the DNA.

Competitor DNA was prepared for FISH hybridization after isolation from the fibroblast cells. NT buffer was added at 500 µL and brought up to 5 mL with sterile, DNase free water. The DNA was sheared through a series of 10 cc syringes: twice through 18 gauge (G), 22 G, and 30 G needles. On ice, 1 µL of DNase I stock solution (1 mg/mL) was added to 1000 µL cold water to make a working dilution. DNase I solution (10 µL) was added to the DNA and incubated at 15°C for 2-12 hours. After 2 hours, the reaction was placed at –20°C to stop the enzyme and 7 µL were run on a 2% agarose gel. If the DNA was not a desirable size (300-500 bp), 10 µL of enzyme was added, the reaction
was incubated again, and run on a gel. This process was repeated until the desired fragment size was reached. (See figure 2.2)

![gel_image](image.png)

**Figure 2.2:** Quality and size verification of *E. grevyi* competitor DNA. Lane 2 contains a 1 Kb ladder. Lane 2 holds competitor DNA. Desirable size is 300 bp.

The reaction was terminated by addition of 125 µL of EDTA and 25 µL of 20% SDS to each 5 mL of DNA solution and heated at 65°C for 20 minutes. The samples were split into 2 Oakridge tubes and precipitated with 1/10 volume 2.5 M sodium acetate and 2.5 volumes of 95% EtOH. The DNA pellets were washed in 70% EtOH, dried overnight under a hood, and resuspended in 400 µL of TE buffer.

C. BAC DNA Preparation

Two MHC class I genes and two MHC class II genes (Table 2.1) were obtained from the James A. Baker Institute for Animal Health at Cornell University as horse BAC clones from the CHORI-241 horse BAC library. The average size of the BAC clones is 13397 base pairs (bp), and the average insert size is 171 Kbp (Vessere 2004). Horse BAC clones were sent from the University on LB agar containing chloramphenicol (12.5 µL/mL). Single colonies were removed to 5 mL of LB broth containing chloramphenicol (12.5µL/mL) and grown 15 hours at 37°C with 230 rpm shaking. When bacterial density was sufficient, 250 µL of culture was added to fresh LB broth and chloramphenicol (125 µL/250 mL medium), and grown as above.
Table 2.1: MHC Horse BAC Clones

<table>
<thead>
<tr>
<th>Clone number</th>
<th>Gene</th>
<th>Comments (Becky Tallmadge, personal communication)</th>
</tr>
</thead>
<tbody>
<tr>
<td>167M09</td>
<td>MHC class I</td>
<td>Hybridizes with α1, α3, and cytoplasmic probes. Appears to contain one classical gene by PCR and sequencing of α1 hypervariable region.</td>
</tr>
<tr>
<td>294E05</td>
<td>MHC class I</td>
<td>Hybridizes with α1, α3, and cytoplasmic probes. Appears to contain two classical genes by PCR and sequencing of hypervariable region.</td>
</tr>
<tr>
<td>288J19</td>
<td>MHC class II</td>
<td>Hybridizes with DQα, DQβ, DRα, and DRβ probes.</td>
</tr>
<tr>
<td>229D23</td>
<td>MHC class II</td>
<td>Hybridizes with DRβ and UM011 microsatellite probes.</td>
</tr>
</tbody>
</table>

The bacterial DNA was isolated by centrifugation of the cultures in sterile 50 µL Oakridge tubes for 15 min (5500 x g). The supernatant was removed and the pellet resuspended in 10 mL of Tris-glucose-EDTA with 10 µL of RNase-It added to 10 mL of sodium hydroxide/ sodium dodecyl sulfate (NaOH/SDS) solution and incubated at room temperature for 5 min. Next, 10 mL of cold Potassium Acetate (KOAc) was added, the tube was inverted to mix the solution, and incubated for 15 min on ice. After centrifugation (20,000 x g) at 4°C for 30 min, the supernatant was decanted to a clean Oakridge tube and centrifuged as before for 15 min. The supernatant was decanted to a clean conical centrifuge tube and purified of proteins and RNA after gravity flow through Qiagen 100 columns (kits #10043 and #12543). Briefly, Qiagen columns were equilibrated with 4 mL of QBT solution, the DNA was applied, and then the columns were washed twice with 10 mL of QC solution. The DNA was eluted into clean 50 mL clear Oakridge tubes five times with 1 mL of 65°C QF solution, to keep the solution at temperature. The DNA was precipitated by adding 10 mL of cold isopropanol to each tube, inverted to mix, and centrifuged (15,000 x g) for 30 min at 4°C. The supernatant was removed and 500 µL of 70% ethanol (EtOH) was used to rinse the pellet off the side of the tube. The DNA was transferred to a 1.5 mL screw-cap microfuge tube and
centrifuged (15,000 x g) for 15 min. After the EtOH was removed, the pellet was air dried 5-10 min and resuspended in 40 µL of TE, pH=8.0. The DNA was run on a 1% agarose gel with ethidium bromide (EtBr) in a ratio of 1 µL EtBr: 40 µL 1x TBE for visualization with the IS-1000 Digital Imaging System (Alpha Innotech Corporation). Purity was checked by looking for a small (less than 50 bp) RNA band and quantified by spotting on a 1% agarose gel with 20, 40, 100, 250, and 500 ng/µL, standards. (See figure 2.3 and 2.4)

**Figure 2.3:** Lanes 1-4 contain the MHC clones to determine purity. Lane 1) 167M09, 2) 299E05, 3) 288J19, 4) 229D23.

**Figure 2.4:** The top squares contain 1 µL of DNA standards in given nanograms. Bottom squares contain 1 µL of MHC clones. I1) 167M09, I2) 299E05, II1) 288J19, II2) 229D23.

**D. BAC Probe Preparation**

Purified MHC clone DNAs were labeled by nick translation with either Biotin or Digoxygen (Dig) following the manufacturers’ instructions (Biotin, Bionick™ Labeling System #18247-015 by Invitrogen; Dig, DIG-Nick Translation Mix #1745816 by Roche). Briefly, for the biotin labeled probes: on ice, 5 µL of 10x dNTP mix, 1 µg of DNA,
distilled water to 45 µL, and 5 µL of 10x enzyme mix were added to a 1.5 mL microfuge tube to make a 50 µL reaction. The mix was incubated at 16°C for 60 min. Three microliters of the reaction were removed to a microfuge tube and placed at 95°C for 5 min. The tubes were then placed on ice for 2 min and 1 µL loading buffer was added. Samples were run next to 3 µL of 1 Kb ladder with 1 µL of loading dye on a 2% agarose gel with EtBr as above. When the tested reaction mixes produced a smear between 100-300 base pairs on a 2% agarose gel, 5 µL of stop buffer was added to stop the reaction. (See figure 2.5)

![Figure 2.5](image)

**Figure 2.5:** Nick translation of biotin probe. Lane 1 holds a 1 Kb ladder. Lane 2 holds the labeled biotin probe. Ideal size is about 300 bp as shown.

Unincorporated nucleotides were separated from the probes by ethanol precipitation: one-tenth volume of 3 M sodium acetate (NaOAc) was added, followed by 2 volumes of cold 100% EtOH, inverted, and placed at -20°C for 2 hours. The tubes were centrifuged (15,000 x g) for 10 min. at 4°C. The supernatant was decanted and the pellets were rinsed in 70% EtOH, and centrifuged as above. The pellets were dried in a vacuum savant for 15 min and resuspended in 40 µL of TE, pH=8.0. The probes were quantified the same fashion as the BAC clone DNA in section B. (See figure 2.6)

![Figure 2.6](image)

**Figure 2.6:** The top row contains DNA standards in nanograms. The bottom row contains biotin labeled probes: 1) 167M09, 2) 299E05, 3) 288J19, 4) 229D23.
Dig probe was labeled using a similar process: 1 µg of template DNA was added up to 16 µL of sterile water; 4 µL of DIG-Nick Translation mix was added and incubated at 15°C for 90 min. DNA was tested by running on a 2% agarose gel and stopped at 200-500 base pairs with 1 µL of 0.5 M EDTA buffer (pH=8.0) and heated to 65°C for 10 min. (See figure 2.6) Ethanol precipitation as described for the biotin labeled probes to remove the unincorporated nucleotides, and the pellet was resuspended in 40 µL of TE, pH=8.0.

E. Probe Hybridization

Hybridizations were done as previously described (Bailey, et al. 1997). Briefly, for every 22 mm x 22 mm slide area, 100 ng of probe, 4 µg of exotic competitor DNA, 4 µg of horse competitor DNA, and 6 µg of salmon sperm DNA were mixed on a shaker for 30 min in a 1.5 mL microfuge tube and ethanol precipitated as above. Formamide (5.5 µL) was added to the pellet and placed on vortex shaker for 30 min. Dextran sulfate/ 2x SSC (5.5 µL) was added and shaken 15 min. The probe mix was placed at 75°C for 10 min to denature the DNA, and 37°C for 60 min for annealing of competitor DNA to repetitive elements.

Slides were removed from -70°C and run through 70%, 80%, and 90% EtOH, respectively, for 2 min each. After air-drying, they were checked for metaphase spread quality and quantity using phase contrast microscopy. Slides were denatured in solution in a coplin jar for 2 min at 70°C (+1°C/slide) and immediately run through a -20°C EtOH series as above (70%, 80%, and then 90%) to stop the denaturation process, allowed to air dry, and placed on 42°C slide warmer with cover slips while the probe was being prepared.

Eleven microliters of probe mix was added to each 22 mm x 22 mm slide area, which was then covered with a glass coverslip, sealed with rubber cement, and allowed to hybridize at 37°C for 48 hours in a humidified, sealed container.

F. Slide Washes and Counterstain

Following slide hybridization for 48 hours, washes were done under low stringency (for heterologous probes) at 37°C by washing three times in 50% formamide/
2x SSC pH=5.3 for 5 min each. This was followed by three 3 min washes in 2x SSC pH=7.0. Slides were placed in 1x PBD pH=8.0 until antibodies were applied. Probe signal was visualized following the manufacturers’ directions (Avidin-FITC, Ventana #51370-9; αDig-Rhodamine, Roche #92128821). Briefly, blocking solution (200 µL) was placed on each slide and incubated in a humidified chamber for 30 min. Sixty microliters of Avidin-FITC or αDig-Rhodamine (diluted 5 µL of antibody stock in 495µL of water or maleic acid solution) was placed on appropriate slides, which were covered with a plastic cover slip and incubated in a humidified chamber for 15 min. The slides were washed 3 times in 1x PBD for 2 min each. Thirty microliters of 4’, 6-Diamidino-2-phenylindole (DAPI) (50 ng/mL) was placed on each slide and covered with a glass coverslip. Placing the slide in a bibulous paper notebook and gently rubbing pressed air bubbles out. DAPI was allowed to absorb for 20-30 min before viewing the slide.

G. Visualization and Analysis

Analysis was done using Zeiss Axioplan2 fluorescent microscope and images were captured using Cytovision©/Genus™ Application Software Version 2.7 (Applied Imaging). The slide was scanned in its entirety for metaphase spreads with elongated, DAPI-banded chromosomes to make chromosome identification easier. No fewer than ten pictures were taken to show the positive hybridization of probe to a chromosome. Chromosomes were identified using the banding patterns from standardized karyotypes from previous studies (OA Ryder 1978) and Marlys Houck (personal communication).

III. Results

All four equine BAC probes hybridized to the metaphase spreads of EBU, EGR, EKI, and EAF. The probes hybridized with minimal background and were visualized as two bright fluorescent points on each sister chromatid. For each species, the four MHC class I and class II genes localized to the same region on their respective chromosomes. The DAPI banding pattern is similar to the G-banding pattern, dark staining areas are G-C rich. Using standardized karyotypes, by studying the banding pattern, chromosome size, and chromosome arm morphology, the chromosome could be narrowed down to one or a few possible chromosomes. The MHC hybridized to the
q arm of chromosome 13 in EBU, the q arm of one of the chromosomes 9-11 in EGR, the q arm of one of the chromosomes in EKI (16-18), and the p arm of chromosome 5 in EAF. (See figure 2.7)

![Figure 2.7: FITC labeled MHC in green and Rhodamine labeled MHC in red. A) EBU13q B) EKI16-18q C) EAF5p D) EGR9-11q](image)

IV. Discussion

This study demonstrated that the MHC class I and class II genes mapped together on the same chromosome arm in each of the four species: EBU13q, EKI16-18q, EAS5p, and EGR9-11. Therefore, the organization of the MHC in these species appear to be more like that found in humans and mice, in one cytogenetic location, and not like that of bony fish (MHC classes on separate chromosomes), cattle (classes separated by a large amount of non-MHC DNA on one arm), or pigs (bisected by the centromere).

Though the gene classes remained together, the different chromosome morphology indicates evolutionary change. In the domestic horse, the MHC maps to an acrocentric chromosome (ECA20), whereas in the other species studied, the location is on a metacentric chromosome. To complicate the matter further, the MHC is on a long arm of the chromosome in the Asiatic asses and zebras, and in the true asses, it resides on a short arm. This chromosome has changed somehow. At this point in the study, it seems that the zebras and Asiatic asses may be more closely related since their chromosome morphology and MHC locations are similar.
Chapter 3: Comparative mapping of ECA20 syntenic genes to the chromosome of nine species of Equidae

I. Introduction

Within Equidae, species exhibit a wide ranges in diploid chromosome number (2n) ranging from 2n=32 in *Equus zebra hartmannae* to 2n=64 in *Equus przewalskii*. This wide range of chromosome number within a closely related species shows that Equidae chromosome morphology and number are changing quickly over time (Bush, et al. 1977). One mechanism for modification is a fission-fusion event, causing a change in chromosome number. Ryder (1978) proposed this idea first based on G-banded chromosomes. Whitehouse and co-workers (1985) found a fission event in *E. burchelli*, while Houck and co-workers (1998) found a centric fission event in the *E. a. somaliensis* pedigree. Myka and co-workers (2003) characterized a centric fission in chromosomes among five of the ten species of Equidae. Comparing genome organization among species within a genus provides information on chromosome evolution. As described here, genes flanking the MHC of the horse were mapped to chromosomes in nine of the species of Equidae. Four genes spanning the length of ECA20 were chosen based on their location. These genes were FISH mapped, and the results were used to propose an ancestral chromosome and what evolutionary changes that chromosome may have undergone.

II. Materials and Methods

A. Chromosome Preparation

Metaphase chromosomes were prepared by CRES (except *E. caballus* and *E. asinus* which were prepared from lymphocytes at the University of Kentucky) as described in the Materials and Methods section of Chapter 2. Animals used for this study were referenced by their laboratory of origin accession number: *Equus caballus* (ECA) #EQP71, *E. przewalskii* (EPR) #1237, *E. asinus* (EAS) #EQP6, *E. hemionus onager* (EHO) #4791, *E. h. kulan* (EHK) #5431, *E. h. kiang* (EKI) #13338, *E. grevyi* (EGR) #4931, *E. burchelli* (EBU) #12781, and *E. zebra hartmannae* (EZH) #10833. Lymphocytes were collected from blood. Briefly, 5 cc heparinized whole blood was
collected by sterile technique and transferred to a heparinized vacutainer. The blood was centrifuged at 120 x g for 10-15 min. The buffy coat and plasma layer (ap/bc) were removed using a 3 cc syringe with an 18 or 20 G needle. For each 0.5 cc of ap/bc recovered a T25 flask was set up with the following components: 9.5 cc media, 100 µl sodium heparin, 0.5 cc ap/bc, and 250 µl phorbol. Cells were cultured, harvested, and dropped on slides as described in the Materials and Methods section of Chapter 2.

B. BAC DNA Preparation

One of the clones from Chapter 2 of this manuscript was used to represent the MHC, which functions in basic immune response. The genes mapped include: endothelin 1 (EDN1) is located near the centromere in horse and functions as a vasoconstrictor; inositol 1,4,5-triphosphate receptor, type 3 (ITPR3), located distal to both EDN1 and the MHC, mediates the release of intracellular calcium; methylmalonyl coenzyme A mutase (MUT) is located distal to EDN1, MHC, and ITPR3 and has differing functions between species but primarily acts to degrade protein. EDN1 was mapped to ECA20 by Caetano and co-workers (1999), several genes were mapped from MHC to this chromosome by Newkirk and Skow (unpublished data), Lear and co-workers (2001) mapped ITPR3 to this chromosome, and Myka and co-workers (2003) confirmed MUT location and determined the same chromosome was involved in five of the equid species.

Figure 3.1: The mapped order of the four genes used in this experiment. EDN1, MHC, ITPR3, and MUT.

FISH mapping these four genes described earlier permitted comparison of genome organization around the MHC region in the Equidae.
Equine BAC DNA was prepared as described previously in the Materials and
Methods section of Chapter 2. Since all four MHC horse BAC clones hybridized to the
same location presented in Chapter 2, one clone (288J19) was chosen to represent the
gene. ITPR3 (INRA66C7) and MUT (INRA360A12) were previously stored in our –70°C
freezer. Primers were developed for EDN1 from Venta et al (1996) and tested on
genomic DNA with FastStart Taq DNA Polymerase (Roche #2 032 937 250). The PCR
conditions as follows. The following solution with the final concentrations was made:
2mM 10x PCR buffer, 1.5-4mM magnesium chloride solution, 200 µM dATP, 200 µM
dCTP, 200 µM dGTP, 200 µM dTTP, 0.2-1 µM forward primer, 0.2-1 µM reverse primer,
q.s. to 50 µL with distilled water, 2 U of FastStart Taq DNA Polymerase, and 500 ng of
template DNA. The PTC-200 Peltier Thermal Cycler (MJ Research) was used with the
following program: 1) Denaturation/activation took place for 10 seconds (sec) at 95°C,
2) Denaturation again for 30 sec at 95°C, 3) Annealing was set at 59°C for 30 sec, 4)
Elongation at 72°C for 30 sec, 5) Repeat steps 2-4 x39, 6) Final extension at 72°C for
10 min, and 7) Cool 1°C/sec to 4°C. Two microliters of the PCR product were run on a
1% gel with one microliter of loading dye next to a 1 Kb ladder to make sure the PCR
product ran at the expected size. (See figure 3.2)

Figure 3.2: A 1 Kb ladder is in lane 1. PCR product for EDN1 is in lane 2.
Single band shows proper amplification. A duplicate was run in lane 3.

The following cocktail was made for the sequencing reaction with Big Dye
(Applied Biosystems #4336774): 8.0 µL of Big Dye sequencing mix, q.s. to 20 µL with
distilled water, 3 ng of template DNA, 3.2 pmol of forward or reverse primer (make
separate cocktails for each primer). Cocktails were placed in a thermal cycler for the
following sequence: 1) 1°C/sec up to 98°C, 2) 98°C for 15 sec, 3) 1°C/sec to 50°C, 4)
50°C for 5 sec, 5) 1°C/sec to 60°C, 6) 60°C for 4 min, 7) repeat steps 1-6 x35, 8) 4°C.
These products were then cleaned of the Dye terminators using the Centri-sep (Princeton Separations #CS-901) protocol.

Briefly, columns were hydrated with .8 mL of distilled water for 30 min. Air bubbles were removed by tapping, and the top then bottom caps were removed. Column was allowed to drain of excess water and centrifuged for 2 min at 750 x g. Twenty microliters of the product was transferred to the top of the columns, directly onto the center of the gel bed. The columns were placed into the sample collection tubes and centrifuged as before. The samples were dried in a vacuum until completely dry, approximately 15 min. The samples were rehydrated in 20 µL of formamide, heated at 95°C for 2 min and placed on ice for 1 min. The products were placed in the AB310 for sequencing, and successfully compared to the Basic Local Alignment Search Tool (BLAST) on Pubmed (http://www.ncbi.nlm.nih.gov/BLAST/) (seen in Appendix 3).

The primers were sent to Colette Abbey, research associate of the Animal Science department at Texas A&M, to be pulled from the CHORI-241 horse BAC library. The clone positive for EDN1 was sent to us on LB agar and chloramphenicol and prepared as described in the Materials and Methods section of Chapter 2. (See image 3.3)

![Figure 3.3: Quantitation of BAC clones ITPR3 and MUT next to standards (in nanograms) on a 0.4% gel. The 10 ng standard was concentrated in the tube. ITPR3 is estimated at 20 ng, MUT is estimated at 150 ng.](image)

C. BAC Probe Preparation

DNA from each of the four BAC clones was labeled with a different color fluorochrome to better visualize the order of the genes. The MHC gene was prepared with biotin (Bionick™ Labeling System #18247-015 by Invitrogen) as in the Materials
and Methods section of Chapter 2. The EDN1, ITPR3, and MUT genes were labeled using the Vysis Nick Translation Kit (# 32-801300) following the manufacturers directions. EDN1 was labeled with SpectrumRed™ (#30-803400), MUT with SpectrumGreen™ (#30-803200), and ITPR3 with SpectrumOrange™ (#30-803000). Briefly, in a chilled microfuge tube in the order listed, the following ingredients were added; (17.5-x) µL of nuclease free water; x µL of BAC DNA (1µg); 2.5 µL of 0.2 mM SpectrumGreen™, SpectrumRed™, or SpectrumOrange™ dUTP; 5 µL of 0.1 mM dTTP; 10 µL of dNTP mix; 5 µL of 10x nick translation buffer; and 10 µL of nick translation enzyme to make a total volume of 50 µL. The Spectrum™ fluorochromes were kept in the dark at all times. The contents were vortexed, briefly centrifuged, and incubated at 15ºC until the DNA smear (tested as described in the Materials and Methods section of Chapter 2) was in the 300 bp range. (See figure 3.4) If the smear was larger than 300 bp, additional enzyme (10 µL) was added and the reaction was run an additional 2 hours. The reaction was stopped by heating at 70ºC for 10 min and then stored at −20ºC until it could be used for hybridization.

![Figure 3.4: Lane 1 holds a 1 Kb ladder. Lane 2 holds an ideal Spectrum fluorochrome DNA smear between 200-500 bp.](image)

**Figure 3.4:** Lane 1 holds a 1 Kb ladder. Lane 2 holds an ideal Spectrum fluorochrome DNA smear between 200-500 bp.

**D. Probe Hybridization**

Probe hybridization was done as described before in the Materials and Methods section in Chapter 2. The entire process was done in reduced light to prevent the Spectrum fluorochromes from fading. Exotic competitor DNA (4 ng) was added to the proper probe mix along with 4 ng of domestic horse competitor DNA to prevent excessive background hybridization to repetitive areas of the chromosome.
E. Slide Washes and Counterstain

Slide washes and counterstain were done in reduced light, but otherwise as described in the Materials and Methods section of Chapter 2. After placement in 1x PBD, 200 µL blocking solution (Appendix 2) was placed on the slide prior to a plastic cover slip and incubated in a humidified chamber for 30 min at 37°C. Avidin-Cy-5 fluorochrome (Jackson # 016-170-084) was diluted to 2 µg/mL Cy-5 in 495 µL water or maleic acid solution. This dilution was added to the slides (60 µL), the slide was coverslipped and incubated as above for 15 min. After the hybridization of the fluorochrome, slides were washed three times in 1x PBD for 2 min per wash. The slides were stained with DAPI as described previously.

F. Visualization and Analysis

Visualization and analysis was done as previously described in the Materials and Methods section of Chapter 2 using the Cytovision©/Genus™ Application Software Version 3.1. Karyotypes for chromosome identification were from Ryder (1978) and Marlys Houck (personal communication).

III. Results

The four BAC clones hybridized successfully to the chromosomes of each Equidae species. As can be seen in Figure 3.5, the gene order was conserved throughout the family Equidae. The clones hybridized to ECA20 as expected. Some non-specific hybridization of unincorporated nucleotides did occur, causing an occasional “blotchy” appearance of the fluorochrome. Often, this non-specific hybridization would take place at the centromeres. Some of the pictures shown below may have been enhanced to show the proper results by reducing background or correcting the registration of the fluorochrome. All four probes hybridized to EPR18, an acrocentric chromosome as in ECA20. In domestic donkeys, the clones hybridized to EAS8, a metacentric chromosome. The four BAC clones in each EHO16, EHK14, and EKI16; the clones hybridized to four specific points along the length of metacentric chromosomes. The chromosome number was deduced by using karyotypes and inverting the image and changing to black and white on the Cytovision©/Genus™.
The higauss filter 9x9 also helped in making the images more clear. The morphology of the zebra chromosomes were also metacentric, but the BACs hybridized to either the p or the q arm. Both Grevy's and Burchell’s zebra chromosomes had probe hybridization to the q arms (EGR11 and EBU13, respectively), while Hartmann’s zebra chromosomes had hybridization to the p arm, EZH10. Interphase spreads were observed, however they did not allow for a better resolution of the gene order.

**Figure 3.5:** Chromosomes of nine species of Equidae labeled with the following fluorescent probes: Spectrum Red™/EDN1, Cy-5/MHC, Spectrum Orange™/ITPR3, and Spectrum Green™/MUT.  
A) *Equus caballus* (ECA20)  
B) *E. przewalskii* (EPR18)  
C) *E. asinus* (EAS8)  
D) *E. hemionus* onager (EHO16)  
E) *E. h. kulan* (EHK14)  
F) *E. kiang* (EK16)  
G) *E. zebra* hartmannae (EZH10)  
H) *E. grevyi* (EGR11)  
I) *E. burchelli* (EBU13)

**IV. Discussion**

As seen in figure 3.5, the order of the genes was conserved among the species of Equidae. The two horse species were the only phylogenetic group to maintain an
acrocentric chromosome. The other seven species studied shared morphology in the form of a metacentric chromosome. This mapping data is consistent with the data from several previous studies. Myka and co-workers (2003) mapped the MUT gene to the acrocentric chromosome EPR18. Yang and co-workers (2003) used chromosome paints to compare HSA6 to both EBU13 and EZH10. Studies by Richard and co-workers (2001) confirmed homology of EZH10p with HSA6p. Raudsepp and co-workers (2001) and Yang (2004) have reported homology between HSA6 and EAS8 via FISH mapping and reciprocal chromosome painting, respectively. *E. a. somaliensis* was found in the previous chapter to have the MHC probe localize to the EAS5p. In this experiment, its closest relative *E. asinus* (and some debate there is no species difference) has the MHC probe localized very close to the centromere. This could be due to the registration being offset by a slider bar on the microscope for the Cy-5 fluorochrome. As mentioned in the results, hybridization of repetitive elements in the probe to the similar sequence in the chromosomes occurred. In the future, an increased amount of competitor DNA should be used to reduce this complication.

Extensive comparative mapping studies have not been done with the Asiatic asses. Based on observations of the metaphase spreads of these three animals, and those of the true asses, there is a possibility of a neocentromere having been formed in these chromosome homologues due to the retained gene order and new centromere location. Little is known about the formation of neocentromeres, however, previous work done in primates (Montefalcone, et al. 1999), the red-legged partridge (Kasai, et al. 2003), and humans (Amor, et al. 2004) showed this process can take place during evolution.

Based on the results here, and following the rules of parsimony, the morphology of the ancestral equid chromosome possessing the MHC cannot be precisely predicted. The ancestral equid chromosome may have been an acrocentric chromosome as found in ECA and EPR. It may have fused with a short or long acrocentric to form the metacentric chromosome seen in zebras and true asses. If a neocentromeric formation occurred in the chromosomes of the Asiatic asses, it would be simple to make that transition directly from the ancestral chromosome by shifting the centromere from the end of the chromosome to the middle. (See figure 3.6) However, the ancestral
chromosome also could have been a metacentric chromosome with the four genes occurring on a q arm, similar to EGR or EBU. Human chromosome 6 also shares this morphology. A fission event would have had to occur, resulting in the formation of the chromosomes of ECA and EPR. At this point, this acrocentric chromosome could have fused with a longer acrocentric to provide the morphology as in EZH. It also could have developed a neocentromere to produce the morphology in EHK, EKI, and EHO (see figure 3.7).

**Figure 3.6:** Diagram of ancestral acrocentric. The mapped genes are indicated by colored bars: EDN1 (red), MHC (purple), ITPR3 (orange), and MUT (green). This diagram demonstrates that three events could have occurred to establish the modern forms of the homologous chromosomes.
Figure 3.7: Diagram of ancestral metacentric. The mapped genes are indicated by colored bars: EDN1 (red), MHC (purple), ITPR3 (orange), and MUT (green). This diagram demonstrates that three events could have occurred to establish the modern forms of the homologous chromosomes.
Chapter 4: Comparison of *Tapirus indicus* and *Rhinoceros unicornis* with ECA20 and other Equidae homologues

I. Introduction

The order Perissodactyla, or the odd-toed Ungulates, is made up of three families: Equidae, Tapiridae, and Rhinocerotidae. Tapirs inhabit tropical forests and grasslands in South and Central America as well as parts of Southeast Asia (Nowak 1999). Tapiridae consist of four species, each with a different chromosome number: Malayan tapir (*T. indicus*) 2n=52, South American tapir (*T. terrestris*) 2n=80, mountain tapir (*T. pinchaque*) 2n=76, and Baird’s tapir (*T. bairdii*) 2n=80 (Houck, et al. 2000).

Rhinoceroses inhabit a variety of landscapes, varying from tropical rainforest to grasslands to swamps (Nowak 1999). Their chromosome number ranges in different species: White rhinoceros (*Ceratotherium simum*) 2n=82, Black rhinoceros (*Diceros bicornis*) 2n=84, Sumatran rhinoceros (*Dicerorhinus sumatrensis*) 2n=82, Indian rhinoceros (*Rhinoceros unicornis*) 2n=82, and the diploid number is unknown for the rare Javan rhinoceros (*R. sondaicus*) (Houck, et al. 1995). Trifonov and co-workers (2003) discussed the possibility of there being very high degree of conservation between the chromosomes of these animals due to the similar and close number of chromosomes. Rhinoceros chromosomes have a heterochromatic p-arm morphology, making it unique and difficult to determine chromosome number. The heterochromatic p-arms vary between individuals; homologous chromosomes look similar based on their banding pattern rather than their morphology (Houck, et al. 1995).

The purpose of this study was to observe the order of the genes surrounding the MHC in two species one from each of the two other orders of Perissodactyla.

II. Materials and Methods

A. Chromosome Preparation

Metaphase chromosomes were prepared by CRES as described in the Materials and Methods section of Chapter 2. Animals used for this study were referenced by their accession numbers: *Tapirus indicus* #8676 and *Rhinoceros unicornis* #12915.
**B. Competitor DNA Preparation**

Tapir competitor DNA was prepared as described in Chapter 2B. The cells were harvested directly from whole blood sent to us by the Audubon zoo in New Orleans. Blood was isolated using a Qiagen FlexiGene DNA kit - 50 mL (#51204). Blood (8 mL) was added to 20 mL FG1 buffer in a 50 mL centrifuge tube. The solution was centrifuged for 5 min at 2000 x g. The supernatant was discarded. The protease (16 µL) was mixed in 4 mL FG2 buffer and added to pellet. The pellet was vortexed until completely reconstituted. The solution was incubated at 65°C for 10 min. Isopropanol was added (4 mL) and mixed by inversion until DNA was visible. The solution was centrifuged at 2000 x g for 3 min. The pellet was washed with 70% EtOH and vortexed for 5 seconds. The pellet was centrifuged as above and drained of supernatant. The pellet was air dried for at least 5 min. Buffer FG3 was added (1 mL), the tube was vortexed at low speed for 5 seconds, and then incubated for 1 hour at 65°C. An OD reading was used for quantification.

**C. BAC DNA Preparation**

Equine BAC DNA was prepared as before in the Materials and Methods section of Chapter 3. MHC BAC clone 167M09 was used for T. indicus and clone 229D23 was used for R. unicornis.

**D. BAC Probe Preparation**

BAC probes were prepared as previously described in the Materials and Methods section of Chapter 3. The EDN1, ITPR3, and MUT genes were labeled using the Vysis Nick Translation Kit (# 32-801300). EDN1 was labeled with SpectrumRed™ (#30-803400), MUT with SpectrumGreen™ (#30-803200), and ITPR3 with SpectrumOrange™ (#30-803000).

**E. Probe Hybridization**

Probes were hybridized as previously described in the Materials and Methods section of Chapter 3, using 4 ng of T. terrestris competitor DNA for the tapir
hybridization mix and 4 ng *R. unicornis* competitor DNA for the rhinoceros hybridization mix. Both the tapir and rhinoceros mixes contained 4 ng of *E. caballus* competitor DNA.

F. Slide Washes and Counterstain

Slide washes and counterstaining were done as previously described in the Materials and Methods section of Chapter 3. Following processes were done in reduced light to prevent quenching of the fluorochromes. Blocking solution, and Avidin-Cy-5 dilutions were added to the slides separately and incubated between each step. After the hybridization of the fluorochrome, slides were washed three times in 1x PBD and DAPI was added as before.

G. Visualization and Analysis

Visualization and analysis were done as previously described in the Materials and Methods section of Chapter 3 using the Cytovision©/Genus™ Application Software Version 3.1. Karyotypes for comparison were from Ryder (1978) and Marlys Houck (personal communication).

III. Results

The probes successfully hybridized to the chromosomes of both *T. indicus* and *R. unicornis*. There was a significant amount of background however, so if this experiment were to be repeated, more competitor DNA should be added. Figure 4.1 below shows the successful hybridization to TIN4, a metacentric chromosome, and figure 4.2 shows both RUN chromosomes (one of 12, 14, 15, or 22) from a single spread, one acrocentric and one with a heterochromatic p arm.
Figure 4.1: Chromosomes of *T. indicus* labeled with the following fluorescent probes: Spectrum Red™/EDN1, Cy-5/MHC, Spectrum Orange™/ITPR3, and Spectrum Green™/MUT.

Figure 4.2: Homologous chromosomes of *R. unicornis* from the same cell, labeled with the following fluorescent probes: Spectrum Red™/EDN1, Cy-5/MHC, Spectrum Orange™/ITPR3, and Spectrum Green™/MUT.

IV. Discussion

The results show that the Tapiridae chromosome containing the MHC is metacentric and may have formed a neocentromere. The organization of the genes surrounding the MHC in this species and in the *E. hemionus* and *E. asinus* are similar. However, this characteristic alone cannot indicate relatedness between the species. According to Henry and co-workers (1991) the tapir and equid pancreatic polypeptide is more closely related to each other than to the rhinoceros. This metacentric chromosome morphology may support that protein study data. The chromosome morphology of *T. indicus* is similar to that of the Asiatic and true asses. Possibly, the neocentromere formed in their common ancestor. It is also possible that neocentromeres formed in the two species independently. If this is the case, perhaps there is a characteristic of this chromosome allowing it to form neocentromeres more
readily. Another possibility is that the ancestral chromosome was this metacentric form found in the tapir and the asses, and the development of the neocentromere was at the telomere end. Further studies of the other species would contribute to the information gathered here. Since there was such a significant difference between the species of Equidae it is possible that the other three species of Tapiridae may have different morphology.

The rhinoceros chromosome pair to which the probes hybridized was acrocentric and more similar to the homologous chromosomes of *E. caballus* and *E. przewalskii*. Though one of the chromosomes contains a p-arm, this arm is largely heterochromatic. These results indicate that the inversion of the MHC with respect to the centromere may have occurred prior to the divergence of the Perissodactyla from a common mammalian ancestor. There are five species of rhinoceros and only one was looked at for this study. Other species would need to be studied before a theory of chromosome evolution could be established.
Chapter 5: In Summary

This research was done to determine the orientation of genes surrounding the MHC in the extant equids. The orientation on these genes along the corresponding human chromosome is reversed with respect to the centromere. The hypothesis was that an ancestral chromosome might be inferred from this information. Although the ancestral chromosome could not be determined from the results, valuable information about the Equidae, Tapiridae, and Rhinocerotidae was gathered, contributing to the knowledge of this syntenic region and the changes it has gone through.

During the progression of the research an interesting discovery was made, several of the animals in the family Perissodactyla (four asses and one tapir) have chromosomes that appear to have formed a neocentromere. Or in the reverse scenario, the zebras, horses, and rhinoceroses have the neocentromere in the form of an acrocentric chromosome. Hopefully this data will be valuable to researchers studying neocentromeres. Another chromosome feature was identified in the rhinoceros, a heterochromatic p-arm on one of the chromosomes. This information will be useful for identifying chromosomes during karyotype standardization.

It seemed logical in the beginning of the project that not only would an ancestral chromosome be devisable, but also that a simple theory of parsimony would be able to be applied to the changes taking place in these animals. After collecting data on all of the members of Equidae, two simple plans could resolve both the ancestral chromosome and the most parsimonious method of devising the differing morphologies. Additional data from a tapir and rhinoceros might help to identify the ancestral chromosome, and was subsequently collected. However, the results for the tapir and rhinoceros were dissimilar and did not identify the most parsimonious evolutionary route. If the mitochondrial DNA “timeline” is correct, then a neocentromere must have been formed in two completely separate events, once in the Tapiridae, and once in the Equidae (asses). The possibility exists that if the tapir chromosome was the ancestral form then two separate neocentromeres on the acrocentric chromosomes evolved, once in the rhinoceroses, and once in the horses (EPR, ECA).
More data needs to be collected to infer what chromosome type is the ancestral form, how these chromosomes might have evolved through time, and what characteristic of this chromosome has permitted it to evolve by two completely independent events. Although the ancestral form of this chromosome was not concluded, the data is conclusive about the order of the genes surrounding the MHC in the studied species, and provides information about chromosome evolution in the order Perissodactyla.
### APPENDIX 1: HORSE BAC DONORS

<table>
<thead>
<tr>
<th>Gene</th>
<th>Source</th>
<th>Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major Histocompatibility Complex (MHC)</td>
<td>USDA Horse BAC library</td>
<td>James A. Baker Institute for Animal Health at Cornell University</td>
</tr>
<tr>
<td>Endothelin 1 (EDN1)</td>
<td>CHORI library</td>
<td>Texas A&amp;M</td>
</tr>
<tr>
<td>methylmalonyl Coenzyme A mutase (MUT)</td>
<td>CHORI library</td>
<td></td>
</tr>
<tr>
<td>inositol 1,4,5-triphosphate receptor, type 3 (ITPR3)</td>
<td>CHORI library</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX 2: SOLUTIONS

0.5 M EDTA buffer:
18.61 g EDTA
q.s. to 100 mL with distilled water
Adjust pH to 8.0 with ~2 g sodium hydroxide, autoclave.

1x PBD, pH=8.0:
0.1 M sodium phosphate
0.1 M di-sodium hydrogen phosphate
0.1% Nonadet P40
Store at 4°C.

10x Nick Translation Buffer:
0.5 M Tris-Cl pH=7.8-8.0
50 mM MgCl2
0.5 mg./mL BSA

2x SSC, pH=7.0:
300 mM sodium chloride
30 mM sodium citrate
Store at 4°C.

20x SSC, pH=5.3:
175.3 g 3.0 M sodium chloride
88.2 g 0.3 M sodium citrate
800 mL distilled water
pH to 5.3 then q.s. to 1000 mL distilled water

50% formamide/ 2x SSC wash solution, pH=5.3:
20 mL formamide
4 mL 20x SSC, pH=5.3
8 mL double distilled water
Make fresh each time.

70% formamide/ 2x SSC denaturation solution, pH=7.3:
28 mL formamide
4 mL 20x SSC, pH=5.3
8 mL double distilled water
Make fresh each time.

Blocking stock solution:
Dissolve 10 g casein in 100 mL maleic acid solution. Store at –20°C.

Blocking working solution for biotin labeled probes:
Dilute blocking stock solution 1:2 with maleic acid solution.
Blocking working solution for Dig or Fitc labeled probes:
Dilute blocking stock solution 1:10 with maleic acid solution.

CHIASM (chloroform:isoamyl alcohol):
Prepare in a 24:1 dilution chloroform:isoamyl alcohol.

Colcemid:
Gibco # 23014-020

Dextran sulfate/ 2x SSC:
50 µL 40% dextran sulfate stock
10 µL 20x SSC
40 µL sterile filtered water

Maleic acid solution:
11.61 g maleic acid (0.1 M)
8.76 g sodium chloride (0.15 M)
q.s. to 1 L

MEM medium:
1% L-glutamine
1% Antibiotic-antimycotic
10% FBS
9.5 mL per T25 flask

Pokeweed Mitogen:
Gibco #15360-019

Potassium Acetate (KOAc), pH=4.8:
19.5 g potassium (K)
10.5 mL glacial acetic acid
150 mL double distilled water
adjust with glacial acetic acid, may take several mL

SE-buffer:
7.5 mL 5.0 M sodium chloride
25.0 mL 0.5 M EDTA, pH=8.0
50 mL 10% SDS
q.s. to 500mL with distilled water, store at 4°C

Sodium hydroxide/sodium dodecyl sulfate (NaOH/SDS):
2.5 mL 20% sodium dodecyl sulfate (SDS)
46.5 mL double distilled water
1.0 mL 10 M sodium hydroxide (NaOH)
filter sterilize, make fresh after one week
TBE:
9,500 mL distilled water
108 g Tris base
55 g boric acid
40 mL 0.5 M EDTA pH=8.0
q.s. to 1 L

Tris-EDTA buffer (TE):
1 mL 1 M Tris-HCl, pH=8.0
.2 mL 0.5 M EDTA , pH=8.0
99.8 mL distilled water

Tris-glucose-edetate disodium (TGE): 50 mM glucose/ 10 mM edetate disodium (EDTA), pH=8.0/ 25 mM Tris-Cl, pH=8.0
5.0 mL 1 M glucose stock
2.0 mL 0.5 M EDTA, pH=8.0
2.5 mL 1 M Tris-Cl, pH=8.0
q.s. to 100 mL and autoclave or filter sterilize
APPENDIX 3: BLAST SEQUENCE

Forward primer sequence:

```
gi|6855655|gb|AF130760.1|AF130760  Equus caballus chromosome 20 EDN1 gene, partial sequence
Length = 266
Score =  165 bits (83), Expect = 4e-38
Identities = 83/83 (100%)
Strand = Plus / Plus

Query: 120 agcacctgaagttaccattgctgaaatgtttttccctatgtgtatatttaacaggaccaa 179
|............................................................|
Sbjct: 155 agcacctgaagttaccattgctgaaatgtttttccctatgtgtatatttaacaggaccaa 214

Query: 180 gacactatggagaaaggctggaa 202
|.................................|
Sbjct: 215 gacactatggagaaaggctggaa 237
```

Reverse primer sequence:

```
gi|6855655|gb|AF130760.1|AF130760  Equus caballus chromosome 20 EDN1 gene, partial sequence
Length = 266
Score =  287 bits (145), Expect = 4e-75
Identities = 152/153 (99%), Gaps = 1/153 (0%)
Strand = Plus / Minus

Query: 29 tttcagcaatggtaacttcaggtgctctcc-cgtctctgctaatctctcttaatcaagaa 87
|............................................................|
Sbjct: 180 tttcagcaatggtaacttcaggtgctctccacgtctctgctaatctctcttaatcaagaa 121

Query: 88 ggcaatcccttaacagaagggagaccaggaggctgtaaaactgattgaaagtaaagtgtt 147
|............................................................|
Sbjct: 120 ggcaatcccttaacagaagggagaccaggaggctgtaaaactgattgaaagtaaagtgtt 61

Query: 148 tctactcacctgagtttttttcttgcttgcaaa 180
|............................................................|
Sbjct: 60 tctactcacctgagtttttttcttgcttgcaaa 28
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Notes: [mailto:gvessere@chori.org](mailto:gvessere@chori.org)


Name: Christine Marie Mains

Date of Birth: November 15, 1978

Place of birth: Green Bay, WI

Undergraduate Degree:
  Institution: University of Kentucky
  Date: May 2002
  Degree: Bachelor of Science
  Animal Science major
  Biological Science minor

Scholastic honors:
  Dean's list: Fall 1999, Spring 2001, and Spring 2002

Professional Publications: