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FISH analysis comparing genome organization in the domestic horse *(Equus caballus)* to that of the Mongolian wild horse *(E. przewalskii)*

J.L. Myka,[a] T.L. Lear,[a] M.L. Houck,[b] O.A. Ryder[b] and E. Bailey[a]

[a] M.H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY;
[b] Center for Reproduction of Endangered Species, Zoological Society of San Diego, San Diego, CA (USA)

Abstract. Przewalski's wild horse (*E. przewalskii*, EPR) has a diploid chromosome number of 2n = 66 while the domestic horse (*E. caballus*, ECA) has a diploid chromosome number of 2n = 64. Discussions about their phylogenetic relationship and taxonomic classification have hinged on comparisons of their skeletal morphology, protein and mitochondrial DNA similarities, their ability to produce fertile hybrid offspring, and on comparison of their chromosome morphology and banding patterns. Previous studies of GTG-banded karyotypes suggested that the chromosomes of both equids were homologous and the difference in chromosome number was due to a Robertsonian event involving two pairs of acrocentric chromosomes in EPR and one pair of metacentric chromosomes in ECA (ECA5). To determine which EPR chromosomes were homologous to ECA5 and to confirm the predicted chromosome homologies based on GTG banding, we constructed a comparative gene map between ECA and EPR by FISH mapping 46 domestic horse-derived BAC clones containing genes previously mapped to ECA chromosomes. The results indicated that all ECA and EPR chromosomes were homologous as predicted by GTG banding, but provide new information in that the EPR acrocentric chromosomes EPR23 and EPR24 were shown to be homologues of the ECA metacentric chromosome ECA5.

Przewalski's wild horse (*Equus przewalskii*, EPR) is the only extant wild horse and historically lived in an area that is now comprised of sections of Mongolia, Khazakstan, and the Xinjiang-Uygur Autonomous Region of China (Ryder, 1993). All living Przewalski's wild horses are descendants of 13 individuals (Ryder, 1994) and are now found only in captive settings such as zoos and where reintroduced into wildlife preserves.

A close relationship between domestic horses (*Equus caballus*, ECA) and EPR has been shown by many researchers. Skull measurements do not distinguish between the two species (Eisenmann and Baylac, 2000), while other skeletal features are distinct (Sasaki et al., 1999). Protein polymorphism studies support the close relationship (Kaminski, 1979; Lowenstein and Ryder, 1985; Bowling and Ryder, 1987), as do molecular DNA studies (Oakenfull and Clegg, 1998) and amino acid sequences (Pirhonen et al., 2002). Studies of mitochondrial DNA and 12S ribosomal RNA gene sequences show little or no differences between ECA and EPR (George and Ryder, 1986; Ishida et al., 1995; Oakenfull and Ryder, 1998; Oakenfull et al., 2000; Jansen et al., 2002). Additionally, domestic horse/Przewalski's horse hybrids are viable and can produce offspring (Short et al., 1974), while hybrids of domestic horses with other equids are usually viable but almost always infertile.

Analyses of chromosome number and morphology are of use in characterizing and defining species. EPR has a diploid chromosome number of 2n = 66, in contrast to 2n = 64 in ECA (Benirschke et al., 1965; Benirschke and Malouf, 1967). Examination of the karyotypes of EPR and ECA revealed that the difference in diploid chromosome number could be explained

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Request reprints from Dr. Teri L. Lear, M.H. Gluck Equine Research Center
Department of Veterinary Science, University of Kentucky
Lexington, KY 40546-0099 (USA); telephone: 859-257-4757
fax: 859-257-8542; e-mail: equigene@uky.edu

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by EPR containing two additional pairs of acrocentric chromosomes and one less metacentric chromosome pair than ECA, with a Robertsonian fusion suspected in ECA (Ryder et al., 1978). Ryder suggested that the metacentric chromosome pair ECA5 was homologous to two pairs of acrocentric chromosomes in EPR (Ryder et al., 1978).

This study was initiated to a) specifically determine if ECA5 homologues were involved in the Robertsonian rearrangements associated with the two equids, and b) further investigate homology between EPR and ECA chromosomes by fluorescence in situ hybridization (FISH) mapping. Large insert equine probes have been successfully used to identify horse chromosome homology with donkey chromosomes (Raudsepp et al., 2001). Therefore, this approach was selected for comparative mapping since the domestic horse and Przewalski's wild horse are closely related.

Materials and methods

Chromosome preparations

Metaphase chromosome spreads were prepared by the CRES laboratory at the Zoological Society of San Diego. Fibroblast cell lines of EPR accession numbers KB7413 and KB12925, from the Frozen Zoo®, were used to prepare metaphase spreads as previously described (Kumamoto et al., 1996). Briefly, cells were harvested after exposure to colcemid (final concentration 0.025 μg/ml) for 105 min, and subsequently exposed to 0.067 M KCl for 30 min prior to fixation in methanol:acetic acid.

Probes

DNA was prepared from horse bacterial artificial chromosome (BAC) clones, obtained from Institut National de la Recherche Agronomique (INRA) (Godard et al., 1998; Milenkovic et al., 2002) and the USDA CHORI-241 Equine BAC library (http://www.chori.org/bacpac/equine241.htm). Forty-six domestic horse-derived BAC clones, previously mapped to ECA, were selected from 38 of 44 autosomal chromosome arms in the ECA karyotype plus ECAX (Table 1). Of the total loci mapped, 44 were specific equine genes, one contained equine DNA in the form of an anonymous BAC, and one was an expressed sequence tag (EST).

FISH mapping and analysis

DNA labeling and FISH was performed as previously described (Lear et al., 2001).

Results

All 46 horse BACs hybridized to EPR chromosomes. The 46 BACs included at least one probe from 38 of the 44 ECA autosomal chromosome arms and both arms of ECAX. A summary of BAC localizations in ECA, EPR and human genomes can be found in Table 1.

Horse BAC clones containing the genes DIA1 (ECA5q17), LAMC2 (ECA5p17-p16), LAMB3 (ECA5p15), UOX (ECA5q15-q16), VCAM1 (ECA5q14), and VDUP1 (ECA5p12) were FISH mapped to Przewalski's horse chromosomes (Fig. 1c). BAC probes containing genes from ECA5p and ECA5q hybridized to two separate Przewalski's horse acrocentric chromosome pairs, EPR23 and EPR24, respectively. For example, VDUP1 and VCAM1 identified two separate acrocentric chromosome pairs (Fig. 1a). The identification of the ECA5 homologues as EPR23 and EPR24 was based on GTG-banding patterns (Fig. 1b). No other rearrangements were found. With the exception of the differences involving ECA5 and its homologues EPR23 and EPR24, the distribution and order of the genes used in this study appeared to be the same for both species. Each ECA chromosome has one EPR homologue, with the exception of ECA5, which was shown to have two homologues, as described above.

Discussion

Based on mitochondrial DNA sequence diversity, domestic horses and Przewalski's wild horses are thought to have diverged from a common ancestor within the past 500,000 to 1 million years (Ishida et al., 1995; Oakenfull et al., 2000, respectively). Indeed, the karyotypes of these two species appear very similar and the hypothesis was advanced that they differ only by a single Robertsonian translocation appearing as a metacentric chromosome in ECA and two small acrocentric chromosomes in EPR (Ryder et al., 1978). Here we demonstrate that the genetic material from the metacentric ECA5 is located on two acrocentric chromosome pairs in EPR, EPR23 and EPR24. While a single marker does not prove homology between entire chromosome arms, this interpretation is consistent with chromosome banding patterns, size and morphology of the chromosomes involved.

These data do not distinguish between a fusion of ancestral acrocentric chromosomes to form ECA5 or a fission of the ancestral ECA5 homologue to create EPR23 and EPR 24. All the genomic material on ECA5 is derivative from HSA1 homologous DNA. Proposed ancestral mammalian karyotypes suggest that the majority of HSA1 homologous genetic material was originally found on one ancestral mammalian chromosome (Murphy et al., 2001; Yang et al., 2003). Consequently, while fusion or fission may equally explain the differences between these two horse karyotypes, the most parsimonious explanation for this phenomenon favors the fission of an ancestral equid chromosome containing HSA1 homologous genomic material to yield two acrocentrics ancestral to EPR23 and EPR24.

However, parsimony does not constitute proof and to resolve this question more comparative gene mapping needs to be conducted. The argument of parsimony assumes that fusion of chromosomes occurs at random and that random chance does not favor the same fusions of homologous acrocentric chromosomes in multiple species. The situation for equids with regard to HSA1 homologous DNA is complicated by two observations. First, at least three horse chromosomes, ECA2, ECA5, ECA30 show homology to HSA1 genes (Raudsepp et al., 1996); Second, the gene order on ECA5 indicates multiple rearrangements relative to the human gene order (Milenkovic et al., 2002). Indeed, neither configuration may represent an ancestral phenotype and both configurations may be derivative through multiple chromosome rearrangements.

This study did not identify any other exceptions to chromosome homology between Przewalski's horse and domestic horse. The results are consistent with the hypothesis that a very close phylogenetic relationship exists between the two species.

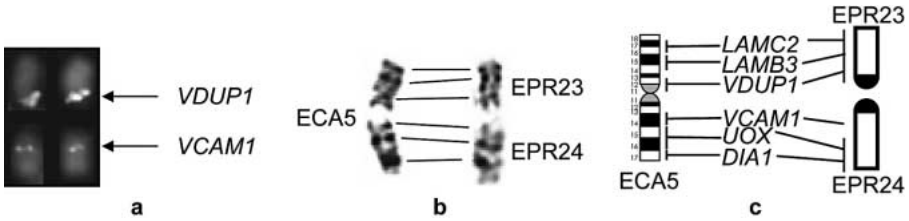

Fig. 1. (**a**) BAC clones containing VDUP1 (ECA5p12) and VCAM1 (ECA5q14) hybridized to *E. przewalskii* chromosomes. VDUP1 (EPR23) was visualized with FITC, and VCAM1 (EPR24) was visualized with Rhodamine Red-X. Chromosomes were counterstained with DAPI. (**b**) EPR23 and EPR24 are the ECA5 homologues. EPR23 and EPR24 are arranged next to ECA5, illustrating the similarities in the GTG-banding patterns. (**c**) Schematic presentation of ECA5 marker locations on ECA5, EPR23, and EPR24.

Table 1. List of FISH mapped markers with their chromosome location in EPR, ECA, and human (*Homo sapiens*; HSA). Equine map locations with references represent previously published mapping data. Human map locations for corresponding genes were retrieved from (http://www.ncbi.nlm.nih.gov). A question mark (?) indicates map position unknown.

Symbol	Locus name	Chromosome location in		
		EPR	ECA	HSA
A4	Anonymous BAC	1p	1p (Lear, unpublished data)	?
FES	v-fes feline sarcoma viral oncogene homolog	1q	1q (Lear et al., 2000)	15q26.1
PKM	Pyruvate kinase muscle type 2 (PKM2)	1q	1q21 (Lear et al., 2000)	15q22
ALPL	Alkaline phosphatase, liver/bone/kidney	2p	2p14 (Mariat et al., 2001)	1p36.1-p34
SMARCA5	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5	2q	2q21 (Lear et al., 2001)	4q31.1-q31.2
GLG1	Golgi apparatus protein 1	3p	3p13-p12 (Lear et al., 2001)	16q22-q23
UCHL1	Ubiquitin carboxyl-terminal esterase L1	3q	3q22 (Lear et al., 2001)	4p14
TCRG	T cell receptor gamma	4p	4p15-p14 (Lear et al., 2001)	7p15-p14
EN2	Engrailed homolog 2	4q	4q27 (Lear et al., 2001)	7q36
VDUP1	Vitamin D up-regulated protein 1	23	5p12 (Lear et al., 2001)	1
LAMB3	Laminin, beta 3 (nicein, kalinin)	23	5p15 (Mariat et al., 2001)	1q32
LAMC2	Laminin gamma 2 chain	23	5p17-p16 (Mariat et al., 2001)	1q25-q31
VCAM1	Vascular cell adhesion molecule 1	24	5q14 (Lear et al., 2001)	1p32-p31
UOX	Urate oxidase	24	5q15-q16 (Godard et al., 2000)	1p22
DIA1	Diaphorase	24	5q17 (Mariat et al., 2001)	22q13.2-q13.31
INHA	Inhibin, alpha subunit	5p	6p14 (Mariat et al., 2001)	2q33-q36
KRAS2	v-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog	5q	6q21 (Lear, unpublished data)	12p12.1
LDHA	Lactate dehydrogenase A	8p	7p14.1-p13(Milenkovic et al., 2002)	11p15.4
LYVE-1	Lymphatic vessel endothelial hyaluronen receptor 1	8q	7q16-q18 (Chowdhary et al., 2003)	11
SART3	Squamous cell carcinoma antigen recognized by T cells 3	6p	8p16-p15 (Lear et al., 2001)	12q24.1
TYMS	Thymidylate synthase	6q	8q12 (Lear et al., 2000)	18p11.32
SLC7A10	Solute carrier family 7, member 10	7p	10p15 (Hanzawa et al., 2002)	19q13.1
AMD1	s-Adenosylmethionine decarboxylase 1	7q	10q21 (Lear et al., 2001)	6q21-q22
DDX5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	10p	11p13 (Lear et al., 2001)	17q23-q25
GH	Growth hormone	10p	11p13 (Lear, unpublished data)	17q22-q24
CHRM1	Acetylcholine receptor, muscarinic 1	11q	12q14 (Milenkovic et al., 2002)	11q13
POR	P-450 (cytochrome) oxidoreductase	12p	13p13 (Milenkovic et al., 2002)	7q11.2
PRM1	Protamine 1	12q	13q14-q16 (Lindgren et al., 2001)	16p13.2
LOX	Lysyl oxidase	13	14q22 (Lear et al., 2001)	5q23-q31
Septin 2-like	Septin 2-like cell division control protein	14	15q12 (Lear, unpublished data)	?
GLB1	Galactosidase, beta-1	15	16q22 (Lear, unpublished data)	3p21.33
ALOX5AP	Arachidonate 5-lipoxygenase-activating protein	16	17q14-q15 (Mariat et al., 2001)	3q12
CHRNA	Cholinergic receptor, nicotinic, alpha	17	18q24-q25 (Lear, unpublished data)	2q24-q32
PROS1	Protein S (alpha)	19	19q21 (Milenkovic et al., 2002)	3p11-q11.2
MUT	Methylmalonyl CoA mutase	18	20q21 (Lear et al., 2001)	6p21
GZMA	Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	20	21q13-q14 (Chowdhary et al., 2003)	5q11-q12
RPN2	Ribophorin II	21	22q17 (Chowdhary et al., 2003)	5q11-q12
IFNB1	Interferon, beta 1, fibroblast	22	23q16-q17 (Lear et al., 2001)	9p21
GGTA1	Glycoprotein, alpha-galactosyltransferase 1	26	25q17-q18 (Milenkovic et al., 2002)	9q33-q34
SOD1	Superoxide dismutase 1	27	26q15 (Godard et al., 2000)	21q22.1
KITLG	KIT ligand	29	28q13 (Terry et al., 2002)	3p14.1-p12.3
HESTG05	EST	30	29qter (Godard et al., 2000)	?
TGFB2	Transforming growth factor, beta 2	31	30q14 (Milenkovic et al., 2002), 6q21 (Lear, unpublished data)	1q41
PLG	Plasminogen	32	31q12-q14 (Lear et al., 2000)	6q26
TRAP170	Thyroid hormone receptor associated protein complex component	Xp	Xp15-p14 (Raudsepp et al., 2002)	Xp11.4-p11.2
PGK	Phosphoglycerate kinase 1 (PGK1)	Xq	Xq13-q14 (Milenkovic et al., 2002)	Xq13.3

However, the resolution of FISH mapping a single marker to each chromosome arm will not necessarily lead to the identification of intrachromosomal inversions or small translocations. It is possible that other rearrangements exist that would identify differences in genome organization. Rearrangements not detected by our low density comparative map might be observed by increasing the density of domestic horse markers on the Przewalski horse chromosomes. Studying the synaptonemal complexes of ECA/EPR hybrids might identify putative chromosomal inversions, following the approach of Switonski and Stranzinger (1998). However, these species are closely related and it is possible that no inversions exist. Consequently, characterization of these two horses as different species may revolve about the differences in repetitive elements found between the two types of horses (Wichman et al., 1991).

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