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Application of IGF2 – Specific Identifier Probe for Cytogenetic Study of Somatic and Sperm Cells in Horses*

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The development of molecular techniques with fluorochromes has had an invaluable impact on discovering the nature of chromatin structure. Here, we show the application of a locus specific identifier probe (LSI) for precise and selective visualization of the horse IGF2 gene in the metaphase, interphase nuclei and sperm cells. Our study may be helpful for interpretation of results of interphase fluorescence *in situ* hybridization (I-FISH). We analyze and discuss the variation in the number and localization of FISH signals in somatic and sperm cells of horse.

Key words: horse, IGF2, FISH, chromosome, somatic cells, sperm cells.

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Insulin-like growth factor 2 (somatomedin A) is a small mitogenic peptide and is the one of the most ubiquitous embryo and fetal growth factors in mammals (BIRAN *et al.* 1994; O'NEILL *et al.* 2000; REIK *et al.* 2001; NAGATA *et al.* 2003; OGAWA *et al.* 2003). The IGF2 gene plays an important role in the control of processes involved in cell growth, differentiation, and reproduction (BIRAN *et al.* 1994; REIK *et al.* 2001). Therefore, it may be crucial for animal production (RON & WELLER 2007).

The physical localization of the IGF2 gene in domestic horse showed fluorescence signals on chromosome 12 at q13 (RAUDSEPP *et al.* 1997). This region is homologous to segments within human chromosome 11. The equine genomic IGF2 sequence was found to be homologous in the IGF2 coding region (OTTE *et al.* 1998) when compared to other animal IGF2 sequences from mouse, rat, sheep. In this work, we focused on applying a locus specific identifier IGF2 probe in order to analyze the timing of replication in interphase horse nuclei and to identify the chromosome position in sperm cells.

Material and Methods

Probes for IGF2 gene were amplified from horse genomic DNA by long template PCR (ExpandTM Long Template PCR System Kit, Roche Diagnostics GmbH, Mannheim, Germany) with specific primers that were designed according to the horse genomic sequence. Obtained PCR products of ~ 2.5 kb were fragmented by heating at 95°C for 30 min. The fragmented PCR products were labeled

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using FISHBright™ Nucleic Acid Labeling Kit – 547 Orange – Kreatech's Universal Linkage System, (Kreatech Diagnostics**,** Amsterdam, Netherlands) with the standard protocol according to the manufacturer's instructions. Metaphase spreads and interphase lymphocytes were obtained from routine 72-h lymphocyte cultures with colchicine (1 μg/ml) (Sigma, USA), respectively, followed by a 10-min treatment with 75 mM KCl at 37°C and fixation in methanol:glacial acetic acid (3:1). Preparations of horse sperm cells were obtained with DTT papain solution according to the procedure described by BUGNO-PONIEWIERSKA *et al*. (2009). The slides with fixed cells (lymphocytes – metaphase and interphase nuclei and sperm cells) were treated with 100 μg/ml RNAse in 2xSSC buffer in a humidified chamber at 37°C for 1h. Next, the slides were washed three times with 2xSSC buffer and were treated with 1% pepsin in 10 mM HCl in a coplin jar at 37°C for 10 min. Then, the slides were washed twice with PBS, once with PBS supplemented with 50 mM $MgCl_2$ and were passed through a set of ethanol solutions (70%, 80% and 95%) at room temperature for 3 min. Co-denaturation of sample and probe on a microscope slide was carried out on a hot plate at 75°C for 5 and 10 min. for metaphase/interphase nuclei and sperm cells, respectively. Hybridization was performed in a humidified chamber at 37°C overnight. The slides were washed with 1xPost-Wash Buffer I (0.4xSSC/0.3% Tween 20) for 2 min. at 72°C (±1şC) and next washed with 1xWash Buffer II (2 x SSC/Tween 20) at room temperature for 1 min. For DNA visualization, the slides were counterstained with a drop of mounting medium with 4',6'-diamino-2-phenylindole (DAPI II) counterstain and were analyzed in an Olympus BX61 fluorescence microscope equipped with a DP72 CCD camera and Olympus CellF software.

Results and Discussion

We have shown that the FISH technique with a LSI probe can be used for precise and selective visualization of the horse IGF2 gene in metaphase, interphase nuclei and sperm cells (Fig. 1). Our probes were able to hybridize to sequences localized at terminal parts of two small submetacentric chromosomes, in agreement with previous data (RAUDSEPP *et al.* 1997). On the basis of morphology, size and DAPI staining, the labeled chromosomes were identified as ECA12 (BOWLING *et al.* 1997).

Additionally, the fluorescence *in situ* signals of the IGF2 probe were analyzed in interphase nuclei of lymphocytes. Fluorescence signals from moderate to bright intensity in interphase nuclei were seen. In the nuclei of standard cells, two distinct signals were observed (approximately in 79% of all cases). These signals are aligned close to each other. Moreover, cytogenetic analysis showed that the LSI probe can appear as three or four signals (approximately in 21 % of nuclei) which may be due to the degree of DNA condensation and relative distance between chromatids. According to this, three signals are characteristic for early replication (Fig. 1D) and four for late and post replication state (Fig. 1E, F). Probably, the timing of replication of the gene depends on the nuclear position of homologous chromosomes. The development of fluorochrome-based molecular techniques has had an invaluable impact on discovering the structure of the nucleus (DORRITIE *et al.* 2004; BUGNO *et al.* 2009). With fluorescence *in situ* hybridization (FISH), it was possible to conclude that each chromosome, occupying a finite, mutually exclusive fraction of the nuclear volume, represents a structural unit
(LUKASOVA *et al.* 2002; PARADA & MISTELI 2002; WILLIAMS 2003). It is still a matter of discussion what is more important for proper nuclear position of chromosomes: chromosome size or

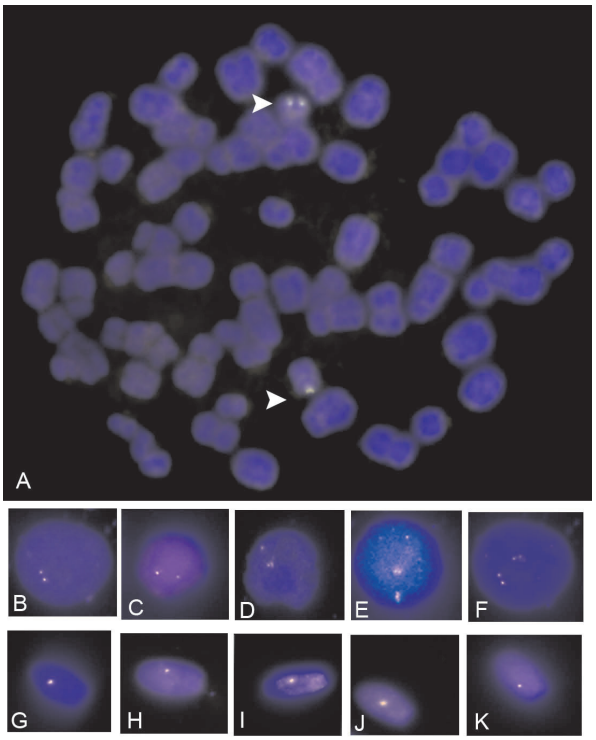

Fig. 1. Horse IGF2 gene in metaphase, interphase nuclei and sperm cells. The normal mare metaphase spread with IGF2 signal in the terminal parts of two small submetacentric chromosomes (A). Replication timing analysis by fluorescence *in situ* hybridization with a LSI probe. The microphotographs (B-F) show representative interphase cell nuclei which were hybridized with a probe specific to the IGF2 loci. A nucleus with two unreplicated alleles (B and C), one replicated and one unreplicated allele (D), and two replicated alleles (E and F). Localization of IGF2 locus in sperm cell (G-K).

density (KUPPER *et al.* 2007). It is postulated that during the cell cycle chromosomes can move and change their topology which can in turn regulate gene expression. The mechanisms underlying non-random chromosome positioning are essential for understanding many biological processes such as changes in gene expression during stem cell differentiation and reprogramming of nuclei in cloning by nuclear transfer (CREMER & CREMER 2001; EDELMANN *et al.* 2001). Contrary to somatic cells, the sperm nucleus has a unique chromatin structure with highly condensed and protamine-associated DNA. Human data with suspension fluorescence *in situ* hybridization and MCB analysis also indicated a specific distribution of chromosomes in the sperm nucleus. This observation may be due to the inactive state of the sperm nucleus and has implications for the early stages of fertilization (PRESTON 1994; MANVELYAN *et al.* 2008). We observed a single fluorescence of moderate to bright intensity in each analyzed sperm cell. The single IGF2 signal was detected in the peripheral part of the sperm head (Fig. 1G-K). Also, we showed that the sensitivity of detected signals is due to successful decondensation of chromatin of sperm cells as described previously (BUGNO-PONIEWIERSKA *et al.* 2009). Further analysis of gene and chromosome positioning would be useful to understand the structure of animal chromatin and its biological and clinical implications.

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