

Comparative analysis of vertebrate *EIF2AK2* (*PKR*) genes and assignment of the equine gene to ECA15q24-q25 and the bovine gene to BTA11q12-q15

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(Received 19 January 2006; accepted 13 April 2006)

Abstract – The structures of the canine, rabbit, bovine and equine *EIF2AK2* genes were determined. Each of these genes has a 5' non-coding exon as well as 15 coding exons. All of the canine, bovine and equine *EIF2AK2* introns have consensus donor and acceptor splice sites. In the equine *EIF2AK2* gene, a unique single nucleotide polymorphism that encoded a Tyr329Cys substitution was detected. Regulatory elements predicted in the promoter region were conserved in ungulates, primates, rodents, Afrotheria (elephant) and Insectifora (shrew). Western clawed frog and fugu *EIF2AK2* gene sequences were detected in the USCS Genome Browser and compared to those of other vertebrate *EIF2AK2* genes. A comparison of *EIF2AK2* protein domains in vertebrates indicates that the kinase catalytic domains were evolutionarily more conserved than the nucleic acid-binding motifs. Nucleotide substitution rates were uniform among the vertebrate sequences with the exception of the zebrafish and goldfish *EIF2AK2* genes, which showed substitution rates about 20% higher than those of other vertebrates. FISH was used to physically assign the horse and cattle genes to chromosome locations, ECA15q24-q25 and BTA11q12-15, respectively. Comparative mapping data confirmed conservation of synteny between ungulates, humans and rodents.

translation initiation factor / innate immunity / phylogenetic analysis / FISH mapping

1. INTRODUCTION

The eukaryotic translation initiation factor 2-alpha kinase 2 (*EIF2AK2*, also known as *PKR* or *PRKR*) is an important component of the host innate immune

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antiviral response [20]. Double-stranded RNA (dsRNA) synthesized during viral infection binds to and activates EIF2AK2. Activation by dsRNA causes autophosphorylation of EIF2AK2 and allows this kinase to phosphorylate its natural substrate, the 1-alpha subunit of eukaryotic translation initiation factor-2 (EIF2S1, also known as eIF-2alpha). Phosphorylation of this initiation factor results in inhibition of protein translation and viral replication [25].

Previous reports described the structures of the human and mouse genes encoding the translation initiation factor 2-alpha kinase 2. The human (*Homo sapiens*; HSA) *EIF2AK2* gene (*HSAEIF2AK2*) consists of 19 exons including four non-coding exons (1, 2, 18, and 19). Three alternative splicing acceptors in the second *HSAEIF2AK2* exon have been reported [12]. Additional alternative splice variants that differ in their 3' non-coding regions were previously listed in the Celera database; the *HSAEIF2AK2* mRNA BM473760 contains exon 17 extended with a significant portion of un-spliced intron 17 but exons 18 and 19 are absent in this transcript, while the transcript AA639687, contains exons 17, 18 and 19 but not intron 17. The product of the *HSAEIF2AK2* gene is a 68 kDa protein that is ubiquitously expressed at low levels. The N-terminal part of this protein contains two dsRNA binding motifs (dsRBM) [6, 18]. The C-terminal EIF2S1 kinase domain includes 12 sub-domains that are conserved in several species [9, 25].

The mouse (*Mus musculus*; MMU) *MMUEif2ak2* gene consists of 16 exons. Exon 1 is not translated [28]. The equivalent of the *HSAEIF2AK2* second exon is absent in the *MMUEif2ak2* gene [16]. Orthologous cDNA sequences were previously reported for the rat (*Rattus norvegicus*; RNO) [19], cattle (*Bos taurus*; BTA) and pig (*Sus scrofa*; SSC) *EIF2AK2* genes [2].

We report here characterization of the structures of the equine (*Equus caballus*; ECA), canine (*Canis familiaris*; CFA) and rabbit (*Oryctolagus cuniculus*; OCU) *EIF2AK2* genes. In addition, we performed a phylogenetic comparison of the *EIF2AK2* genes of the horse, dog, cattle, pig, primates, rodents, chicken and gold fish. The equine and bovine *EIF2AK2* genes were localized on horse and cattle chromosomes, respectively, by fluorescent in situ hybridization (FISH) with several bacterial artificial chromosome (BAC) clones.

2. MATERIALS AND METHODS

2.1. Animals

Necropsy tissue samples from 1 Arabian, 1 Hanoverian, 1 Paint, 4 Quarter, 3 Thoroughbred and 6 mix-breed horses were used for genomic DNA extraction by the standard phenol and chloroform method [24].

Table I. Primers used to amplify full-length *CFAEIF2AK2*, *OCUEIF2AK2* and partial *ECAEIF2AK2* cDNA as well as to sequence exon/intron junction regions in the *ECAEIF2AK2* gene.

Primer name	Primer sequence	Primer name	Primer sequence
CfaPRKR-F	GTTACTAGAGGAAATGGCCAATC	CfaPRKR-R	TTGAGCCTTCTAAAATGTAGTGAA
OcuPRKR-F	AATGGCCAATGATCTTTCACCAGG	OcuPRKR-R	GGATGACCCCTCTGAAGTATTCGT
EcaPRKR-F	GSTGAAGGTARATCAAAGAAGGA	EcaPRKR-R	ACAAGTCCAAARTCTCCAATCTT
EcaPKR-ex1F	ACCTGGGGACACGATCGTGT	EcaPKR-ex1R	CTAGAAATCTTCGCGGGGA
EcaPKR-ex2F	CTTTCACCAGGTGTCTTCAT	EcaPKR-ex2R	AATCATGTGGAGGTCCTGTC
EcaPKR-ex3F	CCGGAAGCTGAAGGTTAAATC	EcaPKR-ex3R	AGCTTCGGAAAATCTCTCC
EcaPKR-ex4F	CCACTGCTGACGAAAGATAC	EcaPKR-ex4R	CCCGCCATCCCTTGATTCAT
EcaPKR-ex5F	GTTCCACAAAACAGGAGGCA	EcaPKR-ex5R	TGCCCTCTGTTTTGTGGAAC
EcaPKR-ex7F	GTGCTTATGAATCATCACCTG	EcaPKR-ex7R	CGTTGCTGTCAACAATTTCTT
EcaPKR-ex8F	AGAGCAGCCGCAAGAAGCC	EcaPKR-ex8R	ACTTTGTGGCTTCTTGCG
EcaPKR-ex9F	AAGTTTGGCACCTACTTTCAG	EcaPKR-ex9R	CCCTTTGCCACGGTATACT
EcaPKR-ex10F	GCTCAGGTGGATTTGGCCAG	EcaPKR-ex10R	CGTGTTAAATATGACCGTGAG
EcaPKR-ex11F	TGGCAGAGCTTGATCATCCA	EcaPKR-ex11R	CCCAACCAACAATTATAGTAG
EcaPKR-ex12F	CGTTAGAGCAATGGATCGAC	EcaPKR-ex12R	GTGATCCATTGCTCTAACG
EcaPKR-ex13F	GGACTCGTAAACATCCCTGAA	EcaPKR-ex13R	TTCAGGATGTTACGAGTCC
EcaPKR-ex14F	AAATGAAGTGGACATCTATGC	EcaPKR-ex14R	TCTCTAGCAGTGGGACATATG
EcaPKR-ex16F	CTGTGGACGTTGAAGGAATG	EcaPKR-ex16R	CATTCCTCAACGTCCACAG

2.2. cDNA sources

Using an RNeasy Mini Kit (Qiagen, Valencia, CA), total RNA was extracted from peripheral white blood cells (PWBC) from a Quarter horse and converted into first-strand cDNA with ThermoScript RNase H⁻ Reverse Transcriptase (Invitrogen, Carlsbad, CA) using an oligo-dT primer. This single-stranded cDNA and a commercial dog kidney cDNA (BioChain, Hayward, CA) were utilized to amplify partial equine and canine EIF2AK2 cDNA sequences and extend them using a DNA Walking SpeedUp Kit (Seegene USA, Del Mar, CA) according to the manufacturer's protocol. Full-length EIF2AK2 cDNA was generated from rabbit kidney cDNA (Seegene) using the 3'RACE OcuPRKR-F and 5'RACE OcuPRKR-R primers (Tab. I) according to the manufacturer's protocol.

2.3. BAC clones

Four high-density filters for segment 1 of the CHORI-241 equine genomic BAC library were purchased from the Children's Hospital Oakland Research Institute (CHORI), Oakland, CA. These filters were screened using a ³²P-labeled equine EIF2AK2 cDNA probe according to the supplier's protocol. Positive equine BAC clones as well as the bovine CH240_360J16 BAC clone were purchased from CHORI. Each of the BAC clones was grown individually in 500 mL of LB media. BAC DNA was isolated using the NucleoBond BAC Maxi Kit (BD Biosciences Clontech, Palo Alto, CA) and used as the template

for direct partial sequencing with a BigDye terminator v1.1 Cycle Sequencing Kit on an ABI 3100 Genetic Analyzer according to the manufacturer's recommendations. The ends of the BAC clones were sequenced using universal SP6 or T7 primers. Additionally, the BAC clone inserts were removed by NotI digestion and the insert lengths were estimated by pulsed-field electrophoresis.

2.4. Exon sequencing

The sequences of the human *EIF2AK2* exons (www.ensembl.org) were aligned with equine cDNA using the *bl2seq* program (www.ncbi.nlm.nih.gov) to predict a potential exon structure for the equine *EIF2AK2* gene. Predicted exon sequences were used to design the exon-specific primer pairs listed in Table I that were utilized to partially sequence the BAC clones. Since *ECAEIF2AK2* exons 5, 6 and 7 are located close to each other in genomic DNA, exon/intron junctions in exon 6 were sequenced using primers EcaPKR-ex5F and EcaPKR-ex7R. Similarly, the two closely located 3' terminal exons, 15 and 16 were amplified from the *ECAEIF2AK2* gene and sequenced using primers EcaPKR-ex15F and EcaPKR-ex16R.

2.5. Analyses of DNA sequences

The TFSEARCH computer program (www.cbrc.jp) was used to search for potential promoters upstream of the *EIF2AK2* genes. The GenBank gss database was searched against sequences of the equine and bovine *EIF2AK2* cDNA using the *blastn* program [1]. The *njtree* program was used to construct a phylogenetic tree with distances calculated by standard methods [13, 17] and tree topology was inferred by the Neighbor-Joining algorithm [23]. Pairwise distances between sequences were calculated according to Gu and Zhang [7] based on gamma distribution by assuming the heterogeneity of substitution rates. The bootstrap algorithm [30] with 1000 replications was used to estimate the confidence of each node. The programs used in this study are available on request.

2.6. FISH mapping

DNA from equine BAC clones CHORI241_81I21 and CH241_117I21, as well as from bovine BAC clone CH240_360J16 were labeled with Spectrum OrangeTM-dUTP following the manufacturer's directions (Vysis, Downer's

Grove, IL). DNA from these clones was used for FISH to equine or bovine metaphase spreads, respectively, as described previously [3]. Briefly, the hybridization solution contained 100 ng of labeled probe, 6 μ g of competitor DNA, 4 μ g sonicated salmon sperm DNA in 50% formamide, 10% dextran sulfate, and 1X SSC. Hybridizations proceeded for approximately 16–18 h. Post-hybridization washes were done at 42 °C. The chromosomes were counterstained with DAPI prior to analysis. International cytogenetic nomenclature of the domestic horse (ISCNH1997) [11] and cattle current standard (ISCNDB2000) [10] were used to identify individual horse and cattle chromosomes, respectively. The identification of BTA11 was confirmed by S. Charter (Center for Reproduction of Endangered Species, San Diego Zoological Society).

3. RESULTS AND DISCUSSION

3.1. Identification of the dog *CFAEIF2AK2* gene

The GenBank canine database was searched using the *HSAEIF2AK2* cDNA sequence and the *Canis familiaris* chromosome 17 genomic contig NW_876263, which contains the *CFAEIF2AK2* gene, was identified. The blast alignment revealed that this contig includes all of the *CFAEIF2AK2* coding exons. Gene-specific primers CfaPRKR-F and CfaPRKR-R (Tab. I) were used to amplify a 1739 bp PCR fragment from dog kidney cDNA. This fragment was sequenced directly and its 5' end was extended using a DNA Walking SpeedUp Kit. A full-length cDNA sequence of the *CFAEIF2AK2* gene was deposited in GenBank under accession number AY906960. Alignment of this cDNA sequence and the NW_876263 genomic sequence revealed 17 exons in the *CFAEIF2AK2* gene.

3.2. Identification of the rabbit *OCUEIF2AK2* gene

The GenBank rabbit database was searched using the *HSAEIF2AK2* cDNA sequence and several genomic contigs containing the *OCUEIF2AK2* gene were identified. The sequence of the AAGW01139267 contig was used to design 3' and 5' RACE primers (Tab. I) that were utilized to amplify the full-length *OCUEIF2AK2* cDNA. This cDNA was sequenced directly and the sequence obtained was deposited in GenBank under accession number DQ115394.

3.3. Identification and analysis of the horse *ECAEIF2AK2* gene

Bovine, canine, human and porcine *EIF2AK2* cDNA sequences were downloaded from GenBank and aligned using the MegAlign program. Based on this alignment, two degenerative primers EcaPRKR-F and EcaPRKR-R (Tab. I) were designed and used to amplify a middle portion of the equine *EIF2AK2* transcript from a Quarter horse single-stranded cDNA prepared as described in Materials and Methods. The 1094 bp PCR fragment obtained was sequenced directly. An "A/G" single nucleotide polymorphism (SNP) was detected at position 824. To confirm this equine polymorphism, the SNP region was amplified and directly sequenced in an additional sixteen unrelated horses of various breeds. Ten horses were found to be AA homozygous and six were AG heterozygous.

This cDNA sequence was extended to full-length using a DNA Walking Kit and the sequence was submitted to GenBank under accession number AY850106. The "A/G" SNP detected encodes a unique Tyr329Cys substitution in the equine EIF2AK2 protein. A GenBank search of mammalian databases revealed only the "A" nucleotide at the same *EIF2AK2* location in humans (27 subjects), African green monkeys (*Cercopithecus aethiops*; CAE) chimpanzees (*Pan troglodytes*; PTR), orangutans (*Pongo pygmaeus*; PPY), rhesus monkeys (*Macaca mulatta*; MML), dogs, cattle, pigs, rabbits, mice (9 subjects) and rats (4 subjects). Currently 13 *EIF2AK2* SNP are listed in the mouse GenBank database and 187 in the human GenBank database, but the A/G SNP found in the horse gene was not found in either the mouse or human SNP databases, suggesting a recent origin of this polymorphism in horses.

The 1094 bp fragment of *ECAEIF2AK2* cDNA was next used to screen the CHORI-241 equine BAC library and four positive clones, 81I21, 117I21, 179F14 and 179H4, were identified. The exon/intron junctions of the equine *EIF2AK2* gene in two of these BAC clones, 81I21 and 117I21, were directly sequenced using primers listed in Table I. Alignment of genomic and cDNA sequences revealed 16 exons in this gene, including a non-coding 5'-terminal exon. The donor and acceptor splice sites of all of the introns corresponded to the GU/AG rule. Comparison of bovine and canine *EIF2AK2* cDNA sequences with genomic contigs available in GenBank (Fig. 1, published in electronic-only form at <http://www.edpsciences.org/gse>) revealed similar exon/intron structures for these genes. The lengths of the exons within the *EIF2AK2* open reading frame (ORF) were compared for several mammalian species. Although the number of coding *EIF2AK2* exons is conserved among several mammalian species, the numbers of non-coding exons differ between primates (HSA, PPY and PTR) and other mammals. Primates contain two 5' and two 3' non-coding

Table II. Lengths of coding exons (in bp) in mammalian *EIF2AK2* genes.

Species	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Cattle	119	121	149	121	62	100	35	66	123	117	181	129	105	51	123
Dog	119	121	158	127	71	79	35	63	123	119	178	129	102	54	120
Horse	119	118	146	127	80	97	23	63	123	120	181	129	102	54	141
Primates	119	121	149	127	77	94	35	63	123	159	181	129	102	54	123
Mouse	116	121	137	121	80	49	35	51	123	120	181	132	102	57	123
Rat	116	121	137	124	65	46	32	51	123	129	181	135	102	57	123

EIF2AK2 exons, while the other mammals studied to date have only a single 5' non-coding exon. The coding exons were designated A through O. The lengths of exons A, B, D, and I through O, which encode functional motifs, are less variable than those of the other exons with the exception of exon J (Tab. II). Exon J, which encodes the C-terminal EIF2-alpha kinase subdomains III and IV, varies significantly in length from 117 bp in cattle to 159 bp in primates. The equine exon O contains a short insert located downstream from the region, encoding sub-domain XI. Due to variation in the size of the coding exons, the lengths of the corresponding proteins are also variable (Fig. 2, published in electronic-only form at <http://www.edpsciences.org/gse>).

3.4. Analyses of promoter regions

Analysis of potential *cis*-acting elements in genomic sequences located 300 bp upstream of the 5'-terminal exons of seven mammalian *EIF2AK2* genes revealed conservation of both the kinase conserved sequence (KCS) and the interferon-stimulated response element (ISRE) (Fig. 2), which were previously reported in the human and mouse promoters [15]. ISRE is involved in type I interferon inducibility, while KCS functions as a constitutive activation element. These two elements are separated from each other by four bp in all mammalian *EIF2AK2* promoters studied to date (Fig. 3). These two regulatory elements are also located close to the *EIF2AK2* transcription start in primate and ungulate species. A search of the current dog genome draft did not reveal either the first 5' *EIF2AK2* exon or the KCS and ISRE promoter elements.

3.5. Comparison of avian, teleostean and amphibian *EIF2AK2* genes

A limited number of *EIF2AK2* sequences were recently reported for birds and fishes. These include chicken (*Gallus gallus*; GGA) [14], goldfish (*Carassius auratus*; CAU) [9] and zebrafish (*Danio rerio*; DRE) [22]. Amphibian *EIF2AK2* gene sequences have not been described previously. Using the

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Human  GGGAAAGGCGGAGTCCaaggGGAAAACGAAACT NT_022184
Chimpanzee GGGAAAGGCGGAGTCCaaggGGAAAACGAAACT NW_103405
Orangutan  NNNNAGGCGGAGTCCaaggGGAAAACGAAACT CR859742
Macaque   GGGAAAGGCGGAGTCCgaggGGAAAACGAAACT CJ459440
Elephant  AGGAAGGCGGGTTCgccgGGAAAACGAAACT AAGU0171837
Cattle    AGGAAGGCGGAGTCCgtcgGGAAAACGAAACC NW_928274
Horse     CGGGAGGCGGAGTCCgccgGGAAGACGAAACC AY946025
Shrew     CCGGGGGCGGAGCCCgccgGGAAAACGAAACC AALT01284110
Mouse     GGGAAAGGCGGAGTCCgccgGGAAAACGAAACA NT_039658
Rat       GGGAAAGGCGGAGTCCgccgGGAAAACGAAACA NW_047764
          ← KCS →           ← ISRE →

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Figure 3. Alignment of the KCS and ISRE sequences (indicated in bold upper case) of the *EIF2AK2* genes of primates, ungulates and rodents.

USCS Genome Browser, the whole genome assemblies of Western clawed frog (*Xenopus tropicalis*; XTR) and fugu pufferfish (*Takifugu rubripes*; TRU) were searched with the BLAT program. In *Xenopus*, three tandemly duplicated copies of the *EIF2AK2* gene were found in the genomic scaffold_41. One of these copies was an incomplete sequence due to gaps in the assembly. The two other copies were complete and were included in the phylogenetic analysis. The *Takifugu* genome contains a single copy of the *EIF2AK2* gene located in the shotgun assembly scaffold_7150 (GenBank accession number CAAB01007140).

The 5'-terminal *EIF2AK2* exons B and D encode two dsRNA binding domains in the mammals, birds (chicken) and amphibians (frog) studied to date (Fig. 2). This region is variable in fishes. The two dsRBM are conserved in the fugu *EIF2AK2* protein, but the 5' ends of the zebrafish and goldfish *EIF2AK2* genes encode two Z-DNA-binding domains [9, 22]. *Danio* and *Carassius* *EIF2AK2* genes contain a large kinase insertion in exon K. In all three fish genes, exons E and F are significantly shorter (9 codons) than those sequences in mammals. Only three amino acid residues (2%) in the N-terminal domains (exons A through D) were invariant and 13 (8%) were functionally similar among the vertebrate *EIF2AK2* genes analyzed to date. The kinase catalytic domain (exons I through O) contained 57 (20%) invariant and 98 (34%) functionally similar amino acid residues. This suggests that in vertebrates the kinase catalytic domains are evolutionarily more conserved than the nucleic acid (dsRNA or Z-DNA) binding motifs.

3.6. Phylogenetic analysis of vertebrate *EIF2AK2* genes

ORF sequences of vertebrate *EIF2AK2* genes were aligned (Fig. 4, published in electronic-only form at <http://www.edpsciences.org/gse>) to build a

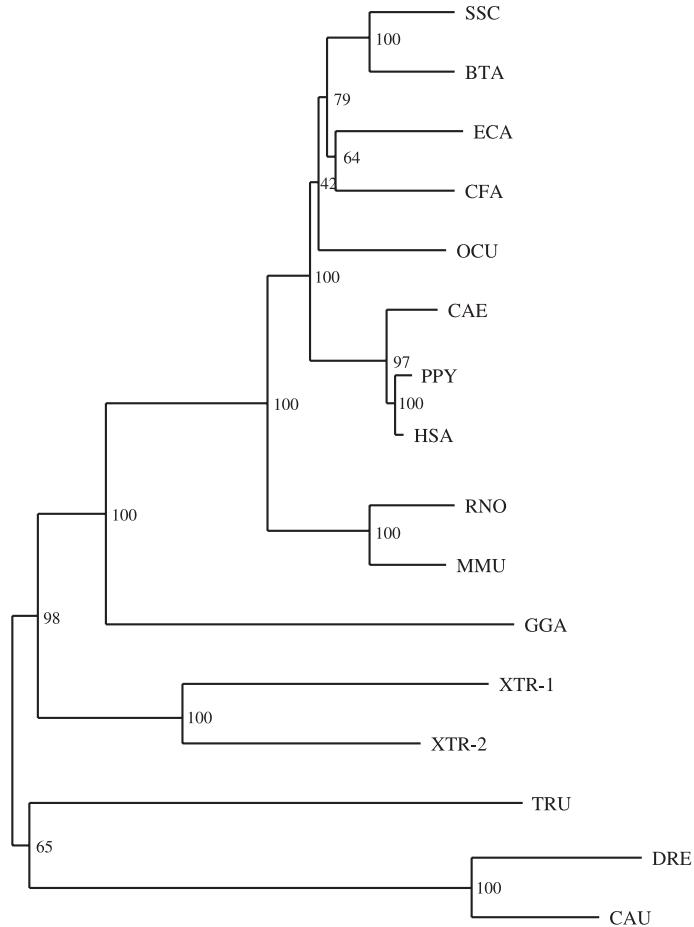


Figure 5. Phylogenetic tree of vertebrate *EIF2AK2* genes. SSC: pig (*Sus scrofa*), BTA: cattle (*Bos taurus*), ECA: horse (*Equus caballus*), CFA: dog (*Canis familiaris*), OCU: rabbit (*Oryctolagus cuniculus*), CAE: African green monkey (*Cercopithecus aethiops*), PPY: orangutan (*Pongo pygmaeus*), HSA: human (*Homo sapiens*), RNO: rat (*Rattus norvegicus*), MMU: mouse (*Mus musculus*), GGA: chicken (*Gallus gallus*), XTR: Western clawed frog (*Xenopus tropicalis*), TRU: fugu pufferfish (*Takifugu rubripes*), DRE: zebrafish (*Danio rerio*), CAU: goldfish (*Carassius auratus*).

phylogenetic tree using the neighbor-joining clustering method with distances calculated by the two-parameter substitution model with a gamma distribution parameter $a = 1.5$. The partial PTR and MML *EIF2AK2* gene sequences were not included in this tree. The structure of the tree (Fig. 5) corresponded to a conventional taxonomy order except in the horse, dog and rabbit branches, which were associated with low bootstrap values. The substitution rates were

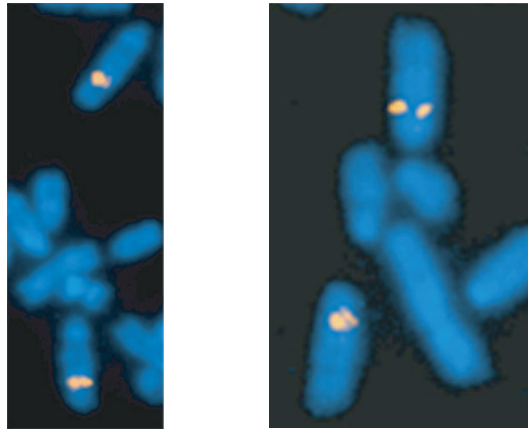


Figure 6. FISH map position of *EIF2AK2* BAC clones (orange) on DAPI counterstained metaphase chromosomes (blue). Left: horse at ECA15q24-q25. Right: cattle at BTA11q12-q15. (This figure is available in colour in electronic form at <http://www.edpsciences.org>).

uniform among the vertebrate sequences except for the *Danio rerio* and *Carassius auratus* cluster, which showed substitution rates that were about 20% higher as compared to those of other vertebrates.

3.7. Chromosomal localization of cattle and horse *EIF2AK2* genes

A search of the GenBank database with cattle genomic contigs containing *BTAEIF2AK2* exons revealed two BAC clones, CH240_360J16 and CH240_380H2. The T7-ends of these clones correspond to the intron 1 and promoter region of the *BTAEIF2AK2* gene, respectively. The cattle CH240_360J16 (~150 kb) clone as well as the horse CH241_81I21 (~160 kb) and CH241_117I21 (~150 kb) BAC clones were used to assign *EIF2AK2* genes to the chromosomal regions BTA11q12-q15 and ECA15q24-q25 (Fig. 6). Orthologous genes are located on human HSA2, chimpanzee PTR2A, dog CFA17, mouse MMU17 (www.ensembl.org) and rat RNO6 (ratmap.gen.gu.se) chromosomes. Using comparative painting (Zoo-FISH), similarities between HSA2 and evolutionarily diverged chromosomes or chromosomal segments of MMU17 [26], BTA11 [8,27], ECA15 [5], CFA17 [4,29] and RNO6 [21] were previously established. Our results further confirm conservation of *EIF2AK2*-containing syntenic chromosomal segments in cattle and horses.

ACKNOWLEDGEMENTS

This work was supported by Centers for Disease Control and Prevention grant CI000216 to MAB/AAP from the National Center for Infectious Diseases. The authors would like to thank J. Lundquist for FISH technical support and Drs. E. Howerth and M. Murphy (University of Georgia, Athens, GA) and T. Thompson (Equine Medicine and Surgery, Douglasville, GA) for providing equine tissue samples and PWBC. Horse gene mapping was supported by a grant from the Morris Animal Foundation (TLL). This work was performed in connection with a project at the University of Kentucky Agricultural Experiment Station and is published as paper: 05-14-114.

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