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High-resolution, human–bovine comparative mapping based on a closed YAC contig spanning the bovine *mh* locus

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Abstract. A closed YAC contig spanning the *mh* locus was assembled by STS content mapping with seven microsatellite markers, eight genes or EST, and nine STS corresponding to YAC ends. The contig comprises 27 YACs, has an average depth of 4.3 YACs, and spans an estimated 1.2 Mb. A linkage map was constructed based on five of the microsatellite markers anchored to the contig and shown to span 7 cM, yielding a ratio of 160 kb/1 cM for the corresponding chromosome region. Comparative mapping data indicate that the constructed contig spans an evolutionary breakpoint connecting two chromosome segments that are syntenic but not adjacent in the human. Consolidation of human gene order by means of whole genome radiation hybrids and its comparison with the bovine order as inferred from the contig confirm conservation of gene order within segments.

Introduction

In recent years, we have witnessed the multiplication of efforts to map economic trait loci (ETL) in livestock with strategies based on linkage analysis (for example, Georges 1998). Although mapping data can be exploited via Marker Assisted Selection (Visscher et al. 1998), maximal valorization probably requires actual cloning of the causal genes (Andersson 1998). When attempting to positionally clone ETL in livestock, animal geneticists benefit to a great extent from the remarkable progress achieved in characterizing the human and mouse genomes. Indeed, conserved chromosome segments (that is, segments within which gene order is conserved for two or more species) are estimated to be of the order of 10 cM on average between human and mouse (Copeland et al. 1993) and possibly larger between man and most livestock species. Identifying these conserved segments and their boundaries in the region of interest therefore allows for subsequent "comparative positional candidate cloning" (Archibald 1998) by exploiting human or mouse transcript maps.

Inference about the extent of gene order conservation is, however, affected by the resolution of the applied mapping methods. Indeed, refined mapping methods are susceptible to reveal rearrangements that could not be resolved by coarser methods. To verify whether micro-rearrangements might be common within previously defined conserved segments, we have generated a high-resolution, bovine–human comparative map surrounding the myostatin (MSTN) locus.

Recently, independent efforts led to the demonstration that loss-of-function mutations in the *MSTN* gene cause double-muscling in mouse and cattle (McPherron et al. 1997; Grobet et al. 1997, 1998; Kambadur et al. 1997; McPherron and Lee 1997). One of these studies used a comparative positional candidate strategy to identify the culprit bovine gene (Grobet et al. 1997). In this study, the bovine BTA2q1 linkage map and human HSA2q31-32 transcript RH-map were aligned using coincident bovine YACs, that is, large insert clones that contained both a bovine microsatellite marker and the bovine ortholog of a mapped human EST. This led to the identification of an interval on the human transcript RH-map that was likely to contain the double-muscling gene. The demonstration that *MSTN*, known to cause a muscular hyperplasia when knocked out in mice, mapped to that very interval, and the subsequent identification of loss-of-function mutations among double-muscled individuals demonstrated the role of *MSTN* in the determination of the double-muscling phenotype.

In this paper, we describe the completion of a YAC-based contig spanning the bovine *MSTN* locus. Bovine microsatellites anchored to this contig allow for a comparison of the linkage and physical map of the region. ESTs mapping to the corresponding region on the human map have been positioned on the bovine contig, allowing for a high-resolution comparison of both maps.

Materials and methods

YAC contig construction. Two distinct bovine YAC libraries representing a total of 12 genome equivalents were used in this work (Schoeberlein et al. unpublished; Takeda et al. 1998). PCR screening of the YAC libraries was performed with three dimensional pooling schemes as described (Libert et al. 1993; Takeda et al. 1998). High-density filters of the Schoeberlein YAC library were generated and used to screen the library by hybridization according to Cai and associates (1996). YAC ends were isolated using "bubble-PCR" following Libert and colleagues (1993). Microsatellite markers were isolated from YACs according to Cornelis and coworkers (1992). Map position of the YAC ends and microsatellites was examined by genotyping either a panel of bovine-rodent somatic cell hybrids (Dietz et al. 1992) or a panel of bovine-rodent radiation hybrids (Womack et al. 1997). Primer pairs used for PCR amplification of the sequence-tagged sites (STS) utilized for contig construction are as described (Grobet et al. 1997; Sonstegard et al. 1997) or reported in Table 1. FISH hybridization with total YAC clone DNA as probes was performed as previously described (Solinas-Toldo et al. 1995b; Masabanda et al. 1998).

The OPTICONTIG program (Georges unpublished) was used to determine the most likely STS/probe order based on STS/probe content of individual YACs. Starting from a randomized order, OPTICONTIG generates a Markov chain in which the $n + 1$ state differs from the n state by the random reassignment of the position of one randomly selected marker. For each state, a criterion is computed counting the number of negative STS within YAC boundaries given the considered order, and penalizing

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Table 1. Primers pairs used for PCR amplification of YAC ends and genes.

	YAC ends	
117C7-T	TTTAACCTCTGAGCCACCAGGGAAGC	CAGCTATTAGTAGCATTCCTGAGGT
117C7-U	TAATAGGTGTTTGTAAAAGCAAATC	ATCAATATAAAAATGGGCTAAATGCGG
74E11-T	GCATCAATATAAAAATGGGCTAAATGCGG	GTAAAAGCAAATCCACTGTTTTGGGGGAATG
215H8-T	AAGACAGAGTAGGTGGGAATGGGT	ATTCACAGGATAAGGTCAGATCCC
215H8-U	AAGTAAGAAACTAGATAAACTGTC	GTCTCTTGGACTTTTTCAGGTATATG
189A3-U	GACTATATAAATTGTAACTTTCAACAATTTG	ATCCGTAACAAATTACTTGGGCGTGGCGTC
179A3-T	CACCTCCACTAGGTGTGTTCGTAG	AGGAATGCAAGCCTACTTTAATACC
179A3-U	GCCTGGAAGGATGGCACTGCCAGCTTT	CAAAGCTGGCAGTGCCATCCTTCCAGG
178C6-U	CTTTGATCATTTCCCAATTTATGTGC	CAGAGGACCCTGAAGGGTTATAGTGC
	Genes	
NAB1	GTTAAACCCATCCAGAGTAATG	TTGATGCCTTTATCCAACAAGGTGG

orders which would not place STS corresponding to YAC ends at the extremities of the corresponding YAC. OPTICONTIG utilizes simulated annealing (for example, Weir 1996) to identify the order minimizing the value of the criterion. The chain is run several times to monitor repeated convergence to the same optimal order or set of equally optimal orders.

Genetic map construction. Microsatellite genotyping was performed as previously described (Georges et al. 1995). Linkage analyses were performed with the TWOPT and BUILD options of the CRIMAP package (Lander and Green 1987). Double recombinant individuals were identified with the CHROMPIC option and regenotyped.

Construction of a radiation hybrid map. DNA from the Genebridge-4 (Walter et al. 1994) and Stanford TNG whole genome radiation hybrid panels was obtained from Research Genetics (Huntsville, Ala.). Both panels were genotyped for human STS with primer pairs whose sequences were obtained from the Whitehead Institute/MIT Centre for Genome Research (Hudson et al. 1995). The resulting segregation vectors were analyzed with the RHMAXLIK program from the RHMAP package (Lunetta et al. 1995). The analysis utilized information from both panels simultaneously, assuming non-proportional distances between markers among the panels.

Results

Construction of a YAC contig spanning the bovine mh locus. In a previous study (Grobet et al. 1997), we described the construction of two small YAC contigs postulated to flank the *mh* locus. Contig "A", comprising seven YACs, contained microsatellite markers *TGLA44*, *BM81124*, *BULGE23*, and *BULGE27*, and the *INPP1* gene. Contig "B", comprising five YACs, contained microsatellites *BULGE20*, *BULGE28*, and *Col3AI*. The *MSTN* gene, causing the double muscling phenotype in cattle and mice, was predicted to map between these two contigs based on genetic mapping of the *mh* locus as well as on comparative mapping data (see above). One YAC containing *MSTN* (Y179A3) was isolated in the same study but could not be connected with either contig "A" nor "B". Therefore, the actual physical distance separating both contigs remained unknown, and the precise position of the myostatin gene on the bovine map was not formally demonstrated.

To complete the physical map of this genomic region, we therefore screened a six-genome equivalent bovine YAC library (Schoeberlein et al. unpublished) by PCR with two previously described microsatellite markers isolated respectively from contig "A" (*BULGE27*) and contig "B" (*BULGE20*), as well as six novel sequence-tagged sites (STS). Five of these were developed from YAC ends isolated by bubble-PCR from clones reported in Grobet et al. (1997) (117C7-T, 117C7-U, 215H8-T, 179A3-T, and 178C6-U). The remaining one corresponded to an STS amplifying the bovine ortholog of a gene known to map in the vicinity of the INPP1–Col3A1 interval on the human radiation hybrid map: the human NAB1 transcriptional repressor (NAB1; Hudson et al. 1995). The corresponding primers were designed by aligning the human and rat NAB1 sequence and targeting primer sequences towards highly conserved segments of the gene. The same YAC library was also screened by filter hybridization with pooled cDNA clones (Image id.: 149056 and 321388) corresponding to an expressed sequence tag (EST) mapping to the same region on the human map: WI-16551. A second bovine YAC library (Takeda et al. 1998) was subsequently screened by PCR with two additional YAC ends (215H8-U and 179A3-U) as well as with MSTN.

Altogether, we isolated 14 new YACs, yielding a total of 27 YACs in the region. FISH hybridization was used to confirm the localization of 24 of these YACs to the centromeric end of bovine Chromosome (Chr) 2 and identify possible chimerism. All selected YACs yielded a signal at the expected chromosome position, while 17 YACs generated additional signals on one (8) or more (9) other chromosomes. Pulsed field gel electrophoresis followed by Southern blot hybridization to total genomic DNA allowed us to estimate the individual size of each YAC, yielding an average insert size of 548 kb. Figure 1 reports the estimated size for all isolated YACs.

The presence of all STS and probes that were utilized to screen the libraries was confirmed on preparations of individual DNA. Moreover, the 27 YAC DNAs were tested by PCR for the presence of (i) two additional microsatellite markers: *BMC9007* (PROC; Sonstegaard et al. 1997) and a new microsatellite isolated from YAC 215H8: *BULGE18;* (ii) two novel YAC ends isolated by bubble-PCR: 74E11-T and 189A3-U; and (iii) an STS amplifying bovine protein C (*PROC;* Sonstegaard et al. 1997). In addition, the presence of the bovine orthologs of two additional EST (WI-13672, WI-21678) mapping in the vicinity of the INPP1–Col3A1 interval in human (Hudson et al. 1995), was tested on individual YAC DNA by Southern blotting with human cDNA clones (respectively Image clones 33009 and 172303) as probes under low-stringency hybridization conditions.

The STS/probe content allowed us to group all 27 YACs into a single, closed contig as shown in Fig. 1. The STS/probe order minimizing the number of inconsistencies in the YAC contig was identified with the OPTICONTIG program. Six equally parsimonious orders were identified and are reported in Fig. 1. These results form the possibility to flip the adjacent *PROC* and *BMC9007* markers, as expected given the fact that both STS were isolated from the same cosmid (Sonstegaard et al. 1997); as well as three equally parsimonious positions of EST WI-13672. Six of the 27 YACs were found positive for *MSTN*, therefore clearly demonstrating that MSTN indeed maps to this contig. Moreover, STS content data positioned *MSTN* between *INPP1* and *Col3A1* as predicted from the human transcript map (Grobet et al. 1997).

Analysis of Fig. 1 shows that three YACs (117C7, 24G12, and 202F2) are sufficient to define a minimum tiling path spanning the entire contig. Based on the estimated size of these clones (117C7:

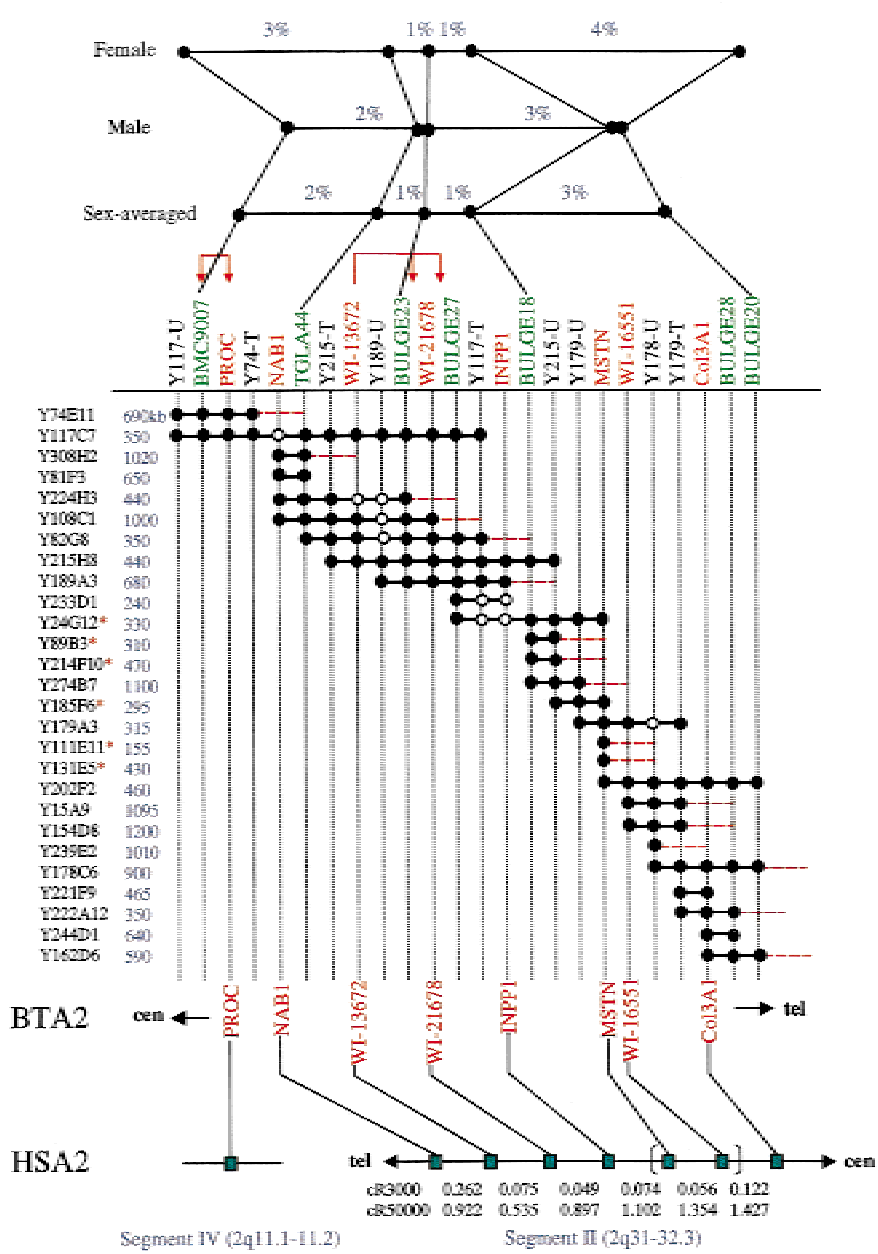

Fig. 1. YAC contig spanning the *MSTN* locus in cattle. STS/probes are represented in the most parsimonious order as determined from the STS content of individual YACs. Genes are represented in red, microsatellites in green, and YAC ends in black. Arrows point towards alternative, equally parsimonious positions for some of the STS. Black bullets report STS content of individual YACs while white bullets represent conflicts in the contig, given marker order. YACs isolated from the Takeda and coworkers (1998) library are marked with a red asterisk. Sizes of individual YACs in kilobases are reported in blue adjacent to YAC names. Chimeric YACs as determined by FISH hybridization are marked with a red stippled line. Sex-averaged, as well as male- and female-specific recombination rates between five microsatellite markers anchored to the contig, are shown above the contig. The gene order obtained in cattle is compared with the gene order determined with radiation hybrids in human at the bottom of the figure. The distance between adjacent genes on the human map is given in centirays as estimated with the Genebridge-4 and TNGF panels. Markers that could not be ordered with odds >100:1 are bounded by red brackets. Genes are assigned to evolutionary conserved chromosome segments II and IV as defined in Sonstegaard and associates (1997). The positions of the centromere and telomere with respect to the gene array show the inversion of segment II when comparing human and bovine.

350 kb; 24G12: 330 kb; 202F2: 460 kb), the corresponding chromosome region might be as small as 1140 kb. It is noteworthy that YAC 24G12 is chimeric, which would lead to an overestimation of the actual physical size of the region. On the other hand, the YACs could carry deletions, as indicated by some inconsistencies in the STS content, which would lead to an underestimation of the total size.

Construction of a high-resolution genetic map spanning the mh locus. The seven microsatellites included in the YAC contig were utilized to genotype 331 individuals from the bovine three-generation IBRP reference family (Hetzel et al. 1993), 255 individuals from the previously described Sart-Tilman backcross pedigree (Charlier et al. 1995), as well as 96 individuals corresponding to five paternal half-sib Maine-Anjou pedigrees segregating for the double-muscling trait (Grobet et al. unpublished). Five of the seven microsatellite markers proved to be polymorphic in this pedigree material.

The corresponding genotypes were used to construct a linkage map with CRIMAP (Lander and Green 1987). The most likely order and sex-averaged recombination rates between adjacent markers are represented in Fig. 1. Odds versus alternative orders were >1000. It can be seen that the marker order resulting from the linkage analysis coincided with the order as determined from the YAC contig. The distance between the outermost markers, *BMC9007* (*PROC*) and *BULGE20*, was estimated at 7 cM. Therefore, this suggests a ratio of the order of 160 kb per cM in this chromosome region.

When allowing for sex-specific recombination rates, we obtained the male and female maps shown in Fig. 1. It can be seen that for three of the four marker intervals, the estimated male recombination rates are smaller than the female estimates. For the *BULGE23–BULGE18* interval, on the contrary, the recombination rate seems larger in the heterogametic meioses. The likelihood of the data under the model of sex-specific recombination rates is, however, only 25 times superior when compared with the sex-

averaged model. More data are, therefore, needed to assess the significance of these sex-specific differences in recombination rates.

High-resolution human/bovine comparative mapping. Zoo-FISH experiments have shown that along most of its length bovine Chr 2 (BTA2q12-42) harbors sequences whose orthologs map to the long arm of Chr 2 in the human. Only the distal end of bovine Chr 2 (BTA2q43-45) seems to escape this rule, the orthologous sequences mapping to distal HSA1p in the human (Solinas-Toldo et al. 1995a). Subsequent work demonstrated that most of BTA2 corresponds to a complex rearrangement of five HSA2q segments (Sonstegard et al. 1997). The precise evolutionary breakpoints as well as the relative orientation of these segments in human and bovine, however, remain poorly defined.

From the genes assigned to the contig described in this work, PROC maps to 2q13-21.3 in the human (segment IV in Sonstegard et al. 1997), while the seven other ones (NAB1, WI13672, WI21678, INPP1, MSTN, WI16551 and COL3AI) are mapping to 2q31-32.3 (segment II in Sonstegard et al. 1997). Therefore, the most proximal YACs from our contig (particularly Y117C7) are bound to contain the corresponding evolutionary breakpoint (Fig. 1).

To more precisely assess the relative orientation of segment II in human and cattle, as well as to determine whether additional micro-rearrangements might have occurred within segment II during evolution, we re-examined the precise order of the seven corresponding genes on the human map. Indeed, while the approximate location of these genes on the human transcript map is known, their ordering based on available data remains ambiguous. We therefore genotyped both a 3000 rad (Genebridge 4; Walter et al. 1994) and a 50,000 rad (Stanford TNG RH panel) human whole genome hybrid panel for the corresponding genes. The resulting segregation vectors were analyzed jointly with the RHMAXLIK option of the RHMAP package (Lunetta et al. 1995). As illustrated in Fig. 1, this analysis resulted in reliable ordering of all but two genes (*MSTN* and *WI16551*), whose position could be flipped without significantly affecting the likelihood of the data. All other orders could be rejected based on odds >100. Therefore, these data point towards a conservation of gene order within the analyzed chromosome microsegment. The orientation of the gene cluster with respect to flanking framework markers included both in linkage maps and in the radiation hybrid maps (Hudson et al. 1995) indicates the inversion of segment II in the bovine with respect to the centromere (Fig. 1).

Discussion

We report the construction of a YAC-based contig spanning the *TGLA44–BULGE20* interval on proximal BTA2q. By assigning the *MSTN* gene to this contig, we confirm the location of the *mh* locus causing double-muscling in cattle to this marker interval as anticipated from linkage studies and human–bovine comparative mapping data (Grobet et al. 1997, Smith et al. 1997).

By comparing estimates of the physical distance (base pairs) covered by this contig with the genetic distance (centimorgan) separating the outermost informative microsatellite markers anchored to the contig (*BMC9007* and *BULGE20*), we calculated a ratio of 160 kb per cM for this chromosome region. This estimate has to be considered cautiously given the evidence for a high incidence of chimerism characterizing the utilized YAC libraries and the possibility for internal deletions affecting some of the YAC clones. Nevertheless, the obtained ratio is remarquably low. Indeed, by analogy with other mammals, the haploid DNA content of the bovine genome is usually estimated at approximately three billion base pairs, while the total sex-averaged genetic map length has been estimated at approximately 30 Morgan (Kappes et al. 1997). Therefore, one would expect a ratio of one million base pairs per centimorgan on average, far superior to what is suggested by our data for the studied chromosome region. In human, recombination rates are known to be inflated towards the telomeric end of chromosome arms, while the opposite tendency is found around centromeres. Our results suggest an inflation of recombination rates in a sub-centromeric region. Note that in bovine all chromosomes are acrocentric.

This work provides us with two independent estimates of the degree of chimerism characterizing the utilized YAC library (Schoeberlein et al. unpublished). Clone extremities were isolated from a number of YACs with bubble-PCR, and their map positions were determined with panels of either bovine–rodent somatic cell hybrids (Dietz et al. 1992) or bovine–rodent radiation hybrids (Womack et al. 1997). YAC end cloning was performed prior to receiving the outcome from the FISH mapping experiments, therefore without bias with regard to possible chimerism. Based on the corresponding results, YACs were sorted in three classes: (i) three nonchimeric YACs for which both extremities were mapping to BTA2q1; (ii) four chimeric YACs for which either both or one extremity was mapped to a chromosome region other than BTA2q1; and (iii) three ambiguous YACs for which one YAC end was shown to map to the right chromosome region, while no data were available for the other extremity. Expressing the likelihood of these data as a simple function of the degree of chimerism, C,

$$L = (1 - C)^3 C^4 (1 - 0.5C)^3$$

yields a maximum likelihood estimate of $C = 52\%$. FISH mapping, on the other hand, yielded an estimate of 17/24, or 71% chimerism. While both estimates may not be significantly different given the limited sample size, the estimate from the FISH hybridization seems unusually high. Examination of the distribution of the signals on the chromosomes other than BTA2q1, however, revealed evidence of non-randomness as five from the 24 recorded signals mapped to 1q31-35, while four mapped to 5q19-25. Although these results have to be considered cautiously, these data might indicate the existence of paralogous loci in the bovine genome. Similar results have recently been reported for the human genome (Eichler et al. 1998).

A previously described 5000 rad whole genome hamster–bovine radiation hybrid panel (Womack et al. 1997) proved to be very valuable to determine whether newly developed STS mapped to the predicted chromosome region. This resource could, however, not be used in this work to assist in ordering STS within the region spanned by the constructed contig owing to a lack of reliable chromosome breaks in this region. This is not unexpected given the limited physical size covered.

Detailed analysis of the map order of seven genes mapping to a conserved chromosome segment in human and cattle (segment II in Sonstegard et al. 1997) indicates interspecies conservation of gene order within this segment. This finding supports the generally admitted assumption that chromosome evolution during mammalian radiation has been accompanied by reshuffling of conserved chromosome segments; however, that within such segments gene order would be faithfully conserved. When comparing human and mice, for instance, the length of these conserved segments has been estimated at 8.8 cM (Copeland et al. 1993). This information is important for future positional candidate cloning efforts in livestock species, as it asserts that once the boundaries of conserved chromosome segments are identified, the order on the human transcript map accurately predicts gene order in the studied animal species.

A microsatellite (*BMC9007*) isolated from a bovine cosmid containing the *PROC* gene was shown to map to the assembled contig. PROC is known to map to HSA2q13-21.3 on the human map, which corresponds to conserved chromosome segment IV as defined by Sonstegard and coworkers (1997). Therefore, our con-

tig must contain the evolutionary breakpoint linking segments II and IV in the bovine. This will eventually facilitate its cloning and molecular characterization.

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