Cloning of a buffalo (*Bubalus bubalis*) prostaglandin $F_{2\alpha}$ receptor: changes in its expression and concentration in the buffalo cow corpus luteum

Shalu Verma-Kumar, S V Srinivas¹, P Muraly, Vijay K Yadav and R Medhamurthy

Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore 560 012, India and ¹Department of Pharmacology and Toxicology, Veterinary College, University of Agricultural Sciences, Bangalore 560 024, India

Correspondence should be addressed to R Medhamurthy; Email: rmm@mrdg.iisc.ernet.in

Abstract

Acting primarily through its specific G protein-coupled receptor termed FPr, prostaglandin (PG) $F_{2\alpha}$ induces regression of the corpus luteum (CL) at the end of a non-fertile oestrous cycle. This study was aimed at cloning a full-length cDNA for FPr and determining its expression and protein concentrations during different stages of CL development in the water buffalo. Serum progesterone and StAR expression were determined to establish temporal relationships between indices of steroidogenesis and changes in FPr expression at different stages of CL development. In contrast to the dairy cow, the stage IV CL (day 20 of the oestrous cycle) did not appear to be functionally regressed in the buffalo. Molecular cloning of a cDNA encoding the buffalo FPr yielded a full length 2193 bp FPr cDNA containing a single open reading frame encoding a 362 amino acid protein with seven putative membrane-spanning domains. The deduced buffalo FPr amino acid sequence possesses a high degree of identity with the other mammalian homologues. Steady state concentration of buffalo FPr transcript increased ($P > 0.05$) from stage I to stage II/III, and declined at 18 h post $PGF_{2\alpha}$ injection. The FPr concentration expressed as fmol/ μ g of plasma membrane protein showed an increase ($P > 0.05$) from stage I (1.98 ± 0.10), through stage II/III (2.42 ± 0.48) to stage IV (2.77 ± 0.18). High affinity FPr was observed in stage I (K_d 4.86 nmol) and stage II/III (K_d 6.28 nmol) while low affinity FPr (K_d 19.44 nmol) was observed in stage IV. In conclusion, we have cloned a full length FPr cDNA from buffalo cow CL and observed that FPr mRNA expression, receptor number and affinity did not vary significantly ($P > 0.05$) within the luteal phase of the oestrous cycle.

Reproduction (2004) 127 705–715

Introduction

The corpus luteum (CL) is a transient ovarian endocrine structure that plays a pivotal role in the control of reproduction in mammals (for reviews see Niswender & Nett 1994, Niswender *et al.* 2000, Diaz *et al.* 2002). In ruminants, CL regression is set in motion by the uterine secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (reviewed in McCracken *et al.* 1999, Niswender *et al.* 2000). Upon binding to its specific receptors (FPr), $PGF_{2\alpha}$ induces luteolysis by way of apoptosis (Juengel *et al.* 1993, McCracken *et al.* 1999, Yadav *et al.* 2002). An intriguing phenomenon characterising the action of $PGF_{2\alpha}$ is the absence of luteolytic action during the early and late stages of the oestrous cycle. Also, in the event of conception the CL on days 13 to 15 of the oestrous cycle appears to be resistant to the luteolytic action of $PGF_{2\alpha}$ (Wiepzig *et al.* 1992). A key determinant of luteolytic action

of $PGF_{2\alpha}$ could be the number and affinity of FPrs present in the CL. In the pig, it has been reported that the concentration of FPr proteins and mRNA levels were lower during the first 12 days of the oestrous cycle (Gadsby *et al.* 1990, Boonyaparakob *et al.* 2003). On the other hand, Wiltbank *et al.* (1995) observed no change in FPr concentration or affinity from day 2 to day 10 of the oestrous cycle in the dairy cow.

The water buffalo is the cornerstone of the livestock production-based agro-economy in many developing countries (Singh *et al.* 2000, FAO 2001). Prostaglandin $F_{2\alpha}$ and its synthetic analogues have been used in the induction of oestrus and/or in the synchronisation of oestrus in normally cyclic buffaloes (Chohan 1998, Brito *et al.* 2002). Therefore, a systematic study of the buffalo oestrous cycle, in particular the luteal phase, to determine the responsiveness to a luteolytic dose of $PGF_{2\alpha}$, is

imperative for the purpose of enhancing reproductive performance by way of induction of oestrus and/or ovulation. Thus, the aims of the present study were to clone cDNA for buffalo FPr and to evaluate the FPr expression and protein levels during different stages of CL development in the buffalo cow. Since StAR gene expression could well be a key determinant of luteal progesterone biosynthesis (for review see Diaz *et al.* 2002) during the normal oestrous cycle, its expression was studied as a marker for the functional status of the CL.

Materials and Methods

Chemicals and reagents

All chemicals used in the study unless otherwise specified were obtained from Sigma Chemical Company, St Louis, MO, USA. Avian myeloblastoma virus (AMV) RT, Taq DNA polymerase, random hexamers, RNAsin, Wizard Plasmid Miniprep purification kits, dNTPs and 100 bp DNA ladder were from Promega, Madison, WI, USA. Oligonucleotide primers were synthesised by Sigma-Genosys, Cambridge, Cambs, UK. Restriction enzymes were obtained from MBI Fermentas, St Leon-Rot, Germany. For random primer labelling, the Random Primer Extension Labelling System and [$\alpha^{32}\text{P}$]dCTP were procured from Perkin Elmer Life Sciences Inc., Boston, MA, USA. GeneRacer Advanced RACE kit, TOPO TA cloning kit for sequencing, Platinum Taq, agarose and Trizol reagent were obtained from Invitrogen, Life Technologies, Carlsbad, CA, USA. [^3H]PGF $_{2\alpha}$ ([5,6,8,9,11,12,14,15- ^3H (N)]PGF $_{2\alpha}$, 200 Ci/mmol) was obtained from New England Nuclear, Boston, MA, USA.

Tissue collection

All animal procedures described in this study were approved by the Institutional Animal Ethics Committee of the Indian Institute of Science. Buffalo cows (*Bubalus bubalis*; Surthi breed) with a known history of normal cyclicity were used in the study and the day of onset of oestrus was designated as day 1 of the oestrous cycle. For collection of CL, the buffalo oestrous cycle was divided into four stages: stage I (days 1–7 of the oestrous cycle), stage II (days 8–10 of the oestrous cycle), stage III (days 11–16 of the oestrous cycle) and stage IV (days 17–20 of the oestrous cycle). Nine buffalo cows were used for collection of CL ($n = 3$ CL/group) from day 7 of the oestrous cycle (stage I), day 20 of the oestrous cycle (stage IV) and 18 h post PGF $_{2\alpha}$ treatment. For collection of PGF $_{2\alpha}$ -treated CL, buffalo cows on day 11 of the oestrous cycle, corresponding to stage III of the oestrous cycle, were injected i.m. with 750 μg Tiaprost (Iliren, Intervet International B.V., Boxmeer, Holland). Blood samples were collected daily or on alternate days from day one of the oestrous cycle until CL collection for progesterone assay. Corpora lutea corresponding to days 8–16 of the oestrous cycle

(stage II/III) were collected from a nearby slaughterhouse according to the morphological criteria established for classification of CL during different days of the oestrous cycle by Ireland *et al.* (1980) in cattle and by Yadav *et al.* (2002) in the water buffalo. For collection of CL tissue, the CL from the ovary was extirpated under sterile conditions, cut into 6–8 pieces, transferred to labelled cryovials, snap frozen in liquid nitrogen and stored at -70°C until RNA or DNA analysis. Similarly, $\sim 100\text{ mg}$ pieces of heart, brain, liver, lungs, spleen, kidney and adrenal gland were also collected and stored at -70°C . Granulosa cells were separated from large and/or pre-ovulatory follicles from non-pregnant buffalo cows at slaughter according to the method of Murdoch *et al.* (1981). Ovaries for granulosa cell collection were snap frozen to allow collection of granulosa cells without RNA degradation. The granulosa cells were recovered from the follicular fluid by centrifugation at 700 g for 5 min at 4°C . Total RNA or genomic DNA extracted from different samples within a group was not pooled and each sample was analysed separately.

Genomic DNA isolation and detection of oligonucleosomes

Genomic DNA isolation, characterisation and analysis of oligonucleosomes were carried out as reported previously (Yadav *et al.* 2002).

RNA isolation

Total RNA was extracted from tissues and granulosa cells using Trizol reagent according to the manufacturer's recommendations. The quality and quantity of each RNA sample were assessed spectrophotometrically.

Oligonucleotide primers

The oligonucleotide primers used (see Table 1) were based on the published cDNA sequence of bovine and ovine FPr (GenBank Accession Numbers D17395 and U73798 respectively) and on the glyceraldehyde 3-phosphate dehydrogenase (G3PDH) genes for the bovine species. Computer searches and sequence alignments were performed at <http://www.ncbi.nlm.nih.gov> and <http://searchlauncher.bcm.tmc.edu/>

5'- and 3'-rapid amplification of cDNA ends (RACE) analysis and cloning of FPr

RNA ligase-mediated (RLM) and oligo-capping rapid amplification of cDNA ends (RACE) methods were used to obtain 5' and 3' ends of the buffalo FPr cDNA using the GeneRacer Advanced RACE kit according to the manufacturer's recommendations. Briefly, 2 μg total RNA from stage II/III CL ($n = 3$ CL, analysed separately without pooling of RNA) were dephosphorylated with calf intestinal alkaline phosphatase and decapped using tobacco acid pyrophosphatase (TAP). The GeneRacer RNA oligo was ligated to the TAP-treated mRNA with T4 RNA ligase and a

Table 1 Oligonucleotide primers used for semi-quantitative RT-PCR and for RLM-RACE, cloning and sequencing of the FPr gene in the water buffalo (*Bubalus bubalis*) CL.

Gene	Primer sequence	Reference
For semi-quantitative RT-PCR		
G3PDH	G3S: 5' TGTTCAGTATGATTCCACCC 3'	Tsai & Wiltbank (1998)
	G3AS: 5' TCCACCACCTGTTGCTGTA 3'	
FPr	S1: 5' CAAAGACTGGGAAGATAGTT 3'	
	AS1: 5' GTAAAAAGGGTTTCACAG 3'	
For RLM-RACE, gene cloning and sequencing		
FPr	S2: 5' CTGGTCTTGAGCCTGTTTAGAA 3'	GenBank Accession Numbers D17395 and U73798
	S3: 5' GAATGGCAACATGGAATCAA 3'	
	AS2: 5' CAACTAGGTGCTTGTGCTG 3'	
	AS3: 5' AGCCAAAAGCAAAAACGATG 3'	
	AS4: 5' GCATTTTCAGAGGAAATGGTG 3'	

S, sense; AS, antisense.

cDNA template generated by reverse transcription using SuperScript II and the GeneRacer oligo dT primer. The 5' and 3' ends were PCR amplified from this cDNA template with the appropriate GeneRacer primers and FPr-specific primers. Reverse transcription and PCR were carried out using a Peltier Thermal Cycler PTC-200 MiniCycler (MJ Research, Waltham, MA, USA). PCR was performed using Platinum Taq DNA polymerase with the following reaction conditions: initial denaturation at 94°C for 2 min, 5 cycles of denaturation at 94°C × 45 s, annealing at 59°C × 45 s and extension at 68°C × 3 min, followed by 25 cycles of touchdown PCR with denaturation at 94°C × 45 s, annealing at 58°C to 56°C × 45 s and extension at 68°C × 3 min. This was followed by a final extension at 68°C × 5 min. Table 1 lists the primers used for the RLM-RACE, cloning and sequencing of the buffalo FPr cDNA. The strategy used to sequence the FPr cDNA is shown in Fig. 2. The RACE and PCR products were gel purified and cloned into pCR4-TOPO vector for sequencing.

DNA sequencing and sequence analysis

The plasmid DNA was isolated using the Wizard Plasmid Miniprep purification system. After using the appropriate restriction enzymes, the clones were sequenced using an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Multiple sequence alignments were obtained at <http://searchlauncher.bcm.tmc.edu>. Hydrophobic analysis of the deduced FPr amino acid sequence using the Kyte-Doolittle method for calculation of hydrophobicity (Kyte & Doolittle 1982) was performed at http://bioinformatics.weizmann.ac.il/hyd-bin/plot_hydroph.pl.

Northern blot analyses of StAR and FPr

Northern blot analyses for StAR and FPr were carried out as reported previously (Yadav *et al.* 2002). For StAR, mouse StAR cDNA (Clark *et al.* 1994) was used to excise a 1456 bp SalI/NotI fragment. For FPr, buffalo FPr clone (906 bp length) corresponding to the N-terminal region was used in the Northern blot analysis. To counter check these results, total ovine FPr cDNA 1719 bp (Graves *et al.* 1995)

was used for excising a 752 bp Hind III/HincII fragment and used in a different set of blots. Normalisation was carried out using the 850 bp G3PDH PCR product as a probe.

RT-PCR

One microgram total RNA was denatured at 65°C and reverse transcribed using the following RT mixture: 200 µM of dNTPs, 40 units RNasin, 1 × RT buffer, 500 ng of random hexamers and 10 units AMV-RT in a total reaction volume of 25 µl. Reverse transcription was carried out for 1 h at 37°C. For PCR, cDNA equivalent to 150 ng total RNA was used. The PCR mix was made up of 200 µM of dNTPs, 1 × Taq buffer, 40 pmol of each gene specific primer and 2 units Taq DNA polymerase in a total reaction volume of 50 µl. PCR for FPr and G3PDH was carried out using the following reaction temperatures: an initial denaturation at 95°C for 2 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. The PCR products were separated on a 1.5% w/v agarose gel containing ethidium bromide and visualised under UV light.

Semi-quantitative RT-PCR for FPr

Semi-quantitative RT-PCR was carried out for FPr cDNA using G3PDH as the internal standard. Pilot experiments demonstrated that FPr was not in saturation until the 35th cycle of amplification while a non-specific band could be visualised after 28 cycles of G3PDH on co-amplification with FPr. To evaluate the effect of increasing RNA input, cDNA equivalent to 50, 100, 150, 300 and 500 ng total RNA was used. Subsequently, co-amplification was carried out using cDNA equivalent to 150 ng total RNA, and 32 cycles for FPr and 22 cycles for G3PDH using the primer dropping method (Wong *et al.* 1994). Thirty microlitres of the PCR products were separated on a 1.5% w/v agarose gel containing ethidium bromide and photographed and quantitated using an Alpha Imager 1200 documentation and analysis system (Alpha Innotech Corp., San Leandro, CA, USA).

Radioreceptor assay

The radioreceptor assay was carried out as previously reported for porcine (Gadsby *et al.* 1990), ovine (Wiepz *et al.* 1992) and bovine (Wiltbank *et al.* 1995) CL. Total membrane protein from each CL was extracted and analysed separately without pooling of membrane protein within each group. Receptor analysis was not performed on CL tissues from PGF_{2α}-treated buffalo cows due to insufficient quantity available for crude membrane preparation. In the present study, pilot studies were carried out to determine maximum binding of [³H]PGF_{2α} to crude membrane preparation of stage II/III CL. Maximal binding was observed with the incubation temperature at 23 °C under acidic (pH 5.75) conditions. Plasma membrane protein (125 µg) of the luteal tissue was incubated with 0.1 pmol [³H]PGF_{2α} and varying concentrations of non-radioactive Tiaprost tromethamine (a synthetic analogue of PGF_{2α}, 2–3 nmol) in a final volume of 145 µl per tube. The tubes were incubated in a water bath shaker at 23 °C for 2 h and radioactivity of bound membrane protein was measured. Number and affinity of receptors of PGF_{2α} in the crude membrane preparation were determined by Scatchard analysis using GPIS program (GraphPad Software Inc., San Diego, CA, USA).

Progesterone assay

The progesterone assay was carried out according to the method of Selvaraj *et al.* (1996) with modifications.

The antiserum GDN#337 kindly provided by Dr G D Niswender (Department of Biomedical Sciences, Colorado State University, Fort Collins, CO, USA) was employed in the assay. The sensitivity of the assay was 0.1 ng/ml and the inter- and intra-assay coefficients of variation were <10%.

Statistical analyses

Data wherever applicable were expressed as means ± S.E.M. The data were analysed by one-way ANOVA followed by Newman–Keuls multiple comparison test, and correlation analyses to determine the *r* value was performed using a GraphPad PRISM program (GraphPad Software Inc.). A *P* value <0.05 was considered statistically significant.

Results

Serum progesterone concentration, expression of StAR mRNA and analysis of DNA for oligonucleosome formation during different stages of CL development and function

Serum progesterone concentrations from buffalo cows at designated stages of CL development are represented in Fig. 1. The concentrations were low (*P* < 0.05) during stage I compared with stage II/III and IV. Administration of PGF_{2α} to mid-oestrous cycle buffalo cows resulted in a significant decrease (*P* < 0.05) in progesterone concentration

Figure 1 (a) Circulating serum progesterone concentrations, (b) mRNA expression of StAR by Northern blot analysis and relative expression of StAR normalised to the internal standard G3PDH and (c) detection of oligonucleosomes in genomic DNA during different stages of CL development (stage I, stage II/III, and stage IV) and from stage II/III buffalo cows 18 h post PGF_{2α} injection. Corpora lutea from twelve buffalo cows (*n* = 3 CL/group) were analysed separately without pooling of RNA or DNA within a group. Bars with different letters are statistically different from each other. Genomic DNA (15 µg/lane) was electrophoresed in 2% agarose gel, stained with ethidium bromide and visualised under UV and a representative picture is shown in (c). Migration (basepairs) of oligonucleosomes is indicated on the right. Note that DNA obtained from CL 18 h post PGF_{2α} injection alone clearly showed the presence of oligonucleosome formation. GC, granulosa cells.

(Fig. 1a). As shown in Fig. 1b, the mouse StAR cDNA probe was observed to hybridise to a major (~3.0 kb) and a minor (~1.6 kb) transcript in the buffalo CL. Besides CL, StAR expression was also observed in the adrenal gland and brain (data not shown). The expression of the major transcript was 1.7-fold higher ($P < 0.05$) in stage II/III CL compared with stage I CL (Fig. 1b); however maximum expression was observed in stage IV CL which coincided with the highest progesterone concentration. A sharp decrease ($P < 0.05$) in StAR expression was observed in CL collected from buffalo cows 18 h post PGF_{2α} injection (Fig. 1b). Basal levels of StAR expression were observed in the granulosa cells (Fig. 1b). A positive correlation with an r value of 0.9673 ($P < 0.05$) was seen between the progesterone profile and StAR expression at each stage of CL development and in PGF_{2α}-treated CL. Examination of genomic DNA in ethidium bromide-stained gels revealed no detectable oligonucleosome formation in CL collected from stage I, stage II/III and stage IV of the oestrous cycle. In contrast, pronounced oligonucleosome formation was observed in CL collected 18 h post PGF_{2α} injection (Fig. 1c).

Molecular cloning of the cDNA encoding the buffalo FPr in the CL

By employing RLM-RACE and RT-PCR techniques, four overlapping fragments of variable sizes (1263, 1037, 910 and 580 bp; Fig. 2) were generated from stage II/III CL RNA and sequenced. As shown in Fig. 3, the full length buffalo FPr cDNA generated was 2193 bp in size, consisting of 171 bp of 5'-untranslated region, 1089 bp of a single open reading frame (ORF) (positions 172–1260 bp) and 933 bp of 3'-untranslated region ending in a poly A tail. The nucleotide sequence from buffalo FPr cDNA shared 98, 98, 90, 87, 86, 83 and 80% identity with the correspond-

ing regions of the ovine, bovine, porcine, feline, human, murine and rat FPr cDNAs respectively. The ORF encoding a 362 amino acid protein with seven putative membrane spanning domains belonging to the family of G protein-coupled receptors is shown in Fig. 3. The deduced buffalo FPr amino acid sequence had 98, 98, 87, 82, 82, 79 and 78% identity to corresponding ovine (Accession Number Q28905), bovine (BAA20871), porcine (AAK95379), feline (AAL36977), human (NP000950), murine (P43117) and rat (AAB19233) FPr proteins respectively. The buffalo FPr cDNA sequence along with its deduced amino acid sequence were recently submitted to GenBank (Accession Number AY346134).

Characterization of FPr expression and FPr protein during different stages of CL development and function

The FPr expression in the CL tissue as assessed by Northern blot analysis (906 bp buffalo FPr cDNA probe) increased significantly from stage I (1.49 ± 0.12) to stage II/III (2.61 ± 0.15) and also stage IV (2.63 ± 0.47) ($P < 0.05$, Fig. 4a). After PGF_{2α} injection, a significant decrease (1.66 ± 0.19) ($P < 0.05$) in FPr expression was observed compared with stage II/III CL (Fig. 4a). FPr expression correlated positively with StAR gene expression ($r = 0.9106$) ($P < 0.05$) and progesterone concentration ($r = 0.9640$) ($P < 0.05$) at each stage of CL development and after PGF_{2α} injection.

Partial PCR products for FPr (267 bp) and G3PDH (850 bp) were amplified and sequenced. Semi-quantitative RT-PCR results indicated that CL from stage I of the luteal phase showed relative FPr expression of 1.04 ± 0.43 , which increased ($P > 0.05$) to 2.89 ± 0.70 at stage II/III, while stage IV CL showed a relative expression of 1.53 ± 0.57 ($P > 0.05$). The FPr expression in the CL collected 18 h post PGF_{2α} injection was 1.05 ± 0.039 ($P > 0.05$) (Fig. 4b). Using both semi-quantitative RT-PCR and Northern blot analyses, FPr expression was observed to be very low in granulosa cells collected from the pre-ovulatory follicles (Fig. 4a,b). Northern blot analysis for FPr using the buffalo cDNA probe revealed a major ~5.0 kb mRNA transcript and a second transcript ~6.0 kb in size. Similar results were obtained using the ovine FPr cDNA probe (data not shown). Different tissues such as heart, liver, kidney and CL showed the specific FPr PCR product while no amplification was seen in granulosa cells (Fig. 4c); however, by Northern blot analysis the FPr expression was observed only in CL (Fig. 4d).

The FPr concentration calculated from Scatchard analysis and expressed as fmol/μg of plasma membrane protein showed an increase ($P > 0.05$) from stage I (1.98 ± 0.10), through stage II/III (2.42 ± 0.48) to stage IV (2.77 ± 0.18) (Fig. 5a) irrespective of the affinity. In this study, two classes of FPr with high and low affinities were recorded in the buffalo CL. High affinity FPr was observed in stage I (K_d 4.86 nmol) and stage II/III (K_d 6.28 nmol) CL, while

Figure 2 Cloning and sequencing strategy for isolation of the water buffalo FPr gene using RT-PCR and 5' and 3' RLM-RACE. The relative position of specific primers (sense, S2 to S3; antisense, AS1 to AS4) is shown. Primers were designed within the conserved regions of bovine and ovine FPr mRNAs (Accession Numbers D17395 and U73798), and the nucleotide sequences of the overlapping cDNA clones were determined. The size of the PCR fragments obtained from each primer/primer set is indicated in parentheses.

10	20	30	40	50	60	70	80	90
CAAGACAGAT	GAGAAAGCTT	GGAAGGAAGA	AGAGAAATTC	CCCAGAGTGG	AGCTTTTGTG	GGGGCCCTCC	GGGAGCAGGG	CCAATACAA
100	110	120	130	140	150	160	170	
AACCTCACA	GATCTCTGGA	CTGCAAGCCT	AAACTCTTGA	GAGGGAGATG	ACTTGAGTGG	CCAGCTCTTA	TCTCCATAAC	A
172	ATG	TCC	ACG	AAC	AGT	TCT	ATA	CAG
	M	S	T	N	S	S	I	Q
241	CTG	GAA	GAA	GAC	CTT	TCA	ATA	TCT
	L	E	E	D	L	S	I	S
310	GCC	ATT	GCT	ATT	CTC	ATG	AAG	GCA
	A	I	A	I	L	M	K	A
379	GCT	AGT	GCT	CTA	GTA	ATC	ACA	GAT
	A	S	A	L	V	I	T	D
448	GCT	TCT	GAT	AAA	GAC	TGG	ATC	TAC
	A	S	D	K	D	W	I	Y
517	GTG	TTC	TCT	GGT	CTG	TGC	CCA	CTT
	V	F	S	G	L	C	P	L
586	AAA	CCA	ATA	TTT	CAC	TCG	ACA	AAA
	K	P	I	F	H	S	T	K
655	TTT	GCT	GTT	TTT	GTA	GCT	TTG	CTG
	F	A	V	F	V	A	L	L
724	TGG	TGT	TTC	TAC	AAA	ACA	GAT	CAA
	W	C	F	Y	K	T	D	Q
793	CTG	GGG	CTC	CTA	GCC	CTG	GGT	ATT
	L	G	L	L	A	L	G	I
862	AAA	TCT	AAA	AGT	CAG	CAG	CAC	AGA
	K	S	K	S	Q	Q	H	R
931	ATA	ATG	TGT	GTT	TCC	TGC	ATT	TGT
	I	M	C	V	S	C	I	C
1000	CAA	GAT	TTT	AAG	GAT	TCC	TGT	GAA
	Q	D	F	K	D	S	C	E
1069	GAT	CCC	TGG	GTA	TAT	ATT	CTT	CTA
	D	P	W	V	Y	I	L	R
1138	TGT	GGA	GTA	CAT	GTC	ATC	AGC	TTA
	C	G	V	H	V	I	S	L
1207	GCT	ATT	TCT	GAT	TTA	CCA	GTT	ACA
	A	I	S	D	L	P	V	T
1270	1280	1290	1300	1310	1320	1330	1340	
TTGAATAGGA	CGGTAAATCA	GTGTAGGTCT	AGGACCAAAT	TTAAAAAATA	GTGTTGGTAA	TACTTCAGTT	AATGTATATA	
ATAGAGAAAG	TCTAACTGGA	AAAATCAGCC	CTCACCCAGA	TAGTATGGGG	GCACTTTTGT	CAGGCTTGGC	TTTTAAATTT	
GTAAATATAG	CTGTATAATT	ACATATTTTA	ACACGGGTTT	TTCAACTAGA	GGTAAATGTG	ATAAAATAAT	GCCATAGGAG	
TCAAACGAAA	GCAATTTGGG	CCTTATCTGT	GCTATTGATG	GTATTGAAAT	GAGTGACTCT	CTTGAAGCCT	GAAGCGTGTA	
TTTGGTCTAC	TACCTGAACA	GTGAAATGGC	CATTTAGGGG	GTCACCTGCTC	TATGTAGACG	AGGCACAGTG	AAATGGGGCA	
CCCTTTTATG	ATCTGCTCTG	TTTACTCCT	CATTACATAG	CCTCAGTTAA	TACATGCATT	GTCATGTCAC	AGGGGATTGA	
TTGTGGCTTC	TTATAATGAC	TTCCACATAC	GTGGTTGGCT	GTCAGGTTGC	CTGGTCTTGT	GAGCCTGTTT	AGAATGAACC	
TTTCTTTTGT	ATATTTTGACA	GATACAACTG	CTTGCAATTA	TTGATATGAA	GGTCAGTTT	TGTTTGAAGA	TATCTTGT	
AAGTCATAGA	TTTGCACAGT	TTTCAGGTAA	TCCCCATTTT	CTCTGAAGAT	GCTTATGTAT	AGTCAATAGT	TTGTAGAGAA	
ACAAGCCTG	TGTGAGCAGG	TGTAGAGGTG	ACTTGCACTG	TAGCAGTTCC	TAAGGAACCT	AGCCATGGAT	AATGCAAAACA	
AGCAGAAGTT	AAACCCCAAG	TGATGGGTGC	AAAGAACATC	GGTAAAGGTG	CCTTACCTGA	GAACCATTTGA	CACAGTGTCA	
TTTCTGTGTC	AGGGAACAAA	AGAAACATAG	CCTCTAAAAA	AAAAAATAAA	AAA	2193		

Figure 3 Nucleotide and deduced amino acid sequences of FPr from CL tissue of the water buffalo ($n = 3$ CL from stage II/III). The deduced amino acid sequence is shown below the nucleotide sequence. The positions of seven putative transmembrane domains (TM 1–7; based on hydropathicity profile) are indicated by double underlining. Consensus sites for N-linked glycosylation are indicated for amino acid residues 4 and 19 (denoted by N). Also shown is the stop codon denoted by an asterisk.

Figure 4 Relative mRNA expression of buffalo FPr in granulosa cells (GC) during different stages (I–IV) of CL development and from CL collected 18 h post PGF_{2α} injection by (a) Northern blot and (b) RT-PCR analyses, and expression of buffalo FPr in different organs of the water buffalo by both (c) Northern and (d) RT-PCR analyses. Twelve buffalo cows ($n = 3$ animals/group) were used for collection of CL, which were analysed separately without pooling of RNA within a group. Granulosa cells were collected from 12 non-pregnant buffaloes from the slaughterhouse. A representative autoradiograph of a blot containing total RNA (20 μ g) probed with a 906 bp buffalo FPr cDNA and then with G3PDH is shown in panel (a). A representative semiquantitative RT-PCR for FPr gene using G3PDH as internal standard is shown in panel (b). The quality of total RNA used for cDNA synthesis is shown by analysis of 28S and 18S (b). The lower panels in (a) and (b) depict the densitometric analyses of FPr mRNA expression normalised against the internal standard G3PDH, and the data are expressed as means $\pm$ S.E.M. Bars with different letters are statistically different ($P < 0.05$) from each other. For FPr, in addition to a major (~ 5.0 kb) transcript, a minor transcript (~ 6.0 kb) was seen in (a) and (c). Representative Northern analysis (c) (lane 1, CL; lane 2, heart; lane 3, liver; lane 4, lungs; lane 5, granulosa cells) and RT-PCR (d) (M, 100 bp ladder; lane 1, CL; lane 2, heart; lane 3, liver; lane 4, lungs; lane 5, granulosa cells) for various buffalo tissues showed maximal FPr expression in CL.

low affinity FPr (K_d 19.44 nmol) was observed in the stage IV CL (Fig. 5b). In the buffalo cow, the specific binding of PGF_{2α}, expressed as counts/min, was 5842, 350 and 401 in the CL, lungs and liver respectively. The specific binding in other tissues such as heart, spleen and small intestine was below the non-specific binding. That the binding of [³H]PGF_{2α} was specific in the crude membrane preparation was confirmed by addition of increased cholesterol and PGE₂ concentrations, which failed to displace [³H]PGF_{2α} binding (data not shown).

Discussion

Water buffaloes are well suited to subtropical and tropical climates and possess great potential as milch, draught and meat animals (Valle 1994, Singh *et al.* 2000, Brito 2002).

FAO (2001) catalogues the world buffalo population at 167 million, which constitutes 12.5% of the whole bovine population. Considering the large buffalo population, there is a need to enhance efficiency of reproductive performance in these animals. Prostaglandin F_{2α} and its synthetic analogues are being extensively used for manipulation of the buffalo oestrous cycle, but information on buffalo oestrous cycles is, at best, fragmentary. The FPr is a key determinant in luteal sensitivity to the luteolytic actions of PGF_{2α} and thus it becomes imperative to study the temporal and spatial dynamics of FPr in the water buffalo.

The key regulated step in luteal progesterone production appears to be regulation of transport of cholesterol to the inner mitochondrial membrane apparently mediated by StAR (Stocco, 2001). In the present study, we evaluated

Figure 5 FPr concentrations and affinity throughout the luteal phase in the water buffalo. Nine buffalo cows ($n = 3$ animals/group) were used for collection of CL ($n = 3$ CL/group), which were analysed separately without pooling of membrane protein within a group (the FPr concentration in CL of buffalo cows collected 18 h post PGF_{2α} injection could not be evaluated) and the data were expressed as means $\pm$ S.E.M. Representative Scatchard analyses of binding of PGF_{2α} to receptors at different stages – (a) stage I, (b) stage II/III and (c) stage IV of CL development are shown in the upper panels. Bar diagrams represent PGF_{2α} receptor concentration (d) and affinity of [³H]PGF_{2α} to FPr (e) during different stages of CL (stage I, II/III and stage IV) development. The affinity of receptors at stage IV was significantly lower ($P < 0.05$) compared with CL at other stages.

StAR gene expression and progesterone concentrations as indices of steroidogenesis and functionality of the CL. A positive correlation was observed between these two indices throughout the oestrous cycle in the buffalo. Surprisingly, we observed maximum StAR expression and high serum progesterone concentrations at stage IV corresponding to day 20 of the oestrous cycle. The DNA analysis for detection of oligonucleosomes also revealed the absence of oligonucleosome formation at this stage, indicating a functional and not regressing CL at this stage. In contrast, in dairy cattle Pescador *et al.* (1996) found lower expression of StAR in stage IV CL obtained from the slaughterhouse, in which the stage of CL was assessed only by morphological evaluation. The plausible reasons for this discrepancy lie in the determination of the onset of oestrus and dating of the cycle in the present study. Although the length of the buffalo oestrous cycle is determined to be ~ 21 days, the length of the cycle may be longer in these animals. Studies are underway to determine the time of functional (decreased steroidogenesis)

regression of CL during spontaneous luteolysis at the end of a normal oestrous cycle in the buffalo cow. Yadav *et al.* (2002) documented that serum progesterone concentrations fell within 4 h and decreased maximally by 18 h post PGF_{2α} treatment, with a concomitant decrease in the levels of StAR mRNA and protein observed at 12–18 h post PGF_{2α} treatment. Studies on the effect of PGF_{2α} on StAR mRNA expression in ovine (Juengel *et al.* 1995) and bovine (Pescador *et al.* 1996) CL *in vivo* and human CL *in vitro* (Chung *et al.* 1998) also suggest a significant time-dependent decrease in StAR mRNA expression coinciding with the decrease in serum progesterone concentrations.

The cloned FPr cDNA in the buffalo CL encodes for a 362 amino acid protein with seven putative membrane spanning domains. Sequence alignment of the nucleotide and the deduced amino acid sequence of the buffalo FPr show a high degree of identity with the previously cloned FPr in other species including the cow (reviewed in Anderson *et al.* 2001). In the present study, Northern blot analysis using a buffalo FPr cDNA (906 bp length) clone

corresponding to the N-terminal region of FPr revealed two transcripts in the buffalo CL. The presence of two transcripts for FPr becomes interesting in the light of the report by Pierce *et al.* (1997) on the cloning of an FP prostanoïd receptor isoform termed FP (B) that differed from the FP (A) in the carboxyl terminus. The FP (A) isoform appears to arise by the failure to utilise a potential splice site, while a 3.2-kilobase pair intron is spliced out from the FP gene to generate the FP (B) isoform mRNA. The two isoforms have indistinguishable radioligand binding properties, but seem to differ in functional coupling to phosphatidylinositol hydrolysis. However, on cloning the FPr using RT-PCR and 5'- and 3'-RACE analyses, cDNA equivalent to a single mRNA transcript was obtained and this may be due to the strategy used to isolate the cDNA fragments reported here. The primer sets were designed to optimise the isolation of full length transcript based on the reported sequence of other mammalian FPr cDNAs, and the detection of shorter or less abundant cDNA in the stage II/III CL tissue could have been missed in the present study. In contrast, a single transcript of ~6.0 kb has been reported in the ovine CL (Graves *et al.* 1995) and of ~5.0 kb in the porcine CL (Boonyaparakob *et al.* 2003). Although a single gene appears to encode for FPr in all the species investigated thus far, multiple transcription initiation sites have been identified in the murine and bovine FPr genes. Ezashi *et al.* (1997) demonstrated that a single copy gene in the haploid genome encoded for the bovine FPr, and two mRNA forms (I and II) were generated by different transcription start points.

While two transcripts for FPr could be demonstrated by Northern analyses in the buffalo CL, the relative expression of the major (~5 kb) transcript is presented in the data. However, the FPr primers used in semi-quantitative RT-PCR did not distinguish between the two transcripts. Northern analysis for FPr showed a positive correlation with StAR mRNA expression and progesterone secretion at each stage of CL development examined and in PGF_{2α}-treated CL. We observed a significant increase in FPr expression from stage I to stage II/III CL with the levels maintained at stage IV. Similar results have been documented in cattle (Sakamoto *et al.* 1995), rabbit (Boiti *et al.* 2001) and pig (Boonyaparakob *et al.* 2003). However, our semiquantitative RT-PCR data revealed that while FPr expression was significantly higher throughout the luteal phase, it did not vary significantly at any stage of CL development. This observation is consistent with that of Juengel *et al.* (1996) in sheep and Tsai *et al.* (1996) in cattle who documented few, if any, significant changes in luteal FPr concentrations throughout the oestrous cycle.

The optimal conditions for *in vitro* radioligand binding assays employed in this study were based on assay conditions reported for porcine luteal cells (Gadsby *et al.* 1990), ovine luteal cells (Wiepz *et al.* 1992) and cow CL tissue homogenates (Wiltbank *et al.* 1995). That the receptor numbers in this study revealed no significant difference

during different stages of the CL agree with the findings in the dairy cow (Wiltbank *et al.* 1995). However, we have documented two classes of FPr, with high and low affinities, in the buffalo cow. The low affinity receptors were observed in the late luteal phase (stage IV). The receptor population has not been examined during the late luteal phase in the dairy cow, only in stage I and stage II/III (Wiltbank *et al.* 1995). The intraluteal concentrations of PGF_{2α} may be quite high during luteolysis and may reach concentrations at which occupancy of the low affinity site is significant (Diaz *et al.* 2002). This might explain the presence of low affinity FPr during the stage IV luteal phase. Indeed, luteal tissue taken from ewes 4 h after induction of luteolysis by exogenous administration of PGF_{2α} secreted multifold higher amounts of PGF_{2α} *in vitro* than corresponding luteal tissue from untreated ewes (Rexroad & Guthrie 1979). Alternatively, it is possible that the low affinity PGF_{2α} binding site may be a receptor for another species of eicosanoids and that PGF_{2α} may exhibit low affinity binding to the receptor as a result of its structural similarity (Kunapuli *et al.* 1997). Nonetheless, the significance of this finding requires further investigation, and it should be noted that more than one class of receptors has also been reported in CL from other species (Orlicky 1990, Chung *et al.* 1998). No significant differences in either affinity or concentration of FPr between stage I and stage II/III were recorded in the present study. Thus, as observed in several farm animals including the buffalo cow (reviewed in Jainudeen & Hafez 1993, McCracken *et al.* 1999, Niswender *et al.* 2000), lack of responsiveness to PGF_{2α} during the early luteal phase appears not to be related to low FPr concentrations. It has been reported that there are distinct physiological changes during early and mid-cycle bovine CL. The expression of mRNA encoding prostaglandin endoperoxide G/H synthase-2 is decreased in the early luteal phase while it is increased in the mid-luteal phase following PGF_{2α} treatment (Tsai & Wiltbank 1998). Also, there appears to be a distinct haemodynamic change in response to PGF_{2α} injection between the early (no change in blood flow) and mid-luteal (decreased blood flow) phases (Acosta *et al.* 2002). There is increasing evidence to suggest that intraluteal PGF_{2α} production appears to be one of the key factors that determines the luteolytic effect of exogenously administered PGF_{2α} (reviewed by Diaz *et al.* 2002).

The full length FPr cDNA generated in the present study was not subjected to further studies such as receptor binding and functional characterisation following its expression, since findings such as deduced amino acid sequence, Northern blot analysis, RT-PCR results and radioligand displacement assays provide strong evidence that the cloned receptor was FPr.

In summary, an FPr from the buffalo cow CL was cloned and sequenced. Although an increase in steady state concentration of buffalo FPr transcript from preovulatory granulosa cells to stage II/III was recorded, the expression did not vary significantly throughout the luteal phase. The

receptor number and affinity across different stages of CL development revealed no significant differences, but two classes (high and low affinity) of receptors were observed in the stage IV buffalo CL. The results show that the receptor population does not appear to be responsible for the lack of luteolytic response observed during the early stage of CL development.

Acknowledgements

The Department of Biotechnology and the Indian Council of Medical Research funded this work. We thank the Department of Biotechnology, the University Grants Commission and the Indian Council for Medical Research for financial support. We are grateful to Prof. Patricia Hoyer (Department of Physiology, College of Medicine, University of Arizona, AZ, USA) and Prof. D M Stocco (Texas Tech University Health Sciences Center, Texas, USA) for kindly providing the ovine FPR and mouse StAR cDNA respectively.

References

- Acosta TJ, Yoshizawa N, Ohtani M & Miyamoto A 2002 Local changes in blood flow within the early and midcycle corpus luteum after prostaglandin F₂α injection in the cow. *Biology of Reproduction* **66** 651–658.
- Anderson LE, Wu YL, Tsai SJ & Wiltbank MC 2001 Prostaglandin F(2α) receptor in the corpus luteum: recent information on the gene, messenger ribonucleic acid, and protein. *Biology of Reproduction* **64** 1041–1047.
- Boiti C, Zampini D, Zerani M, Guelfi G & Gobbetti A 2001 Prostaglandin receptors and role of G protein-activated pathways on corpora lutea of pseudopregnant rabbit *in vitro*. *Journal of Endocrinology* **168** 141–151.
- Boonyaparakob U, Gadsby JE, Hedgpeth V, Routh P & Almond GW 2003 Cloning of pig prostaglandin F₂α FP receptor cDNA and expression of its mRNA in the corpora lutea. *Reproduction* **125** 53–64.
- Brito LF, Satrapa R, Marson EP & Kastelic JP 2002 Efficacy of PGF_{2α} to synchronize estrus in water buffalo cows (*Bubalus bubalis*) is dependent upon plasma progesterone concentration, corpus luteum size and ovarian follicular status before treatment. *Animal Reproduction Science* **3** 23–35.
- Chohan KR 1998 Estrus synchronization with lower dose of PGF_{2α} and subsequent fertility in subestrus buffalo. *Theriogenology* **50** 1101–1108.
- Chung PH, Sandhoff TW & McLean MP 1998 Hormone and prostaglandin F₂α regulation of messenger ribonucleic acid encoding steroidogenic acute regulatory protein in human corpora lutea. *Endocrine* **8** 153–160.
- Clark BJ, Wells J, King SR & Stocco DM 1994 The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). *Journal of Biological Chemistry* **269** 28314–28322.
- Diaz FJ, Anderson LE, Wu YL, Rabot A, Tsai SJ & Wiltbank MC 2002 Regulation of progesterone and prostaglandin F (2α) production in the CL. *Molecular and Cellular Endocrinology* **91** 65–80.
- Ezashi T, Sakamoto K, Miwa K, Okuda-Ashitaka E, Ito S & Hayaishi O 1997 Genomic organization and characterization of the gene encoding bovine prostaglandin F₂α receptor. *Gene* **190** 271–278.
- FAO 2001 <http://www.fao.org>.
- Gadsby JE, Balapure AK, Britt JH & Fitz TA 1990 Prostaglandin F₂α receptors on enzyme-dissociated pig luteal cells throughout the estrous cycle. *Endocrinology* **126** 787–795.
- Graves PE, Pierce KL, Bailey TJ, Rueda BR, Gil W, Woodward DF, Yool AJ, Hoyer PB & Regan JW 1995 Cloning of a receptor for prostaglandin F₂α from the ovine corpus luteum. *Endocrinology* **136** 3430–3436.
- Ireland JJ, Murphee RL & Coulson PB 1980 Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. *Journal of Dairy Science* **63** 155–160.
- Jainudeen MR & Hafez ESE 1993 Cattle and buffalo. In *Reproduction in Farm Animals*, pp 315–329. Ed. ESE Hafez. Philadelphia: Lea Febiger Press.
- Juengel JL, Garverick HA, Johnson AL, Youngquist RS & Smith MF 1993 Apoptosis during luteal regression in cattle. *Endocrinology* **132** 249–254.
- Juengel JL, Meberg BM, Turzillo AM, Nett TM & Niswender GD 1995 Hormonal regulation of messenger ribonucleic acid encoding steroidogenic acute regulatory protein in ovine corpora lutea. *Endocrinology* **136** 5423–5429.
- Juengel JL, Wiltbank MC, Meberg BM & Niswender GD 1996 Regulation of steady-state concentrations of messenger ribonucleic acid encoding prostaglandin F₂α receptor in ovine corpus luteum. *Biology of Reproduction* **54** 1096–1102.
- Kunapuli P, Lawson JA, Rokach J & FitzGerald GA 1997 Functional characterization of the ocular prostaglandin F₂α (PGF₂α) receptor. Activation by the isoprostane, 12-iso-PGF₂α. *Journal of Biological Chemistry* **272** 27147–27154.
- Kyte J & Doolittle RF 1982 A simple method for displaying the hydrophobic character of a protein. *Journal of Molecular Biology* **157** 105–132.
- McCracken JA, Custer EE & Lamsa JC 1999 Luteolysis: a neuroendocrine-mediated event. *Physiological Review* **79** 263–323.
- Murdoch WJ, Dailey RA & Inskeep EK 1981 Preovulatory changes in prostaglandins E₂ and F_{2α} in ovine follicles. *Journal of Animal Science* **53** 192–205.
- Niswender GD & Nett TM 1994 Corpus luteum and its control in infraprimate species. In *The Physiology of Reproduction*, pp 781–816. Eds E Knobil & JD Neill. New York: Raven Press.
- Niswender GD, Juengel JL, Silva PJ, Rollyson MK & McIntush EW 2000 Mechanisms controlling the function and life span of the corpus luteum. *Physiological Review* **80** 1–29.
- Orlicky DJ 1990 [³H]Prostaglandin F_{2α} membrane binding reexamined. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **40** 181–190.
- Pescador N, Soumano K, Stocco DM, Price CA & Murphy BD 1996 Steroidogenic acute regulatory protein in bovine corpora lutea. *Biology of Reproduction* **55** 485–491.
- Pierce KL, Bailey TJ, Hoyer PB, Gil DW, Woodward DF & Regan JW 1997 Cloning of a carboxyl-terminal isoform of the prostanoid FP receptor. *Journal of Biological Chemistry* **272** 883–887.
- Rexroad CE Jr & Guthrie HD 1979 Prostaglandin F₂α and progesterone release *in vitro* by ovine luteal tissue during induced luteolysis. *Advances in Experimental Medicine and Biology* **112** 639–644.
- Sakamoto K, Miwa K, Ezashi T, Okuda-Ashitaka E, Okuda K, Houtani T, Sugimoto T, Ito S & Hayaishi O 1995 Expression of mRNA encoding the prostaglandin F₂α receptor in bovine corpora lutea throughout the estrous cycle and pregnancy. *Journal of Reproduction and Fertility* **103** 99–105.
- Selvaraj N, Medhamurthy R, Ramachandra SG, Sairam MR & Moudgal NR 1996 Assessment of luteal rescue and desensitization of macaque corpus luteum brought about by human chorionic gonadotrophin and deglycosylated human chorionic gonadotrophin treatment. *Journal of Biosciences* **21** 497–510.
- Singh J, Nanda AS & Adams GP 2000 The reproductive pattern and efficiency of female buffaloes. *Animal Reproduction Science* **60** 593–604.

- Stocco DM** 2001 StAR protein and the regulation of steroid hormone biosynthesis. *Annual Review of Physiology* **63** 193–213.
- Tsai SJ & Wiltbank MC** 1998 Prostaglandin F2alpha regulates distinct physiological changes in early and mid-cycle bovine corpora lutea. *Biology of Reproduction* **58** 346–352.
- Tsai SJ, Wiltbank MC & Bodensteiner KJ** 1996 Distinct mechanisms regulate induction of messenger ribonucleic acid for prostaglandin (PG) G/H synthase-2, PGE (EP3) receptor, and PGF2alpha receptor in bovine preovulatory follicles. *Endocrinology* **137** 3348–3355.
- Valle WG** 1994 Reproductive management of water buffalo under Amazon conditions. *Buffalo Journal* **10** 85–90.
- Wiepz GJ, Wiltbank MC, Nett TM, Niswender GD & Sawyer HR** 1992 Receptors for prostaglandins F2alpha and E2 in ovine corpora lutea during maternal recognition of pregnancy. *Biology of Reproduction* **47** 984–991.
- Wiltbank MC, Shiao TF, Bergfelt DR & Ginther OJ** 1995 Prostaglandin F2alpha receptors in the early bovine corpus luteum. *Biology of Reproduction* **52** 74–78.
- Wong H, Anderson WD, Cheng T & Riabowol KT** 1994 Monitoring mRNA expression by polymerase chain reaction: the 'primer-dropping' method. *Analytical Biochemistry* **223** 251–258.
- Yadav VK, Sudhagar RR & Medhamurthy R** 2002 Apoptosis during spontaneous and prostaglandin F2alpha-induced luteal regression in the buffalo cow (*Bubalus bubalis*): involvement of mitogen-activated protein kinases. *Biology of Reproduction* **67** 752–759.

Received 18 November 2003

First decision 9 February 2004

Accepted 10 March 2004