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The search for the Booroola (*FecB*) mutation

G. W. Montgomery, J. M. Penty, E. A. Lord and M. F. Broom

AgResearch Molecular Biology Unit, Department of Biochemistry and Centre for Gene Research, University of Otago, PO Box 56, Dunedin, New Zealand

Sheep derived from the Booroola Merino strain carry an autosomal mutation (*FecB*) that increases ovulation rate and litter size. One approach to characterize the genetic mutation is to locate the gene using positional cloning. The locus has been mapped to a region between genes for secreted phosphoprotein 1 (SPP1) and epidermal growth factor (EGF) on sheep chromosome 6. Analysis of possible candidate genes have excluded a number of genes associated with control of reproduction including genes from chromosome 6. Attempts to define close flanking markers and clone the region of DNA containing the mutation are now in progress. We have cloned additional markers and developed a linkage map showing that the *FecB* locus maps towards the centromere on chromosome 6. We have developed a yeast artificial chromosome (YAC) library for the sheep and begun screening the library to identify large DNA clones spanning the *FecB* region. These will be used to locate the mutation and shed light on how the mutation increases ovulation rate in Booroola sheep.

Introduction

Sheep derived from the Booroola Merino strain provide an important model to study mechanisms controlling ovulation rate in mammals because they carry an autosomal mutation that increases ovulation rate and litter size (Davis *et al.*, 1982; Piper and Bindon, 1982). The effect of the locus (*FecB*; COGNOSAG, 1989) is additive for ovulation rate, but partially dominant for litter size because of embryonic loss in homozygous carriers with high ovulation rates (Piper *et al.*, 1985).

Carriers of the *FecB* mutation can be identified only by phenotypic effects in females. One goal of research in Booroola sheep has been to identify a simple test for carriers of the *FecB* allele by finding a biochemical or genetic marker linked to the gene (McNatty *et al.*, 1991; Montgomery *et al.*, 1992a). A suitable marker would provide a rapid and accurate means of predicting the Booroola genotype of males, and of lambs before puberty.

A linked genetic marker is also essential as the first step towards molecular characterization of the gene and its function using genetic studies. Strategies for finding and characterizing genes without any prior knowledge of the gene function are now well developed and referred to as positional cloning (Collins, 1992). Positional cloning is initiated by locating the gene to a chromosome. Successive narrowing of the location and cloning of expressed genes in the region will eventually result in the identification of the gene. Positional cloning provides a powerful alternative to functional studies and has been used successfully to identify a number of genes responsible for inherited disorders in humans and mice.

The first step of finding markers linked to the *FecB* locus has now been completed. The locus maps near the centromere on sheep chromosome 6 (Montgomery *et al.*, 1993, 1994a). This article reviews studies to map the *FecB* locus and progress towards locating and characterizing the mutation responsible for the increased ovulation rate in Booroola gene carriers.

Genetic Marker Studies

Protein polymorphisms

The theoretical basis for linkage mapping has been developed for many years, but the application in livestock species has been limited by a lack of suitable genetic markers. Genetic markers can include polymorphisms associated with proteins, anonymous DNA segments or gene loci. Locus names and symbols used to describe gene loci follow the rules for nomenclature in sheep (Echard *et al.*, 1994).

Initial studies in Booroola flocks focused on the biochemical analysis of variation in proteins. Analysis of carrier and non-carrier groups of Booroola sheep suggested an association between alleles of β haemoglobin (HBB) and the *FecB* locus (Dratch *et al.*, 1986). Subsequent linkage studies did not support this observation and the *FecB* locus was clearly excluded from the region of HBB (Montgomery *et al.*, 1990; Tate *et al.*, 1992). Screening all the common protein polymorphisms in sheep (including transferrin and the ovine major histocompatibility complex) failed to provide any evidence of genetic linkage to the *FecB* locus (Mulsant *et al.*, 1991; Tate *et al.*, 1992). The number of protein polymorphisms described for sheep is limited and many of the alleles are rare. Consequently these markers excluded only a small proportion of the genome (Tate *et al.*, 1992).

DNA markers

Systematic genome searches have become possible only with the development of methods to detect large numbers of DNA markers and the construction of genetic linkage maps. The first markers were restriction fragment length polymorphisms (RFLPs) that exploit the ability of restriction enzymes to detect single base differences (or point mutations) in DNA sequences (Botstein *et al.*, 1980). Other markers exploit variation in the copy number of repetitive DNA elements between individuals that are common throughout the genome. Highly polymorphic DNA minisatellite sequences, composed of arrays of variable number of tandem repeats (VNTR) scattered over the genome, produce multi-band DNA fingerprinting patterns (Jeffreys *et al.*, 1985). Microsatellite markers detect variation in the length of small simple sequence repeats such as the dinucleotide AC/GT (Weber and May, 1989).

Markers associated with gene sequences, such as RFLPs, provide important links with genetic maps of other species. However, many RFLPs are not very informative. Microsatellite markers are highly polymorphic and informative in linkage studies. They have become the markers of choice for livestock linkage mapping projects (Bishop *et al.*, 1994; Crawford *et al.*, 1994; Rohrer *et al.*, 1994). The disadvantage is that they provide map location information only for closely related species.

In 1987, we began a project to map the *FecB* locus. At that time, there was no linkage map for sheep and the strategy adopted was to combine the use of RFLP and anonymous markers. We developed RFLP markers using cDNA probes for cloned genes from humans and cattle (Montgomery *et al.*, 1992c). Concurrently, we analysed DNA fingerprinting probes in the Booroola families. The probes used in our flocks failed to detect linkage to the *FecB* locus (Crawford *et al.*, 1993).

DNA fingerprinting experiments were discontinued in favour of microsatellite markers (Crawford *et al.*, 1994) because these markers are locus specific and can be detected easily using the polymerase chain reaction (PCR). Analysis of linkage between RFLP and microsatellite markers demonstrated that the markers were dispersed throughout the sheep genome and provided the basis for an initial genetic linkage map for sheep (Crawford *et al.*, 1994).

Linkage Mapping of the *FecB* Locus

Linkage to the Booroola gene was first detected after screening 145 markers (Montgomery *et al.*, 1993). An anonymous microsatellite marker (OarAE101) was linked to *FecB* with a maximum lod score of 17.33 at 13 cM. Immediately afterwards, a second microsatellite (OarHH55) was also linked to *FecB* (Montgomery *et al.*, 1993). These markers could not assign a chromosomal location to *FecB* because their map position was unknown.

The microsatellite markers were linked to an RFLP for secreted phosphoprotein 1 (SPP1, Montgomery *et al.*, 1993). In humans, SPP1 maps to human chromosome 4q. The order of many genes from human chromosome 4 is similar to genes on either mouse chromosomes 3 or 5. We were able to use this extensive gene mapping information from the human and the mouse to identify additional genes that should map to the region of the *FecB* locus. RFLPs were developed for a number of genes in the region and *FecB* was shown to be linked to epidermal growth factor (EGF) and complement factor I (IF; Montgomery *et al.*, 1993). These genes provided markers on the opposite site of the *FecB* locus from OarAE101 and SPP1. The additional linkage information also confirmed that this region of the sheep genome has extensive homology or synteny with human chromosome 4q and mouse chromosomes 3 and 5.

An approach using DNA fingerprinting was successful in French Booroola flocks in identifying markers linked to the *FecB* locus (Lanneluc *et al.*, 1994). The segregation of a large number of loci can be sampled in a single experiment using VNTR markers. Analysis of 12 minisatellite probes demonstrated that, as in other species, the minisatellites in sheep tend to be organised in clusters (Lanneluc *et al.*, 1994). One cluster of seven minisatellite bands was linked to the *FecB* locus (Lanneluc *et al.*, 1994). This cluster was also strongly linked to the A blood group in sheep. The distance between the A blood group and the *FecB* locus was estimated at 38 cM and mapped on the opposite side of the fingerprint bands from the *FecB* locus. There was no direct evidence for linkage between the *FecB* locus and the A blood group in agreement with the earlier studies (Mulsant *et al.*, 1991). The chromosomal location of the *FecB* locus could not be determined from these results (Lanneluc *et al.*, 1994). The DNA fingerprint bands do not provide a unique location on the genome and the location of the A blood group had not been determined.

Mapping *FecB* to Sheep Chromosome 6

Comparing the gene maps of humans with cattle and sheep suggested that *FecB* should map to sheep chromosome 6 (Montgomery *et al.*, 1994a). In sheep, chromosomes 4 and 6 are difficult to distinguish and there is some confusion in the numbering of chromosome 4 and 6 in different standards for the sheep karyotype. The gene for β casein (CSN2) has been assigned by *in situ* hybridization to sheep chromosome 6q23–q31 (Ansari *et al.*, 1992) and sheep chromosome 4 (Hayes *et al.*, 1992). One method to distinguish chromosomes 4 and 6 clearly is to analyse chromosomes from sheep carrying the Robertsonian translocation (designated as t1; *rob* 6;24) where chromosome 6 is centrically fused to chromosome 24 (Ansari *et al.*, 1992). *In situ* hybridization demonstrates that CSN2 maps to chromosome 6, the long arm of the Robertsonian translocation t1 (Ansari *et al.*, 1992).

FecB was assigned to sheep chromosome 6 by two methods (Montgomery *et al.*, 1994a). The linkage group, including *FecB*, OarAE101, SPP1 and OarHH55, was extended by analysis of additional markers. Genes for platelet-derived growth factor receptor alpha (PDGFRA) and αS1-casein (CSN1S1) were linked to this group of markers. In cattle and sheep the casein genes are tightly linked (Ferretti *et al.*, 1990; Threadgill and Womack, 1990; Leveziel *et al.*, 1991; Lien *et al.*, 1993). Inclusion of the casein gene cluster in the *FecB* linkage group assigns the *FecB* locus to sheep chromosome 6 (Montgomery *et al.*, 1994a).

An alternative approach used a partial panel of sheep × hamster somatic cell hybrids (Burkin *et al.*, 1993). Markers from the linkage group, including the microsatellites OarAE101 and BM143, and the genes EGF, SPP1 and PDGFRA, were mapped in the hybrid panel. All markers identified bands specific to somatic cell hybrids containing sheep chromosome 6 (Montgomery *et al.*, 1994a).

Markers from the region of the *FecB* locus can be used for a direct test for carriers of the mutation. The marker OarAE101 has been used successfully to identify *FecB* carriers in Booroola × Awassi ewes (Gootwine *et al.*, 1994).

Candidate Genes

Physiological studies with homozygous (BB), heterozygous (B+) and non-carriers (++) of the *FecB* mutation have shown that there are differences between genotypes in ovarian follicular development, concentrations of pituitary hormones in plasma and pituitary responses to exogenous GnRH (McNatty

Table 1. Genes in the reproductive pathway excluded as possible sites of the Booroola (*FecB*) mutation in sheep

Locus	Symbol	Chromosome Location	
		Sheep	Human
Epidermal growth factor	EGF	6	4q25
Follicle-stimulating hormone, β subunit	FSHB	15q24–qter	11p13
Follicle-stimulating hormone, receptor	FSHR	—	2p21–p16
Follistatin	FSA	—	—
Glycoprotein hormones, α subunit	CGA	—	6q14–q21
Hardy-Zuckerman 4 feline sarcoma viral (v-*kit*) oncogene homologue	KIT	6	4q12
Luteinising hormone, β subunit	LHB	14	19q13.3
Luteinising hormone receptor	LHCGR	—	2p21
Inhibin, α	INHA	2q	2q33–q34
Inhibin, βA polypeptide	INHBA	4q27–q31	7p15–p13
Insulin-like growth factor I	IGFI	3	12q22–q23
Insulin-like growth factor II	IGFII	21q21–qter	11p15.5
Oestrogen receptor	ESR	8q25–q27	6q25.1

et al., 1991; Montgomery *et al.*, 1992a). The increased concentrations of follicle-stimulating hormone (FSH) in plasma in BB and B+ ewes may advance the maturation of gonadotrophin-responsive and gonadotrophin-dependent follicles to increase ovulation rate (Scaramuzzi *et al.*, 1993). In addition, the *FecB* mutation increases the expression of one or more of the βA-inhibin mRNA species and may influence inhibin or activin synthesis (Fleming *et al.*, 1992). Consequently, genes in the reproductive pathway that regulate release and homeostatic control of FSH could be considered as potential candidate genes for the site of the *FecB* mutation.

Polymorphisms for a number of these candidate genes were identified and screened in the Booroola families (Table 1). Genes specifically excluded from linkage to the *FecB* locus include the α and β subunits of FSH (Montgomery *et al.*, 1990, 1992b), the β subunit of luteinizing hormone (LH, G. W. Montgomery, unpublished), genes for α-inhibin, βA inhibin, and follistatin (Montgomery *et al.*, 1994b), the receptors of FSH and LH (G. W. Montgomery and R. Rohan, unpublished) and insulin-like growth factor I (IGF-I, Mulsant *et al.*, 1991).

Other candidate genes outside the pathways of gonadotrophin feedback control may also be implicated. There are differences in the ovarian follicles in *FecB* carriers in the absence of gonadotrophins (McNatty *et al.*, 1990). The mutation is expressed during fetal life influencing germ cell development and follicle formation (Smith *et al.*, 1993, 1994).

Once the *FecB* locus was assigned to sheep chromosome 6, genes that map outside of this region are excluded as sites of the *FecB* mutation (Table 1). For example, a mutation in the oestrogen receptor in Meishan pigs is thought to increase litter size by one piglet (Rothchild *et al.*, 1994). In sheep, the oestrogen receptor maps to sheep chromosome 8q (Pearce *et al.*, 1994) and cannot be implicated as the site of the *FecB* mutation.

Gene maps of all mammals were compared to identify genes that influence reproductive development from the equivalent regions to sheep chromosome 6 (Montgomery *et al.*, 1993). The white spotting (W) locus in mice encodes the proto-oncogene kit (KIT, Charbot *et al.*, 1988; Geissler *et al.*, 1988; Nocka *et al.*, 1989). Mice homozygous for mutations at the W locus are deficient in three classes of cells: germ cells, melanocytes and hematopoietic cells (Russell, 1979; Silvers, 1979). EGF influences the growth and differentiation of granulosa cells (Carson *et al.*, 1989; Bendell and Dorrington, 1990; Roy and Greenwald, 1991). RFLP markers for KIT and EGF clearly excluded both these loci as the sites of the *FecB* mutation, although EGF was linked to the mutation at a distance of 26 cM.

Recently, the GnRH receptor was mapped to human chromosome 4q13.1–q21.1 (Kaiser *et al.*, 1994). Linkage analysis in a mouse backcross line mapped the gene to mouse chromosome 5 in the region of

Table 2. Gene loci that map to sheep chromosome 6. This chromosome shows extensive conservation with human chromosome 4

Locus name	Symbol
Albumin	ALB
Bone morphogenetic protein 3	BMP3
Casein, α-S1	CSN1S1
Complement factor 1	IF
Epidermal growth factor	EGF
GRO1 oncogene (melanoma growth stimulating activity, α)	GRO1
Hardy-Zuckerman 4 feline sarcoma viral (v-*kit*) oncogene homologue	KIT
Phosphodiesterase, cyclic GMP, β polypeptide	PDEB
Platelet-derived growth factor receptor, α polypeptide	PDGFRA
Secreted phosphoprotein 1	SPP1

the W locus (KIT) and PDGFRA. The GnRH receptor could be considered to be a strong candidate for the *FecB* locus because of the associated changes in plasma gonadotrophin concentrations in carrier ewes. However, KIT and PDGFRA map to the same location in sheep and are more than 50 cM away from the position of the *FecB* locus. It is therefore unlikely that the *FecB* locus is a mutant form of the GnRH receptor.

A Linkage Map for Sheep Chromosome 6

The next step in fixing the position of the *FecB* locus and developing additional markers close to the gene was to target chromosome 6 specific markers and develop a linkage map. We used the sheep–hamster cell hybrid containing the t1 translocation (chromosomes 6 and 24). Partial genomic libraries were constructed from DNA extracted from the cell lines containing chromosome 6 (Montgomery *et al.*, 1994c). Few clones in the libraries contained sheep DNA, while most clones contained hamster DNA. Discriminating between sheep and hamster clones was easier with the large DNA fragments in the cosmid vectors. Thirteen sheep-specific markers were generated from the clones screened out of the libraries. Seven clones mapped to sheep chromosome 6 and two clones mapped to sheep chromosome 24 (the other chromosome involved in the t1 translocation). The other four clones mapped to sheep chromosome 26 suggesting that the cell line also contains a fragment of sheep chromosome 26.

The microsatellite markers derived from the library were analysed in three generation pedigrees to provide a linkage map of chromosome 6 (Lord *et al.*, 1994). In addition, RFLP markers for genes from the syntenic regions in mouse and humans were also analysed for inclusion in the map (Table 2). A map showing the position of these genes together with some of the microsatellite markers is shown in Fig. 1. The map extends from the locus for phosphodiesterase, cyclic GMP (rod receptor), beta polypeptide (PDEB) to OarCP125 and covers 154 cM. PDEB has been mapped by *in situ* hybridization to the telomere of the chromosome (Lord *et al.*, 1994). The *FecB* locus maps to the region between OarAE101 and EGF/IF (Montgomery *et al.*, 1993) towards the centromere of chromosome 6.

Comparison of the sheep map with maps of other species demonstrates that sheep chromosome 6 contains genes from a large portion of human chromosome 4 extending from 4p16 to 4q26. The linkage maps of sheep chromosome 6 and cattle chromosome 6 are similar with the order and distances of common markers preserved. Genes from sheep chromosome 6 map to mouse chromosomes 3 and 5 with a break point close to SPP1. Similarly, genes from PDGFRA to SPP1 are located on pig chromosome 8 and there is a break point close to SPP1. It is interesting to note that the region around the *FecB* locus is retained intact in humans, sheep and cattle that generally have single or twin births, but two species with high litter size, mouse and pig, have chromosomal break points in this region.

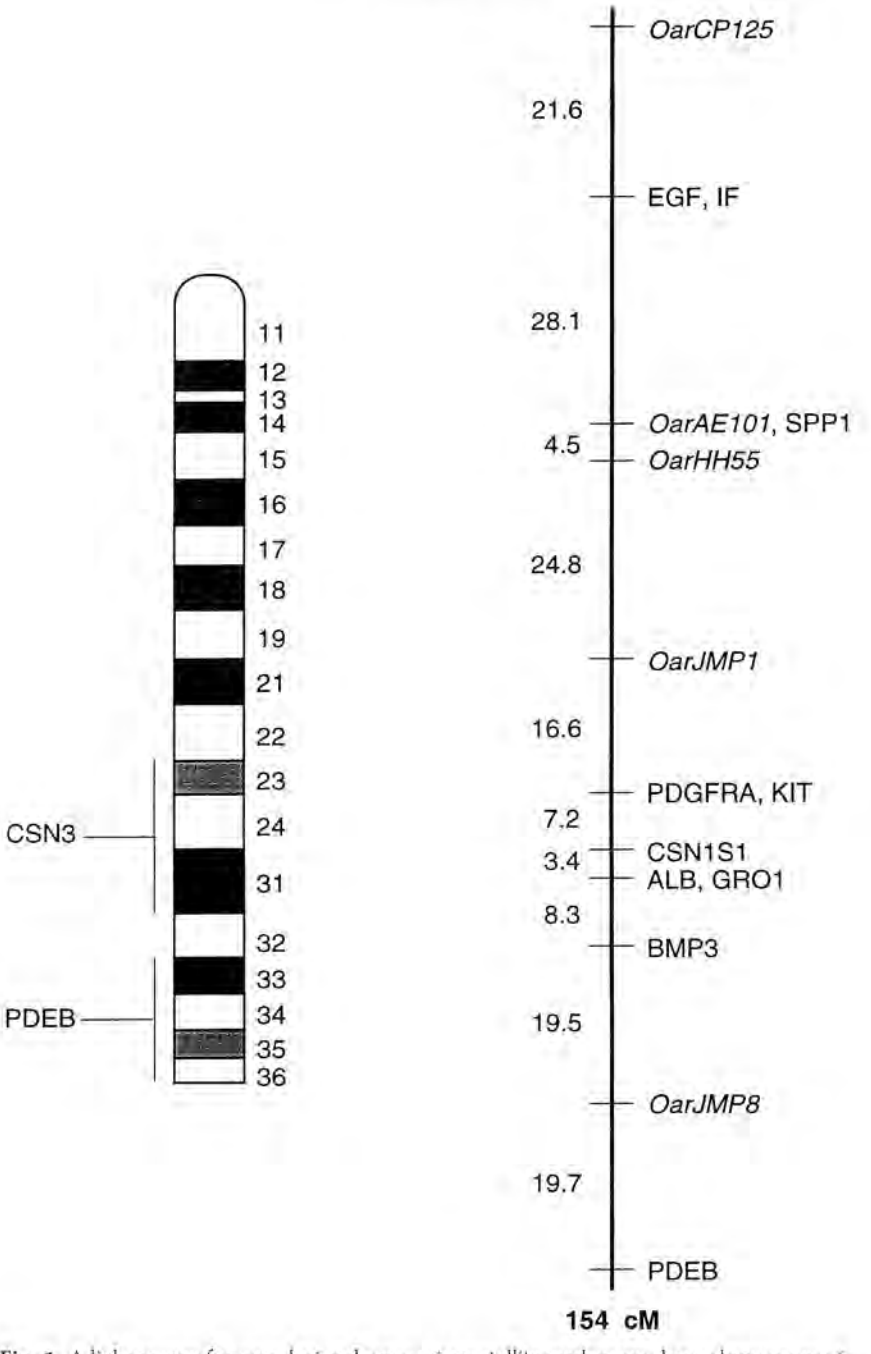

Fig. 1. A linkage map for gene loci and some microsatellite markers on sheep chromosome 6. Gene names corresponding to the symbols on the map are given in Table 2. PDEB has been mapped to the telomere of chromosome 6 by *in situ* hybridization providing an orientation for the linkage map.

A Physical Map of the *FecB* Region

The strategy adopted to locate and identify the *FecB* mutation was to develop a physical map of cloned DNA across the region. For this purpose we developed a genomic library of large fragment size in a YAC vector (pYAC4). The library was constructed in several parts with a total of 39 000 clones. On the basis of average size of the clones, the library contains approximately eight copies of the genome. The first part of the library consists of 13 000 clones with an average insert size of 680 kb. Thirteen clones from the library were analysed by fluorescence *in situ* hybridization (FISH) to chromosome spreads. Two clones were shown to be chimaeric, one clone failed to give clear results and the other ten clones were derived from a single chromosome location.

We have begun screening the library and identified a YAC clone with a probe for IF. Restriction analysis demonstrates the same restriction pattern for both the YAC and genomic DNA. We have completed the expansion of the library and it has been gridded out for screening using filters and PCR pools. The library will be screened with markers closer to the *FecB* locus to build the physical map over this region.

Other Genes Affecting Ovulation Rate

Major genes that increase ovulation rate and litter size have been reported in Inverdale (*FecXI*, Davis *et al.*, 1991, 1992), Javanese (*FecJ*, Bradford *et al.*, 1991), Icelandic (Eythorsdottir *et al.*, 1991), Polish (Martyniuk and Radomska, 1991) and Cambridge (Hanrahan, 1991) sheep. The Inverdale gene is on the sheep X chromosome, but it is thought that the other genes are autosomal. The situation in the Cambridge sheep appears complex with more than one gene segregating in the flock. It has been suggested that the mutation responsible for the high ovulation rate in the Javanese sheep may have originated from the same source as the *FecB* mutation, but it is unlikely that all the genes are from the same origin. If the autosomal mutations are different alleles at the same locus, there are likely to be several independent mutations similar to the W locus described in mice. Crosses between these flocks have not been possible because the strains of sheep are in different countries. Studies are in progress to analyse markers from sheep chromosome 6 in flocks where these genes are segregating. A preliminary study in Javanese sheep reported linkage disequilibrium with markers around the *FecB* locus suggesting that the *FecB* and *FecJ* loci may be allelic (Purwadarir *et al.*, 1994).

Conclusions

The *FecB* locus in sheep has been mapped to sheep chromosome 6 in the region between SPP1 and EGF/IF. A linkage map of chromosome 6 has been developed that will facilitate direct testing for carriers of the *FecB* gene and other major genes affecting ovulation rate in sheep.

The step of identifying the mutation responsible for the increased ovulation rate in Booroola gene carriers will not be easy. A YAC library has been developed to produce a long range physical map of the region. Together with information from the gene maps of other species, expressed sequences must be identified and screened as possible genes responsible for the effect.

The study of the Booroola gene should lead to the identity of a gene with important effects on ovulation rate in mammals and may suggest new treatments for both fertility enhancement and fertility control.

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