Polymorphism of the Promoter Region of Hsp70 Gene and Its Relationship with the Expression of HSP70mRNA, HSF1mRNA, Bcl-2mrna and Bax-AMrna in Lymphocytes in Peripheral Blood of Heat Shocked Dairy Cows

Yafei Cai[1, 2], Qinghua Liu[1], Guangdong Xing[1], Lei Zhou[1], Yuanyuan Yang[1], Lijun Zhang[1] Jing Li[3] and Genlin Wang[1, *]

[1] College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, P. R. China

ABSTRACT : The blood samples were collected from dairy cows at the same milking stage. The single-strand conformation polymorphism (PCR-SSCP) method was used to analyze for polymorphism at the 5'-flanking region of the *hsp70* gene. The mRNA expression levels of HSP70, HSF1, Bcl-2 and Bax-α at different daily-mean-temperature were analyzed by relative quantitative RT-PCR. The DNA content, cell phase and the ratio of apoptosis of lymphocytes in peripheral blood of dairy cattle at different daily-mean-temperature were determined by FCM. The PCR-SSCP products of primer pair 1 showed polymorphisms and could be divided into four genotypes: aa, ab, ac, cc, with the cis-acting element (CCAAT box) included. Mutations in the *hsp70* 5'-flanking region (468-752 bp) had different effects on mRNA expression of HSP70, HSF1, Bcl-2 and Bax-α. The ac genotypic cows showed higher expressions of HSP70mRNA, HSF1mRNA and Bcl-2mRNA/Bax-αmRNA and lower ratio of apoptosis. These mutation sites can be used as molecular genetic markers to assist selection for anti-heat stress cows. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 5 : 734-740)***

Key Words : Polymorphism, *hsp70* Gene, Dairy Cow, HSP70mRNA, HSF1mRNA, Bcl-2mRNA/Bax-αmRNA

INTRODUCTION

Milking of dairy cow is greatly affected by some hormones and nutrients provision. Many factors are involved in milking. In Southern China, most cows show heat shock response in summer because of high temperature and humidity. Many evidences indicate that the optimum temperature of milking Holstein cows is -5°C~20°C. Cows suffer heat shock when the temperature is over 20°C, and the milk yield decline significantly (Holter et al., 1996,1997; Silvia et al., 2003). A few of reports explored the relationship between genetic polymorphism of some genes and milk yield and quality (Chrenck et al., 2003; Badola et al., 2004). Previous reports concentrated on reduction of heat stress through medication and physical adjustment. But little is known about the molecular mechanism of the heat shock response of dairy cows. During the activation of heat-shock protein expression, a very primitive response, denatured proteins, are thought to serve as stimuli to induce the expression of the heat-shock protein. Heat-shock proteins are bound to heat-shock factors (HSFs) within the cytosol in normal situations and can be dissociated by denatured proteins induced by heat shock. Once dissociated, heat-shock proteins are free to bind to denatured proteins. This reaction requires ATP. More heat-shock proteins are generated when HSF1 are phosphorylated and trimerized. These trimers then enter the nucleus and bind to heat-shock elements (HSEs) within the promoter of the hsp gene, leading to transcription and synthesis of more heat-shock proteins (Ahn, 2003). The ratio of apoptosis decreases while the level of HSP increases (Mosser, 1997). The mechanism of apoptosis induced by the heat shock response is still unknown. Many studies show that the ratio of apoptosis can increase up to the proportion of Bcl-2/Bax proteins. Bax protein participates in cell apoptosis and Ca^{2+} and p53 are thought to be involved in the regulation of the Bax protein (Yic, 1997; Ross, 1998; Villunger, 2003; Perfettini, 2004). We do not know much about the expression of the genes mentioned above in the dairy cows undergoing heat stress. In order to explore the transmission molecular signals of bovine heat shock response, we designed four pairs of primers and analyzed both the ratio of apoptosis and the expression level of HSP70mRNA, HSF1mRNA and Bcl-2mRNA/Bax-αmRNA of the lymphocytes in the peripheral blood of dairy cattle at different daily-mean-temperatures.

Brahman preimplantation embryos are less affected by exposure to heat shock than Holstein embryos, in other words, inheritance of resistance is shown in dairy cattle (Shimizu, 1996). It is apparent that the impact of the heat-shock responses on individual dairy cows differs significantly. The heat-shock response maybe act as a quantitative trait. If we can find a molecular genetic marker linked to this quantitative trait, it would be possible to select for thermotolerance cows and improve the milk yield in

* Corresponding Author: Genlin Wang. Tel: +86-25-84395045, Fax: +86-25-84395314, E-mail: genlinwang@hotmail.com

[2] College of Life Science, Anhui Normal University, Wuhu, 241000, P. R. China.

[3] Center for Molecular Recognition, Columbia University, New York, NY 10032, USA.

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Table 1. Five pairs of primers based on the sequences of the 5'-flanking region of *hsp70*

Primers	Sequences of primers	Sites of amplification (bp)	Size of PCR product (bp)	Annealing temperature (°C)
Primer pair 1	Upstream: 5'-CGCTAAGAACCCAATCAA-3' Downstream: 5'-GCTGGCAATAGGCAAGAC-3'	468-752	285	52
Primer pair 2	Upstream: 5'-ATCCAGTTTGATACGGTTCG-3' Downstream: 5'-ACTTCGGGCGGTGGTGTTG-3'	802-1,025	224	52
Primer pair 3	Upstream: 5'-GCAGGACTTGAGGCGAAAC-3' Downstream: 5'-TCTCCAGGCTGCTGTTTC-3'	1,161-1,308	142	55
Primer pair 4	Upstream: 5'-CGCTAAGAACCCAATCAA-3' Downstream: 5'-CCGAAATCTCAACAATC-3'	282-452	171	51
Primer pair 5	Upstream: 5'-TGATACGGTTCGGATGGG-3' Downstream: 5'-ACTTCGGGCGGTGGTGTTG-3'	810-1,025	216	58

The 5'-flanking region of *hsp70* gene amplified with 5 pairs of primers, showing the sequences of primers, the sites of amplification, the predicted size of PCR and annealing temperatures.

summer. Polymorphisms in *hsp* promoters and quantitative modifications in HSP70 expression have been correlated with variations in thermotolerance in fish, insects and mammalian cell lines, and with differences in adaptation to environmental conditions in plants and animals and susceptibility or resistance to diseases in human being. Nucleotide substitutions, deletions and insertions have been shown to modulate promoter strength and transcriptional activity (Deguchi, 1990; Dhillon, 1993; Shimizu, 1996; Favatier, 1997). This prompted us to investigate a possible relationship between the polymorphism in the *hsp70* gene promoter region and HS-induced HSP expression in dairy cows. We designed a series of primers to amplify the 5'-flanking region (promoter region) of the *hsp70* gene of Holstein cows. PCR-SSCP was used to detect single nucleotide polymorphisms (SNPs) in this region. We have found polymorphisms in this region and determined a possible association with the HS response of dairy cows.

MATERIALS AND METHODS

The analysis of the polymorphism in the 5'-flanking region (promoter region) of *hsp70* gene

All of the cows (90 individuals) share the same season of the last deliveries and the same time of birth. The blood samples were collected and transferred to 10 ml Eppendorf tube and 4.5 ml lysis buffer (50 mM Tris, 100 mM EDTA, 100 mM NaCl, 0.5% SDS, 10 mg/ml proteinase K) were added and incubated for 40 h at 55°C until the blood sample was transparent. An equal volume of Tris phenol was added to the blood sample after lysis and it was centrifuged for 10 min at 5,000 rpm at 4°C. The sample was extracted two times with phenol, once each with phenol:choroform (1:1) and choroform:isoamylol (1:1). The DNA was precipitated with two volumes of 100% ethanol and 1/10 of 3 M (pH 4.8) sodium acetate and rinsed with 1 ml at 70% ethanol. The DNA was finally dissolved in $T_{10}E_1$ for 16-20 h at 50°C and kept at -20°C.

Five pairs of primers based on the DNA sequence AY149619 (Genebank accession, 8,566 bp) were designed to amplify the 5'-flanking region of the *hsp70* gene. PCR-SSCP was used to analyze the polymorphism of the 5'-flanking region (1×TBE buffer, 200 V, 20 h). The sequences of the primers, amplification site, predicted size of PCR products and annealing temperature are shown in Table 1. 50 ng genomic DNA extracts were used as templates in the PCR reactions. The PCR products were run on 12% polyacrylamide gels and stained with $AgNO_3$. The frequencies of the genotypes and genes were calculated to analyze the Hardy-Weiberg equilibrium of the mutation sites.

The diversity and identity of the sequences of the PCR fragments from individuals with different genotypes individuals were analyzed with the software *DNAstar 2.0 MegAlign*. Nucleotide substitutions, deletions and insertions were analyzed as well.

The DNA content, cell phase and rate of apoptosis of lymphocytes in peripheral blood of dairy cattle at different daily mean temperatures

Blood samples were collected three times from the cows at different daily-mean-temperature (37.5°C, high temperature in the summer; 26.5°C critical high temperature in the summer; 5.5°C, optimum temperature in the winter). The blood samples were anti-coagulated with sodium heparin. Ficoll reagent (Shanghai Hengxin Co., Ltd.) was used to dissociated lymphocytes from peripheral blood of dairy cattle at different daily-mean-temperatures. The dissociated lymphocytes were rinsed with PBS (Phosphate Buffer) and filtered with nylon net (400 holes) and diluted into (1-5)× 10^6 cell/ml, fixed with 70% ethanol (4°C) for 1-2 h and rinsed with PBS (5 mins×3). The cells were stained with 1 ml propidium iodide (including RNAase) for 30 mins and analyzed by FCM (Flow Cytometry). Argon irons were used to accelerate the fluorescence. The wave-length of the laser was 488 nm and that of the emitted light was beyond 630 nm. A histogram was used to assay the red intensity of

Table 2. Four pairs of relative RT-PCR Primers in this study

Genebank accession	Amplified gene	Sequence of primers	Size of pcr product (bp)	Annealing temperature (°C)
BTU09861	HSP70 mRNA	Upstream: 5'-CGGCTTAGTCCGTGAGAACA-3'	280	62
		Downstream: 5'-CCGCTCGGTATCGGTGAA-3'		
AJ318490	HSF1 mRNA	Upstream: 5'-AGCAGGGAGATGGTGGG-3'	166	56
		Downstream: 5'-ACTCAGGGAAGCAGTTGGT-3'		
U92434	Bcl-2 mRNA	Upstream: 5'-GTGTGGAGAGCGTCAACC-3'	180	63
		Downstream: 5'-CAGAGACAGCCAGGAGAAATCA-3'		
U92569	Bax-α mRNA	Upstream: 5'-CCCGAGAGGTCTTTTTC-3'	250	54.5
		Downstream: 5'-GAGCACTCCAGCCACA-3'		

Four pairs of primers, showing the genebank accessions of mRNA, the sequences of primers, the predicted size of PCR and the annealing temperatures.

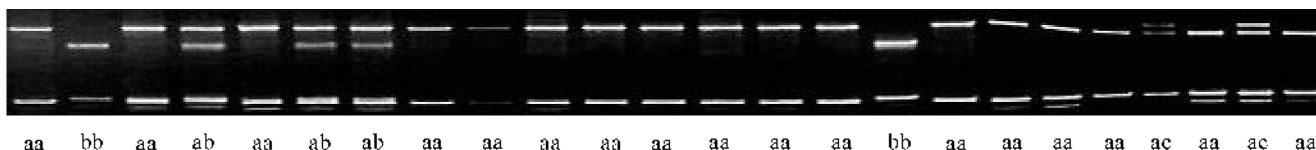

Figure 1. PCR-SSCP products of primer pair 1 stained with $AgNO_3$, showing genotypes aa, bb, ab and ac.

propidium iodide. Software *Cell Quest* and *Modfit LF* were used to assay the phase (G_0/G_1, S and G_2/M) and apoptosis of 10,000 cells. The PI represents the activation of cell proliferation and is calculated as follows:

$$PI = (S+G_2/M)/(G_0/G_1+S+G_2/M)\times 100\%.$$

Relative quantitative PCR analysis of expression of HSP70mRNA, HSF1mRNA, Bcl-2mRNA and Bax-αmRNA

Relative quantitative PCR was used to analyse the expression of HSP70mRNA, HSF1mRNA, Bcl-2mRNA and Bax-αmRNA. Total RNA was extracted from 5-10×10^6 dissociated lymphocytes with Triozol reagent (Invitrogen Co., Ltd.). RNA was run on 1% degenerated sepharose. The RT reaction was carried out according to the RT reaction kit (Promega Co., Ltd.): A 20 μl reaction was prepared by adding the following reagents in the order listed: 25 mM $MgCl_2$ 4 μl; reverse transcription 10×buffer, 2 μl; 10 mM dNTP mixture 2 μl; recombinant Rnasin ribonuclease inhibitor 0.5 μl; AMV reverse transcriptase 15 U; random primers 0.5 μg; Total RNA 1 μg. The 20 μl RT reaction system was incubated at 42°C for 15 mins and heated at 99°C for 5 mins, then incubated at 5°C. Reverse transcription-PCR (RT-PCR) was conducted in a 12.5 μl reaction system. The PCR primers used are as follows (Table 2).

The expression levels of these four genes were assayed with relative quantitative RT-PCR. 18s rRNA was used as internal standard (18s rRNA kit, Ambion Co., Ltd.). The size of amplified with 18s rRNA primers was 488 bp. The PCR system is shown in Table 2. The frequcies of the different genes were calculated by using the *Labimage* software.

Analysis of data

Data on the abundances of HSP70mRNA, HSF1mRNA and Bcl-2mRNA/Bax-αmRNA were not independent, therefore, the transformation of the square root was used to normalize the data of the three groups in different genotypes to meet the requirements. To test variance of three groups among them, independent-samples F Test was conducted. Data shown are the mean±SD abundances of different genes. The single way ANOVA was carried out to analyze abundances of data with SPSS 11.5 software.

RESULTS

Analysis of 5'-flanking region of *hsp70* gene

The size of the 5'-flanking region was 1,467 bp including 5'-UTR (1,217 bp-1,467 bp), promoter sequence (1,175 bp-1,210 bp), TATA box (1,267 bp-1,272 bp), CACCC box (884 bp-895 bp), CCAAT box (478 bp-482 bp), SP1 binding site (1,008 bp-1,031 bp) and HSE (301 bp-320 bp).

Polymorphism in the 5'-flanking region (promoter region) of *hsp70* gene

The predicted size of the PCR products was less than 300 bp. PCR-SSCP assay of the PCR products indicated that the segments amplified with primer pair 1 had polymorphisms (Figure 1). The size of PCR product was about 280 bp and there was a CCAAT box in the amplified product (280 bp). The banding patterns could be divided into four genotypes: aa, bb, ab and ac. The frequencies of different genotypes were 58.9%, 11.1%, 7.78% and 22.22% respectively. The frequencies of alleles a, b and c were: 0.739, 0.150 and 0.111 respectively. X^2 test showed significant difference at this mutation site, (X^2=45.742,

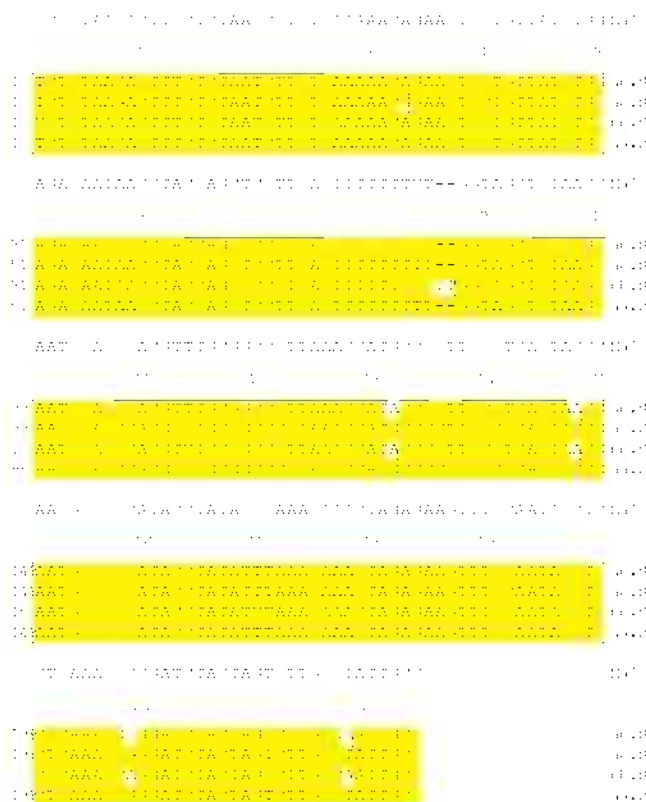

Figure 2. The alignment of the sequences of the four genotypes (aa, ab, bb and ac) done with the software *DNAstar2.0 MegAlign* by using the Clustalxmethod, showing substitutions, transversions and deletion of bases.

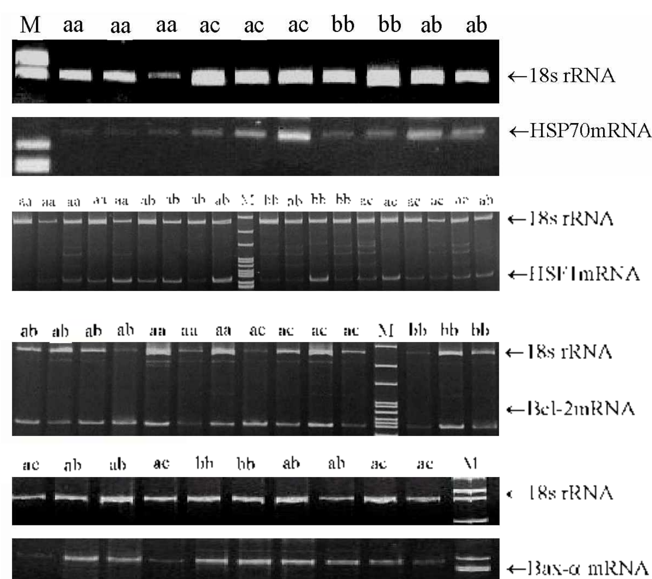

Figure 3. Relative quantity RT-PCR for HSP70mRNA, HSF1mRNA, Bcl-2mRNA and Bax-αmRNA and 18srRNA of lymphocyte cell in dairy cows. Samples were run on 18% polyacrylamide gels and stained with EtBr. 18srRNA of 488 bp was used as internal standard, the sizes of the four genes amplified in the PCR reactions were as follows: 280 bp, 166 bp, 180 bp, 250 bp.

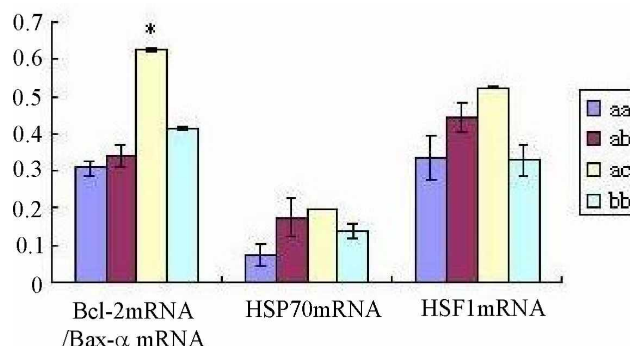

Figure 4. The mRNA levels of HSP70, HSF1, Bcl-2/Bax-α in dairy cow with different genotypes at 37.5, showing the higher expressions of HSP70mRNA, HSF1mRNA and Bcl-2mRNA/Bax-α mRNA in the cows with the ac genotype.

$p<0.01$ X (0.01, 5)=15.09). This mutation site was not in Hardy-Weinberg equilibrium.

Sequence analysis of the four genotypes

The DNA sequences of four genotypes were analyzed with the help of DNAstar MegAlign software. Two nucleotide insertions (TT) at the 86th and 87th sites were found in the bb genotype. Also, nucleotide substitutions (G→A) at the 132th, 148th and 209th sites and a nucleotide transversion (T→A) at the 208th site were identified in both the bb and ab genotypes. A nucleotide substitution (A→G) was found at the 33th site in the ac genotype as well (Figure 2).

Different mRNA levels of HSP70, HSF1, Bcl-2, Bax-α and Bcl-2/Bax-α in peripheral blood of different genotypic dairy cows exposed to heat stress

The expressions of HSP70mRNA, HSF1mRNA, Bcl-2mRNA and Bax-αmRNA in dairy cows exposed to heat stress were investigated by using relative quantitative RT-PCR. The sizes of the PCR products were: 280 bp, 166 bp, 180 bp and 250 bp. 18s rRNA acted as an internal standard and the products were about 480 bp (Figure 3). The expression of Bcl-2mRNA/Bax-αmRNA (0.6245±0.0035, HSP70mRNA (0.1954±0.0013) and HSF1mRNA (0.2034 +0.0023) was highest in ac genotype cows among the four genotypes. Only the expression of HSP70mRNA in ac genotype differs significantly from other genotypes ($p<0.05$) (Figure 4).

The expression of HSP70mRNA at 37.5°C was much higher than at the two other daily-mean-temperatures ($p<0.01$). The expression of HSP70mRNA and Bcl-2mRNA declined when the temperature dropped and the expression of HSF1mRNA was highest at 5.5°C. The ratio of Bcl-2mRNA/Bax-αmRNA was lowest at 26.5°C, which was

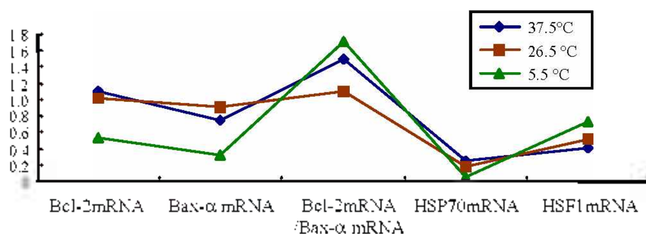

Figure 5. The expressions of Bcl-2mRNA, Bax-αmRNA, Bcl-2 mRNA/Bax-αmRNA, HSP70mRNA and HSF1mRNA at different temperatures.

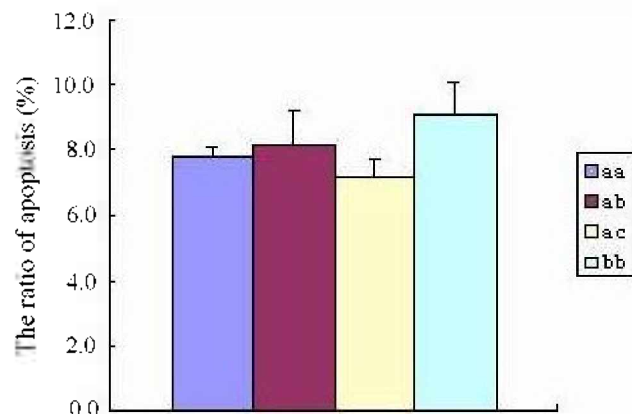

Figure 6. The ratio of apoptosis of lymphocytes in dairy cows with different genotypic, showing that the lowest ratio of apoptosis was in ac genotypic cow at 37.5°C.

significantly different from two other daily-mean-temperatures ($p<0.05$) (Figure 5).

The ratios of apoptosis of cows with different genotypes at 37.5°C by FCM

FCM (Flow Cytometry) results indicated that the apoptosis ratios in different genotypic cows were variable. The lowest apoptosis ratio was found in ac genotypic cow and not different from other genotypes ($p>0.05$). The highest ratio (9.13±0.01%) was obtained in bb genotype and significantly different from other groups ($p<0.05$) (Figure 6). The ratio of apoptosis of lymphocytes was 10.60% at 26.5°C and the highest among those of other three temperatures. The rate of apoptosis of lymphocytes was 2.70% at 5.5°C and the lowest and significantly different from those at the two other temperature ($p<0.05$) (Figure 7).

DISSCUSSION

Polymorphisms in the regulatory region of the hsp gene maybe related to animal susceptibility to disease

Favatier (1999) found that the described bi-allelic polymorphism in the 5% regulatory region of the *hsp70-1* gene did not generate differential gene expression. Using 5%-labelled synthetic oligonucleotides featuring the A to C

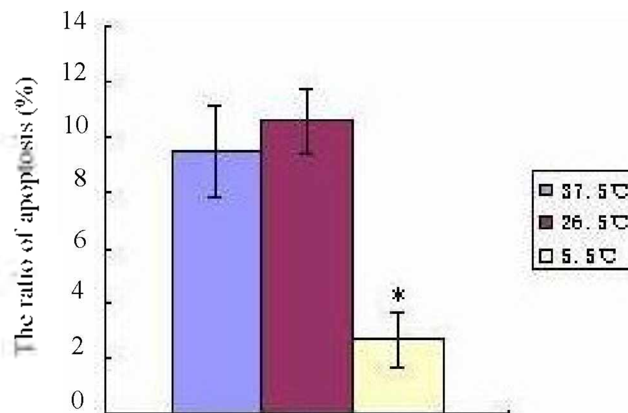

Figure 7. The ratio of apoptosis of bovine lymphocyte determined by FCM.

mutation within the HSE, they found no difference in HSF affinity for HSEa or HSEc. There were no significant differences in HSP70-1mRNA accumulation and HSP expression between *hsp70-1C* and *hsp70-1A* homozygous cell lines. Their results thus suggested that the *hsp70-1A* and *hsp70-1C* bi-allelic promoter polymorphism did not affect HSP expression. Other authors reported a polymorphism in the coding region of the *hsp70-2* gene and its association with diseases such as insulin-dependent diabetes mellitus (Pociot, 1993). This polymorphism consisted of an A→G transition at position 1,267 which generated a PstI restriction site. Furthermore, a significant linkage disequilibrium between the *hsp70-1C* allele and the *hsp70-2* allele containing a PstI restriction site (PstI) was previously observed (Milner, 1992; Aron, 1999). These two *hsp70* genes also shared a similar HSE box, with *hsp70-2* having a C at nucleotide-110 (Milner, 1990), which suggested that they could have similar affinities for HSF. A functional implication of this polymorphism was tested for by Pociot et al. (1993) through studying HSP70-2 mRNA expression in heat-shocked peripheral blood mononuclear cells from individuals with different *hsp70-2* genotypes. Their data showed that PstI-homozygous individuals had slightly lower HSP70-2mRNA expression than heterozygous and PstI-homozygous individuals. Thus, inter-individual and interracial differences in HSP70 expression could relate to regulatory mechanisms distinct from transcriptional regulation, as was also suggested by Lyashko et al. (1994).

The difference in HSP synthesis observed by Lyashko (1994) might either relate to post-translational modifications or involve a distinct as yet undefined polymorphism. Favatier (1999) suggested that differences in *hsp* gene regulation and HSP expression more likely related to distinct cell types and specific metabolic activities than to promoter polymorphism, since the natural polymorphism of the promoter region of the *hsp70-1* gene

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1    TTCAGAGTCC CTTGGACTGC AAGGAGATCC AACCAGTCCA TTCTGAAGGA GATCAGCCCT
61   GGGATTTCTT TGGAAGGAAT GATGCTAAAG CTGAAACTCC AGTACTTTGG CCACCTCATG
121  CGAAGAGTTG ACTCATTGGA AGACTCTGAT GCTGGGAGGG ATTGGGGGCA GGAGAAGGGG
181  ACGACAGAGG ATGAGATGGC TGGATGGCAT CACTGACTCG ATGGACGTGG GTCTGAGTGA
241  ACTCCGGGAG TTGGTGATGG ACAGGGGGCG AGTCATGGGG TCGCAAAGAG TAGGACACGA
301  CTGAATGAAC TGAACTGAAT ATGGTACAAA TCTCCTGCTG AACTAATTAT TTTAAGTTCT
361  AGTCTCCTCA AGAGACATCA CTCTCCACAA GTGGGTTTAG CAACCCTGTC GGTCGTCTTT
421  TACCCTCTAT TAGGGATTGT TGAGATTTCG GCCAGTCTGA AGAATGCCGC TAAGAACCCA
481  ATCAAGCCCT CATGAGTTTT CTCAGGTAAG GTGGTGCTCT CTAAGACTTT CTCTAATCTT
541  CTCAAAAACA GAACTCCTGT TATCTGGAGA CAATAACCTA CCAGCTCCTT CACTTTTTTT
601  TTGGTAGCTC TAAGCAATCC AGCCACGTTT GCGGCCCTTA AAGGATGCCC CTTCCCTGAC
661  TAGCCAATGC CCCCATACCT ATATTTAAAC AAACTAGAGA AGTTTCCAAT ACCTCCTCAA
721  ACCGGGATCT ACTAGTCTTG CCTATTGCCA GCCCATTCCA GTCACAGCAC TTGTGACTCA
781  CCTAGTGTCC CGCCGCCTTC GATCCAGTTT GATACGGTTC GGATGGGGAG CCCCTATAAC
841  TTGGGGCTCC TTCAATAGCC AAACTGCGCA GGCGGTGTCC CCCCACCCCC CACCCGCTCC
901  GCCCTGCGAC TTTGAGCTGG GTCCAGACCT AGCATCCTAA TTCTCTACTA GCCCGTGAGG
961  TCAGAGGCAG CACCTCCATT GTAACGCGAC TAGAGCA GG CG CGTCAAC ACCACCGCCC
1021 GAAGTCCCGA CCCACCAGCC CCTCCTACCG CTTCCCCTCC CATTACCCCT TTCCGAGACA
1141 GCCGATTGGT CCCAGAAAGC CAGGGGGCAG GACTTGAGGC GAAACCCCTG GAATATTCCC
1201GACCTGGCAG CCCCACTGAA CTCGGTCATT GGCTGACGAG GGAAAAGGCG GGGCTTGATG
1261 AAGAATTATA AACACAGAGC CGCCTGAGGA GAAACAGCAG CCTGGAGAAA GCTGATAAAA
1321CTTGCGGCTT AGTCCGTGAG AACAGCTTCC GCAGACCCGC TATCTCCAAG GACCGCCCCG
1381 AGGGGCACCA GAGCTTCACG TCGTTGATCC TGTGGGCCGT TTTCAGGTTT GAAGCTTATC
1441 TCGGAGCCGA AAAGGCAGGG CACCGGC
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Figure 8. Analysis of 5'-flanking region in *hsp70*, showing cis-acting elements, the sequences of cis-acting elements were shown as follows: HSE: CTGAAT. CCAAT box: CCAAT. CACCC box: CACCC. SP1 binding site: G CG . Promoter: TGAGGC GAAACCCCTG GAATATTCCCGACCTGGCAG. TATA box: TATA AA.

was found to have no effect on HSP70 levels. It is possible that there are unknown polymorphisms in other *hsp* genes, including *hsp70* genes, which might provide individual variability in protection against environmental changes.

Polymorphism in 5'-flanking region was found in dairy cows and correlated with HSP70 expression and apoptosis of lymphocyte in peripheral blood

The trait susceptibility to heat stress of dairy cows might be a quantitative trait with low heredity making it difficult to breed according to phenotype. Marker assisted selection (MAS) would be an efficient way to resolve this, thus making it helpful to find out a genetic marker linked with the QTL.

In this experiment, we chose the 5'-flanking region of the *hsp70* gene to study. PCR-SSCP assay showed that a mutation site and a CCAAT box existed in this region. The expressions of HSP70mRNA and Bcl-2 mRNA/Bax-α mRNA in ac genotype cows were significantly higher than those of the other genotypes. In addition, the ratio of apoptosis in ac cows was lower than those of the other genotypes (aa, bb, ab). The results indicated that this mutation site could be related with the trait susceptibility to heat stress. There was a nucleotide substitution (A→G) in the ac genotype at the 33th site compared with that in the aa genotype. In the 5'-flanking region, many cis-acting elements (Figure 8) regulated the expression of HSP70mRNA. Dairy cows with the single mutation at the 33th site seemed to be resistant to heat stress during our study. It is possible that other genes linked with this molecular genetic marker affected the susceptibility to heat stress. Further studies are needed to address these possibilities.

The genetic equilibrium of some genes in animal population could be affected by many factors such as: mutation, migration, genetic drift and selection. Artificial selection is one of the important factors affecting the genetic balance. The genetic equilibrium linked with QTL is lost when one trait is selected for artificially. In this experiment, the *hsp70* gene were not in the Hardy-Weinberg equilibrium. The possible effect of artificial selection on the QTL of heat stress was also suggested by our study.

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