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Research Article

Biochemical and molecular investigations on qualitative and quantitative Hb polymorphism in the river buffalo (*Bubalus bubalis L.*) population reared in Southern Italy

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Abstract

On 398 river buffalo samples, randomly collected in distinct breeding areas of the Campania region, high-resolution analytical systems were used to identify both qualitative and quantitative variations of the Hb phenotype. Polyacrylamide gel isoelectric focusing and HPLC were used to determine the ratio between HBA1 and HBA2 globin chains; restriction endonuclease analysis was performed to assess whether quantitative variations in Hb bands were related to an unusual number of α-globin genes. In the two buffalo subpopulations, allele frequencies of the alpha and beta globin systems were calculated, and F statistics (FIS, FIT and FST) were estimated as parameters of genetic diversity. The results suggest that: i) as shown by RFLP analysis, only a couple of associated α globin genes account for the quantitative variations recorded at the phenotypic level; ii) as expected, in the α globin gene system (*HBA*), the frequency of haplotype *B* (*HBA-B)* largely exceeded that of haplotype *A* (*HBA-A)* (95.1% *vs* 4.9%); iii) the frequency of the usual allele at the beta locus is 0.6, as opposed to 0.4 of the slow variant; iiii) the most significant component of variation of the genetic system of hemoglobin is between individuals within the same location.

Key words: phenotype, RFLP, globin genes, haplotype, gene frequencies.

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Introduction

The biochemical polymorphism of hemoglobin in the water buffalo has been previously described, both by investigating the possible adaptive significance of buffalo hemoglobin structure and thermodynamic properties (Giardina *et al*., 1992) and by analysing the variation in the alpha globin system and beta globin locus (Di Luccia *et al*., 1989; Di Luccia *et al*., 1991a; Di Luccia *et al*., 1991b).

In buffaloes, as in humans and most other mammals, there are two alfa globin genes (Iα and IIα), which are expressed at different levels, the upstream gene being the most efficient. Thus, when the loci show characteristic differences in the average level of globin production, many genotypes can be deduced by the relative densities of the electrophoretic bands.

The buffalo duplicated haplotypes *HBA-A* and *HBA-B* produce four alpha globin chains, corresponding to an equal number of electrophoretically distinguishable Hbs. Isoelectric focusing patterns in ultra-narrow pH gradients show three phenotypes, the most frequent of which (BB) consists of a major (Hb2) and a minor (Hb4) hemoglobin. The rarer phenotype AA also consists of a major hemoglobin (Hb1) and a minor one (Hb3), whilst phenotype (AB) exhibits the four hemoglobins (Di Luccia *et al*., 1989).

With the finding of an electrophoretically slower β-globin chain, provisionally called beta slow, the number of hemoglobins which can be found in one individual raises up to eight (Di Luccia *et al*., 1991a).

The Campania region (Southern Italy) has long been a home to river buffalo breeding, strictly connected with the cheese-making of the traditional "mozzarella". Of the 250,000 buffaloes living in Italy, more than 80% are bred in the provinces of Salerno and Caserta (ANASB, 2001). These two breeding areas are different from each other, as far as both animal management and breeding systems are concerned. Thus, a survey was carried out on the two river buffalo subpopulations, to estimate the amount of variation

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at the alpha and beta globin loci and to check the homogeneity of the hemoglobin system in the overall buffalo population living in Campania.

This paper reports the results obtained from 398 samples, randomly collected in the two breeding areas and processed as follows: i) high-resolution analytical systems were used in order to identify both qualitative and quantitative variations of the Hb phenotype; ii) restriction endonuclease analysis was performed, to assess whether quantitative variations in Hb bands were related to an unusual number of α-globin genes; iii) in the two buffalo sub-populations, allele frequencies of the alpha and beta globin systems were calculated, and F statistics (FIS, FIT and FST) were estimated as parameters of genetic diversity.

Material and Methods

Sampling and sample preparation

Within the buffalo population, 398 individuals of both sexes were randomly sampled in the two breeding areas. Based on prior evidence, the sampling protocol was designed to detect both a gene frequency of 4% and of 40%, the former percentage concerning the alpha globin genetic system and the latter one the beta locus.

Setting the level of confidence at 0.95, the sample size was determined by the following formula

$$n = \frac{d^2}{1.96^2 \times P_{exp}(1 - P_{exp})}$$

where: n = required sample size; P_{exp} = expected frequency; d = desired precision (Thrusfield, 1995).

Blood samples were treated by standard procedures, as described by Di Luccia *et al*. (1991b) and Huisman (1986).

Electrophoresis and densitometry of hemoglobins

To investigate the Hb polymorphism and define Hb phenotypes, hemolysates were analyzed by isoelectric focusing (IEF) on immobilized ultra-narrow pH (7.1-7.5) gradient (IPG), as previously described (Di Luccia *et al*., 1991b; Di Luccia *et al*., 1989). Routine analyses were performed on gel slabs from conventional IEF in a pH 6.7-7.7 gradient (Di Luccia *et al*., 1991a) and scanned with a computerized enhanced laser densitometer Ultroscan XL equipped with Gelscan 2.0 software from Pharmacia-LKB (Uppsala, Sweden).

RP-HPLC of alpha and beta globins

As the densitometric evaluation of Hb bands showed a large variability and did not allow to fit for certain all the phenotypes in the frame of the globin expression ratio expected on the basis of alpha duplicated arrangements, all the hemolysates were analysed by RP-HPLC, in order to control the recorded variation at the quantitative tetramer level. Globin chains were separated by reversed-phase HPLC, according to Shelton *et al*. (1984), with appropriate modifications for the study of non-human globin chains (Di Luccia *et al*., 1991b; Manca *et al*., 1990; Schroeder *et al*., 1985).

RFLP of alpha globin genes

RP-HPLC of alpha and beta globin confirmed the existence of a certain number of individuals whose Hb band proportions were out of the normal range as expected on the basis of a normally duplicated α globin gene arrangement.

Thus, RFLP analysis was used to establish whether abnormal hemoglobin phenotypes resulted from duplicated or from multiple genes. To check the number of α genes, all homozygotes at the β locus were chosen, total genomic DNA was extracted from their leukocytes and then completely digested with HindIII, EcoRI, BamHI, KpnI and BstEII enzymes. Digested DNA was fractionated in 0.8% agarose gels in 40 mM Tris-borate buffer containing 50 mM EDTA, pH 8, and transferred to Gene-bind 45 membranes (Pharmacia Biotech), as described by Goossens and Kan (1981). Plasmid pGα2 (Southern, 1975) containing the goat α2-globin gene was digested with EcoRI to obtain a 1.3 kb fragment containing the α2 goat globin gene, that was then labeled with ^{32}P (MegaprimeTM DNA labeling system - Amersham) and used to probe the nylon filters.

Statistics

The effects of genotype as shown by densitometric evaluation of single Hb bands and chromatographic determination of globin peaks were evaluated by least square variance analysis, in order to compare the observed alpha globin and Hb proportions with those expected based on the differential gene expression in duplicated α globin gene arrangements in Mammals.

The frequencies of the *A* and *B* alpha haplotypes and of the *B* and B^S beta alleles were estimated by the GENEPOP program (Raymond and Rousset, 1995), also utilized to check the Hardy-Weinberg equilibrium.

Genetic diversity and its partition within and among subpopulations were quantified by F_{ST}, F_{IS} e F_{IT} (Wright, 1965), and their statistical significance was evaluated by the F-STAT program (Goudet, 1995).

Results

Nomenclature

The different Hb phenotypes found in the river buffalo refer only to variations in the alpha globin genetic system, as the beta locus appeared monomorphic ever since the new variant was detected. The standard gene nomenclature rules for ruminants refer to the alpha globin genetic system as *HBA* and to the beta locus as *HBB* (Andresen *et al*., 1991). Accordingly, and analogously to the horse nomen-

clature (Sandberg and Cothran, 2000), the buffalo *HBA* haplotypes were named *A* and *B,* and the presence of the beta slow variant was indicated by simply adding a superscripted letter "s" to the usual allele *HBB* (*HBB*^{s}) or to the HBA phenotypes (ABA^{s}B^{s}, A^{s}B^{s}, B^{s}B^{s}). More accurate indications will be suggested as soon as the protein amino acid sequence is available and the amino acid substitutions are known. Presently we can only conclude that the small differences in migration of the tetramers carrying different beta alleles (Figure 1 and Figure 2) suggest that the HBB^{S} variant is the result of a conservative mutation which, based on HPLC analyses, should be a neutral *vs* neutral amino-acid substitution (Figure 3).

Qualitative hemoglobin phenotypes

Isoelectrofocusing analysis of river buffalo hemoglobins with ultra-narrow immobilized pH (7.1-7.5) gradients revealed eight tetramers (Figure 1), six of which focused on the anodal side with minute differences in their isoelectric points (pI), while the remaining two focused on the cathodal side. Single phenotypes showed from two to seven tetramers.

The phenotype AA (lane 4) has two hemoglobins, (Hb1 and Hb3) which differ in pI by about 0.02 pH units, due to the amino acid substitutions 129 Leu → Phe and 131 Ser → Asn in the α chain (Ferranti *et al*., 2001).

The phenotype BB (lane 2) consists of Hb2 and Hb4, which differ in 10 Val → Ile and 11Gln → Lys amino acid substitutions. The Hb4 pI is higher than the Hb2 pI by about 0.2 pH units, due to the charged amino acid substitution 11Gln → Lys. The pI of Hb2 is between that of Hb 1 and that of Hb 3, being 0.008 pH higher than Hb1, as a result of two neutral amino acid substitutions at position 10 (Val → Ile) and 64 (Asn → Ala) in the α chain (Ferranti *et al*., 2001).

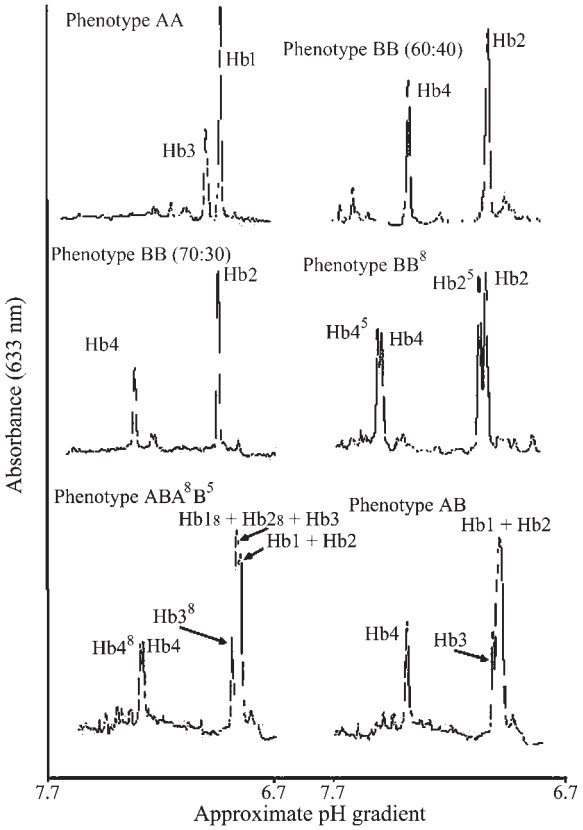

Figure 2 - Representative hemoglobin phenotypes, shown as densitometric scans of ultrathin polyacrylamide gels after isoelectric focusing over 1 pH unit (6.7-7.7).

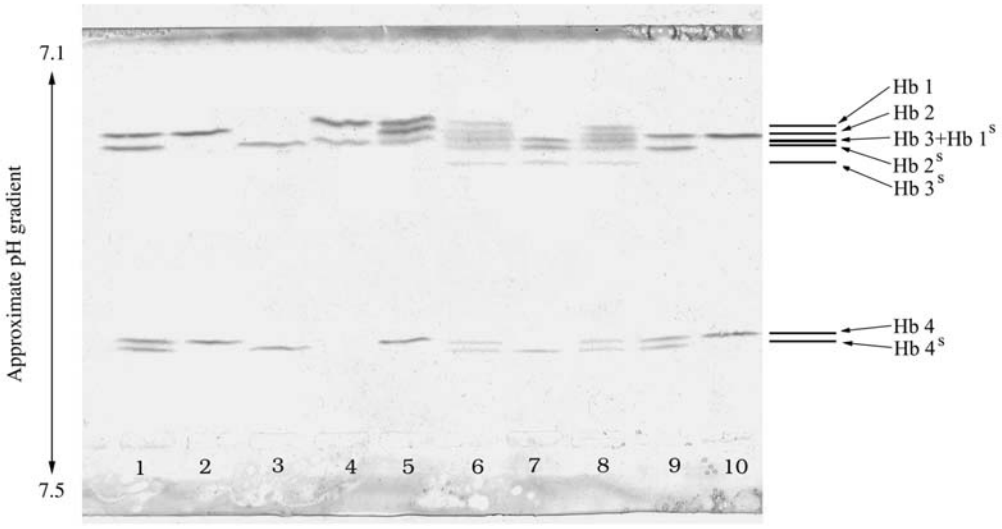

Figure 1 - Isoelectric focusing of the river buffalo hemoglobin on immobilized pH gradients (7.1 - 7.5). Lanes 1 and 9 - heterozygote BB^{s}; lanes 2 and 10 - homozygote BB; lane 3 - homozygote B^{s}B^{s}; lane 4 - homozygote AA; lanes 5 - heterozygote AB; lanes 6 and 8 - heterozygote ABA^{s}B^{s}; lane 7 - heterozygote A^{s}B^{s}. Superscript "s" assigned to Hb and its phenotypes indicates the presence of one of the two variant β-globin chain.

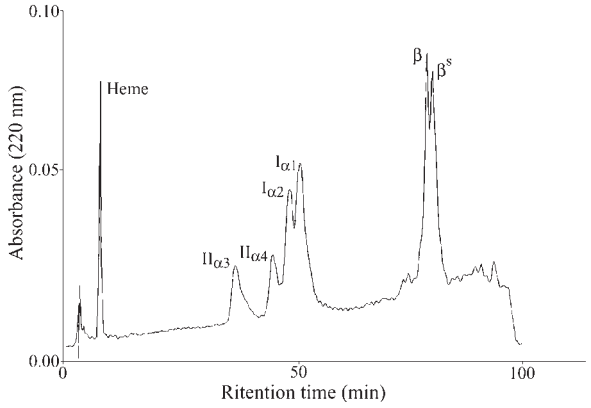

Figure 3 - Reversed-phase high performance liquid chromatography of lysates of ABA^sB^s Hb phenotype. The numbering of the α chains is the same as that of the associated Hb tetramers in Figure 1.

The B^SB^S phenotype shown in lane 3 consists of $Hb2^S$ and $Hb4^S$, both with a pI slightly higher than the Hbs of the BB phenotype, due to the presence of the beta slow globin chain. Lanes 1 and 9 show the BB^S pattern, and lanes 6 and 8 exhibit the pattern of the ABA^SB^S phenotype. This phenotype, never reported before, shows seven of the expected eight hemoglobins, because $Hb2^S$ is characterized by a pI that is very similar to that of Hb3, differing in pH by less than 0.004 pH units.

RFLP analysis

The results of the RFLP analysis confirmed the presence of only duplicated haplotypes in all the examined samples. Total genomic DNA from three BB individuals with different proportions of α-globin chains was digested with five restriction endonucleases: EcoRI, HindIII, BamHI, KpnI and BstEII. The different patterns obtained are shown in Figure 4. The restriction enzymes HindIII and BamHI produced a single band of 12 kb and 10 kb, respectively, while two bands were found with EcoRI (9.4 kb and 1.7 kb) and KpnI (6.0 kb and 1.3 Kb). Only BstEII cleaved DNA within the gene, as reported in goats and sheep (Schon *et al*., 1981; Sambrook *et al*, 1989), producing two bands of 2.7 kb and 2.3 kb, respectively. Figure 4 clearly illustrates the overall results in that, despite the different proportions recorded at the levels of hemoglobin and alpha globin (in the samples above), there are undoubtedly no differences between their restriction patterns.

As to the whole α-globin gene cluster, Figure 5 shows the restriction enzyme map obtained after double-digestions. The HindIII and BamHI digests contain at least two genes, while EcoRI and KpnI show intergenic sites.

Quantitative hemoglobin and alpha and beta globin

Once the number of alpha globin genes was ascertained, all the quantitative data were considered in the frame of double arrangements.

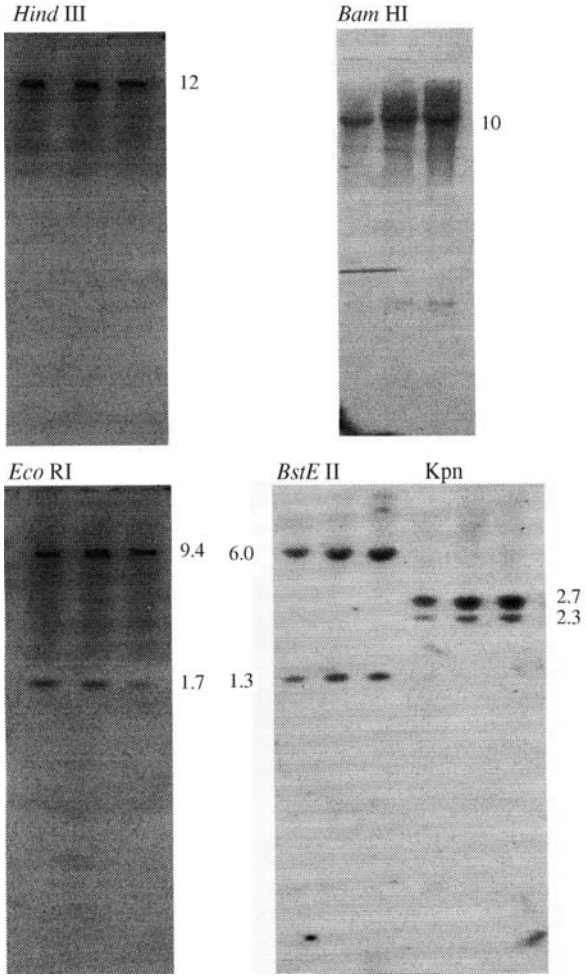

Figure 4 - Southern blot analysis of genomic buffalo DNA digested with restriction endonucleases. The samples were of phenotype BB that contained different Hb2:Hb4 ratios (approx. 55:45, 65:35 and 75:25). The fragment sizes are given in kb.

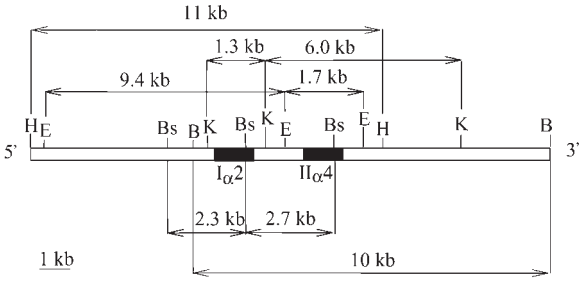

Figure 5 - Schematic representation of the restriction endonuclease map for 13 sites in the river buffalo α-globin gene from BB hemoglobin phenotypes. B = *Bam*HI; Bs = *Bst*EII; E = *Eco*RI; H = *Hind*III; K = *Kpn*I.

The means and standard deviations obtained by statistical analysis of the Hb band densitometric values are shown in Table 1, making it clear that Hb2 bands are about 1.8 fold more intense when compared to those of the Hb4

Table 1 - Mean values and standard deviation of densitometric data obtained analysing buffalo Hb bands.

Phenotype	BB	B^SB^S	BB^S	
N	133	47	182	
Hb2	64.34 ± 4.12	64.33 ± 4.08	33.31 ± 2.07 32.37 ± 2.03	65.62 ± 4.09
Hb4	34.93 ± 3.46	34.40 ± 3.96	17.60 ± 2.5 16.98 ± 2.53	34.58 ± 3.67

bands. The data refer only to 362 single samples which were homozygous for the alpha gene arrangement B, but both homozygous and heterozygous at the beta locus (B and B^S alleles). Least square variance analysis showed that the effect of the genotype on the percentage values was non-significant.

Percent gene efficiencies (mean ± sd) from the 5' to the 3' end, as calculated by densitometric data in the buffalo, are reported in Table 2, compared to those found in sheep (Pieragostini *et al*., 2003).

Allele frequencies and population parameters

Table 3 shows the alpha and beta allele frequencies for river buffalo hemoglobins. No allelic frequency deviation from the Hardy-Weinberg equilibrium was found, and the comparison between expected and observed heterozygotes (Table 3) shows no signs of decline in heterozygosity. Table 4 shows the F statistics used as heterozygosity calculations to estimate the differentiation among subpopulations. Fis and Fit are relatively higher than Fst for both loci, indicating that local inbreeding and population subdivision may have some influence, while the variance of allele frequencies among populations is very low; the F_{ST} value indicates that only 1% of the variance is due to genetic differentiation among sub-populations, while 99% of the variance lies within the population.

Table 2 - Percent gene efficiencies (mean ± sd), from the 5' to the 3' end as calculated by densitometric data in buffalo and sheep.

Specie	N	Iα	IIα	Iα/IIα	Reference
Buffalo	362	32.56 ± 3.12	17.5 ± 2.6	1.86	Present paper
Sheep	30	32.2 ± 2.68	17.6 ± 1.91	1.83	Pieragostini *et al*., 2002

Table 3 - Allele frequencies at the alpha (*HBA*) and beta (*HBB*) systems and observed *vs* expected heterozygotes in the two province subpopulations and in the total population.

Breedig area	Sample size	Allele	Allele Frequency	Ho	He
Caserta	175	*HBA-A*	0.031	0.063	0.061
		HBA-B	0.969		
		HBB	0.611	0.503	0.477
		HBB^S	0.389		
Salerno	223	*HBA-A*	0.054	0.108	0.102
		HBA-B	0.946		
		HBB	0.630	0.498	0.467
		HBB^S	0.370		
Total	398	*HBA-A*	0.044	0.088	0.084
		HBA-B	0.956		
		HBB	0.622	0.500	0.470
		HBB^S	0.378		

Discussion

The main points emerging from these results are two. The first one refers to the allele polymorphism and the genetic differentiation of the examined buffalo subpopulations as to their hemoglobin system. The other concerns the high variability of the quantitative data, *i.e.*, of the output of alpha genes, as deduced from the concentration of different α globin chains obtained by integrating RP-HPLC chromatographic peaks and from the densitometric evaluation of the respective Hb PAGIF bands.

a) Allele polymorphism

As to the *HBA* allele frequencies in the buffalo populations world-wide, haplotype A is generally less common than haplotype B. The findings reported in this paper, indicating a 4.9% frequency for haplotype HBA-A and 95.1% for haplotype HBA-B, offer confirmation both of the above trend and of the results obtained in previous surveys of the Italian buffalo population (Iorio and Annunziata, 1986; Masina *et al*., 1977).

Conversely, there are no reference data as to *HBB*, this locus being previously thought to be monomorphic. The β allele frequencies found in this screening were 62.4% for the wild type and 37.6% for the so-called slow allele, indicating that the latter must be considered a common allele.

The F-statistics estimates based on hemoglobin genetic systems showed no significant departure from the Hardy -Weinberg expectation for the buffalo population. The estimates of FST population differentiation were not significantly greater than zero, indicating that the most significant component of variation in the hemoglobin genetic system is among individuals within the same location. Based on these results, the variation between local subpopulations seems to be negligible, but the hemoglobin system does not contain sufficiently informative variables to

Table 4 - F-statistics estimates.

	F_{IS}	F_{ST}	F_{IT}
HBA	-0.047	0.004	-0.043
HBB	-0.061	0.002	-0.063
Total	-0.059	0.001	-0.060

draw final conclusions on the genetic structure of the water buffalo population of Campania.

b) Quantitative data

Different proportions of Hb tetramers may have multiple causes.

Firstly, there are possible structural origins such as those ascribed to the presence of alpha globin extra genes found in different mammalian species; indeed, multiple copies of α-globin genes have been observed in humans (Goossens *et al*., 1980; Higgs *et al*., 1980; Liebhaber *et al*., 1986)), horses (Bowling *et al*., 1988), apes (Zimmer *et al*., 1980), goat (Schon *et al*., 1982; Bergesen *et al*., 1991), sheep (Vestri *et al*., 1983; Ristaldi *et al*., 1995) and cattle (Scaloni *et al*., 1998). The presence of a trend of expression was widely confirmed; particularly in sheep triplicated and quadruplicated alpha-globin haplotypes, it was found that the alpha-chain output of the downstream genes progressively decreases (Vestri *et al*., 1991, 1994).

In the case of the river buffalo, the variation of the ratios Hb2:Hb4 among phenotypes cannot be explained by the presence of extra genes, because RFLP analysis clearly showed that there were no differences in the restriction patterns of the examined samples. Only two non-allelic genes were therefore responsible for the quantitative differences in hemoglobin.

Anyway, looking at the mean standard deviations in Table 1, the wide range of variability of the data is a fact. Indeed, the most striking aspect of the results reported in this paper is the size of the data set of quantitative records and, most of all, the general correspondence between the records obtained for the same individual by different analytical procedures. This kind of variation has already been observed in previous studies on sheep with the same duplicated gene arrangements (Pieragostini *et al*., 2003), where the mean values recorded, as well as the variability ranges, were very similar to those of the buffalo (Table 2). It cannot be excluded in any case that the variation may be due to analytical or pre-analytical errors, but the apparent repeatability of the phenomenon may indicate that the possible presence of additional mechanisms of gene-expression regulation should not be neglected.

A certain range of quantitative hemoglobin phenotypes may be justified either by individual differences at "regulatory" loci closely linked to structural α-globin genes or by a different gene regulation occurring in *tandemly repeated* genes.

In Sonoran deer mouse and Gambel's deer mouse hemoglobins, the Hb ratios varied widely among individuals within the same population, and extensive inheritance studies on mice have shown that this residual quantitative variation is under fine-scale genetic control (Snyder, 1980).

Proudfoot (1986) demonstrated that transcriptional interference substantially inhibits the downstream α gene by stimulating the transcription of the upstream α gene. This inhibition is modulated by transcriptional termination signals located between the two α genes. Recently, Shewchuk and Hardison (1997) have shown that the size of CpG islands in the α-globin gene cluster, which includes both 5' flanking and intragenic regions, positively correlates with the level of gene expression, also suggesting that a mechanism at the chromatin structure level may be involved.

Apart from these considerations which invoke genetic mechanisms, even variables such as protein stability, efficiency of hemoglobin tetramer formation, and other factors can affect the steady-state levels of globin variants and the consequent repeatability of quantitative evaluation, both of globins and hemoglobins. In conclusion, the crucial point in this discussion is whether the quantitative variability recorded for the alpha globin system and the related hemoglobins in buffalo is of genetic or non-genetic origin. In our opinion, there are no answers to this question at present, and further investigations are needed.

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