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A Comparison of the β^A- and β^B-Globin Gene Clusters of Sheep

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Summary. Domestic sheep have two common alleles at the adult β-globin locus, β^A and β^B. Here we report the structure of the β-globin locus of A-haplotype sheep. The locus consists of 12 genes, organized as a triplicated 4-gene set: 5′ ϵ^I-ϵ^{II}-$\psi\beta^I$-β^C-ϵ^{III}-ϵ^{IV}-$\psi\beta^{II}$-β^A-ϵ^V-ϵ^{VI}-$\psi\beta^{III}$-β^F 3′. This arrangement is identical to that of the closely related goat locus. Sheep with the B haplotype have a locus arrangement consisting of a duplicated four-gene set, lacking the β^C gene as well as three other genes present in A sheep and goats. In order to understand the evolutionary history of the B sheep locus, we have sequenced the β^B gene from these sheep, and the β^C gene from A-haplotype sheep, and compared the sequences to those of the sheep β^A, goat β^C, and β^A, and cow adult β genes. Our results indicate that the β^B gene has diverged recently from the β^A gene, and therefore the β^B locus structure may have resulted from a recent deletion from a triplicated locus.

Key words: Sheep — Globin genes — Evolution — Gene duplication

Introduction

The ruminant globin clusters have unusual evolutionary histories among mammals. The β-globin genes of all mammals have descended from an ancestral mammalian cluster consisting of five genes, 5′ ϵ-γ-η-δ-β 3′, whose descendents have undergone various duplications, deletions, inactivations, and changes in developmental regulation in different orders and species of mammals over the course of evolution (Goodman et al. 1984). In the ancestral ruminant, the γ-globin gene was lost and the δ-globin gene became defective. The remaining four-gene set, ϵ-$\epsilon(\eta)$-$\psi\beta(\delta)$-β, was duplicated, and the β-globin gene in each block assumed a new function and pattern of developmental regulation.

In goats (*Capra hircus*), the original four-gene set was triplicated: 5′ ϵ^I-ϵ^{II}-$\psi\beta^X$-β^C-ϵ^{III}-ϵ^{IV}-$\psi\beta^Z$-β^A-ϵ^V-ϵ^{VI}-$\psi\beta^Y$-β^F 3′ (Townes et al. 1984). The β^F gene is expressed in the fetus, the β^C protein is found in juveniles from birth to about 6 months of age, and the β^A globin gene is expressed in adults (Huisman et al. 1969). The β^C and β^A switch is reversible under conditions of anemia or hypoxia, or following injection of erythropoietin (Huisman et al. 1967; Boyer et al. 1968).

The bovine (*Bos taurus*) β-globin cluster also consists of a duplicated four-gene set, with the addition of a small-scale duplication of one of the pseudogenes: 5′ ϵ^3-ϵ^4-ψ^3-β-ϵ^1-ϵ^2-ψ^1-ψ^2-γ 3′ (Schimenti and Duncan 1985b). The cow γ gene is more closely related to the β-globin gene than to the γ-globin gene of the ancestral mammalian β-globin cluster, although it is expressed in the fetus.

Sheep (*Ovis aries*) have two alleles at the β-globin locus, β^A and β^B (Huisman et al. 1958). Sheep with the β^A allele have a developmental β-globin switching pattern resembling that of the goat (Hammerberg et al. 1974). Since the triplication event that created the β^C-globin gene preceded the divergence of sheep and goats (Li and Gojobori 1983), it would be expected that their β-globin loci would be similar.

In this paper we describe the cloning and mapping of the A sheep locus, and confirm that no major rearrangements have occurred since the divergence of A sheep and goats. Verification of the A sheep locus structure is necessary in order to understand the evolution of the B sheep locus. B sheep lack the

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β^C-globin gene (Benz et al. 1977) and their β-globin switching pattern is like that of the cow. One or more embryonic globins are replaced by a fetal globin, which is followed by an adult globin called β^B whose expression is not affected by anemia (van Vliet and Huisman 1964). We have previously shown that the B sheep β-globin locus consists of a duplicated four-gene set: 5′ ϵ^I-ϵ^{II}-$\psi\beta^I$-β^B-ϵ^{III}-ϵ^{IV}-$\psi\beta^{II}$-β^F 3′ (Garner and Lingrel 1988). Thus, these sheep lack the β^C globin gene as well as three other genes that are present in the goat and A-haplotype sheep. Two models can be proposed to explain the evolutionary history of this locus. Possibly the ancestors of sheep and goats were polymorphic for duplicated and triplicated loci, with both haplotypes being retained in sheep but only the triplicated locus remaining in goats. Alternatively, the ancestors of sheep and goats may all have had a triplicated locus, and some sheep later deleted a four-gene set.

Sequence comparisons could verify one of these models. If the β^B sheep locus is descended directly from the ancestral eight-gene haplotype, then the β^B-globin gene would be expected to be equally similar to the β^A- and β^C-globin genes. However, if the β^B-globin gene arose recently, after the triplication event that produced the β^A-globin and β^C-globin genes, then the β^Bgene would be more similar to either the β^A- or the β^C-globin gene. Therefore we have sequenced the β^B-globin gene from B sheep, which we had isolated previously, and the β^C-globin gene from A sheep, and compared these sequences with related ruminant juvenile and adult gene sequences. Our results favor the deletion model.

Materials and Methods

Probes for Library Screening and Southern Blots. Probes were obtained from the cloned goat ϵ^V-, ϵ^{IV}-, $\psi\beta^Z$-, and β^F-globin genes. Each of these probes is specific for the descendants of one member of the ruminant ancestral four-gene set. The ϵ^V probe hybridizes to the goat ϵ^I-, ϵ^{III}-, and ϵ^V-globin genes; the ϵ^{IV} probe hybridizes to the ϵ^{II}-, ϵ^{IV}-, and ϵ^{VI}-globin genes; the $\psi\beta^Z$ probe hybridizes to the pseudogenes, and the β^F-globin probe hybridizes to the β^C, β^A, and β^F genes. These probes have been described in detail elsewhere (Garner and Lingrel, 1988).

Construction of a Genomic Phage Library. Genomic DNA from a homozygous A-haplotype sheep was used to construct a partial Sau3aI library in the lambda vector EMBL4 using standard methods (Maniatis et al. 1982). EMBL4 phage arms were prepared by digestion with BamHI and SalI and ethanol precipitation to remove the small linker fragments. The genomic DNA was ligated to the phage arms, packaged, and plated without amplification. The library was screened with the goat ϵ^{IV} and β^F probes described above, using methods described by Maniatis et al. (1978). At low stringency wash conditions these probes cross-react with the ϵ^I-, ϵ^{III}-, and ϵ^V-globin genes and the pseudogenes, respectively.

DNA from 40 positive phage clones was digested with EcoRI and BamHI or HindIII and combinations of these enzymes. Comparisons of these digests and hybridization patterns found by Southern blot analysis of the digests allowed most of the clones to be ordered in groups on the basis of shared fragments. Comparisons of double digests allowed tentative overlapping fragments to be identified at the ends of clones 14 and 18, and 24 and 49. These overlaps were confirmed by sequencing. Southern blot analysis using the four goat probes was used to locate the genes within the locus.

Nucleotide Sequencing. The gene sequences were determined using a variation (Duncan 1985) of the dideoxy chain termination method (Sanger et al. 1977). Subclones for sequencing were obtained using the deletion method of Dale et al. (1985).

Sequence Analysis. Sequences were aligned using the Micro Genie programs from Beckman. Percent divergences were calculated by counting each mismatch between two sequences and dividing by the length of the longer sequence. Each insertion or deletion was counted as one mismatch regardless of size. Silent and replacement changes within coding regions were calculated by the method of Perler et al. (1980) using a computer program written by F. Fuller and supplied to us by A. Efstratiadis.

Results

Linkage Arrangement of the A Sheep Locus

Forty overlapping clones spanning the A sheep β-globin locus were isolated (Fig. 1). The locations of the genes were determined by Southen blot hybridizations using the four goat gene probes described above. The results obtained with EcoRI digestions are shown in Fig. 2. The goat ϵ^V probe hybridizes to three regions in the A sheep locus, at about 10, 50, and 92 kb from the 5′ end of the cloned region (Fig. 2A, clones 16, 4, 38, 27, and 42). As in the goat and B sheep, the goat ϵ^{IV} probe hybridizes to regions downstream of each ϵ^V-hybridizing region, at 19, 58, and 100 kb from the 5′ end of the locus (Fig. 2B, clones 4, 18, 27, 49, and 35). Similarly the goat $\psi\beta^Z$ probe hybridizes to three regions, each downstream of the ϵ^{IV}-hybridizing regions, at 24, 64, and 111 kb from the 5′ end of the cloned region (Fig. 2C, clones 4, 18, 49, 35, and 46). The goat β^F probe also hybridizes to three fragments, at 32, 78, and 124 kb from the 5′ end of the locus (Fig. 2D, clones 14, 24, 36, and 46). Genomic Southern blots of A sheep DNA hybridized with the same four goat probes show the same number of hybridizing fragments (Garner and Lingrel 1988), indicating that the complete A sheep locus is contained within these clones. As expected, the A sheep locus is very similar to that of the goat, consisting of a triplicated four-gene set: 5′ ϵ^I-ϵ^{II}-$\psi\beta^I$-β^C-ϵ^{III}-ϵ^{IV}-$\psi\beta^{II}$-β^A-ϵ^V-ϵ^{VI}-$\psi\beta^{III}$-β^F 3′.

Sequence Features of the β^B- and β^C-Globin Genes

The nucleotide sequences of the sheep β^B- and β^C-globin genes are shown in Fig. 3. The conserved

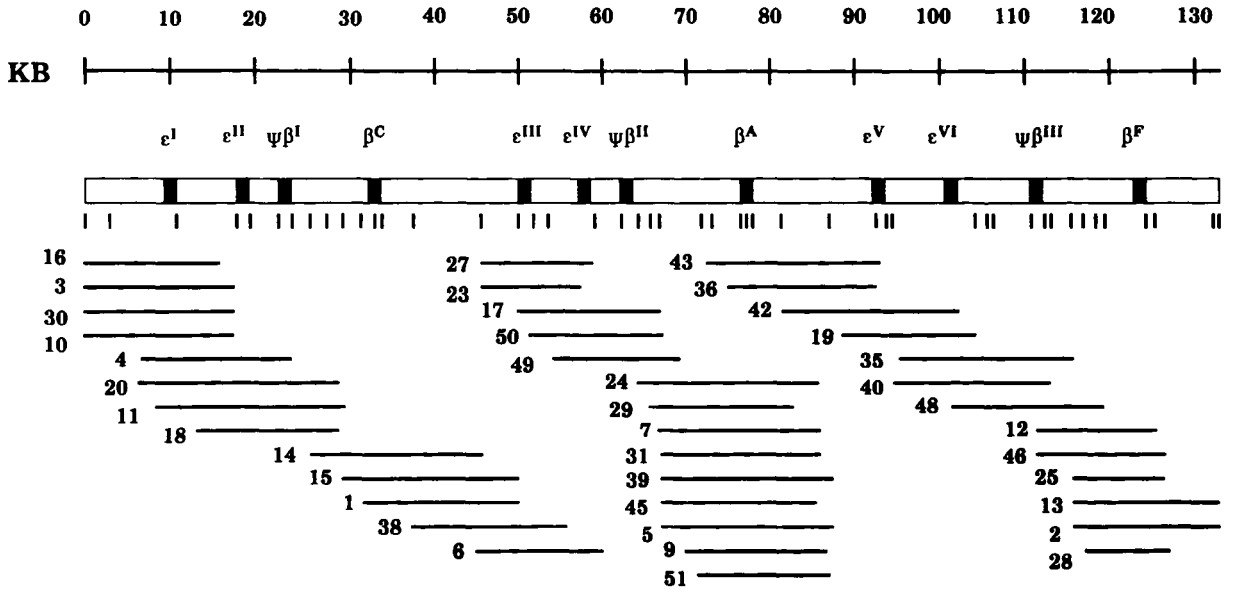

Fig. 1. Linkage map of the β-globin gene cluster of sheep with the β^A allele. EcoRI restriction sites are indicated as vertical bars below the map. Overlapping phage clones spanning the locus are numbered.

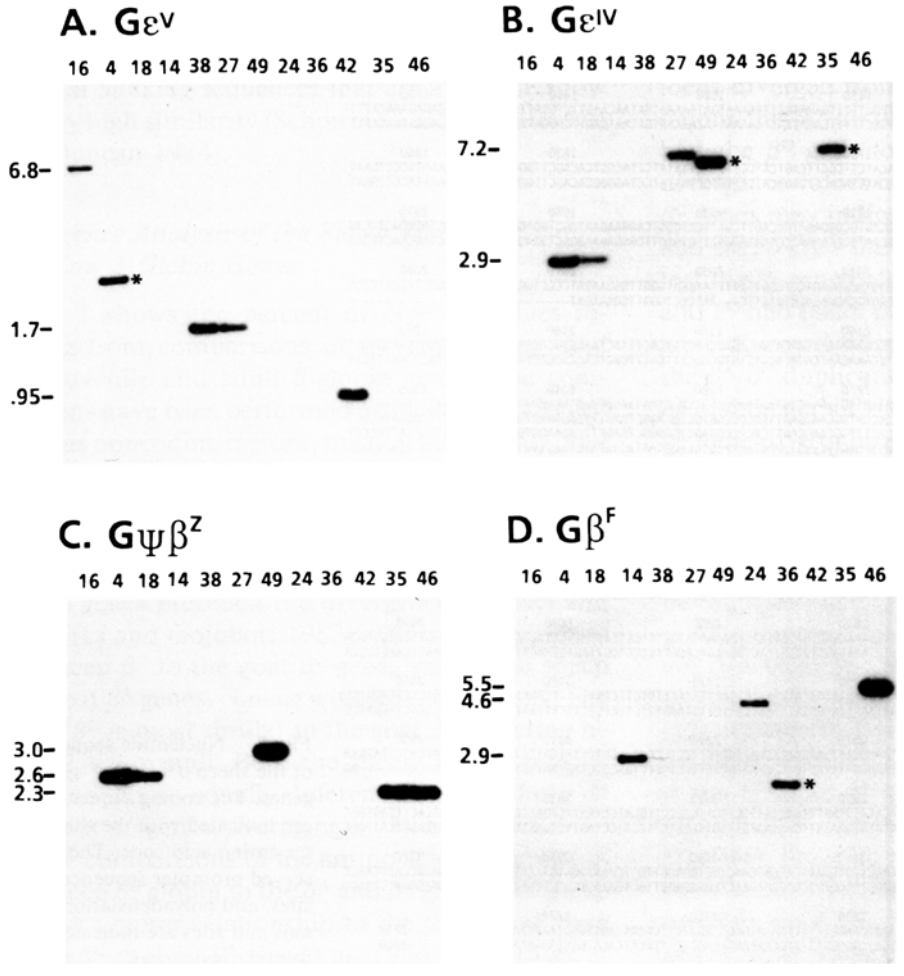

Fig. 2. Southern blot of EcoRI-digested sheep clone DNA hybridized to goat β-globin probes $G\epsilon^V$, $G\epsilon^{IV}$, $G\psi\beta^Z$, and $G\beta^F$. Lanes are labeled with the clone numbers. Sizes of hybridizing fragments are listed in kilobases. Bands marked with an asterisk (*) are EcoRI-partial Sau3AI fragments produced during cloning and are not representative of genomic fragment sizes.

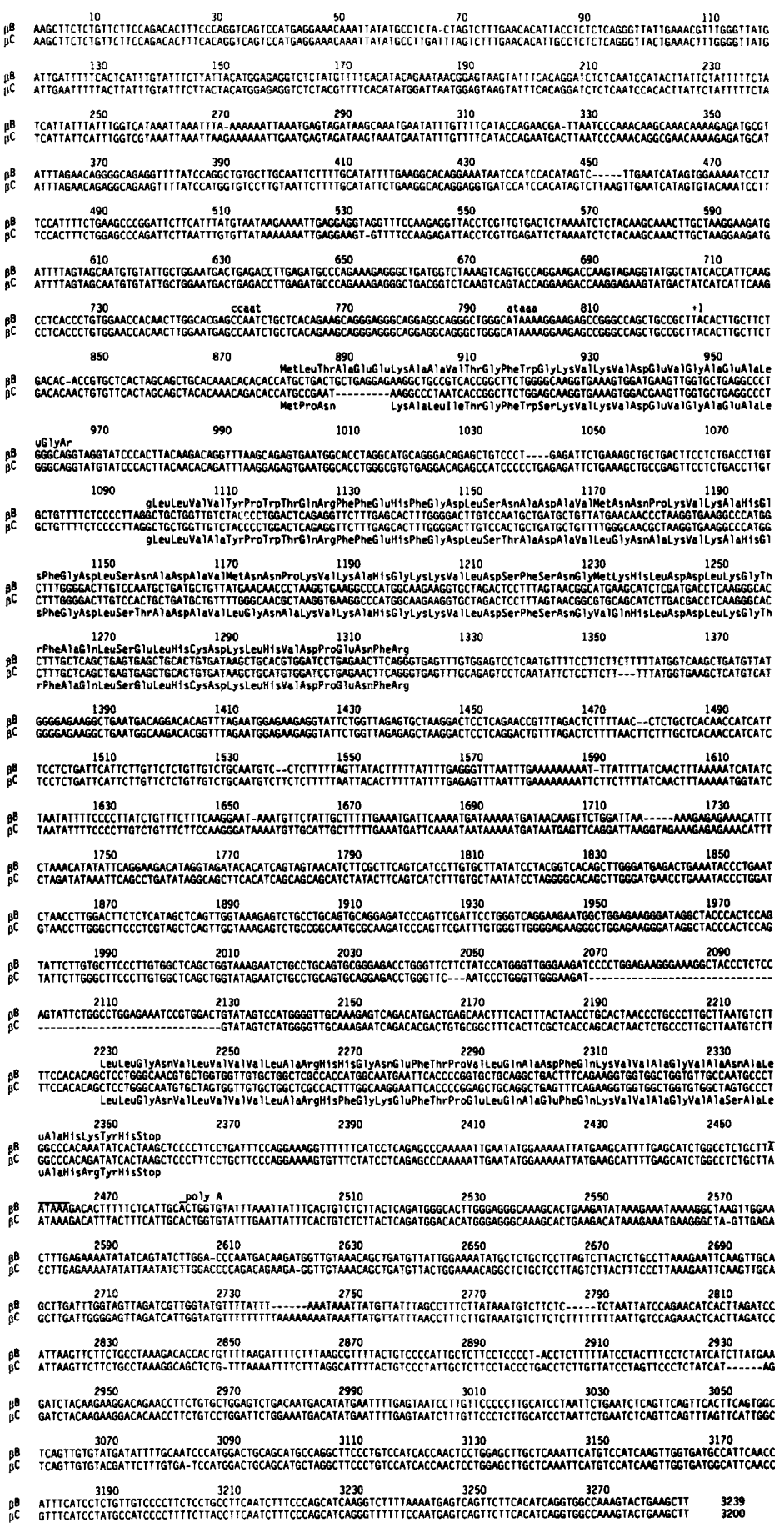

Fig. 3. Nucleotide sequences of the sheep β^B- and β^C-globin genes. The coding sequences are indicated with the three-letter amino acid code. The conserved promoter sequences, cap sites, and polyadenylation signals and sites are indicated over the sequences.

promoter sequences, cap sites, intron and exon boundaries, and polyadenylation signals and sites were located by comparisons to the previously sequenced goat β^A- and β^C-globin genes. The coding region sequences for both genes correspond to the published amino acid sequences (Boyer et al. 1966).

Comparisons of the Sheep, Goat, and Cow β-Globin Genes

The goat β^A- and β^C-, sheep β^A-, β^B-, and β^C-, and cow adult β-globin sequences are all very similar to each other, with overall percent divergence values of 10% or less between any pair. Sequences for all six genes are available from −146 nucleotides from the cap site to the end of the first exon, and from the beginning of the second exon to the poly A addition site 129 nucleotides downstream from the end of the third exon (Kretschmer et al. 1981; Schon et al. 1981; Li and Gojobori 1983; Schimenti and Duncan 1984). The first intron sequence of the sheep β^A-globin gene is not available so this region was excluded from all the comparisons. The sheep β^A-globin sequence also has undetermined bases in three codons in the second exon, so these nucleotides were not included in any of the divergence comparisons, and the corresponding codons were not considered in any of the coding region comparisons. The alignments of these sequences are shown in Fig. 4. Additional flanking sequences that are available show equally high similarity (Schon et al. 1981; Schimenti and Duncan 1984).

Divergence Analysis of the Sheep, Goat, and Cow β-Globin Genes

Table 1 shows the percent divergence values resulting from comparisons of the goat, sheep, and cow juvenile and adult β-globin genes. The comparisons have been performed using the overall coding plus noncoding regions, the noncoding only, and coding only. The results are essentially the same regardless of which regions are considered.

Sequence comparisons to date had indicated that the duplication event that produced the β^A- and β^C-globin genes preceded the divergence of goats and sheep (Li and Gojobori 1983). The comparisons of the sheep β^C to the goat β^C gene, and to the sheep and goat β^A genes, concur with this conclusion. The sheep β^C is most similar to the goat β^C, differing by only 2.4% overall. Both the sheep β^C and goat β^C genes are about equally different from the sheep β^A and goat β^A genes, averaging 8.7% divergence.

The comparisons of the amino acid sequences of the sheep β^B globin to the β^A and β^C globins of sheep suggest a closer relationship to the β^A protein than to the β^C protein (Boyer et al. 1966; Czelusniak et al. 1982). Nucleotide sequence comparisons concur with this suggestion. The percent divergence values of the β^B- versus the β^A-globin genes of goat and sheep are 3.0 and 3.3, respectively, while the values for the β^B versus the goat and sheep β^C comparisons are 8.6 and 8.8%. This suggests that the β^B gene arose from the β^A-globin gene after the β^A and β^C sequences had diverged, probably at about the time of divergence of goats and sheep.

However, it is possible that the sheep β^B gene resembles the β^A gene because of functional constraints on regulation or protein structure. As a control to test this possibility, we included the cow adult β-globin gene sequence in our comparisons. The cow adult β globin is similar to the sheep β^B in regulatory pattern and presumably function, so if conservation of functional sequence features is a significant factor in sequence similarity, the cow β should also resemble the β^A genes more than the β^C-globin genes. As shown in Table 1, the cow β is more similar to the β^A genes (7.1 and 7.4% divergence) than to the β^C genes (9.5 and 9.7% divergence) but only slightly.

The cow diverged from the common ancestor of goats and sheep about 15 to 20 million years ago (Li and Gojobori 1983; Schimenti and Duncan 1985b). The β^A/β^C duplication is estimated to have occurred about 12 million years ago (Li and Gojobori 1983), but given the uncertainties involved in such estimates, it is still unclear whether the cow locus diverged from the goat/sheep ancestral locus before the β^A/β^C duplication, or whether the cow had the β^C gene for a time and later lost it. The divergence values between the cow β and the β^A and β^C genes may indicate that the cow split from goats and sheep after the β^A/β^C duplication occurred, but lost the β^C gene before much divergence between β^A and β^C had taken place. Alternatively, the cow may have diverged from the goat/sheep ancestor before the β^A/β^C duplication event, and the greater extent of divergence of the cow β from the β^C genes may be indicative of more rapid evolution of the β^C genes due to their new function and different regulatory pattern. A third possibility, as mentioned above, is that the cow β and sheep and goat β^A genes, having some similar functions and regulatory patterns, may have conserved sequence elements for these reasons.

The question of the evolutionary relationship of the cow locus to the goat/sheep ancestral locus can be addressed partially by comparisons of pseudogene sequences. Each β-globin gene in the ruminant β-globin clusters has an adjacent pseudogene which is the product of the same duplication event that created each β gene (Cleary et al. 1981; Brunner et al. 1986). Since all the pseudogenes in these clusters share some of the same defects, it appears the ancestor of them all was defective. Thus, as nonfunctional genes, their evolution in theory should have been

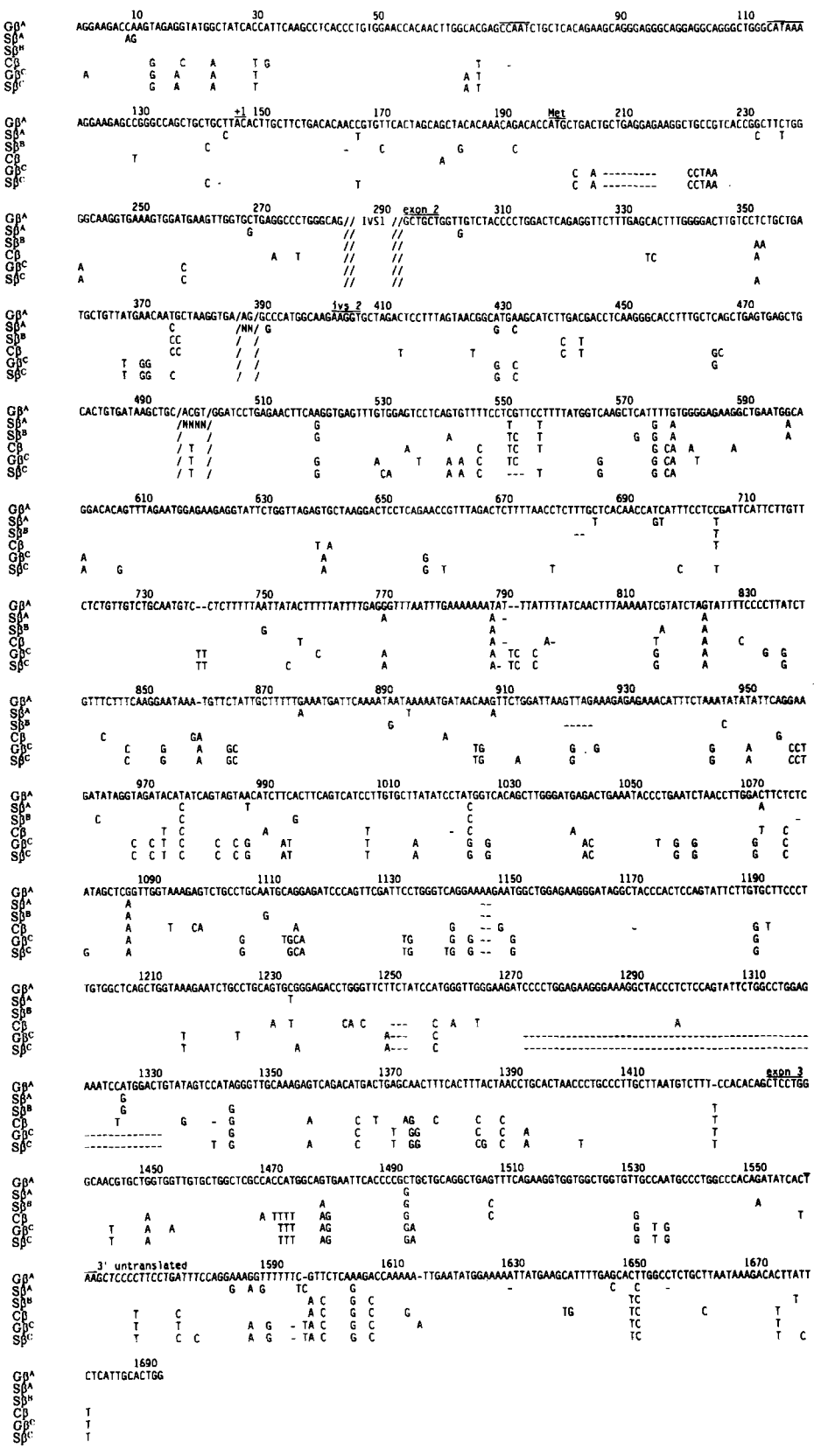

Fig. 4. Alignment of the goat β^A- ($G\beta^A$), sheep β^A- ($S\beta^A$), sheep β^B- ($S\beta^B$), cow β- ($C\beta$), goat β^C- ($G\beta^C$), and sheep β^C- ($S\beta^C$) globin gene sequences. The goat β^A sequence is shown; for the remaining genes only the mismatches are shown. Undetermined nucleotides are indicated as N. Deletions are marked with hyphens (-) and sequence regions excluded from the divergence analyses are marked with slashes (/). Overlines indicate conserved promoter sequences, capsites, initiation codons, and the beginnings of exons, introns, and the 3′ untranslated region.

unaffected by selection for functional sequences. When the cow ψ^3 gene, which is adjacent to the adult β gene, is compared to the $\psi\beta^X$ and $\psi\beta^Z$ genes of goat, which are adjacent to the β^C and β^A genes, respectively, the cow ψ^3/goat $\psi\beta^X$ and cow ψ^3/goat $\psi\beta^Y$ sequences are nearly equally divergent, (9.3 and 8.7%, respectively). The divergence between the goat $\psi\beta^X$ and $\psi\beta^Z$ genes is slightly less (8.1%). Thus, the

Table 1. Percent divergence of the cow, goat, and sheep juvenile and adult β-globin genes

		% Divergence		Coding region changes		
		Overall	Noncoding	Silent	Replacement	Total
Sheep β^B	Goat β^A	3.0	3.2	1	8	11
	Sheep β^A	3.3	3.3	5	9	14
Sheep β^B	Goat β^C	8.6	8.9	8	22	30
	Sheep β^C	8.8	9.4	9	20	29
Sheep β^B	Cow β	6.8	7.5	7	13	20
Cow β	Goat β^A	7.1	7.5	5	15	20
	Sheep β^A	7.4	7.6	7	18	25
Cow β	Goat β^C	9.5	9.8	8	25	33
	Sheep β^C	9.7	10.2	7	23	30
Goat β^A	Sheep β^A	2.4	2.5	3	5	8
Goat β^C	Sheep β^C	2.4	2.9	1	5	6
Goat β^A	Goat β^C	8.3	8.8	6	20	26
	Sheep β^C	8.9	9.7	7	20	27
Sheep β^A	Goat β^C	8.7	9.3	10	17	27
	Sheep β^C	8.8	9.7	9	17	26
Cow ψ^3	Goat $\psi\beta^Z$	8.7				
Cow ψ^3	Goat $\psi\beta^X$	9.3				
Goat $\psi\beta^X$	Goat $\psi\beta^Z$	8.1				

Percent divergences were calculated as described in Methods. The coding region changes indicate number of changes out of 140 codons, calculated as described in Methods. Regions of deleted or undetermined sequence were excluded from all coding region calculations

closer similarity of the cow β to the β^A genes may not extend to the rest of their respective four-gene sets, and may reflect conservation of functionally required sequences or rapid divergence by the β^C genes, rather than a closer evolutionary relationship.

We have considered the possibilities that the sheep β^B resembles the sheep and goat β^A genes because of functional similarities, and that the β^B fails to resemble the β^C genes because the juvenile genes have evolved unusually quickly. These factors probably contribute to the resemblance between the sheep β^B and the goat and sheep β^A genes. However, the simplest explanation for the very high similarity between the β^B and β^A globin genes is that they have diverged recently.

Discussion

The ruminant β-globin gene clusters are unique among globin loci in having undergone recent large-scale block duplication events, followed by the derivation of genes with different developmental functions. Such large regions of repeated highly similar sequences might be expected to be unstable in the face of unequal recombination events. It is easy to imagine that such an event could have created a triplicated locus from two chromosomes carrying duplicated clusters (Fig. 5B), and our sequence data indicate that a comparable deletion event may account for the structure of the B sheep locus (Fig. 5C). Many examples of deletions removing previously duplicated gene regions, presumably by unequal recombination, have been described. These include the γ/δ fusion pseudogene found in lemurs (Jeffreys et al. 1981), and variant human β globins such as Lepore and Kenya (Weatherall and Clegg 1981). Rando et al. (1986) have described great variability in the sheep α-globin locus, in which variants with two, three, or four genes per chromosome can be found in the population. The presence of two alleles at the β-globin locus in Barbary sheep (*Ammotragus lervia*) suggests another variation on the ruminant β-globin locus structure. These animals, like domestic sheep, have a haplotype that expresses a β^C globin during anemia, and a haplotype that lacks the anemic globin. However, in the haplotype lacking the β^C globin, the adult globin is structurally similar to the β^C protein (Huisman and Miller 1972). It is unknown whether this cluster has undergone a deletion, but if it has, the β^A gene may have been the one deleted.

Another consequence of the long tandem duplicated DNA segments in the ruminant β-globin clusters is the high probability of gene conversion or gene correction. Examples of probable gene conversions have been noted previously in these clusters. The goat ϵ^I and ϵ^{II} genes are slightly more similar at their 5′ ends than in the remainder of the genes (Goodman et al. 1984; Menon and Lingrel 1986). The cow ϵ^2 and ϵ^4 are more similar to each other than either is to its orthologue in goat, although the ϵ^2/ϵ^4 duplication necessarily preceded the cow/goat divergence (Schimenti and Duncan 1985a). The cow

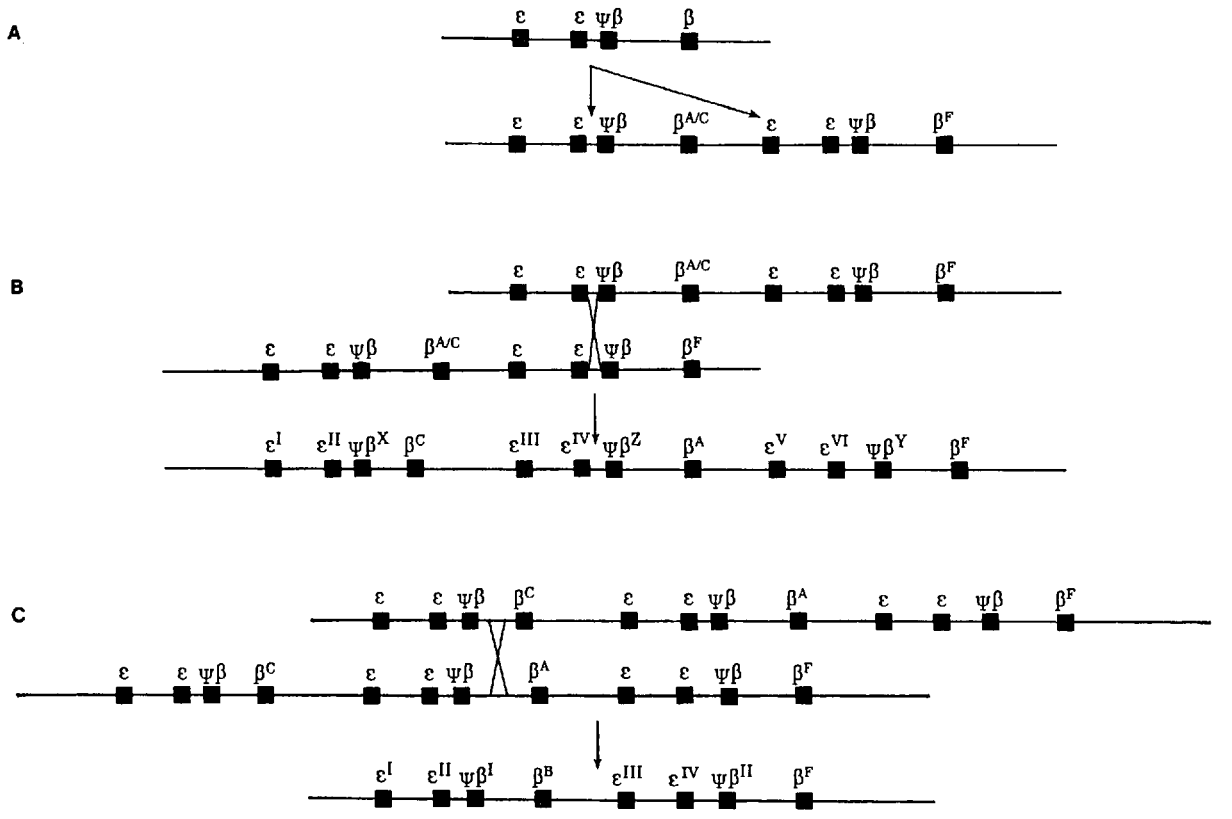

Fig. 5. Schematic diagram of the evolutionary histories of the goat and sheep β-globin gene clusters. **A** Duplication of the ancestral ruminant four-gene cluster resulting in an eight-gene cluster. **B** Unequal crossover leading to the triplicated cluster of goats and β^A sheep. **C** Hypothetical model for the deletion of four genes resulting in the eight-gene cluster of β^B sheep.

pseudogene intervening sequences are unexpectedly similar to the functional β-globin gene intervening sequences, given the evolutionary distances between these genes (Brunner et al. 1986). Most of these cases are more subtle than the examples of very recent events seen on a background of more highly diverged DNA, such as those described by Schon et al. (1982), involving the goat α-globin genes, or by Hill et al. (1984) concerning the extreme similarity at the 5′ ends of the mouse βh0 and βh1 genes. Gene conversions in the distant past may lack clear endpoints. They may be evident only from slight discrepancies in the overall divergence values for different pairs of genes that are known to have diverged at the same time. The possibility of such obscure gene conversions makes it risky to estimate times of gene duplication events or rates of evolution in families of closely related genes. Our sequence results indicate that the sheep β^B gene diverged from the goat and sheep β^A-globin genes very recently, but the possibility cannot be excluded that the similarity between these genes is the result of a recent interchromosomal gene conversion.

A potential problem with the deletion model for the origin of the B sheep locus is that animals that deleted the β^C-globin gene might have been at a selective disadvantage. The sequence comparisons suggest that the β^B gene diverged from the β^A-globin gene at about the same time goats and sheep diverged, or perhaps slightly before. The regulation of the β^A- and β^C-globin genes in the goat/sheep ancestor cannot be known. In present-day sheep, the β^C protein is expressed at very low levels in juveniles, making up less than 10% of the β globin (Huisman et al. 1969), so the loss of the β^C-globin gene might not be deleterious at this stage. In goats, however, the β^C is the major β globin for the first 4–6 months after birth. In both sheep and goats, severe anemia can cause a nearly complete switch from β^A to β^C.

The regulator of the β^A to β^C switch is erythropoietin (Thurmon et al. 1970). Because the β^C-globin genes of sheep and goats are extremely similar, it is possible that the difference in the regulation of β^C in these animals is the result of differences in the regulation of erythropoietin levels, not of the β^C genes. Such changes in regulation in sheep and goats could have occurred after the two species diverged, perhaps relating to the selection of habitats at different altitudes, or with different endemic parasites. The ancestral sheep/goat anemic switch regulation may have been different from that of either present-day goats or sheep. If the switch mechanism was less sensitive in the ancestral animal, an individual that lost the β^C-globin gene might not have been at a selective disadvantage unless it became severely anemic. It is also possible that the β^B allele was

linked to another advantageous trait that allowed it to be selected in spite of some loss of fitness. Evans and Turner (1965) have found that in some breeds of present-day sheep, animals with the β^B allele are more reproductively successful than those with the β^A allele.

The loss of the β^C gene did not necessarily affect the fitness of the animal, however. The deletion that removed the β^C gene may have included regulatory regions involved with the switching off of the adult gene. Alternatively, the switching off of the β^A gene may be triggered directly by increasing levels of the β^C protein. Another possibility is that the β^B gene may have begun to diverge and lose its ability to switch off before the β^C gene was deleted.

In summary, our efforts to understand the evolutionary history of the sheep β^B locus are complicated by evidence of functional constraints on the divergence of the sheep and goat β^A, cow β, and sheep β^B genes, and of the possibility of rapid divergence of the β^C genes. An additional complication is introduced by the possibility of gene conversion events. It seems unlikely that the close similarity between the β^B and β^A genes can be explained by these factors alone. It is more probable that the β^B gene is similar to the β^A genes because these two genes share a recent common ancestor. Sequence comparisons of other regions in the A and B sheep β-globin clusters, such as pseudogenes or intergenic regions, could help answer questions of conservation or rapid divergence of functional sequences, and could provide information on the extent of regions of gene conversion. Such comparisons could possibly confirm the deletion model and could even locate the site of the deletion.

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