Molecular basis of bovine red-cell protein 4.2 polymorphism in Japanese black cattle

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Cattle were divided into three groups according to the red cell-protein 4.2 (P4.2) phenotypes P4.2⁷⁶, P4.2⁷⁵ and P4.2^{76/75}, whose red cells contained M_r 76000 (P4.2/76), 75000 (P4.2/75) and both 76000 and 75000 isoforms respectively. To elucidate the molecular basis that underlies the diversity of P4.2, the gene structures of bovine P4.2/76 and P4.2/75 were investigated. Two P4.2 cDNA clones were isolated from bone-marrow cDNAs of the animal with the P4.2^{76/75} phenotype. These were identical in size (2.2 kb), encoding major erythroid P4.2 with 687 amino acids, but were different in three nucleotides, resulting in changes of amino acids at the 599th, 601st and 627th residues. Analysis of genomic DNA from the three phenotypes demonstrated that

these two clones were derived from gene transcripts by which P4.2/76 and/or P4.2/75 were produced. *In vitro* transcription and translation of P4.2/76 and P4.2/75 cDNAs indeed generated P4.2/76 and P4.2/75 identical in size to the red-cell proteins. These findings demonstrated that polymorphism of the P4.2 gene at codons 599, 601 and 627 of P4.2 cDNA was the cause of the molecular diversity of bovine red-cell P4.2. Although distinct electrophoretic mobilities suggested a structural difference in the two isoforms, this polymorphism appeared to have little effect at least on P4.2 association with band 3, since no significant difference was observed in the amount of P4.2 relative to total membrane proteins despite the phenotype difference for P4.2.

INTRODUCTION

Protein 4.2 (P4.2), one of the major components of the human red-cell membrane with an apparent M_r of 72000, accounts for approx. 5% of the total protein content of the membrane [1]. Despite its relative abundance, little is known about its physiological function in red cells. Human red-cell P4.2 is localized on the inner surface of the plasma membrane and is tightly bound to the membrane by association with the cytoplasmic domain of band 3 protein [1,2] and through N-myristylation at Gly² [3,4]. Studies on P4.2 deficiency, associated with abnormal red-cell shapes and haemolytic anaemia, due to P4.2 gene defects [5,6] or secondary to band 3 gene defects [7,8] suggested that P4.2 was required to stabilize red-cell membrane integrity.

On the other hand, various mammalian red cells contain P4.2, which shows M_r values of 72000–78000 in SDS/PAGE and accounts for 3–5% of the total protein content. The nearly complete lack of P4.2 observed in bovine red cells with hereditary total deficiency of band 3 [9] and a stable association of P4.2 to the membrane skeleton in other animals [10,11] suggested that P4.2 plays important roles in the structural organization of the red-cell membrane in those mammals just as in human red cells. Interestingly, several animal species show molecular diversity of P4.2. The majority of Japanese Sika deer have an M_r 78000 P4.2 isoform, whereas most of the rest have nearly equal quantities of the M_r 78000 and 76000 (P4.2/76) forms [11], and a lesser number have the M_r 76000 isoform alone (Y. Amano, M. Inaba and Y. Maede, unpublished work). Moreover, our recent studies [12] showed that some calves exhibiting anaemia with poikilocytosis and diarrhoea in the neonatal period have a doublet of P4.2 polypeptides of M_r 76000 and 75000 (P4.2/75). It is unclear as yet whether the presence of P4.2 isoforms in red cells is deleterious to the functional organization of the membrane or not, and how, if at all, it affects the membrane integrity. It should

be noted that the presence of human P4.2 isoforms (M_r 74000 and normal M_r 72000 forms) in reduced amounts, probably related to the presence of an alternatively spliced form of P4.2, was reported in several cases of hereditary P4.2 deficiency with haemolytic anaemia [5,6]. It has also been reported that in an autosomal dominant stomatocytosis with haemolytic episodes [13] the patients' red cells contained M_r 74000 and 72000 polypeptides at the normal level as in bovine and deer red cells, indicating the possible significance of structural diversity of P4.2 in red-cell morphological anomaly and resultant haemolytic anaemia.

The purpose of the present study is to elucidate what the molecular basis that underlies P4.2 diversity is and whether the P4.2 diversity affects the constitution of membrane proteins in bovine red cells. The red-cell membrane proteins of more than 200 individuals of several cattle breeds were surveyed to examine the phenotype of P4.2. Then, the difference between the M_r 76000 and 75000 isoforms was investigated in terms of their gene structures.

EXPERIMENTAL

Preparation of red-cell membranes

Red-cell membranes were prepared as described previously [9]. Protein concentrations of membranes were determined by the method of Bradford [14] with a protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.) using BSA as the standard.

Red-cell membrane-protein analysis

Red-cell membrane proteins were separated by SDS/PAGE using Laemmli's buffer system [15] or the Tris/Tricine buffer

Abbreviations used: P4.2, red-cell protein 4.2; P4.2/76 and P4.2/75, P4.2 isoforms with apparent molecular masses from SDS/PAGE of 76000 and 75000 respectively; RACE, rapid amplification of cDNA ends.

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The nucleotide sequences reported in this paper have been deposited in GenBank under the accession numbers AF030029 and AF030030.

Table 1 Primers for PCR amplification and sequencing analysis

p11, p3, p3N and p17 are sense primers; p10, p12, p2, p18 and p16 are antisense primers. Nucleotides are numbered according to bovine P4.2 cDNA with respect to the A of the initiation codon.

Name	Sequence	Position
p11	5'-AGCCAAGTGGATAAGAACGGTCTG-3'	-29--6
p3	5'-GATGGCAGATTCTGCACCCAA-3'	1031-1051
p3N	5'-CTTAAAGGAGGTGAAGTCCTGGAGGCTGTGAT-3'	1057-1089
p17	5'-ATGCCAGAGACAGCAGAGCAACATCA-3'	1768-1793
p10	5'-CTCGATTCCAGGGGTAAAGA-3'	427-407
p12	5'-CAGGCTCTGCCTCTGTGTCTTCT-3'	968-945
p2	5'-ACAGCACTTCAGACCACACA-3'	1194-1174
p18	5'-CTGTAGCTCTTCTCTATGAA-3'	1901-1880
p16	5'-GTTTCTCTTCCCATCATGCTTGGT-3'	2142-2119

system including SDS [16]. Membrane proteins were stained with Coomassie Brilliant Blue and quantified by densitometric scanning using a GS-700 densitometer (Bio-Rad). P4.2 was detected and identified by immunoblotting using rabbit anti-human P4.2 polyclonal antibodies as reported previously [11].

Bone-marrow cells

Sternal bone-marrow cells were obtained under sedation with xylazine from a Japanese black cow whose red cells contained both P4.2/76 and P4.2/75 (phenotype 4.2^{76/75}). Bone-marrow cells rich in erythroid precursor cells were obtained from an anaemic animal with hereditary total deficiency of band 3 [9], although the P4.2 phenotype of the latter animal had not been identified until the molecular basis for P4.2 polymorphism was clarified later, since its red cells also lacked P4.2 secondary to the complete deficiency of band 3.

Cloning of bovine red-cell P4.2 cDNAs

All manipulations of RNA and DNA were performed according to the standard protocol [17]. Total bone-marrow cell RNA was prepared by the guanidine thiocyanate method. Poly(A)⁺ RNA was purified by oligo(dT) cellulose chromatography using an mRNA purification kit (Pharmacia Biotechnologies, Uppsala, Sweden).

Poly(A)⁺ RNAs from P4.2^{76/75} and P4.2⁷⁶ cows were reverse transcribed using a 3' rapid amplification of cDNA ends (RACE) system (Gibco BRL, Gaithersburg, MD, U.S.A.) or Marathon cDNA amplification kit (Clontech Laboratories, Palo Alto, CA, U.S.A.). To ensure isolation of an erythroid P4.2 cDNA clone, we first worked on the cDNAs prepared from bone marrow rich in erythroid progenitors as described above. The cDNAs obtained were amplified by PCR (35 cycles of 1 min at 95 °C, 1 min at 58 °C and 1 min at 72 °C) using a GeneAmp PCR reagents kit (Perkin-Elmer, Norwalk, CT, U.S.A.) with primer pair p3 and p2 (Table 1), prepared based on the cDNA sequence of human P4.2 [18,19]. The amplified p3-p2 fragment was cloned into the pCR II vector using a TA cloning kit (Invitrogen, San Diego, CA, U.S.A.) and sequenced on both strands using a PRISM dye primer cycle sequencing kit with M13 forward and M13 reverse primers on an ABI 373A DNA sequencer (Perkin-Elmer).

The 5' and/or 3' RACE reactions were performed with combinations of primers p3N, p2 and p12, and the adaptor primers using an LA PCR reagents kit (Takara, Otsu, Japan). The resultant overlapping 5' or 3' RACE products were cloned into the pCR II or pCR 2.1 vector (Invitrogen) and sequenced on

both strands. Thus the nucleotide sequence of bovine red-cell P4.2 cDNA (≈ 2.5 kb), encompassing the 5'-untranslated region, coding region and 3'-untranslated region, was determined.

Then bone-marrow cell cDNAs from a P4.2^{76/75} cow were amplified to isolate P4.2 cDNA clones containing the open reading frame corresponding to P4.2/76 and P4.2/75 using primers p11 and p16 complementary to nucleotide sequences in the 5'- and 3'-untranslated regions respectively. Amplified cDNAs were cloned into the pCR 2.1 vector, and the inserts were fully sequenced on both strands.

Analysis of genomic DNA

Genomic DNAs of P4.2⁷⁶, P4.2⁷⁵ and P4.2^{76/75} cows were isolated from buffy-coat cells using a QIAamp blood kit (Qiagen, Hilden, Germany). Genomic DNA was amplified by PCR with primers p17 and p18 and was directly sequenced using a PRISM dye terminator cycle sequencing kit (Perkin-Elmer) to determine genotypes for P4.2.

In vitro synthesis of P4.2/76 and P4.2/75

Bovine P4.2 was synthesized *in vitro* in the presence of [³⁵S]methionine (Tran³⁵S-label, ICN Pharmaceuticals, Irvine, CA, U.S.A.) in the TNT coupled reticulocyte lysate system (Promega, Madison, WI, U.S.A.) using plasmids pBP4.2/76 and pBP4.2/75 containing cDNA inserts (nt -29 to 2142) of P4.2/76 and P4.2/75, respectively, as the templates. Translation products were separated on a Tris/Tricine 10% polyacrylamide gel, and the gel was dried and exposed to Kodak X-OMAT AR film for 5-20 h at room temperature.

RESULTS

Phenotypes for bovine red-cell P4.2

SDS/PAGE analysis of red-cell membrane proteins from 209 healthy adult Japanese black cattle showed that they could be divided into three groups according to the electrophoretic features of polypeptides corresponding to P4.2 (Figure 1). A total of 58% of the individuals had the electrophoretic pattern in which the apparent *M_r* of the P4.2 band was calculated to be 76000, whereas a small population (4%) showed a P4.2 band with an

Figure 1 Identification of two isoforms of P4.2 in bovine red-cell membranes

Bovine red-cell membrane proteins were analysed by Laemmli's SDS/PAGE (A) and Tris/Tricine PAGE (B) followed by staining with Coomassie Blue and by immunoblotting using anti-human P4.2 antibodies (C). Protein contents per lane were 10 µg in (A) and (B) and 2 µg in (C). Lanes 76, 76/75 and 75 show typical electrophoretic profiles of the membrane proteins from phenotypes P4.2⁷⁶, P4.2^{76/75} and P4.2⁷⁵ respectively. P4.2/76 and P4.2/75 are indicated with arrowheads.

Table 2 Membrane-protein profiles of red cells with different P4.2 phenotypes

Data represent means $\pm$ S.D. for the relative abundance of each major membrane protein against total membrane-protein content. Data shown were obtained from densitometric scanning of the proteins separated under the same conditions (SDS/8% polyacrylamide gels), and membranes were from healthy adult (> 2 years old) Japanese black cattle.

Membrane protein	Relative abundance		
	P4.2 ⁷⁶ (n = 41)	P4.2 ^{76/75} (n = 26)	P4.2 ⁷⁵ (n = 7)
Spectrin ($\alpha + \beta$) and ankyrin	34.08 $\pm$ 3.02	33.23 $\pm$ 3.27	33.24 $\pm$ 2.97
Band 3	21.69 $\pm$ 2.09	21.37 $\pm$ 1.85	21.13 $\pm$ 1.70
Protein 4.1 (a + b)	3.15 $\pm$ 0.43	3.15 $\pm$ 0.31	3.18 $\pm$ 0.32
Protein 4.2 (76 + 75)	3.48 $\pm$ 0.29	3.42 $\pm$ 0.27	3.44 $\pm$ 0.27
Actin	3.94 $\pm$ 0.43	4.00 $\pm$ 0.40	4.04 $\pm$ 0.50
GA3PD	2.03 $\pm$ 0.29	1.89 $\pm$ 0.42	2.07 $\pm$ 0.26
B3/P4.2*	5.93 $\pm$ 0.60	5.93 $\pm$ 0.57	5.76 $\pm$ 0.40
4.1a/4.1b†	2.03 $\pm$ 0.81	1.99 $\pm$ 0.57	2.01 $\pm$ 0.46

* Relative ratio of band 3:P4.2.

† Marker for red-cell aging [28].

M_r of 75000 (Figure 1A). The rest appeared to contain equal amounts of both M_r 76000 and 75000 proteins. Variations of P4.2 bands were more readily detected when membrane proteins were subjected to Tris/Tricine PAGE (Figure 1B). These two polypeptides were immunospecifically recognized with anti-human red-cell P4.2 antibodies in immunoblotting (Figure 1C), indicating that the bovine red-cell membrane proteins with M_r values of 76000 and 75000 were sequence-related isoforms of P4.2. In the present study, these two isoforms were designated P4.2/76 and P4.2/75. Therefore, individual animals were phenotypically identified as P4.2⁷⁶, P4.2⁷⁵ and P4.2^{76/75}, indicating that they possessed red cells with P4.2/76, P4.2/75 and both P4.2/76 and P4.2/75 respectively. Phenotypic differences in red-cell P4.2 due to the appearance of P4.2/76 and P4.2/75 were also observed in several other bovine species, including Holstein Friesian and Japanese Shorthorn (results not shown). Densitometric scanning of the separated proteins showed that the relative abundance of P4.2 (P4.2/76 and P4.2/75) in the total membrane proteins was approx. 3.5% and was the same in the three phenotypes (Table 2). Statistically, no significant difference relevant to P4.2 phenotypes was observed in the relative abundance of other major membrane proteins as shown in Table 2.

Cloning of bovine P4.2/76 and P4.2/75 cDNAs

cDNAs of bovine erythroid P4.2 were isolated to elucidate the molecular basis for the diversity of P4.2 molecules. The 5' and 3' RACE products were cloned (Figure 2) and fully sequenced on both strands for several independent clones isolated. The sequences of the 5'-flanking region, overlapping region of 5' and 3' RACE, and 3'-untranslated region were identical in all clones sequenced, except that the lengths of the 5' end in 5' RACE clones were different (nt -146 to -29 relative to the A of the initiator AUG). Thus the nucleotide sequences of nearly full-length cDNAs for bovine P4.2 were determined. The sequence around the initiation codon (GCC AUG GGA) was in Kozak's consensus sequence (A/GCC AUG G) [20].

Using primer p11 and p16 constructed in the 5'- and 3'-untranslated regions, respectively, relevant P4.2 cDNAs ($\approx$ 2.2 kb) were amplified and cloned from bone-marrow cDNAs of the animal with the P4.2^{76/75} phenotype. Sequencing analysis of

Figure 2 Cloning of bovine P4.2 cDNA

The strategy employed for cloning of P4.2/76 and P4.2/75 cDNAs is summarized here. First, a p3-p2 fragment clone (pCR4.2/3-2) was obtained by PCR amplification using primer pair p3 and p2 derived from the sequence of human P4.2 cDNA [18,19]. pCR4.2/3N and pCR4.2/2 were 3' and 5' RACE clones isolated from PCR products amplified using combinations of adaptor primer and p3N and p2 respectively. The elongated 5' sequence was obtained by 5' RACE with adaptor primer and p12. Three independent clones with different length of the 5'-untranslated region (pCR4.2/12.1-3) were obtained. Thus nearly the full length of P4.2 cDNA was sequenced. P4.2 cDNA clones pBP4.2/76 and pBP4.2/75 containing full open reading frames that encoded P4.2/76 and P4.2/75 (thick line), respectively, were obtained by PCR amplification of bone-marrow cDNAs from the P4.2^{76/75} phenotype.

Figure 3 Molecular polymorphism of the P4.2 gene

(A) Genomic DNAs from cattle with P4.2⁷⁶, P4.2⁷⁵ and P4.2^{76/75} phenotypes were amplified by PCR with primer pair p17 and p18, and directly sequenced on both strands using those primers. Nucleotides corresponding to bases 1795, 1803 and 1879 in P4.2 cDNAs were C, G and G in P4.2⁷⁶, A, A and A in P4.2⁷⁵, and C+A, G+A and G+A in P4.2^{76/75} phenotypes respectively (arrowheads). (B) Differences of nucleotide and deduced amino acid sequences in two isoforms of P4.2. Based on the results from analyses of cDNA and genomic DNA, the P4.2 cDNA with codons 599, 601 and 627 of CCC, ATG and GTT was found to encode P4.2/76, whereas that with ACC, ATA and ATT was determined to be P4.2/75 cDNA.

the isolated clones showed that there were two types of cDNA clones differing in three nucleotides (underlined in the following codons) in distinct codons. Nucleotide triplets in one clone at codons 599, 601 and 627 were CCC, ATG and GTT respectively,

Bov. 76	MGQGLGIKSCDFQAARNNAEHHTNDISSQRLFVRRGQPTTSLHFAQPVHTFLRLKKVA	60
Bov. 75	E.....KAL.....I.Y.R.....RA.P.....	60
Human	E.....E.Y.KA.....H.T.....S.....T.N.R.....	60
Murine	H.....E.....Y.KA.....H.T.....S.....T.N.R.....	60
Bov. 76	LIAQTGKQPSKANGTQATFSVSSLGDRKWSAMVEERDDQSWTISVTPADAIIGHYSLL	120
Bov. 75	T.....E.....I.R.....PI.....V.....A.....V.....V.....	120
Human	T.....E.....I.R.....PI.....V.....A.....V.....V.....	120
Murine	T.....E.....I.R.....PI.....V.....A.....V.....V.....	120
Bov. 76	LQISGRKQ-CLGQFTLLFNPNWREDAVFLGNEAQRKEYLNNQGLIFLGTADCIQAEPWD	179
Bov. 75	V.....LL.....K.....M.....Y.....E.....P.....	179
Human	V.....LL.....K.....M.....Y.....E.....P.....	180
Murine	V.....K.....YP.....D.....Q.....E.T.....V.....F.Y.....	180
Bov. 76	FGQLEEDVIDLGLSLLTVDNQVEKWNPNVHVARVLGALLHILKEKSVLPTAKIQTTEERA	239
Bov. 75	F.G.....S.R.....SK.K.....SO.....F.....OR.....POT.A.Q.G.....	239
Human	F.K.....M.S.K.....SM.K.....KD.NQ.A.....V.....A.K.....ISQT.AAQ.G.....	240
Murine	F.K.....M.S.K.....SM.K.....KD.NQ.A.....V.....A.K.....ISQT.AAQ.G.....	240
Bov. 76	LLNKRGSAPILRQWVTGHRPVYEGQAWVLAACVTLRGLGIPARVVTTFTSAQGTGG	299
Bov. 75	Y.....V.....L.....R.....D.....T.....S.....C.....D.....V.....	299
Human	Y.....V.....L.....R.....D.....T.....S.....C.....D.....V.....	300
Murine	Y.....V.....L.....R.....D.....T.....S.....C.....D.....V.....	300
Bov. 76	DLVNVEYNNEGFLQNGEDNRRGAWIFQTSTECWMPDLLEVYDQWQILYPSALKGGEVL	359
Bov. 75	R.....ID.....D.....GO.....H.....V.....V.....N.....SOG.G.....	359
Human	R.....ID.....D.....GO.....H.....V.....V.....N.....SOG.G.....	360
Murine	R.....ID.....D.....GO.....H.....V.....V.....N.....SOG.G.....	360
Bov. 76	EACDLVPVRAVKEGIVLTPAASDIFASINASCVVWKCAGEDGTELTDSNTKYFGNNIST	419
Bov. 75	GS.....S.....ELQ.D.....VPEL.....AV.....C.....K.....N.R.DV.....C.....	419
Human	GS.....S.....ELQ.D.....VPEL.....AV.....C.....K.....N.R.DV.....C.....	420
Murine	GS.....S.....ELQ.D.....VPEL.....AV.....C.....K.....N.R.DV.....C.....	420
Bov. 76	KNVDCD-CHEDITQNYKYPEGSSQEKVMLEKVKQYRM-KHKNDGIYPPCCETDDPLHLFL	477
Bov. 75	G.GS.....R.....V.....L.....E.....R.E.EKERE.DN.....R.....SL.....AS.....V.L.....	477
Human	G.GS.....R.....V.....L.....E.....R.E.EKERE.DN.....R.....SL.....AS.....V.L.....	479
Murine	G.GS.....R.....V.....L.....E.....R.E.EKERE.DN.....R.....SL.....AS.....V.L.....	479
Bov. 76	KAPSSALGKNVEISVNNLNPDTQKEVQLAIGLQAMYNGVLAALKWRKNFVLILSANS	537
Bov. 75	E.S.....IP.SGDGQL.T.I.....E.K.H.V.....A.....L.....G.S.KQLFM.KP.Q.....	537
Human	E.S.....IP.SGDGQL.T.I.....E.K.H.V.....A.....L.....G.S.KQLFM.KP.Q.....	539
Murine	E.S.....IP.SGDGQL.T.I.....E.K.H.V.....A.....L.....G.S.KQLFM.KP.Q.....	539
Bov. 76	AKISTSLFNSNFQSLPENSFLRLTAMATHS-S-LPCFAQQDIARRPHLAIEPETA	595
Bov. 75	E.I.TIG.....F.....RNP.....T.....E.N.S.....E.....C.....K.....K.....	595
Human	E.I.TIG.....F.....RNP.....T.....E.N.S.....E.....C.....K.....K.....	599
Murine	E.I.TIG.....F.....RNP.....T.....E.N.S.....E.....C.....K.....K.....	599
Bov. 76	QHQLMALVSIHNPDLVPLEDCVISIFGKGLHREKSYRVNSVQPRNTLRLTQKFVPMKV	655
Bov. 75	Y.....H.....S.....LQ.S.....A.M.....L.R.....R.....FR.....W.E.....MCAKFO.T.T.HV.....	655
Human	Y.....H.....S.....LQ.S.....A.M.....L.R.....R.....FR.....W.E.....MCAKFO.T.T.HV.....	659
Murine	Y.....H.....S.....LQ.S.....A.M.....L.R.....R.....FR.....W.E.....MCAKFO.T.T.HV.....	659
Bov. 76	GCQRLTVMDCNMFQNLTFNFTVMVAPKSPA	687
Bov. 75	L.....V.....D.....YKS.T.....ELS.....	687
Human	L.....V.....D.....YKS.T.....ELS.....	691
Murine	L.....V.....D.....YKS.T.....ELS.....	691

Figure 4 Multiple alignment of bovine (Bov.), human and murine P4.2

The alignment of P4.2 from bovine, human and murine red cells was performed according to their deduced amino acid sequences (positions numbered at the right). Dots indicate residues identical to those of bovine P4.2/76. The residues differing between P4.2/76 and P4.2/75 are shown by white letters on black. The potential band 3-binding site (#1 shaded region) [23] and ATP-binding site (#2 shaded region) [24] are indicated.

and those in the other clone were ACC, ATA and ATT respectively (Figure 2). These differences created a simultaneous displacement of three amino acids, Pro⁵⁹⁹, Met⁶⁰¹ and Val⁶²⁷ to Thr, Ile and Ile respectively, possibly being the cause of the different electrophoretic mobilities of the two P4.2 isoforms.

To verify that these clones with several substitutions of nucleotides were indeed derived from P4.2/76 and P4.2/75 gene transcripts, corresponding regions of genomic DNA from P4.2⁷⁶, P4.2⁷⁵ and P4.2^{76/75} phenotypes were analysed. Direct sequencing of PCR-amplified DNA fragments showed that those nucleotides, 1795, 1803 and 1879, were C, G and G in P4.2⁷⁶, A, A and A in P4.2⁷⁵, and duplicates of C+A, G+A and G+A in P4.2^{76/75} phenotypes respectively (Figure 3). It was, therefore, concluded that P4.2/76 cDNA possessed codons CCC (Pro⁵⁹⁹), ATG (Met⁶⁰¹) and GTT (Val⁶²⁷), whereas those codons in P4.2/75 cDNA were ACC (Thr⁵⁹⁹), ATA (Ile⁶⁰¹) and ATT (Ile⁶²⁷). These results also indicated that cattle have three genotypes according to the sequence of the P4.2 gene, namely P4.2^{76/76}, P4.2^{75/75} and P4.2^{76/75}. In every analysed cow, the genotype exactly agreed with its own phenotype.

Figure 4 shows the deduced amino acid sequences of bovine P4.2/76 and P4.2/75 cDNAs in alignment with human and murine red-cell P4.2. It was demonstrated that P4.2/76 and P4.2/75 each comprise 687 amino acids. Sequence similarities at the deduced amino acid level were 72.6% and 66.4% to those of human [18,19] and murine [21,22] P4.2 respectively. A

Figure 5 *In vitro* synthesis of P4.2/76 and P4.2/75

P4.2 cDNAs in plasmids pBP4.2/76 (76) and pBP4.2/75 (75) were transcribed and translated in the TnT coupled reticulocyte lysate system in the presence of [³⁵S]methionine, and the products were separated by Tris/Tricine PAGE. Lane 76 + 75 contains a mixture of the translation products of pBP4.2/76 and pBP4.2/75. Lane —plasmid is the product in the absence of exogenous plasmid. P4.2/76 and P4.2/75 are indicated by arrowheads. Bands with less intensity indicated by squares are likely to be N-terminal truncated products (see text).

myristylation site at Gly² [3] and a putative binding site to the cytoplasmic domain of band 3 [23] were well conserved. One of the Gly residues in the consensus sequence GXGXXGR for the ATP-binding sites in human P4.2 [24] and dematin [25] was replaced by Asp at the corresponding site in bovine P4.2 (Gly³¹⁵-Xaa-Asp³¹⁷-Xaa-Xaa-Gly-Arg³²¹). Despite the difference in three amino acids reported above, the theoretical molecular masses of the two isoforms were exactly the same (M_r 76615).

In vitro synthesis of P4.2/76 and P4.2/75

Bovine P4.2 was synthesized *in vitro* in a transcription- and translation-coupled reticulocyte lysate system using pBP4.2/76 and pBP4.2/75 as the templates. ³⁵S-Labelled polypeptides synthesized from pBP4.2/76 and pBP4.2/75 showed migrating positions corresponding to P4.2/76 and P4.2/75 respectively (Figure 5), indicating that the isolated clones definitely represented the major gene transcripts for P4.2/76 and P4.2/75 in bovine erythroid cells. Faint bands with $M_r \approx 10000$ less than P4.2/76 and/or P4.2/75 were also seen. These were considered to be polypeptides, translation of which was initiated at a downstream AUG codon in the same open reading frame (Met⁹³) surrounded by a possible sub-optimal consensus sequence for initiation (GCA AUG G). This result demonstrated that the difference in electrophoretic mobility between P4.2/76 and P4.2/75 was solely due to the difference in their primary structure occurring in a portion adjacent to the C-terminus.

DISCUSSION

The present study demonstrated that bovine red cells possess two molecular isoforms of P4.2 (P4.2/76 and P4.2/75) that are different in their primary structures by substitution of three amino acid residues in the C-terminal region of the polypeptide due to a polymorphism in the P4.2 gene. This polymorphism was attributable to a novel genomic variation occurring simultaneously at non-successive codons 599, 601 and 627. Supposing that the erythroid exon-intron boundary in the bovine P4.2 gene is the same as in the human or murine genome, codons 599, 601 and 627 would be localized in the same exon, exon XII [21,26]. The presence of P4.2 isoforms has been reported in human [5,6,13] and deer [11] red cells. In human red cells with a nearly complete lack of P4.2 with haemolytic anaemia, the presence of an M_r 74000 isoform was observed in small quantities [5,6]. In other cases of autosomal dominant stomatocytosis, the M_r 74000 isoform appeared to be present at the expense of the normal

M_r 72000 P4.2, whose content was remarkably reduced, and the total amount of P4.2 was normal [13]. The M_r 74000 P4.2 appears to be an alternatively spliced form with a 30-amino-acid insert following Gln³ [1,18,19,27]. Reverse transcription-PCR amplification of the 5' region with primers p11 and p10 gave only a single band ($\approx$ 460 bp) corresponding to that part of the isolated clones (results not shown), suggesting the absence of alternatively spliced transcripts in bovine bone-marrow cells. Therefore, the molecular basis that underlies the bovine P4.2 isoforms is totally distinct from that observed in human red cells with a P4.2 anomaly. The variety of P4.2 polypeptides in deer red cells in SDS/polyacrylamide gels appears to have a mechanism similar to the P4.2 polymorphism in cattle, since deer also showed the three phenotypes P4.2⁷⁸, P4.2^{78/76} and P4.2⁷⁶, with abundance in this order, and the M_r 78000 and 76000 forms were equal in amount in the P4.2^{78/76} phenotype ([11]; Y. Amano, M. Inaba and Y. Maede, unpublished work) as was the case in the bovine P4.2^{76/75} phenotype (Figure 1).

Although the predicted change of three amino acid residues due to the P4.2 gene polymorphism, Pro⁵⁹⁹, Met⁶⁰¹ and Val⁶²⁷ in P4.2/76 to Thr⁵⁹⁹, Ile⁶⁰¹ and Ile⁶²⁷, respectively, in P4.2/75, causes neither an alteration in the M_r of the entire protein molecule nor a change in charge, the two P4.2 isoforms present in red cells and synthesized *in vitro* showed a marked difference in electrophoretic mobility in SDS/PAGE and Tris/Tricine PAGE in the presence of SDS (Figures 1 and 5). This finding suggests that P4.2/76 and P4.2/75 could have different tertiary structures, possibly generating dodecylsulphate binding in a different manner or in a different amount to result in distinct migration on the gels.

One of the identified characteristics of P4.2 is to be bound to the cytoplasmic domain of band 3 [2], and band 3 appears to be the only acceptor protein for P4.2 to associate to the plasma membrane in mature red cells, as demonstrated by studies on partial or total deficiency of band 3 [7–9]. The most likely binding site in human P4.2 to band 3 was identified to be within Val³³-Arg-Arg-...-Phe⁴⁵ where an Arg³⁴-Arg motif was essential [23]. The amino acid sequence of this region was found to be well conserved in bovine red-cell P4.2, as shown in Figure 4. Despite the different primary structure and a putative structural difference in the C-terminal region of two P4.2 isoforms, there was no significant phenotypic difference in the amount of P4.2 relative to band 3 in red-cell membranes (Table 2). Moreover, red cells from healthy adult animals with distinct P4.2 phenotypes were morphologically normal (results not shown) and appeared to have a normal life span as judged by the protein 4.1a:4.1b ratio [28] of their membranes (Table 2). These findings suggest that the difference of the primary structure found in P4.2/76 and P4.2/75 is likely to be silent or negligible at least for their binding to band 3.

On the other hand, recent studies have postulated that the membrane association of P4.2 in human red cells is rather complicated, including interaction with ankyrin through binding with band 3 and other unidentified membrane components [23], and that P4.2 might be involved in the formation or function of

intracellular storage vesicles in non-erythroid cells [4,29]. Therefore, we cannot rule out the possibility that the substitution of the three amino acids in the C-terminal region has significance for some unknown properties of P4.2 in red cells or non-erythroid cells in cattle.

We thank Dr. A. Ban, Dr. J. Sakai and Dr. D. Watanabe (Yamagata Prefectural Federation of Agricultural Mutual Relief Association), and Dr. I. Koshino and Dr. N. Matsuki (University of Tokyo) for their assistance. This study was supported by Grants-in-Aid for Scientific Research from Ministry of Education, Science, and Culture of Japan (numbers 06454129, 07456140 and 09460145) and by grants from the Morinaga Hoshikai and the Itoh Memorial Foundation.

REFERENCES

- Cohen, C. M., Dotimas, E. and Korsgren, C. (1993) *Semin. Hematol.* **30**, 119–137
- Korsgren, C. and Cohen, C. M. (1986) *J. Biol. Chem.* **261**, 5536–5543
- Risinger, M. A., Dotimas, E. M. and Cohen, C. M. (1992) *J. Biol. Chem.* **267**, 5680–5685
- Risinger, M. A., Korsgren, C. and Cohen, C. M. (1996) *Exp. Cell Res.* **229**, 421–431
- Rybicki, A. C., Heath, R., Wolf, J. L., Lubin, B. and Schwartz, R. S. (1988) *J. Clin. Invest.* **81**, 893–901
- Bouhassira, E. E., Schwartz, R. S., Yawata, Y., Ata, K., Kanzaki, A., Qiu, J. J., Nagel, R. L. and Rybicki, A. C. (1992) *Blood* **79**, 1846–1854
- Jarolim, P., Palek, J., Rubin, H. L., Prchal, J. T., Korsgren, C. and Cohen, C. M. (1992) *Blood* **80**, 523–529
- Rybicki, A. C., Qiu, J. J., Musto, S., Rosen, N. L., Nagel, R. L. and Schwartz, R. S. (1993) *Blood* **81**, 2155–2165
- Inaba, M., Yawata, A., Koshino, I., Sato, K., Takeuchi, M., Takakuwa, Y., Manno, S., Yawata, Y., Kanzaki, A., Sakai, J. et al. (1996) *J. Clin. Invest.* **97**, 1804–1817
- Inaba, M. and Maede, Y. (1988) *J. Biol. Chem.* **263**, 17763–17771
- Inaba, M., Amano, Y. and Maede, Y. (1990) *Biochim. Biophys. Acta* **1021**, 101–104
- Okabe, J., Tajima, S., Yamato, O., Inaba, M., Hagiwara, S. and Maede, Y. (1996) *J. Vet. Med. Sci.* **58**, 629–634
- Inoue, F., Matsuyama, R. and Tokumine, J. (1990) *Blood* **76**, 9a (suppl.)
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Schagger, H. and Jagow, G. V. (1987) *Anal. Biochem.* **166**, 368–379
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor
- Sung, L. A., Chien, S., Chang, L. S., Lambert, K., Bliss, S. A., Bouhassira, E. E., Nagel, R. L., Schwartz, R. S. and Rybicki, A. C. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 955–959
- Korsgren, C., Lawler, J., Lambert, S., Speicher, D. and Cohen, C. M. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 613–617
- Kozak, M. (1987) *Nucleic Acids Res.* **15**, 8125–8148
- Rybicki, A. C., Schwartz, R. S., Qiu, J. J. and Gilman, J. G. (1994) *Mamm. Genome* **5**, 438–445
- Korsgren, C. and Cohen, C. M. (1994) *Genomics* **21**, 478–485
- Rybicki, A. C., Musto, S. and Schwartz, R. S. (1995) *Biochem. J.* **309**, 677–681
- Azim, A. C., Marfatia, S. M., Korsgren, C., Dotimas, E., Cohen, C. M. and Chishti, A. H. (1996) *Biochemistry* **35**, 3001–3006
- Azim, A. C., Knoll, J. H., Beggs, A. H. and Chishti, A. H. (1995) *J. Biol. Chem.* **270**, 17407–17413
- Korsgren, C. and Cohen, C. M. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4840–4844
- Sung, L. A., Chien, S., Fan, Y. S., Lin, C. C., Lambert, K., Zhu, L., Lam, J. S. and Chang, L. S. (1992) *Blood* **79**, 2763–2770
- Inaba, M., Gupta, K., Kuwabara, M., Takahashi, T., Benz, Jr., E. and Maede, Y. (1992) *Blood* **79**, 3355–3361
- White, R. A., Peters, L. L., Adkison, L. R., Korsgren, C., Cohen, C. M. and Lux, S. E. (1992) *Nature Genetics* **2**, 80–83