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Na,K-ATPase isoform expression in sheep red blood cell precursors

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Isoform expression of mammalian red cell Na,K-ATPase was analyzed using messenger RNA isolated from red cell precursor-enriched bone marrow of anemic sheep. Expression of the catalytic α subunit was analyzed using rat isoform-specific cDNA probes and expression of the β1 subunit, using a sheep β1-specific cDNA probe. RNA isolated from sheep kidney and brain were analyzed concurrently. In the red cell, as in the kidney, messenger RNA encoding only one isoform (α1) of the catalytic subunit is detected; neither of the other isoforms (α2 or α3) could be detected. This holds true for bone marrow of sheep of either the low potassium or high potassium phenotype. Relative to the expression of α1, β subunit-specific message (β1) was extremely low in the red cell compared to either kidney (< 5%) or brain (< 3%). Using a rat cDNA probe specific for a β1-like subunit, β2, message was detected in brain but not in either kidney or bone marrow.

Introduction

For over two decades, the mammalian red blood cell has been a choice cell for studies of active alkali cation transport mediated by the sodium pump. This is particularly true for studies of the different modes of ion translocation and of the side-specificity of cation effects on the enzymic reaction (for review, see Ref. 1) On the other hand, much more active tissues such as kidney and brain have been a focus of efforts to purify the enzyme and study its structure, structure-function relationships and details of the multi-step reaction sequence of the enzyme. In recent years, cDNA cloning and mRNA expression analysis have revealed the existence and structural nature of at least three isoforms of the catalytic subunit of the Na,K-ATPase. In kidney, one isoform of the catalytic subunit, α1, predominates, whereas in other tissues, most notably brain, three forms, α1, α2 and α3 have been reported from studies of both mRNA expression [2], molecular cloning [3] and immunological analysis [4,5].

In view of the paucity of sodium pump sites on the plasma membrane of mature mammalian red cells, structural characterization of the red cell pump with respect to isoform type has been problematic. Although immunoblotting of electrophoretically-separated red cell membrane proteins is consistent with the notion that the α1 subunit is the main isoform in mammalian red cells, at least in dog [6] and sheep [7], it is clear that this technique has limitations in that distinct isoforms are not always well-resolved (for review of isoforms, see Ref. 8). Therefore, the approach used in the present study has been to isolate red cell-specific mRNA and to study its expression using subunit-specific probes. To circumvent the problem that peripheral anemic blood lacks cells sufficiently immature to express Na,K-ATPase-specific message, at least in both human [9] and sheep (Dunham, P.B., personal communication), we have analyzed the expression in bone marrow enriched in red cell precursors.

Relevant to the foregoing is the question of the molecular basis for the genetic polymorphism observed for the sodium pumps of sheep which are of the HK phenotype (high intracellular K^+) or LK phenotype (low intracellular K^+; for a concise review of the HK/LK polymorphism, see Ref. 10). In the present

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study, we have addressed the question as to whether the functional diversity observed in these cells, or more specifically, their messenger RNA-containing precursors, is associated with distinctions in the isoform composition of their sodium pumps.

Methods and Materials

RNA extraction. Sheep brain and kidney were obtained from a freshly killed animal, frozen immediately on dry ice and stored at −70 °C. Bone marrow enriched in nucleated erythrocyte precursors was obtained from HK and LK sheep made anemic by the following procedure: one litre of peripheral blood was withdrawn by jugular phlebotomy on three consecutive days and then on day 5 and day 8. On day 13, the animal was anesthetized and bone marrow was aspirated from the iliac crest. The cells were concentrated by centrifugation and after removal of the plasma the cells were frozen and stored as described above. RNA of the frozen tissues was extracted with guanidinium isothiocyanate and hot phenol essentially as described by Chirgwin et al. [11] with the following modifications. Whereas the solid tissues were first thoroughly disrupted and homogenized in the guanidinium solution, the marrow was added to hot (55 °C) guanidinium followed immediately by addition of hot (55 °C) phenol to disrupt the cells. Poly $(A)^+$ RNA was selected essentially as described by Aviv and Leder [12].

Northern blot analysis. Samples of total and poly$(A)^+$ RNA were analyzed using hybridization to ^{32}P-labelled random-primed cDNA probes. Full length sheep kidney α1 and β1 cDNA probes were gifts from Dr. J. Lingrel, University of Cincinnati College of Medicine. Both were originally contained in pBR322 grown in host HB 101. Full length rat cDNA encoding α1, α2 and α3 cDNA isoforms were gifts from Dr. R. Mercer, Washington University School of Medicine; α1 and α3 were contained in the plasmid pGEM4 and were first linearized by digestion with Nae 1 and Sca 1, respectively, followed by digestion with Sma 1 in the case of α1 and with EcoR1, in the case of α3, The α2 cDNA was was recovered from Bluescript (Stratagene, San Diego, CA) by digestion with EcoR1, The full-length rat β2 probe was a gift from Dr. R. Levenson, Yale University School of Medicine. Analysis was performed on RNA denatured in formamide and subjected to electrophoresis in 1.0% agarose/6% formaldehyde gels. Following electrophoresis the RNA was blotted onto GeneScreen Plus (Du Pont) in 10 × SSC: 1.5 M NaCl and 0.15 M sodium citrate (pH 7.0). Following transfer (2–3 days), the nylon membrane was washed in 5 × SSC for 5 min, air dried and then baked at 80 °C for 2 h in a vacuum oven. Prehybridization and hybridization were carried out essentially according to manufacturers instructions. For hybridization, 25–50 ng heat-denatured cDNA probe per 10 ml solution (approx. 500 000 dpm/ml) were added directly to the prehybridization buffer and hybridization was carried out at 42 °C with constant agitation for 18–24 h. Following hybridization, the filters were washed twice for 10 min at room temperature with 2 × SSC and then twice for 0.5–1.0 h at 65 °C with 2 × SSC containing 1% SDS; for more stringent washing conditions, either 0.5 × or 0.1 × SSC containing 1% SDS were used. Unless indicated otherwise, the last wash was for 1 h at room temperature with 0.1 × SSC. Radioactivity was detected by exposure of the membranes to Kodak X-AR 5 radiographic film at −70 °C, using double Dupont Cronex Lightening Plus intensifying screens. The relative amounts of specific mRNA in Northern blots was quantified by densitometry using a Hopfer scanning densitometer connected to a strip chart recorder.

Results

α subunit expression

Following repeated phlebotomy, bone marrow aspirated from the iliac crest of sheep comprised mature as well as immature blast cells of which the majority (> 70%) were morphologically typical of red cell precursors (not shown). Northern blot analysis of RNA isolated from the marrow as well as from brain and kidney tissues was carried out using the full-length cDNA encoding the sheep kidney α1 isoform.

As shown in Fig. 1a, α1-specific mRNA is expressed in all three tissues. In the experiment shown, the intensities of the bands corresponding to 10 and 30 μg total red cell RNA (bone marrow from an LK animal) are similar to those corresponding to 1.0 and 3.0 μg poly$(A)^+$ RNA, respectively, indicating a 10-fold enrichment of specific message; the intensity corresponding to 0.14 μg kidney total RNA was similar to that of 0.8 μg brain total RNA and to 30 μg red cell total RNA. In a similar experiment carried out with a white cell-enriched fraction obtained from peripheral blood, sodium pump message was too low to be detected (not shown).

Fig. 1b shows the Northern analysis of the same samples as those analyzed in Fig. 1a, except that full-length α isoform-specific cDNA probes isolated from the rat were used. As observed with the sheep α1 probe, the α1 isoform is expressed in all tissues, In fact, as shown below (Table I), the relative intensities are generally similar to those obtained with the sheep α1-specific cDNA probe. In contrast, α2 and α3 isoforms are apparent only in brain tissue, the former as two messages of 3.4 and 5.3 kb and the latter, 3.7 kb, as reported by others in rat tissues [13,14].

Northern analysis of bone marrow obtained from an HK animal is shown in Fig. 2a. Since the relative abundance of α1-specific message was highly variable

among samples of poly(A)$^+$ RNA prepared from bone marrow aspirated from the same anemic animal, quantitative differences in expression of the same isoform among samples from LK versus HK sheep, albeit significant, are not meaningful. Thus, with the particular poly(A)$^+$ RNA sample analyzed in Fig. 2a, α1 subunit-specific message was remarkably abundant, but barely detectable in RNA isolated from marrow aspirates withdrawn at other times from the same anemic HK animal (not shown).

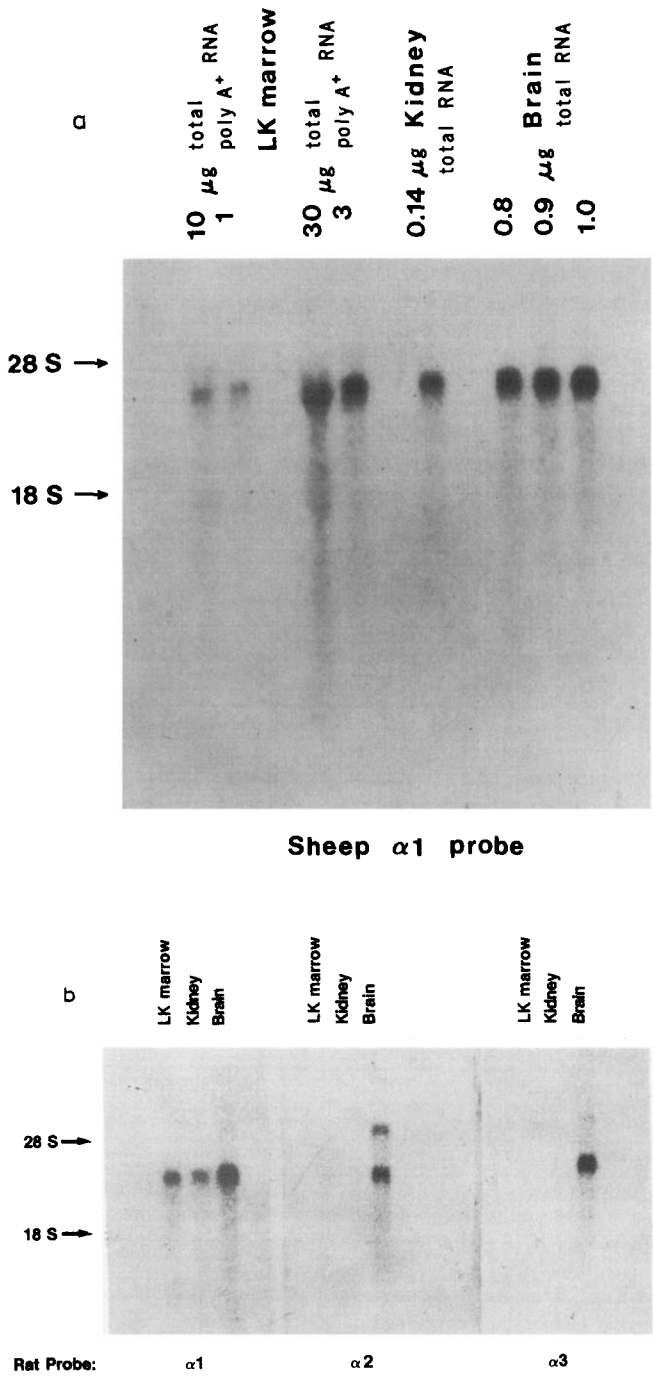

Fig. 1. Northern blot analysis of sodium pump α1 subunit expression in sheep bone marrow (LK animal), kidney and brain. Total RNA was isolated from sheep kidney, brain and bone marrow and poly(A)$^+$ RNA was selected from the total RNA of LK sheep marrow as described in Materials and Methods. After denaturation, the samples were separated in a 1% formaldehyde-agarose gel. Following electrophoresis, the RNA samples were transferred to GeneScreen nylon membrane, followed by hybridization to a random-primed ^{32}P-labelled cDNA probe. The membrane was washed twice for 5 min per wash at room temperature with 2×SSC, 1% SDS, followed by two washes at 65°C for 30 min per wash with 2×SSC and finally for 1 h at room temperature with 0.1×SSC. The blot was exposed overnight at −70°C. (a) Poly(A)$^+$ RNA (1.0 and 3.0 μg) and total RNA (10 and 30 μg) from LK bone marrow, total RNA (0.14 μg) from kidney and and total RNA (0.8, 0.9 and 1.0 μg) from brain were hybridized to ^{32}P-labelled cDNA containing the entire coding sequence of the sheep α1 catalytic subunit (specific activity, $3 \cdot 10^8$ cpm/μg trichloroacetic acid-precipitable DNA). (b) Poly(A)$^+$ RNA (3.2 μg) from LK bone marrow, total RNA (0.3 μg) from kidney and total RNA (1.25 μg) from brain were hybridized to ^{32}P-labelled rat α1, α2 and α3 isoform-specific cDNA probes. Specific activities were $5.5 \cdot 10^8$, $5 \cdot 10^8$ and $9.5 \cdot 10^8$ cpm/μg DNA, respectively.

TABLE I

Comparison of reactivity of α1- and β1-specific mRNA in sheep kidney brain and bone marrow

In each experiment, the relative areas under the peaks of the scans of the radioautographs were normalized to that of the highest amount of kidney mRNA analyzed. For samples analyzed at more than one concentration, average values are shown.

Fig. No.	Probe	Tissue	Reactivity per μg RNA	Relative ratio β1 : α1
1a	Sheep α1	kidney	1.0	
		brain	0.35	
		LK marrow	0.08	
2a	Rat α1	kidney	1.00	
		brain	0.31	
		HK marrow	0.22	
3	Sheep β1	kidney	1.00	1.00
		brain	0.55	1.57 [a]
		LK marrow	trace	
		HK marrow	< 0.01	< 0.05 [a]

[a] Ratios calculated from reactivities/μg RNA of experiments of Figs. 2a and 3.

Fig. 2b shows the comparative pattern of expression of kidney, brain and HK sheep red cell RNA probed with catalytic isoform-specific rat probes. As in the case of the LK bone marrow, with the same amounts of RNA electrophoresed concurrently and probed with isoform-specific rat probes under relatively high stringency conditions, neither the α2 nor α3 isoforms were detected in either the red cell or kidney, whereas all three are present in brain. In the particular experiment shown, α1 is clearly visible with 3 μg RNA, but barely visible with 1 μg due, presumably, to faulty application.

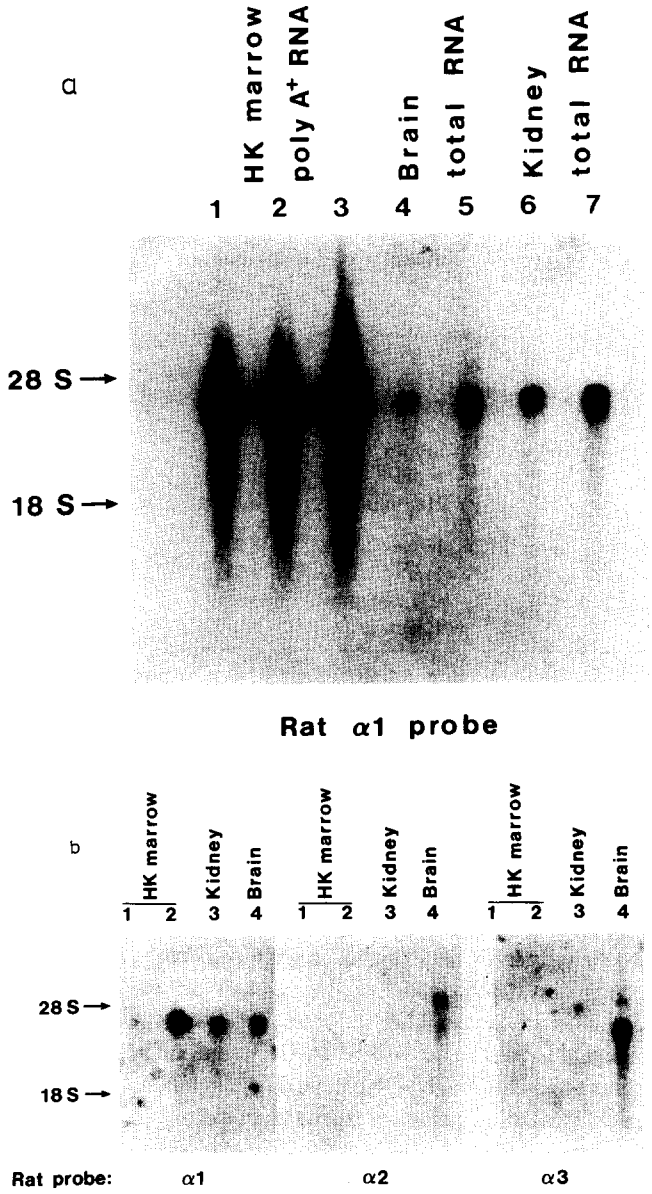

Fig. 2. Northern blot analysis of $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoform expression in sheep bone marrow (HK animal), kidney and brain. Analysis was carried out as described in Fig. 1. (a) Lanes 1, 2 and 3: represent 7.5, 15.0 and 22.0 μg HK marrow poly(A)$^+$ RNA, respectively; lanes 4 and 5: represent 0.65 and 1.3 μg brain total RNA; lanes 6 and 7: represent 0.18 and 0.35 μg kidney total RNA, respectively. Specific activity of the rat $\alpha 1$ cDNA probe, $1.4 \cdot 10^9$ cpm/μg. (b) Lanes 1 and 2: represent 1.0 and 3.0 μg HK marrow poly(A)$^+$ RNA, respectively; lane 3: 0.35 kidney total RNA and lane 4: 1.3 μg brain total RNA. Specific activities of $\alpha 1$, $\alpha 2$ and $\alpha 3$ cDNA probes were $2.1 \cdot 10^9$, $1.8 \cdot 10^9$ and $2.0 \cdot 10^9$ cpm/μg, respectively.

β subunit expression

Fig. 3 shows the results of Northern analysis of kidney, brain and red cell (HK and LK bone marrow) poly(A)$^+$ RNA using full length sheep $\beta 1$-specific cDNA. As shown previously with rat brain [14], two $\beta 1$ mRNA species, 2.7 and 2.35 kb in size, are present in sheep brain and kidney. In fact, the relative reactivity of the $\beta 1$ isoform in brain is greater than that of the α isoforms (cf. Figs. 1 and 2 and Table I below).

As shown in Fig. 3, $\beta 1$ message was weakly visible in the LK sample analyzed at relatively high concentration (10 μg in a mini-gel apparatus). Somewhat higher levels of expression were apparent in the HK sample in which $\alpha 1$ message was also more abundant (see below). It is unlikely that the difference in amounts of message in the two samples is related to the genotype since message was sparse in other marrow aspirates from the same HK animal (not shown).

The possibility of expression of a $\beta 1$-like isoform, namely one similar to that cloned recently by Martin-Vasallo et al. and referred to as $\beta 2$ [15] was also examined in bone marrow, kidney and brain as shown in Fig. 4. In this experiment using a $\beta 2$ isoform-specific rat cDNA probe, message for $\beta 2$ was detected in brain, but not in bone marrow. This holds true even though the relative amounts analyzed were such that $\alpha 1$-specific message was much greater in the bone marrow compared to the brain sample, as in the analysis of $\beta 1$ expression shown in Fig. 3. As expected, kidney was also devoid of $\beta 2$ message [15].

Relative abundance of $\alpha 1$ and $\beta 1$ messages

As mentioned above, the relative abundance of pump message varied among mRNA samples isolated from different marrow aspirates. This variability taken together with the fact that poly(A)$^+$ RNA was analyzed in the case of bone marrow and total RNA, in the cases of kidney and brain, precludes a meaningful comparison of the different tissues with respect to the amounts

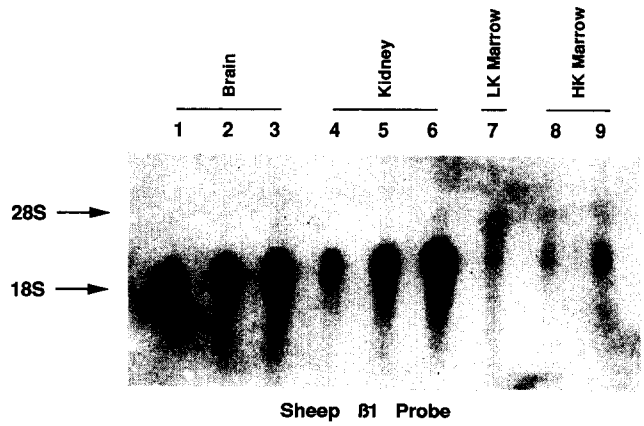

Fig. 3. Northern blot analysis of $\beta 1$ expression in LK and HK sheep bone marrow, kidney and brain. Analysis was carried out as described in Fig. 1. Lanes 1, 2 and 3: represent brain total RNA (0.13, 0.25 and 0.5 μg, respectively); lanes 4, 5 and 6: represent kidney total RNA (0.07, 0.18 and 0.35 μg, respectively); lane 7: represents LK marrow poly(A)$^+$ RNA (10.0 μg RNA); lanes 8 and 9: represents HK marrow poly(A)$^+$ RNA 2.0 and 4.0 μg, respectively). The specific activity of the sheep $\beta 1$-specific cDNA probe was $3 \cdot 10^9$ cpm/μg The nylon membrane was washed once with 2×SSC, 1% SDS for 1 h at 65°C and then with 0.1×SSC, 1% SDS for 1.5 h at 65°C and exposed for 3 days at −70°C.

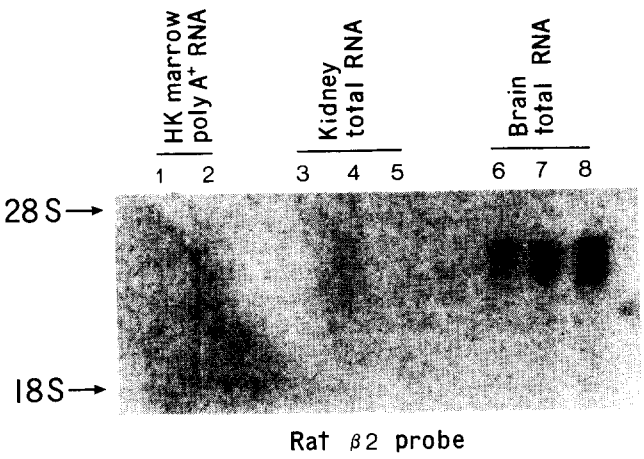

Fig. 4. Northern blot analysis of $\beta 2$ expression in HK sheep bone marrow, kidney and brain. Poly(A)$^+$ RNA from HK marrow (5.0 and 10 μg), total RNA from kidney (0.5, 1.0 and 1.5 μg) and total RNA from brain (0.75, 1.0 and 2.0 μg) were analyzed as described in Fig. 1. The specific activity of the rat $\beta 2$-specific cDNA probe was $1.1 \cdot 10^9$ cpm/μg DNA. The nylon membrane was washed twice with 2×SSC for 10 min per wash, then twice with 1×SSC, 1% SDS at 65°C for 1 h per wash and finally at room temperature for 1 h. The blot was exposed overnight at −70°C.

of either $\alpha 1$- or $\beta 1$-specific message, per se. Nevertheless, it is informative to compare the quantities of isoform-specific mRNAs analyzed concurrently on the same blot so that, for the same tissue, the abundance of $\beta 1$ relative to $\alpha 1$ can be estimated.

Table I shows the relative amounts of isoform-specific mRNAs quantified by scanning densitometry of autoradiographs as described in Methods and Materials. This was carried out for the experiments shown in Figs. 1a, 2a and 3. For each autoradiograph, the amounts shown were expressed per μg RNA and normalized to the amount in kidney. As shown, the $\beta 1 : \alpha 1$ ratio in the HK red cell precursors is $< 5\%$ that of kidney and $< 3\%$ that of brain. Although the weakly reactive bands obtained for the 10-μg LK sample as well the 2-μ HK sample shown in Fig. 3 could not be quantified, it is apparent from inspection of Fig. 3 that their reactivities with the $\beta 1$ probe are similar. Since the reactivity of the same LK sample with the $\alpha 1$ probe is about one-third that of the HK sample (Table I), it appears that the $\beta 1 : \alpha 1$ ratio in the LK sample is $< 2\%$ that of kidney.

Discussion

The question of the structural identity of the sodium pump of the mammalian red blood cell in comparison to more active tissues such as kidney and brain was addressed in the present study using Northern blot analysis. Because of the lack of Na,K-ATPase-specific mRNA not only in mature red cells but also in their precursor reticulocytes in both humans [9] and sheep (Dunham, P.B., personal communication), it was necessary to isolate mRNA from bone marrow of anemic sheep. In these aspirates, the majority ($> 70\%$) of the nucleated cells are red cell precursors. Moreover, it is unlikely that contaminating white cells contribute significantly to the sodium pump-specific mRNA since message in white cell-enriched fractions from peripheral blood was too low to be detected.

From the results of this analysis, it is clear that the catalytic subunit of the Na,K-ATPase expressed in the red cell resembles only that of the kidney. Consequently, any differences in kinetic behavior of the enzyme in these two tissues, for examples differences in the relative activities of the various modes of pump operation or in the Na^+-dependence of ouabain binding [1], cannot be ascribed to differences in structure of the catalytic subunit. Although rat-specific cDNA probes were used, reactivity with the three tissues, erythroid bone marrow, kidney and brain, was similar to that observed with the sheep-specific probe, at least in the case of the $\alpha 1$ cDNA probe. The failure to detect either $\alpha 2$ or $\alpha 3$ message is consistent with the failure also to detect 'α^+' ($\alpha 2$ and/or $\alpha 3$) protein in immunoblots of reticulocyte membranes from sheep [7] and dog [6].

The finding that $\beta 1$ mRNA is barely detectable is consistent also with our failure to detect a β subunit in immunoblots [7]. From studies of expression in rat tissues, it has been found that in most tissues in which $\beta 1$ is absent, expression of a $\beta 1$-like mRNA ($\beta 2$) is observed [15], a notable exception being adult (rat) liver [16]. It now appears that the red cell is another exception. Whether another β-like glycoprotein subunit is present in these cells remains to be determined. Indeed, disparity between mRNA expression and tissue distribution revealed by monoclonal antibodies have led to the prediction of at least three β- as well as α-subunit genes [17].

It may also be concluded from the present study that the genetic dimorphism apparent in sheep whereby the reticulocytes of the one type (HK) mature into red cells with a normal high K^+ content, those of the other (LK), into cells with a low K^+ content, is not likely to be due to differences in isoform expression of the catalytic subunit. Although we cannot rule out the possibility that this genetic distinction is due to an undetected α isoform such that $\alpha 1$ is inactivated selectively during maturation of LK cells resulting in mature HK and LK cells with different isoforms, immunoblot analysis of the membrane protein has failed to detect any difference in their reactivity [18]. It is plausible also that this genetic distinction is the result of a heterofore uncharacterized β-like subunit, distinct from either $\beta 1$ or $\beta 2$, or even the putative γ subunit first described by Forbush and co-workers [19].

We are currently in favor of the possibility that the molecular basis for this polymorphism is the presence of membrane antigens, in particular the L antigen of LK red cells [10]. In fact, it is known that this membrane

antigen appears to have an inhibitory effect on the pump of LK red cells. Thus, antibodies raised against the L antigen release the inhibitory action of the L antigen such that after treatment of LK cells with anti-L, their pumps resemble, to a large extent, the pumps of HK red cells [20].

Acknowledgements

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