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Molecular evidence for increased hematopoietic proliferation in the spleen of the b/b laboratory rat

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Abstract. The splenomegaly and the appearance of a significant number of CFU-E (erythroid colony-forming units) and BFU-E[1] (erythroid burst-forming units) in the Belgrade laboratory rat (b/b) spleen prompted us to analyse further the molecular evidence for increased hematopoietic proliferation in the b/b spleen. Messenger RNAs (mRNAs) specific for globins, proteins for iron transport and deposition and the band 3 protein were used in rat erythropoietic tissues as markers for proliferation and erythroid differentiation. In the b/b spleen, all mRNAs analysed display an erythroid-specific pattern of expression. This analysis also revealed an enhanced level of mRNA for ferritin in the +/b spleen, whereas erythrocyte-specific mRNA production was normal.
Key words. b/b Rat; +/b rat; α-globin; β-globin; band 3 protein; transferrin receptor; ferritin.

The Belgrade laboratory (b/b) rat has an autosomal mutation which in the homozygous state induces severe anemia[2,3]. It is characterized morphologically by red blood cell (RBC) hypochromia, microcytosis and reticulocytosis[3]. Although serum iron is elevated[4], iron uptake into b/b rat reticulocytes is only 20% that of normal[5,6]. The Belgrade laboratory rat reticulocytes and bone marrow have a lower amount of globin polypeptides and half the normal globin mRNA content[7–9].

Thalassemic patients[10] and rats[11] and mice[12] made anemic by bleeding or phenylhydrazine treatment develop splenomegaly with intensive extramedullar erythropoiesis. Consistent splenomegaly is a characteristic of the b/b rat, and Sladić-Simić et al. reported spleen sections with extramedullar erythropoiesis[4]. Hematological analysis of b/b spleen demonstrated a significant number of CFU-E and BFU-E, although the usual hematological method (May-Grünwald Gimsa staining) does not detect late erythroid progenitors[1].

The three basic characteristics of the b/b rat, i.e. incomplete differentiation (low BFU-E and CFU-E)[1], low hemoglobinization (low amounts of globin chains and globin mRNAs)[7–9], and a low iron level[6], prompted us to analyse these features at the molecular level. Therefore, we screened b/b spleen total RNA for the presence of globin mRNAs, iron transport and deposition proteins mRNAs, and mRNAs for proteins related to erythroid cell function but not involved in hemoglobin synthesis. We selected mRNAs for α- and β-globins, the transferrin receptor and ferritin, and band 3 protein, as markers for proliferation and erythroid differentiation

Materials and methods

Animals. Laboratory rats of the Wistar strain (3 months old) were used in all experiments. Belgrade laboratory rats (b/b) were obtained from the original Belgrade colony[4] by crosses between heterozygote females (+/b) and anemic males (b/b) or between two heterozygote animals. The animals were fed a standard laboratory rat diet.

Reticulocytosis was induced in normal (+/+) rats by the removal of 2% of body weight in blood by heart puncture. Animals were killed 3 days later, when reticulocyte counts varied from 30 to 35%.

Reticulocytosis was also induced in (+/+) rats by three daily injections of 1.25% (v/v) phenylhydrazine (PHZ) in isotonic saline (150 mM NaCl) (50 mg/1 g of body weight). Rats were bled by heart puncture on the fifth day, when reticulocyte counts varied from 40 to 55%.

Preparation of total cytoplasmic RNA. RNA was isolated from reticulocytes, spleen and bone marrow in a cesium chloride density gradient[13]. We used a method modified by Chirgwin[14]. The RNA pellet was extracted with guanidine hydrochloride[15] and ethanol precipitated. In order to analyse the intactness and quantity of isolated RNA, 0.8 mg of each RNA sample was electrophoresed on 1.2% agarose gel and 18S and 28S RNA visualized by staining with ethidium bromide[16].

Dot blot hybridization. Serial two-fold dilutions of total RNA in 10 × SSC were applied to Gene Screen sheets according to the method of Cheley[17]. The blots were hybridized for 20 h at 65 °C with 10 cpm of probe per ml of hybridization buffer, washed at 65 °C by standard procedures[18] and autoradiographed. All RNA samples

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Table 1. Spleen weights in +/+, +/b and b/b 3-month-old rats.

Genotype	Spleen weight (g)
+/+	0.40 ± 0.04 (5)*
+/b	0.46 ± 0.11 (5)
b/b	**1.32 ± 0.43 (9)

*Number of rats given in parentheses. Values are mean ± SD for each group of rats. **Significance of the difference from +/+ or +/b, $p < 0.01$.

were applied to the same blot and hybridized with an adequate amount of probe. The levels of specific mRNA (e.g. ferritin) were obtained under identical conditions and therefore comparable. The chicken β-actin cDNA[19] was introduced as in internal control; this probe was used to quantitate mRNAs and was in good agreement with agarose gel electrophoresis of ribosomal RNAs (data not shown). Furthermore, in order to control reproducibility of RNA isolation, dilution, probe labelling and hybridization, each experiment was repeated three times. Manipulations with plasmid DNA were performed following the standard procedures[16]. Double-stranded probes were radioactively labelled using an oligolabelling kit (Pharmacia)[20], while single-stranded probe was 5′ end-labelled using T4 polynucleotide kinase[16].

Probes. Rat β-globin probe was a 350 bp PstI fragment from pBRrgX[21] containing the rat β-globin third exon. Rat α-globin probe was a 30mer oligonucleotide, 5′-CTTGGAGGTAAGCACGGTGCTCACGGAGGC-3′, corresponding to the α-globin cDNA sequence from 130 to 140 aa from plasmid pBRrg 5[21]. Rat ferritin probe was a 30mer oligonucleotide, 5′-ACACCTCATTGCATTCAGCCCGCTCTCCCA-3′, corresponding to the sequence of the heavy chain (H) of rat ferritin from 705 to 715 aa. Band 3 protein probe was a 30mer oligonucleotide, 5′-GGGGTGTCGGAGCTGCTCATCTCCACAGACA-3′, corresponding to the mouse band 3 cDNA sequence from 455 to 464 aa. The probe for transferrin receptor was 3.7 kb SalI-HindIII fragment from pTR10 plasmid. The probe prepared from pA1 was a 2000 bp PstI fragment representing chicken β-actin cDNA[19].

Results

One of the characteristics of the anemic b/b rat is an enlarged spleen (table 1). This indicates an active extramedullar erythropoiesis[11,12]. Therefore, we compared expression of marker genes in erythropoietic tissues (bone marrow and spleen) from control, +/+ and +/b rats, and in the mutant b/b rat.

The heterozygous (+/b) rat carries only one allele of the mutated 'b' gene. It is phenotypically indistinguishable from the control rat except for some minor characteristics[22]. We were interested to test the influence of 'b' mutation (single allele) on the expression of an erythroid-specific set of genes. Rat brain total RNA served as a negative control. Rat +/+ bone marrow total served as the positive control, thus establishing a pattern of expression of erythroid-specific mRNAs in normal rat erythropoietic tissue.

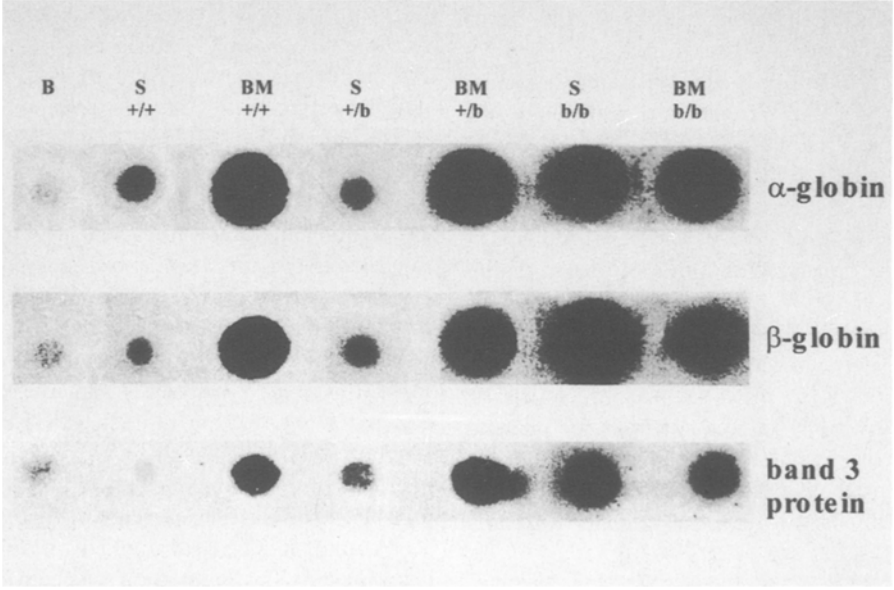

Figure 1. The amounts of α-globin, β-globin and band 3 protein mRNAs per μg of total RNA in spleen (S) and bone marrow (BM) of normal (+/+), heterozygous (+/b) and Belgrade laboratory (b/b) rats. The control was mRNA from rat brain (B). Dot-blot analyses were performed as described in the text, and filters were hybridized to ^{32}P-labelled probes as designated in the text. Two micrograms of total RNA was applied in each dot-blot hybridization.

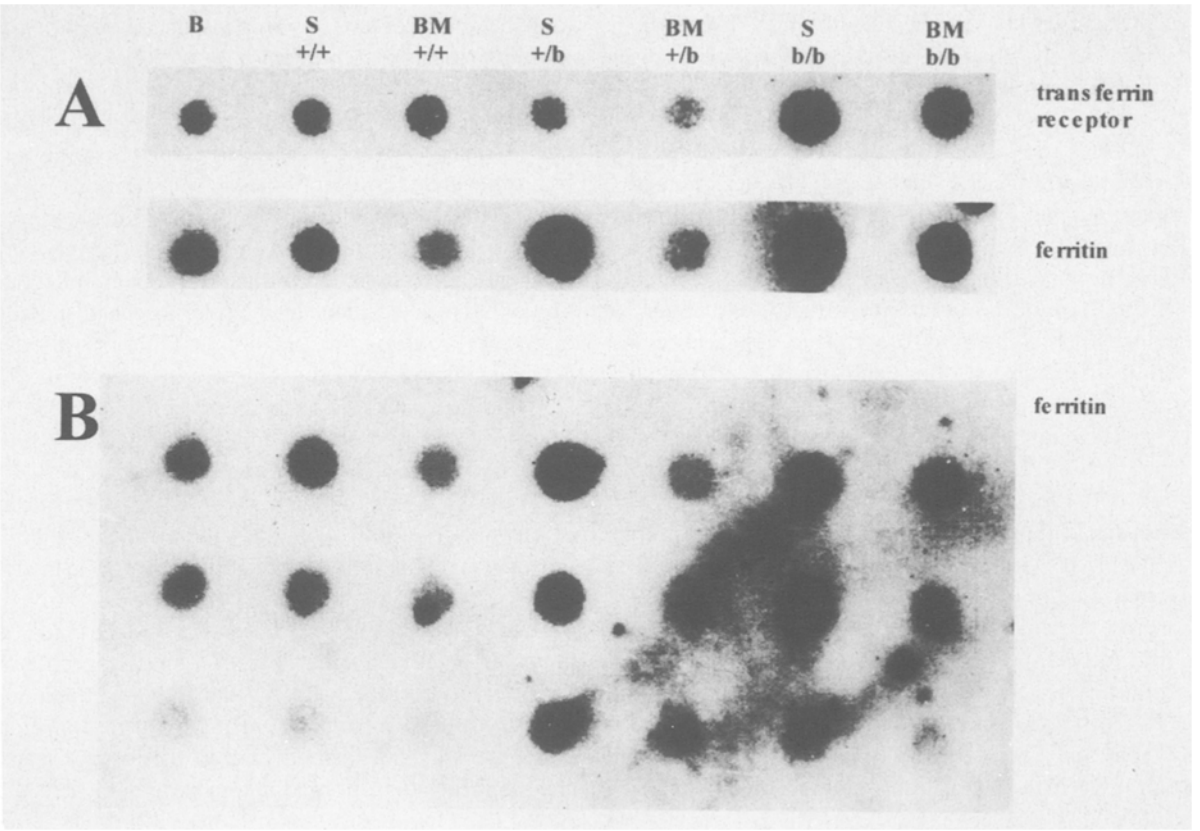

Figure 2. (*A*) The amounts of transferrin receptor and ferritin mRNAs per µg of total RNA in spleen (S) and bone marrow (BM) of normal (+/+), heterozygous (+/b) and Belgrade laboratory (b/b) rats. The control was mRNA from rat brain (B). Dot-blot analyses were performed as described in the text, and filters were hybridized to ^{32}P-labelled probes as designated in the text. Two micrograms of total RNA was applied in each dot-blot hybridization. (*B*) Serial two-fold dilutions of total mRNAs hybridized to ^{32}P-labelled probe for ferritin, i.e. 2 µg, 1 µg, 0.5 µg.

Comparison of α-globin, β-globin and band 3 protein mRNAs. The α-globin, β-globin and band 3 protein mRNAs show similar patterns of expression (fig. 1). The level of all three mRNAs expressed in the b/b spleen is similar to that observed in +/+, +/b and b/b rat bone marrows. Expression of erythroid genes in +/+ and +/b spleen is similar to the nonerythropoietic control (rat brain total RNA). These data prove the expression of erythropoietically active genes in b/b and its absence in +/+ and +/b spleens.

Comparison of transferrin receptor and ferritin mRNAs. Transferrin receptor mRNA is expressed in all tissues investigated, but in the b/b spleen this RNA is significantly increased (fig. 2A). Ferritin mRNA is also present in all tissues analysed (fig. 2A). When spleen ferritin mRNAs are compared, a higher level is detected in both +/b and b/b spleen. Although levels of all markers are normal in heterozygous spleen, and the spleen itself looks normal, ferritin mRNA expression is identical to that of the b/b spleen. The quantitative data are presented in figure 2B.

Discussion

Taking into account previous reports of splenomegaly[4] and the presence of significant numbers of CFU-E and BFU-E[1] in b/b spleen, we decided to analyse erythroid activity of b/b spleen further. We screened total spleen RNA for the presence of α- and β-globin and mRNAs, the transferrin receptor and ferritin mRNAs, and band 3 protein mRNA, as markers for proliferation and erythroid differentiation.

Globin mRNAs are intensively expressed in b/b spleen. When the normal rat and mouse are made anemic by PHZ (phenylhydrazine) treatment, the spleen cells of these animals follow an orderly sequence of cellular and biochemical events[11,12], during which hemoglobin synthesis reaches 85% of total protein synthesized by mouse spleen cells, along with an accumulation of globin mRNAs[23,24]. The b/b spleen behaves in a similar way which is reflected in an increased globin mRNA level.

The expression of band 3 protein is mainly limited to erythrocytes[25]. At the early normoblast stage, the syn-

thesis of the anion transporter is initiated and the protein is inserted into the membrane[25]. Therefore, a significant level of band 3 protein mRNA in b/b spleen clearly demonstrates the normoblast transcription pattern.

It is obvious that some cells in the b/b spleen tend to differentiate toward the final stage. The detection of a considerable number of CFU-E and BFU-E in b/b spleen[1] proves that erythropoiesis starts in the b/b spleen. During further development toward normoblast stage the 'b' mutation interferes with the expression of proteins which contribute to erythroid appearance and function in the spleen. Since the required proteins are not synthesized in erythroid cells of the b/b spleens, these cells cannot be detected either by May-Grünwald-Giemsa or by immunological staining. We demonstrated that genes important for hemoglobin synthesis and erythroid morphology were transcriptionally active in b/b spleen. However, this activity cannot produce mature hemoglobin, enucleation and the formation of a biconcave shape.

Ferritin mRNA is present in all tissues analysed, but it is significantly increased in +/b and b/b spleens. While the +/b rat is considered phenotypically indistinguishable from the +/+ rat[3], the enhanced transcription of ferritin mRNA in +/b spleen points to the influence of the mutated 'b' allele.

It is well established that an increased iron concentration activates the translation of ferritin mRNA[26,27], apparently with little[28] or no[29,30] change in the level of ferritin mRNA. However, in mouse L and hamster CHO cell lines[31], as well as in the human K562 cell line[28], iron-dependent changes in ferritin mRNA levels evidently contribute to the regulation of ferritin synthetic rates. In the case of +/b and b/b spleens, the levels of ferritin mRNA are increased, suggesting regulation at the transcriptional level, although not excluding further possible regulation during translation.

For transcriptional upregulation of ferritin mRNAs in both mutant spleens, a prior increase of iron should be observed in the microenvironment. The source of the iron in the b/b spleen should come from sequestered damaged erythrocytes. The same can be said for the heterozygous rat, since a significant reticulocytosis is found in young animals[22]. Therefore, intensive ferritin mRNA transcription in these spleens may be necessary for the surplus iron storage.

Another possible reason for an increased ferritin mRNA level is that the 'b' mutation affects an as yet unknown protein involved in coordinated regulation of iron transport and storage[32].

Ferritin mRNA accumulation in +/b spleen is an additional feature (significant reticulocytosis in young +/b rat[22] is the first one) that distinguishes it from the +/+ rat. This is another correlative factor of the 'b' mutation and impaired iron pathway[33]. Since the b/b rat's severe hypoxia overshadows the primary defect[34], the +/b rat may be a more suitable animal for investigating the molecular basis of b/b anemia.

The transferrin receptor mRNA is extremely abundant in b/b spleen. The level of transferrin receptor mRNA has been shown to be modulated post-transcriptionally by iron, and transcriptionally by variations in cell growth rate and/or differentiation[35–39]. Furthermore, it has been demonstrated that mRNA for the transferrin receptor is directly induced during erythroid differentiation of MEL cells[40]. Due to the splenomegaly (including extensive proliferation and differentiation), we would expect an increased level of transferrin receptor mRNA. Our finding confirms our assumption.

The increased transcripton in the b/b and +/b spleen raises two additional questions:

1) Is the high level of ferritin mRNA in +/b spleen a direct manifestation of the 'b' mutation, or the consequence of transcriptional activation by the iron overload?

2) If it is true that the b/b spleen is iron-overloaded (as indicated by the ferritin mRNA), that it proliferates (transferrin receptor mRNA) and that it differentiates into erythrocytes (band 3 protein, α- and β-globin mRNAs), why does this overall positive trend lead to anemic rather than normal spleen development?

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1 Pavlović-Kentera, V., Basara, N., Biljanović-Paunović, L., Vasiljevska, M., and Rolović, Z., Exp. Hematol. *17* (1989) 812.
2 Sladić-Simić, Dj., Pavić, D., Živković, N., Marinković, D., and Martinović, P. N., Br. J. Radiol. *36* (1963) 542.
3 Sladić-Simić, Dj., Živković, N., Pavić, D., Marinković, D., Martinović, J., and Martinovic, P. N., Genetics *53* (1966) 1079.
4 Sladić-Simić, Dj., Martinović, P. N., Živković, N., Pavić, D., Martinović, J., Kahn, M., and Ranney, H. M., Ann. N. Y. Acad. Sci. *165* (1969) 93.
5 Edwards, J. A., Garrick, L. M., and Hoke, J. E., Blood *51* (1978) 347.
6 Garrick, M. D., Gniecko, K,. Liu, Y., Cohan, D. S., and Garrick, L. M., J. biol. Chem. *268* (1993) 14867.
7 Chu, M. L., Garrick, L. M., and Garrick, M. D., Biochemistry *17* (1978) 5128.
8 Crkvenjakov, R., Maksimović, V., and Glišin, V., Biochem. biophys. Res. Commun. *105* (1982) 1524.
9 Marjanović, J., Savković, S., Nikčević, G., Ivanović, Z., Milenković, P., and Popović, Z., Biochem. biophys. Res. Commun. *201* (1994) 115.
10 Erslev, A. J., in: Hematology, p. 695. Ed. W. J. Williams, E. Bentler, A. J., Erslev and M. A. Lichtman. McGraw-Hill Publishing Co., New York, NY 1990.
11 Azen, E. A., and Schilling, R. F., J. Lab. clin. Med. *63* (1964) 122.
12 Cheng, T. C., and Kazazian, H. H., Jr., Proc. natl Acad. Sci. USA *73* (1976) 1811.
13 Glišin, V., Crkvenjakov, R., and Byus, C., Biochemistry *13* (1974) 2633.

14 Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J., Biochemistry *18* (1979) 5294.
15 Muller, R., Slamon, D. J., Adamson, E. D., Tremblay, J. M., Muller, D., Cline, M. J., and Verma, I. M., Molec. cell. Biol. *4* (1983) 1062.
16 Sambrook, J., Fritsch, E. F., and Maniatis, T., in: Molecular cloning: a laboratory manual. Ed. C. Nolan. Cold Spring Harbor Laboratory Press 1989.
17 Cheley, S., and Anderson, R., Anal. Biochem. *137* (1984) 15.
18 Church, G. M., and Gilbert, W., Proc. natl Acad. Sci. USA *81* (1984) 1991.
19 Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J., and Kirchner, N. W., Cell *20* (1980) 95.
20 Feinberg, A. P., and Volelstain, B., Anal. Biochem. *123* (1983) 6.
21 Crkvenjakov, R., Bućan M., Konstantinović, M., Fogel, M., Savić, A., and Glišin, V., Hemoglobin *8* (1984) 597.
22 Popović, Z. B., Rajić, N. V., Savković, S. D., and Glišin, V. R., Exp. Hematol. *21* (1993) 21.
23 Kazazian, H. H., Jr., Cheng, T.-C., Polmar, S. K., and Ginder, G. D., Ann. N. Y. Acad. Sci. *241* (1974) 170.
24 Mazur, A., J. clin. Invest. *47* (1968) 2230.
25 Palek J., and Lambert, S., Semin. Hematol. *27* (1990) 290.
26 Zahringer, J., Baliga, B. S., and Munro, H. N., Proc. natl Acad. Sci. USA *73* (1976) 857.
27 Rogers, J., and Munro, H., Proc. natl Acad. Sci. USA *84* (1987) 2277.
28 Mattia, E., den Blaauwen, J., Ashwell, G., and van Renswoude, J., Proc. natl Acad. Sci. USA *86* (1989) 1801.
29 Rouault, T. A., Hentze, M. W., Dancis, A., Caughman, S. W., Harford, J. B., and Klausner, R. D., Proc. natl Acad. Sci. USA *84* (1987) 6335.
30 Hentze, M. W., Rouault, T. A., Caughman, S. W., Dancis, A., Harford, J. B., and Klausner, R. D., Proc. natl Acad. Sci. USA *84* (1987) 6730.
31 Coulson, R. M. R., and Cleveland, D. W., Proc. natl Acad. Sci. USA *90* (1993) 7613.
32 Theil, E. C., J. biol. Chem. *265* (1990) 4771.
33 Edwards, J., Huebers, H., Kunzler, C., and Finch, C., Blood *67* (1986) 623.
34 Rolović, Z., Basara, N., Stojanović, N., Suvajdžić, N., and Pavlović-Kentera, V., Blood *77* (1991) 456.
35 Kuhn, L., Shulman, H. M., and Ponka, P., in: Iron Transport and Storage, p. 149. Ed. P. Ponka, H. M. Schulman and R. C. Woodworth. CRC Press, Boca Raton 1990.
36 Neckers, L. M., Pathobiology *59* (1991) 11.
37 Miskimins, W. K., in: Iron Transport and Storage, p. 119. Ed. P. Ponka, H. M. Schulman and R. C. Woodworth. CRC Press, Boca Raton, 1990.
38 Chan, L. N., and Gerhardt, E. M., J. biol. Chem. *267* (1992) 8254.
39 Murray, M. T., White, K., and Munro, H. N., Proc. natl Acad. Sci. USA *84* (1987) 7438.
40 Chan, R. Y. Y., Seiser, C., Schulman, H. M., Kuhn, L. C., and Ponka, P., Eur. J. Biochem. *220* (1994) 683.