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Expression of the genes for the ferritin H and L subunits in rat liver and heart

Evidence for tissue-specific regulations at pre- and post-translational levels

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The proportion of ferritin light-chain and heavy-chain subunits (L and H) present in the ferritin multimeric shell varies between different tissues. To identify the regulatory mechanisms responsible for the greater amount of L in liver than in heart isoferritins, we analysed ferritin-gene expression at the RNA and protein levels in these two tissues of the rat. In the heart the ratio between the amount of L and H, at the level both of synthesis and accumulation, is about 1 and is the same as the ratio between their respective mRNAs. In contrast, in the liver, the ratio between the L- and H-mRNAs is approx. 2 and cannot entirely explain the large predominance of L in isoferritins in this tissue. Since in the liver the L-mRNA is neither preferentially associated with polyribosomes nor translated more efficiently than its H- counterpart, it seems that the liver-specific isoferritin profile is determined by a combination of pre- and post-translational mechanisms, whereas in heart the post-translational regulation does not seem to be relevant and the tissue-specific pattern is determined at the level of mRNA accumulation.

INTRODUCTION

Ferritin is an iron-containing protein which plays a central role in iron storage and detoxification [1]. Ferritin molecules consist of a spherical shell composed of 24 subunits of two types, heavy (H) and light (L); the assembly into different proportion of the H- and L-chains originates a variety of isoferritins [2]. Each tissue possesses a characteristic H and L content and isoferritin profile, which is modified in iron overload and other pathophysiological conditions. L-subunit-rich isoferritins (LRI) predominate in tissues with high levels of stored iron, such as liver and spleen, whereas H-subunit-rich isoferritins (HRI) are found in organs with no evident iron-storage function, such as heart and pancreas [3]. The various ferritin phenotypes may have different functions which, although not yet clarified, could be related to the different abilities of H and L in the handling of iron [4]. Acute (see [5] for review) and chronic [6] changes in the intracellular levels of iron control ferritin-gene expression, and extensive studies have been performed to analyse the molecular mechanisms of this iron-dependent regulation. Controls at the transcriptional [7–9] post-transcriptional [10,11] and translational [12–15] levels have been reported, the last being quantitatively more relevant and affecting H and L transcripts in the same way [7,12]. The iron-independent mechanisms that regulate the changes in ferritin-gene expression have not been fully investigated, although Northern-blot experiments demonstrated that expression of H and L genes is regulated by pre-translational processes during development [16], differentiation [17], neoplastic transformation [18,19] and stimulation with hormones [20] or cytokines [21]. Little is known of the molecular events that cause the differences in isoferritin profiles and concentrations in different cell types, i.e. in tissue-specific expression of H and L. In the present work we compared ferritin biosynthesis in rat liver and heart, two tissues which have distinct isoferritin profiles and very different proportions of L and H and which have been chosen as models to study at which level the tissue-specific isoferritin pattern is determined.

EXPERIMENTAL

Animals

Male albino rats (Wistar strain) weighing approx. 200 g and fed on a balanced diet *ad libitum* were used.

Ferritin purification and subunit composition

Ferritin was purified from rat liver and heart as described in [2]. Briefly, ferritin was precipitated with 80%-satd. $(NH_4)_2SO_4$ from homogenates of fresh tissues heated at 75 °C and clarified by centrifugation; the precipitate was resuspended and loaded on a Sepharose 6B column. Electrophoretic analysis showed the ferritin to be pure. Subunit composition of ferritin purified from the liver and the heart of three different rats was determined by densitometric scanning of Coomassie Blue-stained bands after separation on 15%-(w/v)-acrylamide/SDS gels [22].

Protein synthesis by tissue slices

Liver and heart slices were prepared and incubated in the presence of 100 μCi of [^{35}S]methionine as described in [23]. At the end of the incubation the slices were homogenized, and equal amounts of radioactivity were taken for ferritin immunoprecipitation.

Immunoprecipitation and electrophoretic analysis of ferritin

Immunoprecipitation of ferritin labelled *in vitro* was performed as described in [23], using anti-(rat liver ferritin) polyclonal antibodies. Immunoprecipitation products were run on 15%-polyacrylamide/SDS gels [22], processed for fluorography [24] and exposed to Amersham Hyperfilm MP. The relative incorporation of radioactivity into each subunit was determined by densitometric scanning of appropriately exposed films.

RNA extraction

RNA was extracted from purified liver polyribosomes prepared by the procedure of Yap *et al.* [25], using the phenol/

Abbreviations used: H, ferritin heavy chain; L, ferritin light chain; HRI, H-subunit-rich isoferritins; LRI, L-subunit-rich isoferritins.

chloroform method as previously described [23]. Total cellular RNA was prepared using the procedure of Chomczynski & Sacchi [26]; the liver was homogenized in a glass Dounce homogenizer, and the heart was homogenized in a Polytron homogenizer.

Northern-blot analysis

Total and polyribosomal RNA were electrophoresed under denaturing conditions, blotted to Hybond C extra (Amersham) nitrocellulose filters and hybridized to ^{32}P-labelled probes as described previously [7]. The DNA probes were labelled by nick-translation using a commercially available kit (Amersham). For the calculation of the absolute amount of ferritin mRNAs, we used a previously described procedure [27] with minor modifications. The H1110 [28] and pRLF3 [29] plasmids containing the cDNAs of H and L were digested with *Rsa*I–*Fnu*DII and *Pst*I respectively to excise the inserts. These fragments encompass the complete coding regions of ferritin chains as well as several nucleotides of untranslated flanking sequences. Various amounts of the gel-purified H- or L-DNA fragments (3–12 ng; adjusted to 30 μg with yeast tRNA) were run in other lanes of the same gels which contained the different RNA samples, blotted and hybridized to the appropriate H- or L-cDNA probe. These DNA restriction fragments had approximately the same size as the ferritin mRNAs and served as hybridization intensity standards to construct a calibration curve after densitometric quantification of the bands. The densitometric values were used to estimate the amount of ferritin subunit mRNAs/μg of total RNA. Autoradiograms were scanned with an LKB laser densitometer, making sure that the exposure of the film was in the linear range. The values were corrected for the amount of rRNA loaded in each lane as measured by hybridization of rRNA with the pXCR7 probe on the same filter.

Probes

The cDNA clones for the rat L and H generously provided by Professor. H. Munro (Department of Applied Biological Sciences, MIT, Cambridge, MA, U.S.A.); the pXCR7 probe containing a *Xenopus laevis* rDNA unit was kindly provided by Dr. I. Bozzoni (Centre for the Study of Nucleic Acids, Rome, Italy).

RESULTS

Ferritins purified from rat liver and heart were analysed by SDS/PAGE, and a typical result is shown in Fig. 1(*a*). In agreement with previous data [2,30–32] we found that purified liver ferritin was predominantly composed of L, whereas heart ferritin contained similar proportions of H and L. Densitometric scanning of the bands obtained after electrophoretic separation of three different preparations for both tissues consistently gave an L/H ratio of about 1:1 for heart ferritin and of about 4:1 for liver ferritin (Table 1). In order to study the molecular mechanisms responsible for this difference in ferritin-subunit composition, we developed a quantitative Northern-blot assay to measure the absolute amounts of L- and H-mRNA in the two tissues. H- or L-DNA fragments were present as hybridization standards on the same filters that contained the RNA samples. Standard curves of the hybridization intensities were generated to estimate the absolute amount of ferritin mRNAs after hybridization with the appropriate H- or L-DNA probe. We found a good linearity of hybridization over the range 1.5–15 ng for the standards and over the range of 10–40 μg of total RNA (results not shown). Replicate experiments with liver RNA preparation allowed one to calculate an L- and H-mRNA content of about 200 and 100 pg/μg of total RNA respectively, with an error below 10%. Further experiments on different animals and tissues were performed using the 3–12 ng range of hybridization standards and 30 μg of total RNA. A representative autoradiogram is shown in Fig. 2; densitometric quantification of three independent sets of experiments is reported in Table 1. Both H- and L-mRNAs were more abundant in the liver than in the heart. In the heart the ratio between the L- and the H-mRNA was 1:1, analogous to that of the accumulated protein (Fig. 1*a*, lane 2, and Table 1); in contrast, in the liver, L and H-mRNA showed a 2:1 ratio, which is half of that predicted by the subunit composition of the purified protein (Fig. 1*a*, lane 1, and Table 1). By using a different procedure, White & Munro [8] found in rat liver an L/H-mRNA ratio (2.4 ± 0.3, as deduced from their graph) very close to the value reported here. Since intracellular distribution of mRNAs plays an important role in the regulation of hepatic ferritin synthesis [12,15], a possible explanation for this phenomenon could be a differential engagement of the two hepatic mRNAs into inactive mRNPs versus actively synthesizing polyribosomes. However, this possibility does not seem to hold in this case, because we found a ratio between L- and H-mRNAs in polyribosomal RNA of 2:1, analogous to that found in total cellular RNA (Fig. 2 and Table 1). In fact about 15% of both mRNAs was found engaged in polyribosomes, confirming previous data that showed high levels of ferritin mRNAs stored as mRNPs [12,15]. These findings suggested that translational controls other than initiation or post-translational events were involved in the preferential accumulation of LRI in rat liver. We studied the rate of synthesis of H and L in tissue slices pulse-labelled *in vitro*, since this system has been demonstrated to be fully representative of the situation *in vivo* [33]. Ferritin synthesized by tissue slices during 1 h of incubation in the presence of radioactive methionine was immunoprecipitated and analysed by SDS/PAGE, and the fluorogram from one representative gel is shown in Fig. 1(*b*). Densitometric quantification of ferritin bands immunoprecipitated from replicate experiments of labelling using tissue slices from different rats gave an L/H ratio of about 1:2 in heart and of about 1:1 in liver. Taking into account the fact that the rat H has twice as many methionine residues as the L [29], the L/H ratios can be calculated as about 1:1 in heart and about 2:1 in liver (Table 1), i.e. proportional to the amount of their mRNAs. Observed half-lives of ferritin varied from 25 to 50 h [5]; therefore the short labelling period used in this work should make the effect of protein turnover on the estimation of synthesis rates negligible.

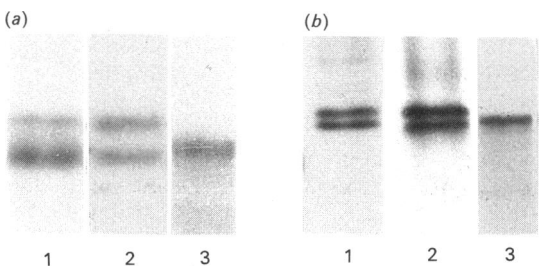

Fig. 1. SDS/PAGE analysis of liver and heart ferritins

Liver and heart ferritins were purified from tissues (*a*) or immunoprecipitated from tissue slices incubated *in vitro* (*b*) as described in the Experimental section and separated on a 15%-polyacrylamide gel. Ferritin bands were revealed by either Coomassie Blue staining (*a*) or fluorography (*b*) of the gels. Lane 1, liver ferritin; lane 2, heart ferritin; lane 3, human recombinant H.

Table 1. H and L mRNAs and subunits in rat liver and heart

(*a* and *b*) The values of ferritin mRNAs were calculated as described in the Experimental section and are expressed as means ± S.E.M. for three separate sets of experiments. For each experiment RNAs extracted from tissue samples of different animals were run on separate gels together with calibration standards and hybridized separately. The values of polyribosomal ferritin mRNAs were calculated assuming 50% of total cellular RNA as polyribosomal [44]. (*c*) Data obtained from lines (*a*) and (*b*). (*d*) The ratios of H and L subunits from three separate experiments are reported. For each experiment slices from different tissue samples were incubated separately in the presence of [^{35}S]methionine and the material immunoprecipitated from homogenates was run on separate gels. The values were obtained by densitometric quantification of differently timed autoradiographic exposures for each gel. The ratio of ferritin subunits in synthesis *de novo* was calculated by taking into account that rat H and L have six and three methionine residues respectively [29]. (*e*) The ratios of H and L in liver and heart ferritins independently purified from the tissues of three different rats are reported. The values were obtained by densitometric scanning of Coomassie Blue-stained SDS-containing gels as described in the text.

	Parameter	Total liver	Total heart	Polyribosomal, liver
(*a*)	L-mRNA (pg/μg of total cellular RNA)	220±18	40±5	34±4
(*b*)	H-mRNA (pg/μg of total cellular RNA)	112±9	36±4	16±3
(*c*)	L/H ratio (mRNAs)	1.94	1.08	2.08
(*d*)	L/H ratio (protein synthesis *de novo*)	2.31	0.83	–
		2.08	1.48	–
		1.97	1.11	–
(*e*)	L/H ratio (accumulated protein)	4.35	1.10	–
		3.70	0.88	–
		3.83	1.17	–

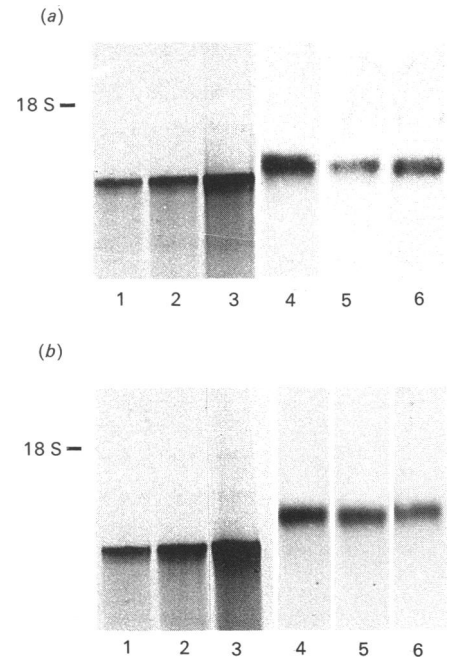

Fig. 2. Levels of ferritin mRNAs in liver and heart

The amount of ferritin L- and H-subunit mRNAs was analysed by Northern-blot analysis using co-electrophoresed L- and H-cDNAs as hybridization standards as described in the Experimental section. (*a*) Hybridization with L probe; (*b*) hybridization with H probe. Lanes 1–3, increasing amounts (3, 6, 12 ng) of L- (*a*) or H- (*b*) cDNAs adjusted to 30 μg with yeast tRNA; lane 4, total liver RNA (30 μg); lane 5, total heart RNA (30 μg); lane 6, polyribosomal liver RNA (30 μg). At he left of each blot the migration position of 18 S rRNA is indicated.

DISCUSSION

Genes which are strictly tissue-specific or exhibit a strong inductive response are, in general, transcriptionally regulated [34,35]. On the other hand, several 'housekeeping' genes are post-transcriptionally regulated [36]. Ferritin genes belong to both groups, since their expression is at the same time ubiquitous and induced by iron [5]. In fact ferritin biosynthesis is regulated at multiple levels, although, in contrast with other genes, post-transcriptional mechanisms seem more important in the response to iron induction, and transcriptional events play a role in other types of iron-independent pathophysiological situations [3]. The aim of this work was to identify the molecular mechanisms responsible for the distinct isoferritin profiles of rat liver and heart. The two ferritins had strikingly different structures, since ferritin purified from rat heart contained equivalent proportions and H and L, whereas hepatic ferritin was richer in L. We believe that the L/H ratios in the two ferritins (4:1 in liver and 1:1 in heart) are representative of the actual isoferritin composition inside the two tissues because: (i) heart and liver ferritin were purified by the same procedure; (ii) different purification procedures gave similar results [2,30–32]; (iii) in humans, where specific anti-H and anti-L antibodies are available for Western-blot experiments, the subunit composition of the purified ferritin provides a reliable index of the total subunit composition in the various tissues [37,38]. By means of quantitative hybridization experiments we found that the liver has levels of H- and L-mRNAs higher than those of the heart, which may explain its larger ferritin accumulation, and has a higher L/H-mRNA ratio, which can partially account for the larger proportion of L in liver isoferritin. The finding that, in the heart, the ratio of the H- and L-mRNAs is analogous to that of the subunits, whereas in the liver the L/H-mRNAs ratio of 2:1 corresponded to an L/H subunit ratio of 4:1 and led us to study the intermediate steps of ferritin biosynthesis. We found that the ratio of L- to H-mRNAs in polyribosomal RNA is similar to that in total cellular RNA, indicating that the different intracellular distribution of ferritin mRNAs that is involved in the response of the cell to both acute and chronic iron load [12,15] is not adequate to explain the preferential accumulation of LRI in rat liver. Fractionation of rat liver postmitochondrial supernatant on sucrose gradients did not reveal differences in distribution between L- and H-mRNA [12,15], making it unlikely that the two ferritin mRNAs

are associated with polyribosomes of different sizes and hence translated with different efficiencies. The results obtained for ferritin synthesis *de novo* in tissue slices confirmed that the two mRNA species are translated with similar efficiency, since in both heart and liver the synthesis of H and L is proportional to the amount of their respective mRNAs.

In conclusion, we found that the heart maintains the same L/H ratio (about 1:1) from mRNA steady-state levels to protein accumulation; in contrast, the liver maintains an L/H ratio of 2:1 from mRNA levels up to subunit synthesis *de novo*, and then it shows a dramatic change to a ratio of 4:1 in the purified protein. These results therefore suggest that the tissue-specific isoferritin expression in rat heart and liver is controlled by at least two mechanisms: a pre-translational one, which leads to differential accumulation of L- and H-mRNAs, and a post-translational one, which determines a further increase in the proportion of L in the liver. The relative increase in L in the accumulated as against the newly synthesized ferritin may be due to a reduced half-life of HRI. In fact, previous studies indicated a longer half-life of hepatic LRI [39,40]. Alternatively, or additionally, a possible regulatory step, largely unexplored, of ferritin synthesis may be localized in the transition of ferritin from a pool of free subunits to assembled shells [41], as also suggested from experiments with monocytes in which iron induced a strong increase in the accumulation of ferritin without a significant enhancement of synthesis *de novo* [42,43]. The lack of differences in subunit ratio between newly synthesized and accumulated ferritin in the heart suggests that either the rate of relative degradation of the two subunits or the assembly into shells, or both, are controlled in a tissue-specific way.

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REFERENCES

1. Drysdale, J. W. (1977) Ciba Found. Symp. **51**, 41–67
2. Arosio, P., Adelman, T. G. & Drysdale, J. W. (1978) J. Biol. Chem. **253**, 4451–4458
3. Drysdale, J. W. (1988) Prog. Nucleic Acids Res. Mol. Biol. **35**, 127–155
4. Levi, S., Luzzago, A., Cesareni, G., Cozzi, A., Franceschinelli, F., Albertini, A. & Arosio, P. (1988) J. Biol. Chem. **263**, 18086–18092
5. Theil, E. C. (1987) Annu. Rev. Biochem. **56**, 289–315
6. Pietrangelo, A., Rocchi, E., Schiaffonati, L., Ventura, A. & Cairo, G. (1990) Hepatology **11**, 798–804
7. Cairo, G., Bardella, L., Schiaffonati, L., Arosio, P., Levi, S. & Bernelli-Zazzera, A. (1985) Biochem. Biophys. Res. Commun. **133**, 314–321
8. White, K. & Munro, H. N. (1988) J. Biol. Chem. **263**, 8938–8942
9. Beaumont, C., Dugast, I., Renaudie, F., Souroujon, M. & Grandchamp, B. (1989) J. Biol. Chem. **264**, 7498–7504
10. Mattia, E., den Blaauwen, J., Ashwell, G. & van Renswoude, J. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 1801–1805
11. Costanzo, F., Bevilasqua, M. A., Giordano, M. & Cimino, F. (1989) Biochem. Biophys. Res. Commun. **161**, 902–909
12. Aziz, N. & Munro, H. N. (1986) Nucleic Acids Res. **14**, 915–927
13. Hentze, M. W., Rouault, T. A., Caughman, S. W., Dancis, A., Harford, J. B. & Klausner, R. D. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 6730–6734
14. Dickey, L. F., Wang, Y.-H., Shull, G. E., Wortman, I. A. & Theil, E. C. (1988) J. Biol. Chem. **263**, 3071–3074
15. Cairo, G., Tacchini, L., Schiaffonati, L., Rappocciolo, E., Ventura, E. & Pietrangelo, A. (1989) Biochem. J. **264**, 925–928
16. Dickey, L. F., Sreedhran, S., Theil, E. C., Didsbury, J. R., Wang, Y.-H. & Kauffman, R. E. (1987) J. Biol. Chem. **262**, 7901–7907
17. Chou, C. C., Gatti, R. A., Fuller, M. L., Concannon, P., Wong, A., Chada, S., Davis, R. C. & Salser, W. A. (1986) Mol. Cell. Biol. **6**, 566–573
18. Cairo, G., Vezzoni, P., Bardella, L., Schiaffonati, L., Rappocciolo, E., Levi, S., Arosio, P. & Bernelli-Zazzera, A. (1986) Biochem. Biophys. Res. Commun. **139**, 652–657
19. Arosio, P., Levi, S., Cairo, G., Cazzola, M. & Fargion, S. (1990) in Iron Transport and Storage (Ponka, P., Schulman, H. M. & Woodworth, R. C., eds.), pp. 201–216, CRC Press, Boca Raton, Ann Arbor and Boston
20. Ursini, M. V. & De Franciscis, V. (1988) Biochem. Biophys. Res. Commun. **150**, 287–295
21. Torti, S. V., Kwak, E. L., Miller, S. C., Miller, L. L., Ringold, G. M., Myambo, K. B., Young, A. P. & Torti, F. M. (1988) J. Biol. Chem. **263**, 12638–12644
22. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
23. Schiaffonati, L., Rappocciolo, E., Tacchini, L., Bardella, L., Arosio, P., Cozzi, A., Cantù, G. B. & Cairo, G. (1988) Exp. Mol. Pathol. **48**, 174–181
24. Laskey, R. A. & Mills, A. D. (1976) Eur. J. Biochem. **56**, 335–345
25. Yap, S. H., Strair, R. K. & Shafritz, D. A. (1978) J. Biol. Chem. **253**, 4944–4950
26. Chomczynski, P. & Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
27. Lowell, C. A., Stearman, R. S. & Morrow, J. F. (1986) J. Biol.Chem. **261**, 8453–8461
28. Murray, M. T., White, K. & Munro, H. N. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 7438–7442
29. Leibold, E. A., Aziz, N., Brown, A. J. & Munro, H. N. (1984) J. Biol. Chem. **259**, 4327–4334
30. Arosio, P., Yokota, M. & Drysdale, J. W. (1976) Cancer Res. **36**, 1735–1738
31. Bomford, A., Lys, Y., McFarlane, I. G. & Williams, R. (1977) Biochem. J. **167**, 309–312
32. Linder, M. C., Nagel, G. M., Roboz, M. & Hungerford, D. M. (1981) J. Biol. Chem. **256**, 9104–9110
33. Clayton, D. F., Harrelson, A. L. & Darnell, J. E., Jr. (1985) Mol. Cell. Biol. **5**, 2623–2632
34. Darnell, J. E. (1982) Nature (London) **297**, 365–371
35. Nevins, J. R. (1983) Annu. Rev. Biochem. **52**, 441–466
36. Hunt, T. (1988) Nature (London) **334**, 567–568
37. Ruggeri, D., Iacobello, C., Albertini, A., Brocchi, E., Levi, S., Gabri, E. & Arosio, P. (1984) in Ferritins and Isoferritins as Biochemical Markers (Albertini, A., Arosio, P., Chiancone, E. & Drysdale, J. W., eds.), pp. 67–78, Elsevier, Amsterdam, New York and Oxford
38. Cozzi, A., Levi, S., Bazzigalupi, E., Ruggeri, G. & Arosio, P. (1989) Chim. Clin. Acta **184**, 197–206
39. Kohgo, Y., Yokota, M. & Drysdale, J. W. (1980) J. Biol. Chem. **255**, 5195–5200
40. Bomford, A., Coulon-Hollingshead, C. & Munro, H. N. (1981) J. Biol. Chem. **256**, 948–955
41. Drysdale, J. W. (1984) in Ferritins and Isoferritins as Biochemical Markers (Albertini, A., Arosio, P., Chiancone, E. & Drysdale, J. W., eds.), pp. 87–96, Elsevier, Amsterdam, New York and Oxford
42. Worwood, M., Hourahane, D. & Jones, B. M. (1984) Br. J. Haematol. **56**, 31–43
43. Summers, M., Worwood, M. & Jacobs, A. (1974) Br. J. Haematol. **28**, 19–27
44. Galau, G. A., Klein, W. H., Britten, R. J. & Davidson, E. H. (1977) Arch. Biochem. Biophys. **179**, 584–599

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