0014-4754/96/060535-05$1.50 + 0.20

In situ hybridization analysis of globin mRNAs in the primitive erythroid cells of the chick embryo

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Received 11 July 1995; received after revision 26 September 1995; accepted 8 November 1995

Abstract. The possibility that the minor embryonic chick hemoglobins might be present in a particular subgroup of primitive erythroid cells has been investigated by in situ hybridization. Probe to detect the mRNA for the α^A globin chain of the minor embryonic hemoglobin was used, and the results of the hybridization were compared with those obtained using as probes the cDNAs for total globin mRNAs. All erythroid cells circulating in a 4-day-old chick embryo gave positive signals with both probes at an approximately constant ratio. This shows that all cells contain a similar assortment of hemoglobin types, excluding the possibility that a subgroup might contain the minor primitive hemoglobins exclusively. However, the cells are not homogeneous, since about 10% of them show a distinctly higher concentration of mRNA of all globin types.

Key words. α^A-Globin; mRNAs; primitive erythroid cells; chick embryo; in situ hybridization.

The chick embryo is a convenient model system to study the switch of hemoglobin types that occurs during the embryonic development of vertebrates. In the chick, a primitive red cell line containing embryonic hemoglobins is present from the start of embryonic development. Later, starting from about day 6 of development, a new red cell line is put into circulation and new hemoglobins, the adult types, are found in the cell lysate[1].

To understand the control of gene expression, an important point is to establish which hemoglobin types are present in the different erythroid cells. There is a wealth of work concerning the hemoglobin types present in the primitive erythroid cells of the chick embryo[1]. Several different fractionation procedures have been used, all showing that these cells express a total of four hemoglobins: the major embryonic hemoglobins Hb P and Hb P′, and the minor embryonic hemoglobins Hb E and Hb M. No adult hemoglobin type is observed in these cells[2–5]. The two major hemoglobins, Hb P and Hb P′, are formed by two embryonic α-like globin chains, π and π' associated with the embryonic β-like globin chain ρ[6–8], while the two minor hemoglobins, Hb E and Hb M, are formed by the two adult α-like chains, α^A and α^D, associated with the minor embryonic β-like globin chain ε[6,8]. The analytical fractionations have never revealed the presence of hybrid hemoglobin molecules formed by major embryonic globin chains interacting with minor embryonic globin chains, except in the lysate of aged erythrocytes[8]. This finding might reflect compartmentalization of minor and major embryonic hemoglobins in different cell lines or on an absolute specificity in the interaction between the different types of α- and β-globin chains. Since the switch from the embryonic to the adult type of hemoglobins, observed in the chick embryo at around day 6, appears to depend on the replacement of the erythroid cell line[2,3,6] it cannot be excluded that the absence of the hybrid hemoglobins in the lysates of primitive erythroid cells might depend on a compartmentalization of the two types of hemoglobins in two different subpopulations of the primitive cell line. It is important to establish the number of different cell types put into circulation by the embryo during development. Immunolabelling experiments aimed at studying hemoglobin compartmentalization did not provide conclusive results[9]. In that investigation, rabbit polyclonal antibodies specific for the α^A- and α^D-globins were prepared and reacted with the primitive erythroid cells of the 5-day-old chick embryos. Fluorescence labelling of the immunoreacted erythrocytes showed that about 10% of those cells had interacted with the antibody preparation with a distinctly high efficiency. Since it was not possible to exclude partial cross reactivity with the major embryonic globins, the results did not establish whether the highly reacting cells were the only ones containing the minor embryonic globins[9].

In situ hybridization studies using specific DNA probes appeared an elegant method to visualize and quantify the α^A-globin mRNA present in the circulating cells of the 4-day-old chick embryo, because its high specificity minimizes interference from the other globin mRNAs. For this reason in situ hybridization was considered a suitable method to provide conclusive evidence of the distribution of embryonic hemoglobins in the primitive

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red cell line. A DNA probe was prepared that was specific for the α^A-globin mRNA, to label only cells containing the minor embryonic hemoglobins. Since the results showed the occurrence of two populations of cells with different mRNA concentrations, the hybridization experiments were also performed using as probes cDNAs prepared from total mRNA isolated from the erythroid cells of the 4-day-old chick embryo. These cells contain almost exclusively globin mRNAs[10], and for this reason the hybridization results obtained using the cDNA probe were considered good reference values for total globin mRNAs.

Materials and methods

In situ hybridization. Fertilized chick eggs were obtained from a local poultry farm. Embryos were grown to the desired stages in a humid incubator at 37.7 °C. Smears of circulating blood of 4-day-old chick embryos were prepared on microscope slides and fixed by exposure to a vapor of 4% p-formaldehyde in 0.9% NaCl in 10 mM sodium phosphate pH 7.2 [phosphate-buffered saline (PBS)]. Smears were washed three times for 15 min at room temperature in PBS and then immersed for 15 min at room temperature in 0.2 M Tris-HCl, 0.1 M glycine pH 7.4. Prehybridization was performed for 60 min at 42 °C in 2 × SSC (SSC = 150 mM NaCl in 15 mM sodium citrate pH 7), 50% formamide, 20 mM L-methionine. The hybridization mix contained 0.5 ng/µl of labelled DNA probe, 50% formamide, 0.5 µg/µl of herring sonicated DNA, 2 µg/µl of *E. coli* tRNA, 1 × Denhardt's solution (0.2 mg Ficoll, 0.2 mg Polyvinylpyrrolidone, 0.2 mg BSA/ml), 20 mM L-methionine, 2 × SSC. Nucleic acids were denatured in the solution used for hybridization by heating for 10 min at 100 °C and then rapidly cooling in an ice bath. Aliquots of 10 µl of the hybridization mix were placed on the blood smears. These were covered with a coverslip and placed in a closed box lined with filter paper soaked with 2 × SSC. Hybridization was carried out overnight at 42 °C. At the end, coverslips were removed and excess probe was washed off by rinsing smears two times for 30 min at room temperature in 2 × SSC, 20 mM L-methionine, then for 15 min at 55 °C in 0.1 × SSC, 20 mM L-methionine, and finally for 15 min at room temperature in 0.1 × SSC. Smears were then sequentially dipped for 30 s in 0.3 M ammonium acetate solutions containing successively 50% ethanol, 70% ethanol and 90% ethanol. At the end, working under a red safelight, air-dried smears were dipped in melted Ilford K5 emulsion diluted 1:1 with H_2O and air-dried for 2.5 h in a dark box at room temperature. Smears were then transferred to a light-tight slide box, and the exposure was continued for 3 to 10 days at 5 °C. Smears were then developed in Ilford IF2 developer for 5 min, fixed in Ilford FIX for 10 min at room temperature, washed for 15–30 min under running water and stained with Giemsa's stain.

Probe preparation and labelling. The α^A chicken globin probe was prepared by double digestion of the plasmid pBRα7-1.7, kindly provided by Dr Engel[11]. This plasmid contains the 1.7 kb *Eco*RI-*Bam*HI fragment of the α^A-globin gene inserted between the *Eco*RI and *Bam*HI restriction sites of pBR322. The plasmid was digested with *Eco*RI and *Bam*HI restriction enzymes, and the 1.7 kb fragment was separated from the vector plasmid by electrophoresis in Tris-EDTA-borate buffer on 1% low-melting agarose gel (BRL), and extracted following a standard protocol[12]. The α^A-globin probe specifically recognized the α^A-globin gene by Southern blot analysis of the DNA from the chicken erythroid cells in the conditions corresponding to those of our experiments. Consequently, this DNA was used as a test probe to detect the minor embryonic hemoglobins in the cells of the primitive erythroid line. The DNA of the plasmid 2.8 kb pGEM-3 (Promega) was used as a negative control probe to reveal possible nonspecific interactions. The plasmid was linearized by digestion with *Eco*RI restriction enzyme (Boehringer), separated from its undigested topological forms by gel electrophoresis in low-melting agarose and extracted from the gel as indicated for the α^A-globin probe. Total poly(A) mRNAs were prepared by standard protocols from the erythrocytes obtained from circulating blood of 4-day-old embryos[12]. Double-strand cDNAs, synthesized from this mRNA preparation using the Amersham cDNA kit, were used as probes to test the total globin mRNAs present in the erythrocytes studied. The specificity of the cDNA globin probe was tested on smears of blood cells from 14-day-old chick embryos that contained both immature and mature red cells. In those smears only the immature cells showed a positive reaction with the cDNA globin probe, demonstrating that the presence of grains in the cytoplasm was not caused by possible in situ hybridization artefacts.

The multiprimer DNA labelling kit (Amersham) and α^{35}S-dCTP, 1000 Ci/mmol, were used to label to high specific activity the three DNA probes, while α^{35}S-dATP, 600 Ci/mmol, was used as precursor to obtain probes at a lower specific activity.

Results

The results of hybridization experiments on primitive erythroid cells isolated from 4-day-old chick embryos are shown in figure 1. Hybridization grains were observed in all the cells of the primitive erythroid line when the 1.7 kb α^A globin gene fragment, labelled with ^{35}S-dCTP, was used as a probe (fig. 1A). The gradients of grain density observed in the proximity of all cells were sharp, indicating that only minor spillage of the mRNAs occurs in the hybridization procedure. The

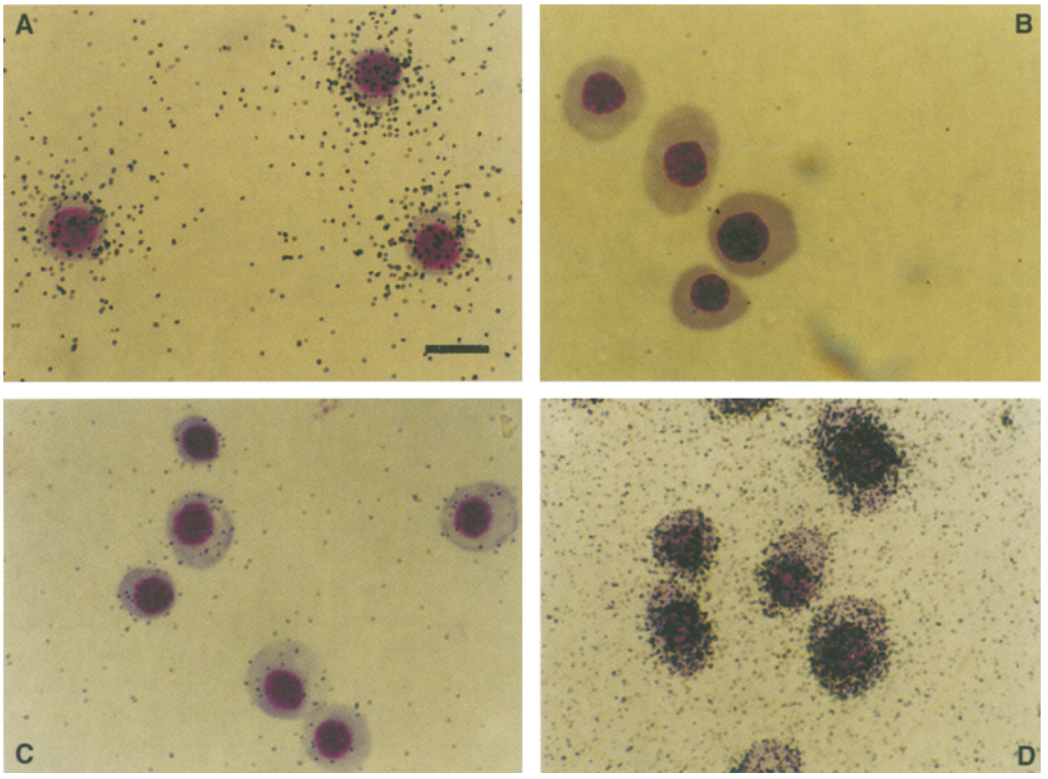

Figure 1. Erythroid cells of 4-day-old chick embryo in situ hybridized with different probes as follows: (*A*) with α^A-globin gene; (*B*) as (*A*), but cells prehybridized with the unlabelled probe; (*C*) with pGEM-3 plasmid DNA; (*D*) with cDNA of all globin mRNAs. Giemsa staining. Original magnification × 780. Bar = 10 µm. Note that in (*A*), (*B*) and (*C*) probes were labelled with α^{35}S-dCTP; in (*D*) the probe was labelled with α^{35}S-dATP. For details, see text.

specificity of probe hybridization was revealed by negative results obtained in the experiments in which hybridization was performed on cells pre-treated with the unlabelled 1.7 kb DNA fragment and by using as a probe the heterologous pGEM-3 DNA, labelled with ^{35}S-dCTP. In the first case there was no evidence of hybridization (fig. 1B). When the heterologous pGEM-3 DNA was used as a probe, the number of grains per cell was only slightly higher than the background (fig. 1C). The possible occurrence of in situ hybridization artefacts was tested in experiments in which the 1.7 kb α^A-globin probe was used to hybridize smears of MEL cells. These are transformed mouse erythroid cells blocked at the proerythroblast stage that can be induced to synthesize hemoglobin by a suitable inducer[13]. Hybridization of MEL cell smears produced the same low density pattern of grains as the heterologous pGEM-3 DNA probe shown in figure 1C. This result confirmed that the presence of grains in the cytoplasm of cells is due to specific interactions.

When the probe was total globin cDNA labelled with α^{35}S-dCTP, the hybridization grains in the erythroid cells were too crowded to be counted accurately. For this reason probes were also prepared with a lower specific activity by using α^{35}S-dATP as labelled precursor. The results of the hybridization with total cDNA labelled with α^{35}S-dATP showed, as expected, the presence of grains in the cytoplasm of all cells (fig. 1D). The decrease in probe sensitivity was sufficient to avoid the excessive crowding of grains per cell that interfered with the accurate determination of the values when the probe was labelled with α^{35}S-dCTP. A higher background level than in panel A is observed in panel B because of the increased number of targets revealed by the mixture of globin cDNAs constituting the probe.

Plots correlating the number of cells containing a particular range of grain numbers with the number of grains per cell have been derived from three experiments in which globin probes were labelled at a lower specific activity with α^{35}S-dATP. The results of the hybridization, when the probe was the α^A-globin gene and labelled only cells containing the mRNA for minor embryonic hemoglobins, confirm the presence of that mRNA in all cells. The plot also shows the occurrence of two types of cells (fig. 2A): one with a lower (55) and one with a higher (108) average number of grains. The

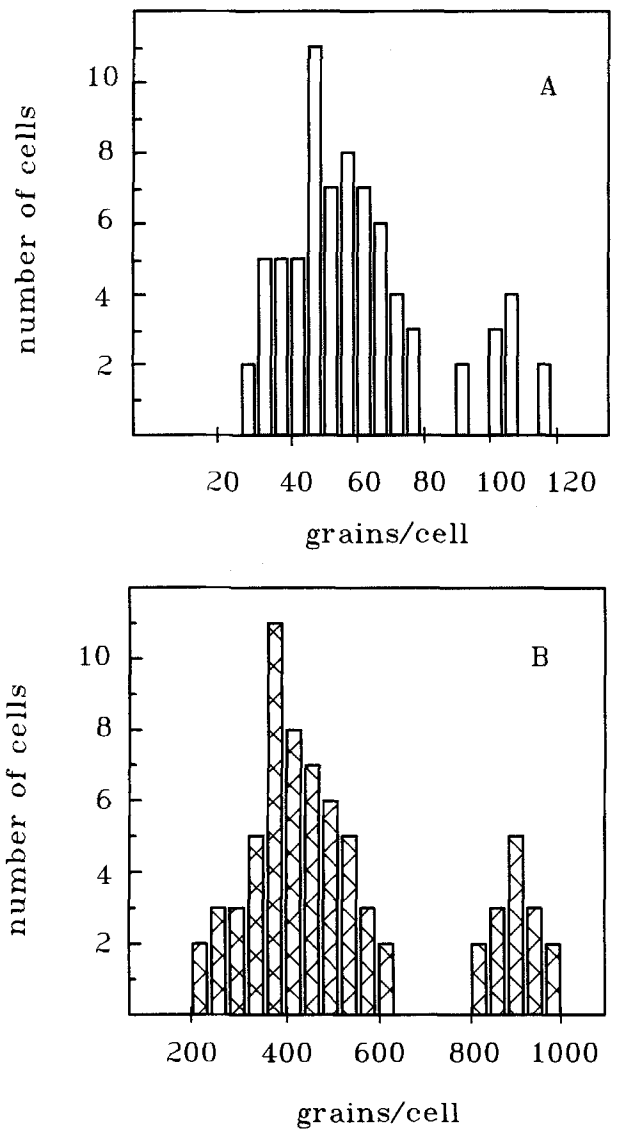

Figure 2. Plots correlating the number of cells showing a particular range of grain number with the range of grain number, as revealed by in situ hybridization. Data are mean values of three different experiments. (A) α^A-globin gene as a probe. Grain density determinations were performed on 74 cells in total. (B) cDNAs for all globin mRNAs as probe. Grain density determinations were performed on 70 cells in total. Unpaired t tests, for the two groups of values of (A) or (B), provide $P < 0.0001$.

pattern of the plot is also bimodal when total globin cDNAs are used as probes (fig. 2B), again indicating the occurrence of two types of cells. In this case the values of grain numbers depend on the concentrations of all possible mRNAs and can be used as reference hybridization values that are not influenced by factors specific to particular mRNAs. Statistical analyses performed using an unpaired t test of the means corresponding to the low and to the high values of the grain number per cell reported in figure 2 provided a two-tailed P value <0.0001 with 16 and 46 degrees of freedom respectively for data of panel A and panel B. Such a P value is extremely significant for the presence of two different groups. It is interesting that the bimodal pattern observed with total globin cDNAs shows a similar percentage of cells displaying a high number of grains to that observed with the probe specific for α^A mRNA. In both experiments there is no correlation between the cell morphology and the number of grains present in the cell, and the patterns do not change if the values are expressed as number of grains per unit area.

Discussion

The results of the in situ hybridization experiments, reported in figure 1A, provide conclusive evidence that the mRNA for the α^A-globin chain of the minor embryonic hemoglobins is present in all the erythroid cells of 4-day-old chick embryos. This is demonstrated by the presence of hybridization grains in all the cells that have been exposed to the radioactively labelled probe specific for the α^A-globin mRNA. Since there is no segregation of the minor embryonic hemoglobins to a particular group of cells, the absence of the hybrid hemoglobin tetramers in the total hemolysate of the primitive erythroid cells is due to the specificity of the reciprocal association of the different globin chains.

Interestingly, the distributions of the values of the number of grains per cell reveal two subpopulations of cells (figure 2A) as was the case in the immunological studies[9]. The presence of two groups of cells in the primitive erythroid line is evident when hybridization is performed to detect the mRNA for the minor embryonic α^A-globin chain and the mRNAs for total globins (fig. 2). In addition, the results do not depend on the specific activity of the probes. The correspondence between the results of the immunolabelling of the erythroid cells, showing differences in the hemoglobin concentrations[9], and those of the in situ hybridization of the mRNA, reported here, suggests that the two subpopulations might in fact be the same group of cells. The possibility that the two groups of cells in panel A and panel B of figure 2 are indeed different subpopulations of the same line is strongly supported by the statistical analysis of the two values. In other words it appears that in the embryonic circulation there is a group of cells containing a higher concentration of mRNAs and a higher concentration of hemoglobins. This group might correspond to the second wave of the primitive red blood cells that are newly synthesized in the yolk sac and released into the circulation as reticulocytes at day 4 of development[14]. It is interesting that the differences in mRNA concentrations do not concern the hemoglobin assortment, as clearly shown by the observation that the ratio between the hybridization values with the probe detecting the mRNA of a minor globin and the probes

detecting total globins is almost constant in the cells of the two groups.

As a final consideration, the results presented here confirm the hypothesis that the cells of the primitive erythroid line are all put into the circulation with a similar specific program of globin gene expression, very likely established at the level of stem cells in the erythropoietic site. The finding that the hemoglobin assortment is the same in cells showing mRNA concentrations differing by a factor of two (fig. 2A, B) indicates that the program for the relative rate of gene expression is not grossly influenced by the efficiency of mRNA synthesis. This is not in conflict with the suggestion that the lineage-specific differences found in the abundance of transcription factors in chick erythroid cells might be, at least in part, a possible cause of the different ρ globin content in primitive and definitive erythrocytes[15]. The minor changes in the relative rates of individual globin synthesis that have been shown to occur in aged erythrocytes[8] appear to have very little influence on the globin composition of the cell. In particular, the differences in protein-synthesizing ability of the aged primitive cells, present at later stages of development and still engaged in transcription of the ρ gene, can be considered the effects of cell senescence because they have only a very marginal influence on the hemoglobin content of those cells[5].

1 Dieterlen-Lievre, F., in: Birds in Vertebrate Blood Cells, p. 257. Eds A. F. Rowley and N. A. Ratcliffe, Cambridge University Press 1988.
2 Beaupain, D., Cell Differentiation *16* (1985) 101.
3 Fucci, L., Vitale, E., Cirotto, C., and Geraci, G., Cell Differentiation *20* (1987) 55.
4 Mahoney, K. A., Hyer, B. J., and Chan, L. L., Devl Biol. *56* (1977) 412.
5 Lois, R., and Martinson, H. G., Biochemistry *28* (1989) 2281.
6 Cirotto, C., Scotto di Tella, A., and Geraci, G., Cell Differentiation *4* (1975) 87.
7 Bruns, G. A. P., and Ingram, V. M., Phil. Trans. R. Soc. London B *226* (1973) 225.
8 Schalekamp, M., and Van Goor, D., J. Embryol. exp. Morph. *84* (1984) 125.
9 Cirotto, C., Panara, F., and Geraci, G., Devl Biol. *61* (1977) 384.
10 Hansen, D. A., Seftor, E. A., Dekloe, J., McCabe, J. B., and Tobin, A. J., Devl Biol. *102* (1984) 278.
11 Dodgson, J. B., and Engel, J. D., J. biol. Chem. *258* (1983) 4623.
12 Sambrook, J., Fritsch, E. F., and Maniatis, T. in: Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press 1989.
13 Friend, C., Scher, W., Holland, J. G., and Sato, T., Proc. natl Acad. Sci. USA *68* (1971) 378.
14 Cirotto, C., Barberini, L., and Arangi, I., Devl Growth Diff. *36* (1994) 111.
15 Minie, M. E., Kimura, T., and Felsenfeld, G., Development *115* (1992) 1149.