

## Expression of the chicken GATA factor family during early erythroid development and differentiation

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### SUMMARY

The DNA motif WGATAR has been identified within transcriptional regulatory domains of globin and other erythroid-specific genes and the activator proteins that bind to this regulatory element, the GATA factors, belong to a multi-gene family that is expressed in chicken erythroid cells. Here we show that, as in chickens, multiple members of the GATA factor family are expressed in human and murine erythroid cells. During the early stages of chicken embryogenesis (well before blood island formation), each of the GATA family members is transcribed with a unique temporal and spatial pattern. In the primitive erythroid lineage, tran-

scription of the embryonic  $\epsilon$ -globin gene parallels GATA-1 expression while the switch to  $\beta$ -globin transcription in definitive erythroid cells is directly preceded by a pronounced increase in GATA-3 accumulation. The timing and pattern of expression of these different mRNAs during avian erythroid development and differentiation suggests that temporally regulated changes in GATA factor expression are required for vertebrate hematopoiesis.

Key words: GATA, transcription factors, red blood cells, embryogenesis

### INTRODUCTION

Erythropoiesis has provided a long established model for studying the developmental regulation of gene expression, and has been extensively investigated in several species. In chicken, precursors to red blood cells are believed to be composed of as much as 25% of the mesodermally derived cells of the posterior zona opaca of early embryos (Groudine and Weintraub, 1981). The earliest erythroid cells of the developing chick embryo are formed in the blood islands, first visible as localized clumps of cells within the extra-embryonic mesoderm at stage 8 of development (Hamburger and Hamilton, 1951) with hemoglobinised primitive erythroid cells detectable between stages 9 and 12 (reviewed by Romanoff, 1960). These primitive erythroid precursors form a cohort of cells that are released into the circulation at embryonic stage 12 (Bruns and Ingram, 1973; Dieterlen-Lievre, 1978). At 5 days of development in ovo, intraembryonically derived definitive erythroid cells begin to appear in the circulation and rapidly accumulate to greater than 50% of the total erythroid cell population by day 8 (Bruns and Ingram, 1973; Dieterlen-Lievre, 1978; Romanoff, 1960).

The GATA DNA binding motif was first identified within transcriptional regulatory domains of erythroid-specific genes as a potentially significant erythroid *cis*-acting element (Emerson et al., 1987; Evans et al., 1988; Wall et al., 1988). Subsequent mutagenesis of consensus GATA

sites within several of these regulatory domains showed that they were indeed required for maximal transcriptional stimulation of the associated genes (Martin et al., 1989; Reitman and Felsenfeld, 1988).

We and others have cloned families of regulatory proteins (termed GATA-1, GATA-2 and GATA-3) that show strikingly similar amino acid sequence identity throughout their DNA binding domains. These factors recognize an erythroid GATA consensus motif and are potent transcriptional activators in transient transfection assays (Yamamoto et al., 1990). The GATA factors have distinct tissue distribution profiles: GATA-1 is expressed in megakaryocytic, mast, erythroid and testis cells, GATA-2 is expressed in a variety of tissues while GATA-3 is most abundantly expressed in neuronal cells and T-lymphocytes (Dorfman et al., 1992; Evans and Felsenfeld, 1989; Ito et al., 1993; Joulin et al., 1991; Ko et al., 1991; Martin et al., 1990; Romeo et al., 1990; Tsai et al., 1989; Yamamoto et al., 1990; Zon et al., 1991; Kornhauser, Leonard, Yamamoto, LaVail, Mayo and Engel, unpublished observations).

In this study, we show first that multiple GATA factors are expressed in human and murine erythroid cells, just as in avian erythroid cells (Yamamoto et al., 1990). Second, we find distinct quantitative changes in cGATA mRNA levels in differentiating definitive chicken erythroid cells. Third, we show that the relative abundance of each GATA family member changes during very early embryonic development, from well before the appearance of the earliest

erythroid progenitors in the yolk sac through to definitive erythropoiesis. During early embryogenesis, there is a spatial and temporal correlation between the expression of cGATA-1 and embryonic  $\beta$ -globin gene transcription and, later in development, between cGATA-3 and adult  $\beta$ -globin transcription. These data suggest that this family of transcription factors may play a broader role in avian erythropoiesis far earlier in development than had previously been anticipated.

## MATERIALS AND METHODS

### Chicken embryos and cells

Developing embryos were isolated from fertilized eggs incubated for varying lengths of time at 39°C and staged by visual inspection and comparison with published examples, or (for later stages) by numbers of somites, since the chronological age of embryos does not necessarily correlate with the developmental age (Hamburger and Hamilton, 1951). Embryos were dissected and immediately disrupted in Buffer D for RNA isolation (Chomczynski and Sacchi, 1987). Cell lines were purchased from the American Type Culture Collection (T-cell lines BW 5147.3 and MOLT-3) or were gifts from V. Patel (uninduced and differentiated MEL cells), S.K. Pierce (CH27 murine B-cells), S.T. Rosen (U266 human B-cells), T. Papayannopoulou (HEL, JK1 and F36 human erythroleukemia

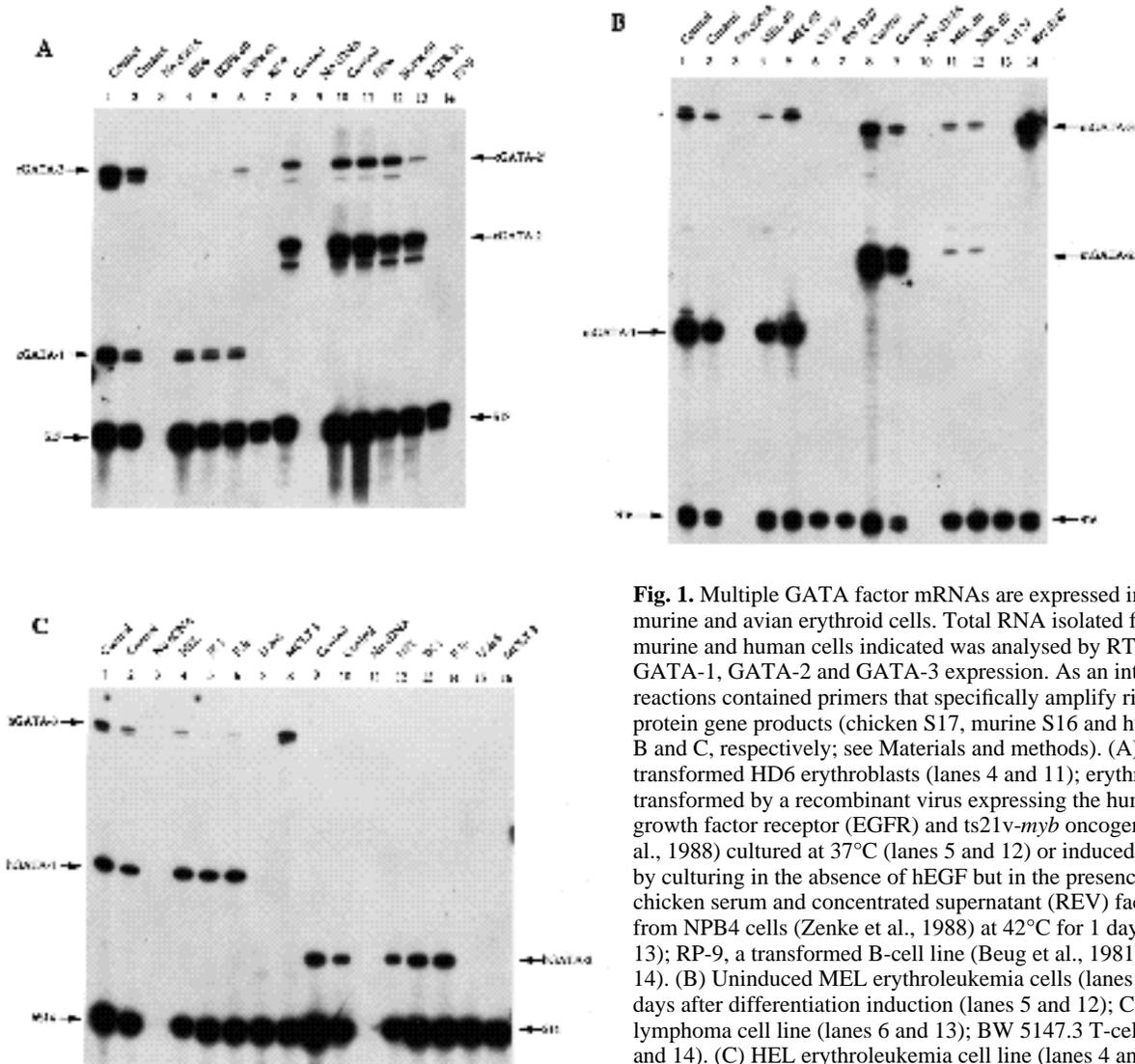
cells), T. Graf, J. Frampton and S. Ness (E26-transformed multipotent chicken progenitor clones, HD50 and HD57, and their myeloid derivatives, clones HD50 27M and HD57 1M). All other cell lines used are as previously described (Yamamoto et al., 1990).

### RT-PCR

Quantitative RT-PCR analysis was performed essentially as described (Camp et al., 1991; Foley and Engel, 1992). Approximately 1  $\mu$ g of denatured total RNA was used as templates in a 20  $\mu$ l cDNA synthesis reaction. In order to reduce variability in cDNA analyses, all RNA samples to be directly compared on a given gel were reverse transcribed into cDNA at the same time, using a single master-mix containing (per sample): 1 $\times$  RT-PCR buffer (50 mM KCl, 20 mM Tris-HCl pH 8.4, 2.5 mM MgCl<sub>2</sub>, 100  $\mu$ g/ml BSA, 2.5 mM DTT, 1 mM each dNTP), 17.5 Units RNasin (Promega), 100 pmol random d(N)<sub>6</sub> primers (Pharmacia) and 8 Units AMV reverse transcriptase (Promega). The reaction was incubated for 10 minutes at 22°C followed by 90 minutes at 42°C. Aliquots of the cDNA reactions were analysed for GATA factor expression in 100  $\mu$ l PCR reactions. Again, to reduce sample to sample variability, all cDNAs to be analysed on a given gel were amplified at the same time using a master-mix containing (per sample): 1 $\times$  Taq DNA polymerase buffer (Promega), 0.2 mM each dNTP, 25 pmol of each primer (see Table 1), 0.2  $\mu$ l [<sup>32</sup>P]dCTP (3,000 Ci/mmol; ICN) and 2.5 Units Taq DNA polymerase (Promega). PCR conditions were 94°C, 2.5 minutes followed by 21–26 cycles (depending on the primers and tissues under analysis) of 94°C, 1 minute; 60–66°C,

**Table 1. PCR Oligonucleotides**

Gene/Primer	DNA Sequence (5' to 3')	Reference	Corresponds to nucleotide
-globin (s)	TCTCC CAACT GTCCG AAC	Dolan et al., 1983	425-442
-globin (as)	TAGGT GCTCC GTGAT CTT	Dolan et al., 1983	1432-1415
-globin (s)	GGGTC CGTGC TCATG GTAAG	Dodgson et al., 1983	332-351
-globin (as)	CTATG GCCAC GGCTG TGCTG	Dodgson et al., 1983	1575-1556
cS17 (s)	TACAC CCGTC TGGGC AACGA C	Trueb et al., 1988	61-81
cS17 (as)	CCGCT GGATG CGCTT CATCA G	Trueb et al., 1988	189-169
cGATA-1 (s)	CTACT GCCAC TCAGC AGCGG G	Evans and Felsenfeld, 1989	323-343
cGATA-1 (as)	TTCTG GCCGT TGAGG CGGTG G	Evans and Felsenfeld, 1989	471-451
cGATA-2 (s)	CTTAC GTGCC GGCTG CCCAT G	Yamamoto et al., 1990	1122-1142
cGATA-2 (as)	CCGGT GCCGT CTCTT CTCCA G	Yamamoto et al., 1990	1302-1282
cGATA-3 (s)	CCACC TCCTC CGCTC ATCAC C	Yamamoto et al., 1990	818-838
cGATA-3 (as)	GCCCG GTGCC GTCTC TTCTC C	Yamamoto et al., 1990	1030-1010
hS14 (s)	GGCAG ACCGA GATGA ATCCT CA	Chen et al., 1986	187-208
hS14 (as)	CAGGT CCAGG GGTCT TGGTC C	Chen et al., 1986	331-311
hGATA-1 (s)	GATCC TGCTC TGGTG TCCTC C	Zon et al., 1990	116-136
hGATA-1 (as)	ACAGT TGAGC AATGG GTACA CC	Zon et al., 1990	298-276
hGATA-2 (s)	CCCTA AGCAG CGCAG CAAGA C	Dorfman et al., 1992	1012-1032
hGATA-2 (as)	GATGA GTGGT CGGTT CTGGC C	Dorfman et al., 1992	1174-1154
hGATA-3 (s)	GTACA GCTCC GGACT CTTCC C	Ko et al., 1991	887-907
hGATA-3 (as)	CTGCT CTCCT GGCTG CAGAC A	Ko et al., 1991	1146-1126
mS16 (s)	AGGAG CGATT TGCTG GTGTG GA	Wagner and Perry, 1985	1451-1471
mS16 (as)	GCTAC CAGGC CTTTG AGATG GA	Wagner and Perry, 1985	1641-1621
mGATA-1 (s)	TGTCA CCGGC AGTGC TTACG G	Tsai et al., 1989	539-559
mGATA-1 (as)	CCACA GTTCA CACAC TCTCT GG	Tsai et al., 1989	691-670
mGATA-2 (s)	CTAGC TACCA TGGGC ACCCA G	F.-Y. Tsai and S. H. Orkin	unpublished
mGATA-2 (as)	CCACA GTTCA CACAC TCCCG G	F.-Y. Tsai and S. H. Orkin	unpublished
mGATA-3 (s)	TCTCA CTCTC GAGGC AGCAT GA	Ko et al., 1991	799-820
mGATA-3 (as)	GGTAC CATCT CGCCG CCACA G	Ko et al., 1991	1044-1024



**Fig. 1.** Multiple GATA factor mRNAs are expressed in human, murine and avian erythroid cells. Total RNA isolated from the avian, murine and human cells indicated was analysed by RT-PCR for GATA-1, GATA-2 and GATA-3 expression. As an internal control, reactions contained primers that specifically amplify ribosomal protein gene products (chicken S17, murine S16 and human S14 in A, B and C, respectively; see Materials and methods). (A) tsAEV-transformed HD6 erythroblasts (lanes 4 and 11); erythroblasts transformed by a recombinant virus expressing the human epidermal growth factor receptor (EGFR) and ts21v-*myb* oncogenes (Khazaie et al., 1988) cultured at 37°C (lanes 5 and 12) or induced to differentiate by culturing in the absence of hEGF but in the presence of anemic chicken serum and concentrated supernatant (REV) factor derived from NPB4 cells (Zenke et al., 1988) at 42°C for 1 day (lanes 6 and 13); RP-9, a transformed B-cell line (Beug et al., 1981; lanes 7 and 14). (B) Uninduced MEL erythroleukemia cells (lanes 4 and 11) or 5 days after differentiation induction (lanes 5 and 12); CH27 B-lymphoma cell line (lanes 6 and 13); BW 5147.3 T-cell line (lanes 7 and 14). (C) HEL erythroleukemia cell line (lanes 4 and 12); JK1 erythroleukemia cell line (lanes 5 and 13); F36 erythroleukemia cell

line (lanes 6 and 14); U266 B-lymphoma (lanes 7 and 15); MOLT-3 T-cell line (lanes 8 and 16). Reactions containing 4× and 1× control cDNAs (A, lanes 1-2, 8; B, lanes 1-2, 8-9; C, lanes 1-2, 9-10) or no control cDNA (A, lanes 3 and 9; B, lanes 3 and 10; C, lanes 3 and 11) were also amplified as positive and negative controls. Amplification products corresponding to cGATA-3, cGATA-1, cGATA-2, cGATA-2 and cS17 (A), mGATA-3, mGATA-1, mGATA-2 and mS16 (B), hGATA-3, hGATA-1, hGATA-2 and hS14 (C) are indicated by arrows.

1 minute; 72°C, 1 minute. Control reactions were performed to ensure that the conditions used were within the linear range of PCR amplification (see control lanes of each figure). All reactions contained, as an internal control, primers to co-amplify ribosomal protein gene products. Samples were analysed on 6% denaturing polyacrylamide gels (loaded for equivalent amounts of internal control) and exposed to autoradiography prior to quantitation on a Molecular Dynamics PhosphorImager. Results shown throughout represent typical data from analysis on at least two separate occasions using at least two independent cDNA preparations.

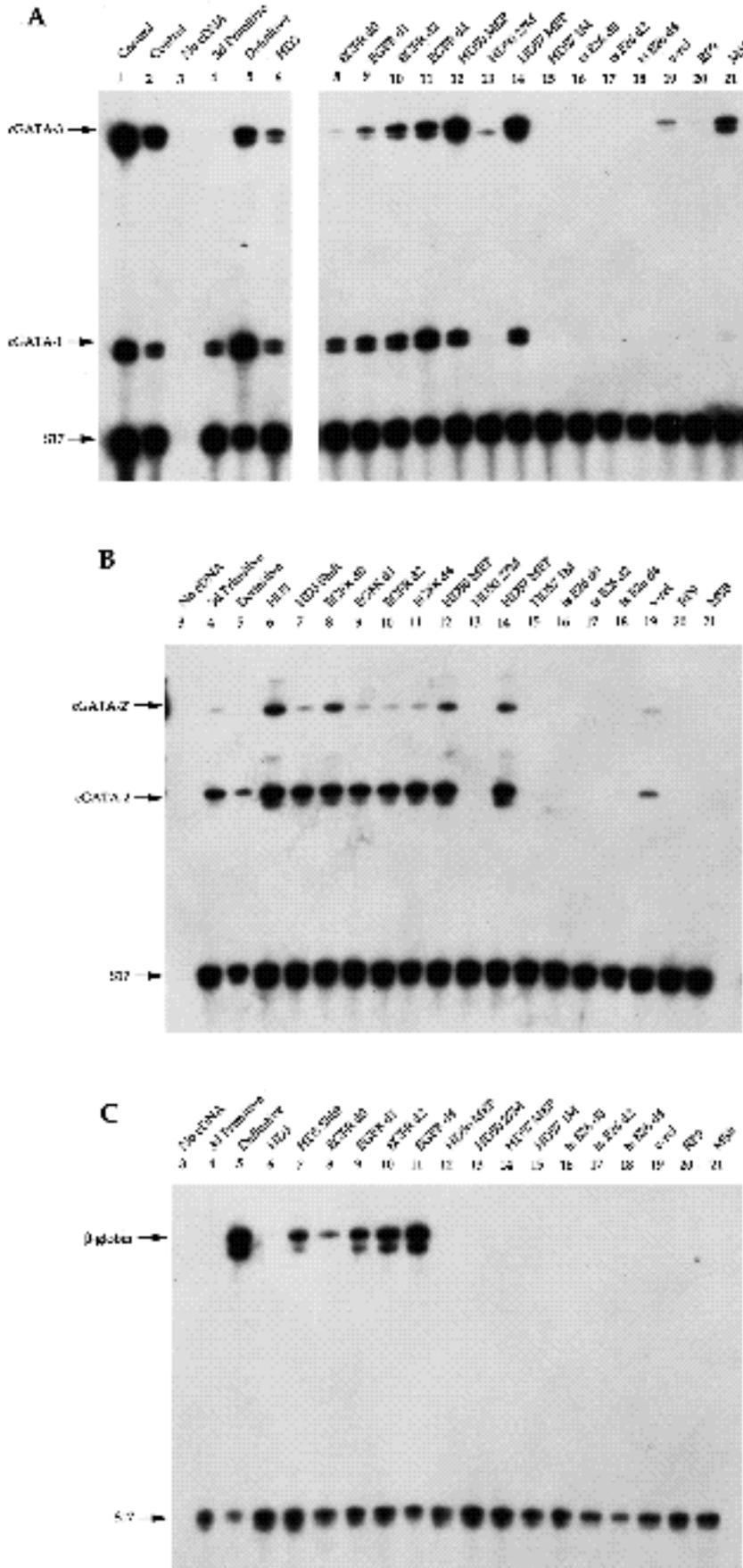
Each pair of sense (s) and antisense (as) primers (see Table 1) was designed to prime within different exons of the respective gene such that only spliced mRNAs are detected in RT-PCR assay. We specifically note that while the assay is designed to permit quantitative comparison of a single GATA or globin mRNA species (normalized to the same ribosomal mRNA) between different samples, the assay does not permit quantitative evaluation of different

mRNA species (e.g. GATA-1 versus GATA-3 mRNAs in Fig. 1 or - versus -globin mRNAs in Fig. 3) within one co-amplified sample. There are several complex reasons for this caveat, including unequal G+C content in the amplified PCR products, differential radioactive incorporation due to the different sizes of the amplified products and different annealing efficiency of primer pairs on the cDNA template during the initial priming event, which is the most critical, uncontrollable variable.

## RESULTS

### Human, murine and chicken erythroid cells express multiple GATA factor mRNAs

Earlier studies had shown that members of the cGATA transcription factor family are expressed in a variety of



**Fig. 2.** Differential expression of cGATA factors in chicken hematopoietic cell lineages. Total RNA was isolated from the hematopoietic cells indicated. The same samples were analysed for cGATA-3 and cGATA-1, cGATA-2 and cGATA-2 and  $\beta$ -globin mRNAs (A, B and C, respectively). All reactions contained primers specific for the S17 ribosomal protein gene product as an internal control. Samples are: primitive red blood cells from 3 day old embryo (lanes 4); definitive red blood cells from anemic adult chickens (lanes 5); tsAEV-transformed HD3 erythroblasts cultured at 35°C or induced to differentiate by culturing at 42°C in the presence of anemic chicken serum (lanes 6 and 7, respectively); erythroblasts transformed by a recombinant virus expressing the human epidermal growth factor receptor (EGFR) and ts21v-myb oncogenes (Khazaie et al., 1988) cultured at 37°C (lanes 8) or induced to differentiate by culturing in the absence of hEGF but in the presence of anemic chicken serum and concentrated supernatant (REV) factor derived from NPB4 cells (Zenke et al., 1988) at 42°C for 1, 2 or 4 days (lanes 9-11, respectively); E26 virus-transformed multipotent progenitor clones HD50 and HD57 (lanes 12 and 14, respectively) and their myeloid derivatives, clones HD50 27M and HD57 1M (lanes 13 and 15, respectively; Graf et al., 1992); ts21 E26-transformed myeloid cells (Beug et al., 1984) cultured at 42°C for 0, 2 or 4 days (lanes 16-18, respectively); v-rel transformed pre-B/pre-T lymphoblast clone (P. Enrietto, unpublished data; lanes 19); RP-9, a transformed B-cell line (Beug et al., 1981; lanes 20); Marek's virus-transformed MSB-1 T-lymphoma cells (Akiyama and Kato, 1974; lanes 21). Samples containing control cDNAs (lanes 1-2 and data not shown) or the PCR master-mix without added cDNA (lanes 3) were also amplified as positive or negative controls. Sources for each of the cell types analysed are cited in the Materials and methods.

hematopoietic lineages and that all three cGATA factors are expressed at differing levels in differentiating erythroid cells (Yamamoto et al., 1990). In the present study, we have used a more sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) amplification technique to extend these investigations to earlier times during erythropoiesis and embryogenesis and to quantify the observed changes in GATA factor mRNA abundance.

To determine whether the expression of multiple GATA factors is a feature unique to avian erythroid cells, RT-PCR analysis was performed on a variety of human, murine and avian erythroid cell types. The results of this analysis confirm that the cGATA, mGATA and hGATA transcription factor families are conserved in their expression pattern during erythropoiesis (Fig. 1A,B and C, respectively). Erythroid cells of all three vertebrate species express GATA-1, GATA-2 and GATA-3. In contrast, B-lymphocytes do not express any GATA factor (Fig. 1A, lanes 7 and 14; B, lanes 6 and 13; C, lanes 7 and 15), thereby demonstrating that the detection of GATA transcripts in erythroid cells is not an artifact of the sensitivity of the methodology employed.

#### The relative abundance of the cGATA factors varies during erythroid differentiation

Previous RNA blot analyses indicated that the cGATA factors are expressed at varying levels during erythroid differentiation (Yamamoto et al., 1990). Because of the limited materials available, those studies were performed using equivalent numbers of differentiating cells, and thus on a per cell basis the abundances of GATA-1 and GATA-2 appeared to decrease as erythroid cells mature (Yamamoto et al., 1990). Minie et al. have similarly observed that the level of cGATA-1 decreases during avian erythroid development when the amount of factor is analysed, as in our earlier studies, on a per cell basis (Minie et al., 1992). However, such studies are complicated by the fact that the total RNA abundance per cell decreases rather precipitously as erythroid cells differentiate (Yamamoto et al., 1990; Zenke et al., 1988), likely reflecting the progression to transcriptional senescence as these cells mature to erythrocytes. In order to resolve this complication in quantitative analysis, we normalized expression levels to that of the small ribosomal protein 17 mRNA (S17; Trueb et al., 1988). Amplification of S17 thus provides an internal control for both the cDNA synthesis step and the total amount of material in the subsequent PCR reaction. A number of recent reports have similarly employed RT-PCR for the analysis of mRNA abundance (Braga et al., 1992; Camp et al., 1991; Foley and Engel, 1992; Keller et al., 1993) and the results obtained have proven concordant with abundances detected by RNA blot analysis when directly compared (e.g. Braga et al., 1992).

We initially examined cGATA expression in a variety of avian hematopoietic cells in order to compare it with prior results as a test of the validity of the RT-PCR methodology. All samples were analysed by PCR on at least two separate occasions using at least two independent cDNA preparations, and where possible, two different RNA preparations. Although the absolute values exhibit slight variability, the relative expression profile of all three GATA factors was found to be consistent both between experiments (when the

same RNA was assayed) and when compared to RNA blot analysis (compare Fig. 2, below, with Fig. 5 in Yamamoto et al., 1990).

cGATA-1 mRNA is present in all erythroid cell lines examined (including E26-transformed multipotent progenitors and tsAEV-transformed HD3 erythroblasts), but is not detected in a number of myeloid or lymphoid cells analysed (Fig. 2A, lanes 6, 12, 14 versus lanes 13, 15-21). When EGFR/*myb*-transformed primary definitive erythroblasts were induced to differentiate, cGATA-1 mRNA levels gradually accumulated to approximately 8-fold greater abundance after 4 days (Fig. 2A, lanes 8-11). This transcript is significantly more abundant in blood isolated from phenylhydrazine-treated adult chickens (definitive lineage cells) than from embryos (primitive lineage cells; Fig. 2A, lanes 5 and 4, respectively).

cGATA-2 is expressed in the same erythroid cell types as cGATA-1. Unlike GATA-1, cGATA-2 mRNA appears to be almost equally abundant in primitive and definitive primary erythroid cells (Fig. 2B, lanes 4-5). In differentiating EGFR/*myb* and AEV-transformed HD3 erythroblasts, the levels of cGATA-2 scarcely change during the course of differentiation (Fig. 2B, lanes 6-11).

An alternatively spliced variant of cGATA-2 (called cGATA-2') that has an additional 33 nucleotides inserted immediately 5' to the amino zinc finger (P. Ting, M. Yamamoto, M. W. L. and J. D. E., unpublished observations) is also expressed in both primitive and definitive primary erythroid cells. Interestingly, when either HD3 or EGFR/*myb* definitive erythroblasts are induced to differentiate terminally, the levels of cGATA-2' decrease within the first 24 hours by 10-fold or greater (Fig. 2B, lanes 6-11). Both cGATA-2 and cGATA-2' are expressed in E26-transformed multipotent progenitor cells as well as in *v-rel*-transformed pre-B/T lymphocytes (and in approximately the same relative abundance; Fig. 2B, lanes 12, 14 and 19), but neither product of the cGATA-2 gene is expressed in mature myeloid, B- or T-cell lineages (Fig. 2B, lanes 13, 15-18, 20-21).

cGATA-3 is hardly detectable in primitive erythroid cells but is abundant in anemic adult reticulocytes (Fig. 2A, lanes 4-5). It is also expressed at very low levels in EGFR/*myb*-transformed definitive erythroblasts but rapidly accumulates as these cells differentiate, being approximately 35-fold more abundant in the 4 day-induced than in the uninduced sample (Fig. 2A, lanes 8-11). HD3 erythroblasts express cGATA-3 at a level comparable to that of 1 day-induced EGFR/*myb* cells (Fig. 2A, compare lanes 6 and 9); in contrast, another (non-differentiating) AEV-transformed cell line, HD6, contains lower levels of cGATA-3 than uninduced EGFR/*myb* cells (Fig. 1A, lanes 4-5). While E26-transformed MEP cells expressed cGATA-3 at very high levels, mature myeloid cells expressed little or no cGATA-3 mRNAs (Fig. 2A, compare lanes 12 and 14 with lanes 13, 15-18). Of the lymphoid cells examined, cGATA-3 is most abundantly expressed in MSB mature T cells, less abundantly in *v-rel*-transformed pre-B/T lymphocytes and is undetectable in RP-9 mature B cells (Fig. 2A, lanes 21, 19 and 20, respectively). Both mGATA-3 and hGATA-3 also share a qualitatively similar expression profile in murine and human lymphopoiesis (Fig. 1; Joulin et al., 1991; Ko et al., 1991).

The chicken  $\gamma$ -globin gene enhancer, which contains two

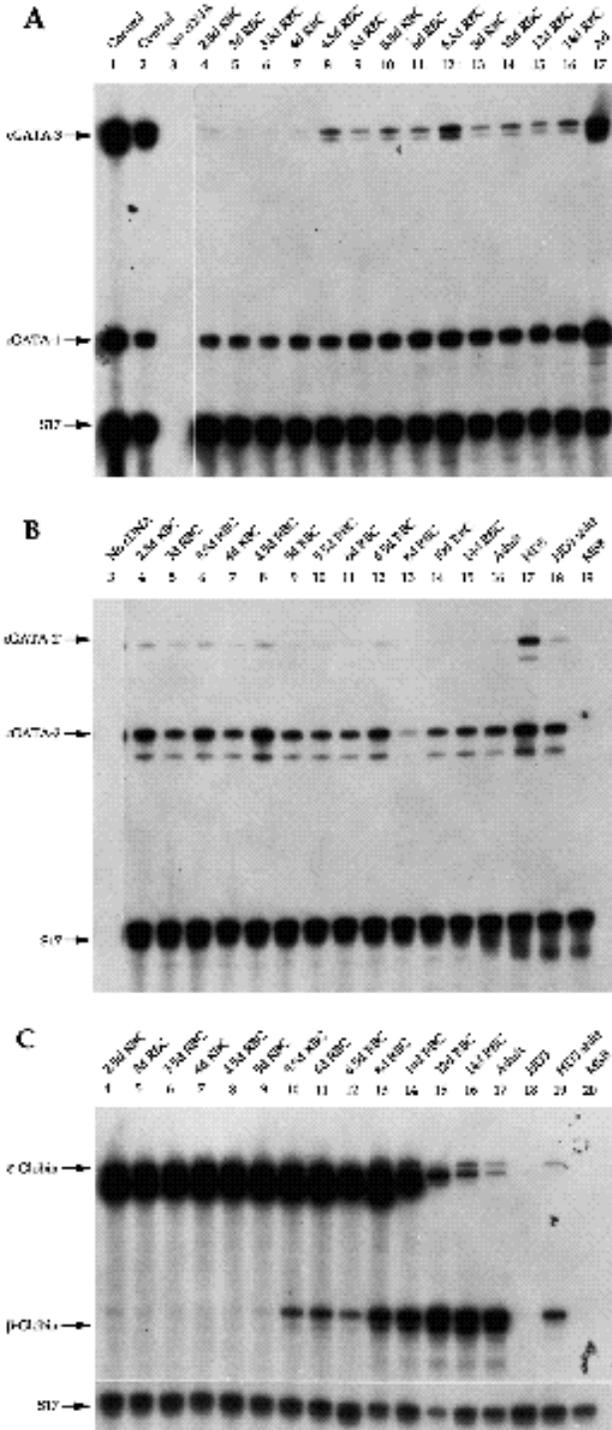
GATA factor binding sites, is functionally required for both embryonic  $\gamma$ - and adult  $\beta$ -globin gene activation (Choi and Engel, 1988; Reitman and Felsenfeld, 1988). In order to determine whether changes in  $\beta$ -globin gene expression within differentiating erythroblasts correlate with changes in the expression of particular GATA family members,  $\beta$ -globin transcription was assayed in the same samples as those analysed for GATA factor expression. The results show, as anticipated, that  $\beta$ -globin mRNA is abundantly

expressed in definitive erythroid cells but is not present in primitive erythroid or any non-erythroid cells examined (Fig. 2C, lanes 4-5, 12-21). When HD3 and EGFR/*myb* cells are induced to undergo differentiation,  $\beta$ -globin transcripts accumulate immediately (Fig. 2C, lanes 6-11). The rate (and profile) of  $\beta$ -globin mRNA accumulation in the latter cells correlates most closely with the increase in cGATA-3 expression.

**GATA-3 induction precedes the onset of definitive chicken erythropoiesis**

To ascertain the temporal expression profile and the relative abundance of GATA factors during erythroid development, total RNA isolated from circulating erythroid cells of day 2.5-day 14 embryos was analysed for cGATA mRNA abundance. All three cGATA factors are expressed in 2.5 day primitive erythroid cells. While the absolute levels of cGATA-2 and cGATA-2 show a small degree of variability from stage to stage, the trend appears to be that both mRNAs decrease slightly in abundance between 2.5 and 14 days of erythroid development (Fig. 3B, lanes 4-15). In contrast, cGATA-1 expression shows a similarly slight but gradual increase (2- to 4-fold) during the same period (Fig. 3A, lanes 4-16). Of the three GATA factors, the accumulation profile of cGATA-3 mRNA is most distinct during this important period of erythropoiesis when globin gene switching occurs (Bruns and Ingram, 1973). cGATA-3 mRNA is barely expressed in 2.5 to 4 day primitive erythroblasts but increases abruptly at day 4.5 and remains at an elevated level (approximately 10-fold greater abundance) through day 14 (Fig. 3A, lanes 4-16). The absolute levels of cGATA-3 also exhibit a slight degree of variability, as observed with cGATA-2. Thus all members of the cGATA family are differentially expressed throughout erythroid development. However, at the time of globin gene switching, only cGATA-3 mRNA levels change significantly.

The RNA samples examined for GATA factor expression during development were also analysed for  $\alpha$ - and  $\beta$ -globin mRNA accumulation. At 2.5 days of embryogenesis, the  $\beta$ -globin gene is already maximally expressed and its abundance begins to decline from day 10 onwards (as primitive erythrocytes are replaced by definitive cells; Fig. 3C, lanes 4-16; Brown and Ingram, 1974). As anticipated,  $\alpha$ -globin mRNA is barely detectable before day 5, but increases



**Fig. 3.** cGATA factor expression during erythroid cell development. Total RNA was prepared from erythroid cells of day 2.5-day 14 embryos. The same samples were analysed for cGATA-3 and cGATA-1, cGATA-2 and cGATA-2,  $\alpha$ - and  $\beta$ -globin RNAs (A, B and C, respectively). All reactions contained primers specific for the S17 ribosomal protein gene product as an internal control. Samples are: circulating erythroid cells isolated from embryos at 2.5 days to 14 days of development (lanes 4-16); definitive red blood cells from anemic adult chickens (lanes 17, A,C; lane 16, B); tsAEV-transformed HD3 erythroblasts cultured at 35°C (lane 17, B; lane 18, C) or induced to differentiate by culturing at 42°C in the presence of anemic chicken serum (lane 18, B; lane 19, C); MSB-1 T-lymphoma cell line (lane 19, B; lane 20, C). Samples containing 4 $\times$  (lanes 1 and data not shown) or 1 $\times$  control cDNAs (lanes 2 and data not shown) or the PCR master-mix without added cDNA (lanes 3 and data not shown) were also amplified as positive and negative controls.

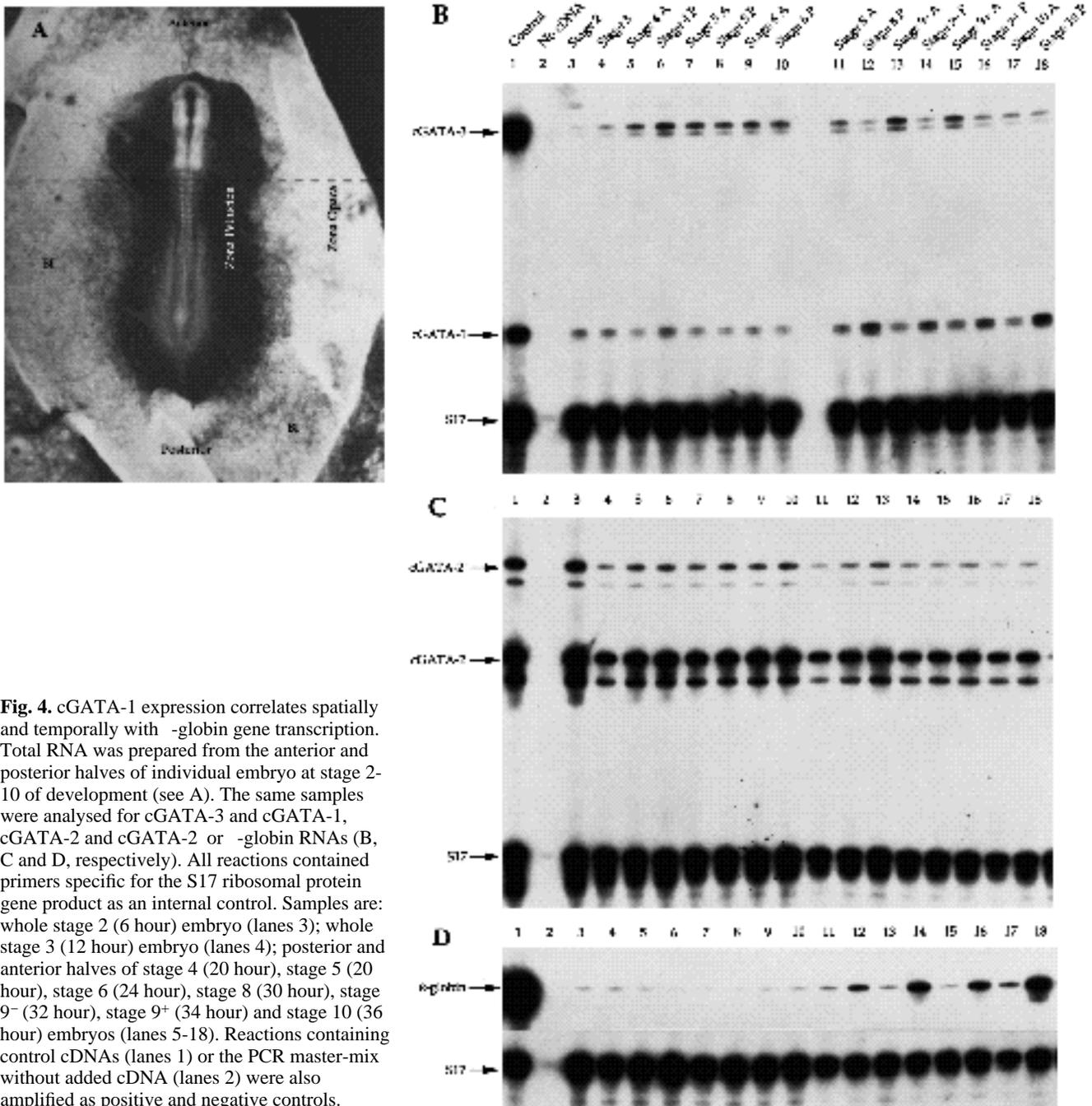
abruptly in abundance at 5.5 days of development (24 hours after cGATA-3 level is up-regulated) and reaches maximal levels by approximately day 8 (Fig. 3C, lanes 4-16).

### Individual GATA factors display unique spatial and temporal expression patterns in the developing embryo

To investigate the temporal appearance of cGATA transcription factors during early embryogenesis, total RNA was isolated from the anterior and posterior halves of stage 2 to 10 embryos and then analysed by RT-PCR. These represent embryos from the initial streak stage (early gastrula) to the

10 somite stage of embryogenesis (Hamburger and Hamilton, 1951).

Between embryonic stages 2 and 10, cGATA-2 and cGATA-2 mRNAs are expressed in essentially equal and constant abundance in the anterior and posterior of each embryo (Fig. 4C). cGATA-3 expression increases steadily between stages 2 and 4 and remains constant (and equally abundant anteriorly and posteriorly) through stage 6 (nominally 24 hours of embryonic development, at the head fold formation stage; Fig. 4B, lanes 3-10). From stage 8-10, GATA-3 mRNA is localized more abundantly in the anterior than posterior of the embryo (Fig. 4B, lanes 11-18), in direct



**Fig. 4.** cGATA-1 expression correlates spatially and temporally with  $\beta$ -globin gene transcription. Total RNA was prepared from the anterior and posterior halves of individual embryo at stage 2-10 of development (see A). The same samples were analysed for cGATA-3 and cGATA-1, cGATA-2 and cGATA-2 or  $\beta$ -globin RNAs (B, C and D, respectively). All reactions contained primers specific for the S17 ribosomal protein gene product as an internal control. Samples are: whole stage 2 (6 hour) embryo (lanes 3); whole stage 3 (12 hour) embryo (lanes 4); posterior and anterior halves of stage 4 (20 hour), stage 5 (20 hour), stage 6 (24 hour), stage 8 (30 hour), stage 9<sup>-</sup> (32 hour), stage 9<sup>+</sup> (34 hour) and stage 10 (36 hour) embryos (lanes 5-18). Reactions containing control cDNAs (lanes 1) or the PCR master-mix without added cDNA (lanes 2) were also amplified as positive and negative controls.

contrast to the accumulation pattern of cGATA-1. cGATA-1 is expressed at a low and essentially constant level in both the anterior and posterior of stage 2-stage 6 embryos (Fig.

4B, lanes 3-10). However, beginning at stage 8 (at the time of overt blood island formation; Romanoff, 1960), cGATA-1 mRNA is more abundantly expressed in the posterior (the

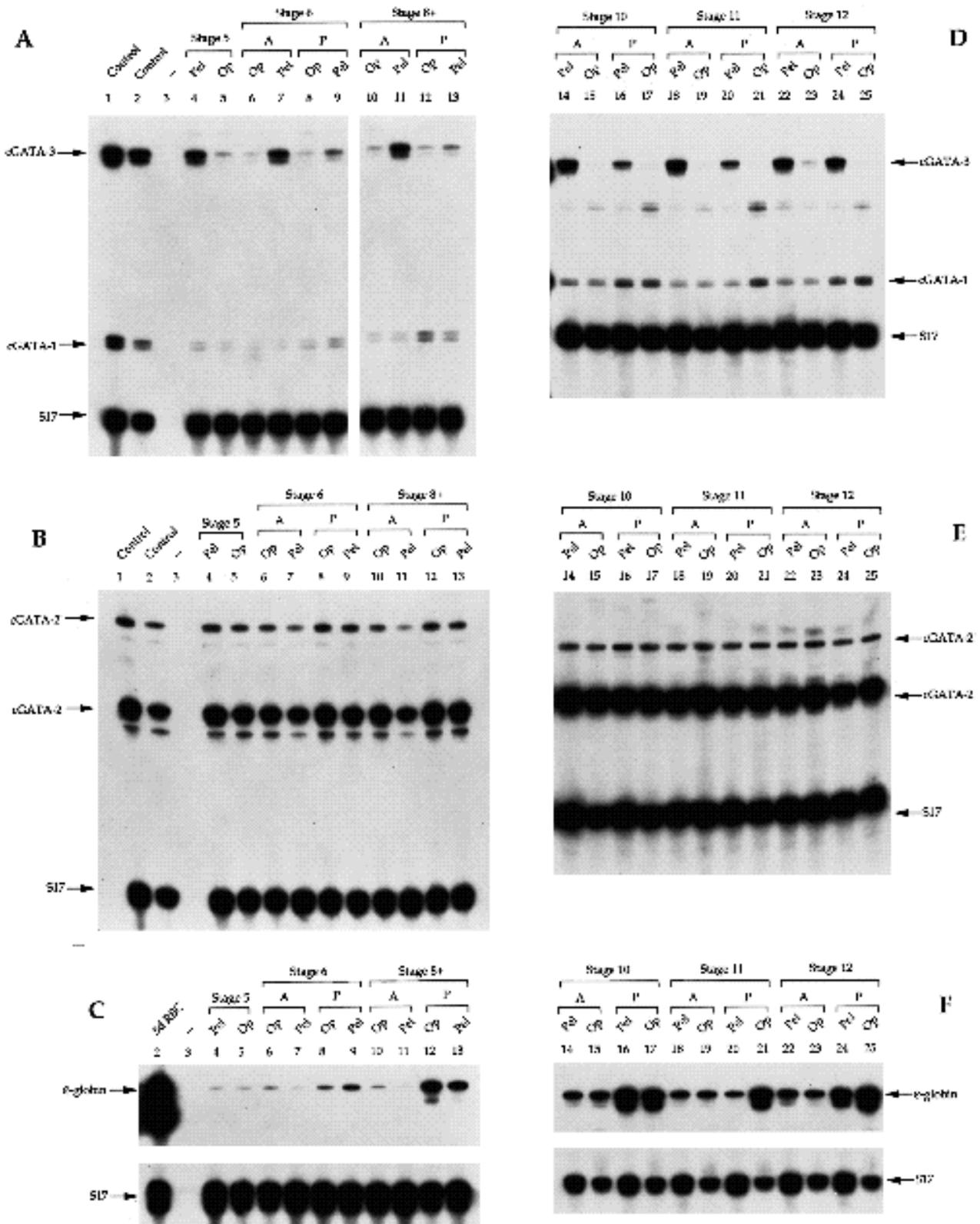


Fig. 5

site of blood island formation) than in the anterior embryonic mesoderm (Fig. 4B, lanes 11-18).

These same RNA samples were also analysed for  $\beta$ -globin expression. This gene is transcribed at a constant (albeit very low) level in stage 2 to stage 6 embryos, but is significantly induced from stage 8 onwards, as anticipated (Fig. 4D, lanes 3-10). While  $\beta$ -globin is not preferentially distributed in either half of the embryo before stage 8, it becomes more abundant in the posterior than the anterior segments later in development (Fig. 4D). Thus  $\beta$ -globin gene activation shows a strong spatial and temporal correlation with that observed for cGATA-1, and is clearly distinct from the expression profiles of cGATA-2 or cGATA-3 mRNAs in these early embryos.

The dissected anterior and posterior halves of the embryo examined in the previous experiment contain both the zona pellucida (the developing embryo proper) and the peripheral zona opaca (containing the developing blood island). To determine if the expression of cGATA factor is restricted to either the embryo proper or to the surrounding embryonic tissue of the zona opaca, stage 5-12 embryos were dissected into anterior and posterior segments and then further dissected into either opaca or pellucida (see Fig. 4A). These samples were then analysed for GATA factor expression.

cGATA-2 and cGATA-3 once again display strikingly uniform expression in each of the dissected portions of the embryo and surrounding tissue (Fig. 5B,E). In contrast, cGATA-1 expression is, in essence, entirely restricted to the zona pellucida (Fig. 5A,D) and is more abundant in the anterior (lanes 7, 11, 14, 18 and 22) than in the posterior (lanes 9, 13, 16, 20 and 24) of the embryo, as shown above. The distribution of cGATA-1 transcripts once again differs from that of the other family members (Fig. 5A,D). To our surprise, cGATA-1 is expressed in all of the dissected segments analysed here, although it tends to be more abundant in the posterior (the region of blood island formation) than in the anterior halves of both the embryo proper and surrounding opaca.

These same samples were also analysed for  $\beta$ -globin transcription, and again the expression of this gene was found to correspond precisely to that of cGATA-1. Thus in the stage 10 embryo shown, both cGATA-1 and  $\beta$ -globin mRNAs (Fig. 5D,F, respectively) are expressed at higher levels in the posterior than in the anterior halves of the embryo (lanes 16-17 versus lanes 14-15). In the stage 11

embryo, both cGATA-1 and  $\beta$ -globin mRNAs (Fig. 5D,F, respectively) are expressed at equivalent levels in both anterior pellucida and opaca and in the posterior pellucida (lanes 18-20), but both are significantly more abundant in the posterior opaca (lanes 21). These results show that each factor has a distinctive temporal and spatial accumulation pattern. Moreover, transcriptional activation of the embryonic  $\beta$ -globin gene correlates precisely with cGATA-1 expression during early embryogenesis. In contrast to our original expectations, cGATA-1 is not only expressed in the classically defined region of blood island formation but throughout the embryo proper.

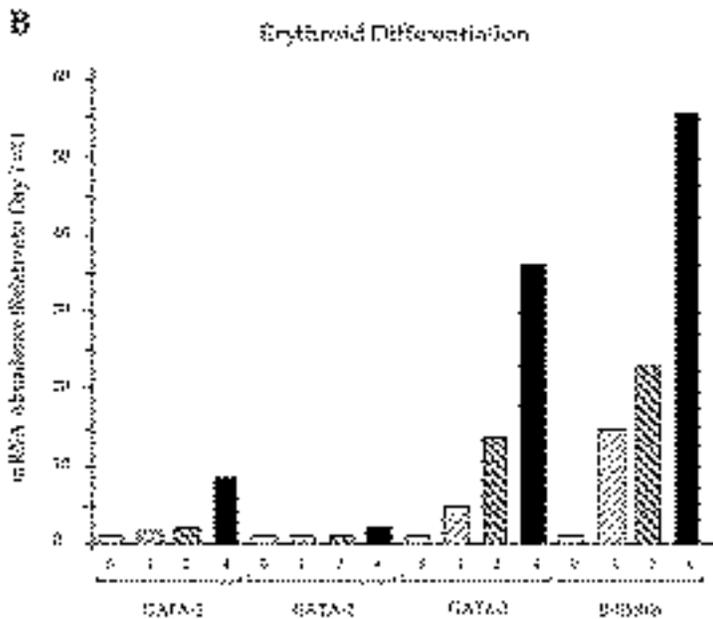
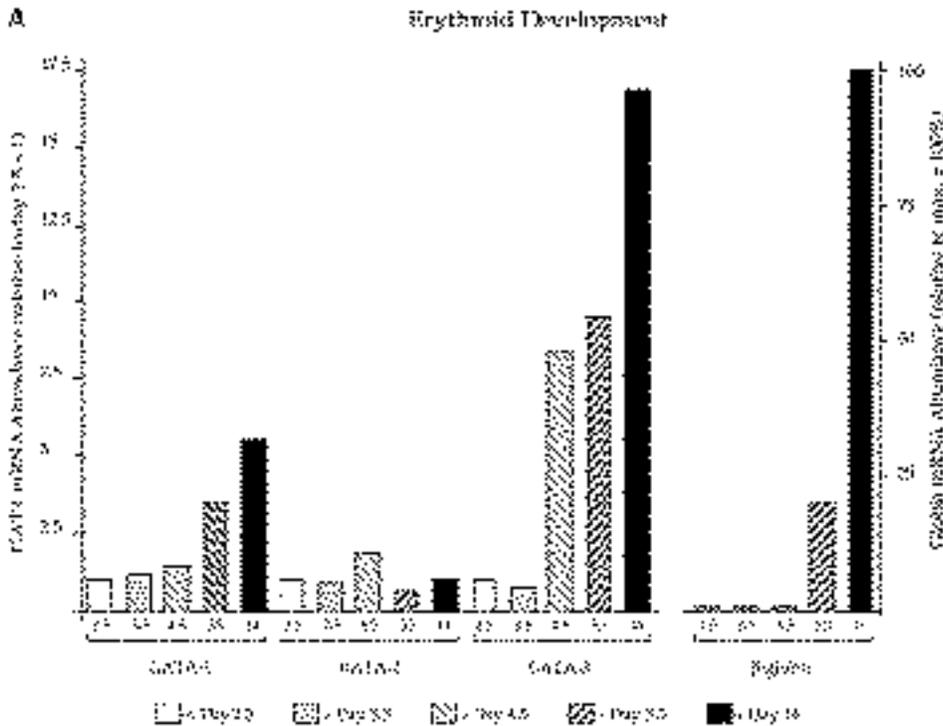
## DISCUSSION

At the time of their first appearance, progenitor cells (which eventually become the distinct tissues of a mature organism) inevitably make up only a minuscule fraction of the whole. Thus their examination requires an assay of extreme sensitivity. While other studies have reported steady state RNA levels or GATA binding activity during erythropoiesis (Minie et al., 1992; Yamamoto et al., 1990), these assays suffer from an inherent insensitivity when examining small numbers of cells such as fractions of a single embryo. In this paper, we have taken an approach based on PCR detection of mRNAs to trace the expression of GATA factors during early chicken embryonic development. Since erythroid cells in the circulating blood of embryos represents a heterogeneous population at a variety of differentiation stages (each of which may express quite different levels of GATA factor mRNA or protein), we compared the level of GATA mRNAs with an internal standard, which is reflective of general cellular metabolism (Keller et al., 1993), as well as to a second internal standard, which is expressed only during late erythroid differentiation (namely globin). Although not without limitations, the increased sensitivity of the RT-PCR assay for very small sample sizes makes it most suitable for the task of detecting GATA factor expression during very early developmental times.

Since the cloning of the erythroid-restricted GATA-1, much effort has focused on elucidating the role of this transcription factor in erythropoiesis. GATA-1 expression is clearly of paramount importance in the generation of a mature erythroid phenotype (Pevny et al., 1991; Simon et al., 1992). However, it is but one member of a multi-gene family expressed in vertebrate red blood cells that can bind to, and activate transcription from, the GATA motif (Yamamoto et al., 1990), immediately raising questions as to the roles that other GATA factors may play in erythroid lineage development. In particular, functionally important GATA binding sites have been identified in a number of erythroid-specific genes, thereby raising the question of which GATA factor actually binds to this motif *in vivo* and whether changes in expression of different members of the GATA factor family might contribute to differential gene activation during development.

The observation that cGATA-1 is more abundantly expressed in erythroid cells of the definitive than the primitive lineage is consistent with previous studies on the accumulation of mGATA-1 mRNA during murine develop-

**Fig. 5.** Individual GATA factors exhibit unique spatial patterns of expression in the developing chicken embryo. Individual embryos at developmental stages 5-12 were dissected into anterior or posterior pellucida or opaca sections (see Fig. 4A) and total RNA was prepared from each section. The same samples were analysed for cGATA-3 and cGATA-1 (A,D), cGATA-2 and cGATA-2 (B,E) or  $\beta$ -globin gene expression (C,F). All reactions contained primers specific for the S17 ribosomal protein gene product as an internal control. Samples are: zona pellucida (embryo proper) and surrounding zona opaca of stage 5 (20 hour) embryo (lanes 4 and 5, respectively); anterior pellucida or opaca and posterior pellucida or opaca of stage 6 (24 hour), stage 8<sup>+</sup> (30 hour), stage 10 (36 hour), stage 11 (44 hour) and stage 12 (48 hour) embryos (lanes 6-25). Reactions containing control cDNAs (lanes 1 and 2) or the PCR master-mix without added cDNA (lanes 3) were also amplified as positive and negative controls.



**Fig. 6.** Relative expression of GATA factors mRNA during erythroid development and differentiation. Results shown in Figs 2 and 3 (and data not shown) were quantitated using a Molecular Dynamics PhosphorImager. The relative abundance of cGATA factor and  $\beta$ -globin mRNAs during erythroid development (A: red blood cells isolated from 2.5 day, 3.5 day, 4.5 day, 5.5 day and 14 day embryos; from Fig. 3, lanes 4, 6, 8, 10 and 16, respectively) and erythroid differentiation (B: EGFR/myb erythroid cells; from Fig. 2, lanes 8-11) are represented graphically. In each case, the amount of GATA factor or globin expression was normalized to the equivalent S17 small ribosomal protein subunit RNA present in the sample.

ment (Whitelaw et al., 1990). Those studies showed that a modest increase in mGATA-1 mRNA level in the fetal liver coincided with the appearance of  $\beta$ -globin gene transcripts, thereby implicating mGATA-1 in the induction of  $\beta$ -globin gene expression (Whitelaw et al., 1990). We detect a similar increase in cGATA-1 expression in erythroid cells during the time of the switch in globin gene transcription. However, when the expression profile of all three cGATA factors is compared in the same samples with the patterns of  $\beta$ - and  $\delta$ -globin gene expression during both erythroid development

and differentiation, the data most strongly infer a role for cGATA-3 (which is expressed only at very low abundance in primitive cells) in activating gene expression in definitive erythroid cells (summarized in Fig. 6).

Both cGATA-1 and cGATA-2 are detected as early as stage 2 of embryogenesis, and are uniformly distributed in the anterior and posterior halves of stage 4-6 embryos. Intriguingly, a low and constant level of  $\beta$ -globin gene expression is also detected in all of these regions of the early chicken embryo as is expression of *c-myc*, a proto-oncogene

present at high levels in immature hematopoietic cells (data not shown; Coll et al., 1983; Gonda et al., 1982; Westin et al., 1982). cGATA-1 and cGATA-2 mRNAs are both abundantly expressed in the posterior region of stage 8 and older embryos (the site and time of first hemoglobinisation; Lillie, 1965); however, a striking spatial and temporal correlation exists between the patterns of cGATA-1 and  $\beta$ -globin gene expression. Zon and colleagues similarly observed GATA-1 expression considerably earlier in amphibian development than the appearance of ventral blood islands (Zon et al., 1991); they also detected *Xenopus* larval globin mRNA expression at a much earlier developmental stage than had previously been demonstrated (again, significantly prior to blood island formation). Such observations suggest that erythroid precursors are present earlier in embryogenesis and are more widely distributed within the extra-embryonic mesoderm than previously determined. Consistent with the pattern of GATA-1 and  $\beta$ -globin expression detected here, previous embryological studies have shown that anterior and posterior portions of the blastoderm, when transplanted prior to blood island formation, are capable of generating erythroid lineage cells (Lillie, 1965). Thus additional inductive signals appear to be required to stabilize the erythroid lineage developmental program in cells initially located in the posterior zona opaca. In a manner analogous to the pattern of cGATA-1 expression described here, Rupp and Weintraub (1991) have similarly reported ubiquitous expression of the muscle-determining factor MyoD throughout the early *Xenopus* embryo, with transcription subsequently stabilized only in the presumptive mesoderm.

We and others have shown that all members of the GATA factor family cloned to date can *trans*-activate GATA-responsive reporter genes (Joulin et al., 1991; Ko et al., 1991; Martin and Orkin, 1990; Yamamoto et al., 1990). Although gel mobility shift assays initially suggested that the cGATA factors bind to consensus GATA motif with similar affinity (Yamamoto et al., 1990), more rigorous examination shows that different members of the family preferentially recognize specific subsets of sequences (Ko and Engel, 1993), including the previously defined erythroid consensus motif (Catala et al., 1989; Evans et al., 1988; Martin et al., 1989; Perkins et al., 1989; Wall et al., 1988). This observation, taken together with the demonstration here that their relative abundance varies during both erythroid development and differentiation, strongly suggests that this family of transcription factors (and not just the erythroid-restricted family member GATA-1) plays a broader and more complex role in the regulation of erythroid-specific gene expression than previously recognized. For example, it has recently been shown that ectopic expression of cGATA-2 in erythroid progenitor cells blocks differentiation (Briegleb et al., 1993).

Changes in the relative abundance of GATA factors could contribute to differential gene regulation as a result of the factors competing for the same DNA sites or specific family members acting at different DNA sites to elicit distinct transcriptional responses. Data accumulated thus far suggest that both mechanisms may contribute to the differentiation program in erythroid cells. The GATA binding sites within the  $\beta$ -globin gene enhancer provide a potential target for such differential GATA factor interactions. The data

presented here are compatible with a model in which GATA-1 binding to the  $\beta$ -globin gene enhancer facilitates interaction with the  $\beta$ -globin gene promoter to drive  $\beta$ -globin transcription in primitive erythroid cells (Choi and Engel, 1988; Foley and Engel, 1992; Gallarda et al., 1989). In definitive erythroid progenitor cells, cGATA-3 might replace cGATA-1 at the  $\beta$ -globin enhancer and, in cooperation with stage-specific promoter-binding factors such as NF-E4, preferentially activate  $\beta$ -globin gene transcription (Choi and Engel, 1988; Gallarda et al., 1989). One can readily envisage how the sequence divergence between GATA-1 and GATA-3 proteins outside of the DNA binding domain (Yamamoto et al., 1990) might mediate distinct interactions between the shared enhancer and either the  $\beta$ - or  $\beta$ -globin gene promoter. However, GATA-1 and GATA-3 exhibit no significant difference in their intrinsic affinity for the chicken  $\beta$ -globin enhancer GATA sites *in vitro* (Ko and Engel, 1993). Thus, such a model would additionally require that differences in the tertiary structure of the proteins provide specificity to their binding to the enhancer GATA sites by steric constraints imposed by other factors bound (adjacent to the GATA sites) within the enhancer module. Potential steric constraints aside, it seems likely that rather than competing with the abundant GATA-1 factor for the same sites, GATA-2 and GATA-3 may elicit distinct transcriptional regulatory responses from a separate array of genes by virtue of their higher binding affinity to newly identified alternative consensus sites, GATCT (Ko and Engel, 1993).

What might be the significance of the differential cGATA-3 expression detected in these early embryos? One of the recently identified sites of prominent GATA-3 expression is the brain, and recent studies have confirmed that cGATA-2 and cGATA-3 are expressed in a specific pattern in the chicken (particularly within the optic lobes) from 4 days of embryogenesis onwards (Kornhauser, Leonard, Yamamoto, LaVail, Mayo and Engel, unpublished observations). We show here that at the time of onset of mid-brain differentiation (stage 5-6, approximately 20-24 hours; Romanoff, 1960), high level of cGATA-3 expression is essentially restricted to the pellucida region (the embryo proper) and is more abundant anteriorly than posteriorly. Furthermore, this transcription factor is expressed at only low levels in early gastrula (stage 2) but increases in abundance in the definitive streak (stage 4) and later embryos. Taken together, these data suggest a role for cGATA-3 in neurogenesis from the very earliest stages of development.

In summary, the present studies show that expression of the GATA transcription factor family varies in a precise fashion during early avian embryonic development and erythroid differentiation. GATA-1 and GATA-2 are expressed from the earliest stages of embryogenesis and in erythroid cells throughout development. Although co-expression of GATA-1 and GATA-2 may be important for the regulation of the erythroid lineage, activation of the embryonic  $\beta$ -globin gene during embryogenesis clearly correlates most strongly with increased GATA-1 transcription. In contrast, cGATA-3 is expressed at very low levels in the blood island region and in primitive erythroid cells but is dramatically up-regulated at the onset of definitive erythropoiesis, clearly preceding the induction of adult  $\beta$ -globin

gene transcription. A similar induction of GATA-3 expression is also detected during terminal differentiation of definitive erythroblasts. We postulate that changes in the relative abundance of GATA family members may influence which factor occupies a given erythroid consensus or alternative consensus GATA site (Ko and Engel, 1993) to mediate differential gene regulation during erythropoiesis. As described here, GATA factor binding to the chicken  $\gamma$ -globin gene enhancer is therefore a potential cellular target for this activity. Finally, we show that GATA-1, GATA-2 and GATA-3 are expressed in human and murine erythroid cells, as in chickens. This observation suggests a possibly conserved role for this family of transcription factors in vertebrate erythropoiesis, and aspects of the differential gene activation role postulated for the avian GATA family might also be applicable to murine and human erythropoiesis.

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