

Regulation and function of transcription factor GATA-1 during red blood cell differentiation

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SUMMARY

The tissue-specific transcription factor GATA-1 is a key regulator of red blood cell differentiation. One seemingly contradictory aspect of GATA-1 function is that, while it is abundant in erythroid progenitor cells prior to the onset of overt differentiation, it does not significantly activate known GATA-1 target genes in those cells. To investigate the mechanisms underlying GATA-1 function during the transition from early to late erythropoiesis, we have examined its expression and activity in normal avian erythroid progenitor cells before and after induction of differentiation. In these primary progenitor cells, GATA-1 protein was predominantly located in the cytoplasm, while induction of differentiation caused its rapid relocalization to the nucleus, suggesting that nuclear translocation constitutes an important regulatory step in GATA-1 activation. As an alternative way of addressing the same question, we also ectopically expressed a GATA-1/estrogen receptor

fusion protein (GATA-1/ER) in red blood cell progenitors, where nuclear translocation of, and transcriptional activation by, this hybrid factor are conditionally controlled by estrogen. We found that hormone-activated GATA-1/ER protein accelerated red blood cell differentiation, and concomitantly suppressed cell proliferation. These phenotypic effects were accompanied by a simultaneous suppression of *c-myb* and GATA-2 transcription, two genes thought to be involved in the proliferative capacity of hematopoietic progenitor cells. Thus, GATA-1 appears to promote differentiation in committed erythroid progenitor cells both by inducing differentiation-specific genes and by simultaneously suppressing genes involved in cell proliferation.

Key words: GATA-1 transcription factor, nuclear translocation, phosphorylation, erythroid differentiation, red blood cell, transcription factor

INTRODUCTION

During erythropoiesis, erythroid progenitor cells are controlled by different sets of growth and differentiation factors which induce the complex signalling cascades required both for limited proliferation and for terminal differentiation. Several groups of transcription factors are thought to translate the information from various signalling pathways into changes in erythroid gene expression. One major group are erythroid-specific zinc finger-type transcription factors, such as the GATA factor family (Yamamoto et al., 1990) and EKLF, a homologue of the *Drosophila* Krüppel protein (Miller and Bieker, 1993; Nuez et al., 1995; Perkin et al., 1995). In addition, members of the steroid/thyroid nuclear hormone receptor family [e.g. the thyroid hormone receptor *c-erbA* type α (*c-erbA/TR- α*), the retinoic acid receptors (RAR, RXR) and estrogen receptor (ER)] positively or negatively regulate erythroid differentiation (Zenke et al., 1990; Schroeder et al., 1992, 1993; Baretino et al., 1993). Furthermore, both the basic

helix-loop-helix (bHLH) transcription factor Tal-1/SCL and the LIM domain transcription factor LMO2 (previously called *rbt-2*) are essential for establishment of the erythroid lineage (Aplan et al., 1992; Warren et al., 1994; Shivdasani et al., 1995; Robb et al., 1995). Finally, the nuclear proto-oncoprotein *c-myb* is required for the generation of definitive red blood cells (Mucenski et al., 1991).

GATA-1, the first major erythroid lineage-restricted transcription factor to be identified, binds to the consensus DNA motif WGATAR that is contained in virtually all erythroid-specific genes (reviewed in Weiss and Orkin, 1995). Together with two other members of the GATA factor family, GATA-2 and GATA-3, GATA-1 shows a distinct and highly conserved expression pattern during red blood cell differentiation (Evans et al., 1988; Evans and Felsenfeld, 1989; Tsai et al., 1989; Yamamoto et al., 1990; Leonard et al., 1993). GATA-1 is also expressed in megakaryocytes, mast cells, granulocytes and the testis (Martin et al., 1990; Romeo et al., 1990; Ito et al., 1993; Zon et al., 1993). GATA-2 is abundantly

expressed in immature erythroid progenitors and declines during the course of maturation (Orkin, 1992; Briegel et al., 1993; Leonard et al., 1993), but it is also found in many other hematopoietic and nonhematopoietic tissues (Yamamoto et al., 1990; Zon et al., 1991b). GATA-3 expression is restricted mainly to T lymphocytes and to specific neurons (Yamamoto et al., 1990; Ko et al., 1991, Kornhauser et al., 1994; George et al., 1994). Additionally, the recently described GATA-4/5/6 define a new subfamily of GATA factors which are expressed in developing heart and gut (Laverriere et al., 1994).

The GATA factors play essential roles in erythroid differentiation: on one hand, GATA-1 represents a major regulator of red cell differentiation as demonstrated by gene inactivation and rescue experiments in vivo and in vitro (Pevny et al., 1991; Simon et al., 1992). GATA-2, on the other hand, has been implicated in self-renewal and maintenance of the immature state of erythroid progenitor cells (Briegel et al., 1993). Additionally, more recent gene inactivation studies demonstrate a more pervasive effect of GATA-2 on all hematopoietic cells (Tsai et al., 1994), while targeted disruption of GATA-3 appears to specifically abolish definitive erythropoiesis (Pandolfi et al., 1995).

GATA-1 mRNA is abundantly expressed in immature erythroid progenitors prior to the onset of terminal differentiation (Yamamoto et al., 1990; Whitelaw et al. 1990; Daylot et al., 1993, Leonard et al., 1993). This observation has raised the possibility that the activity of the GATA-1 protein might be regulated during erythroid cell development. Although recent work reported that the phosphorylation pattern of GATA-1 changes in differentiation-induced mouse erythroleukemia (MEL) cells, no biological effect could be attributed to this event (Crossley and Orkin, 1994). A major difficulty in elucidating details of the molecular events underlying GATA-1 function has been that most studies are based on in vitro erythroid culture systems that principally rely on established cell lines, where the functional properties of GATA-1 might be aberrantly altered.

We report here a detailed analysis of GATA-1 protein activity, employing primary avian erythroid progenitors (Beug et al., 1994, 1995), wherein normal erythropoiesis can be reconstituted in tissue culture. Previously, temperature-sensitive (ts)-oncogene-transformed and normal erythroid progenitor cells of chicken, whose self-renewal and terminal differentiation can be precisely modulated (Knight et al., 1988; Pain et al., 1991; Hayman et al., 1993; Steinlein et al., 1995), have provided useful systems to explore GATA-2 function and the activity of the nuclear hormone receptors *c-erbA*/T3R, RAR and RXR, and estrogen receptor (ER), during erythropoiesis (Barettino et al., 1993; Briegel et al., 1993; Schroeder et al., 1993). In this study, we addressed the question as to whether or not the activity and subcellular distribution of the GATA-1 protein might be altered during differentiation of these cells and, if so, what the causes and functional consequences of these changes might be. In addition, to further characterize the role that GATA-1 might play in red blood cell maturation, we ectopically expressed a conditional GATA-1/estrogen receptor (ER) fusion protein in *ts-v-sea* oncogene-transformed erythroid precursors, a strategy successfully employed for analysis of GATA-2 function (Briegel et al., 1993).

Here we show that GATA-1 is post-translationally regulated

by an (as yet undefined) nuclear translocation process in primary erythroid progenitor cells. We found that GATA-1 is predominantly located in the cytoplasm in these proliferating progenitors, while induction of erythroid differentiation causes GATA-1 to translocate into the nucleus. Most interestingly, nuclear GATA-1 was found to be hyperphosphorylated. Furthermore, we show that the chimeric GATA-1/ER protein accelerates terminal red blood cell differentiation in a strictly estrogen-responsive fashion. The accelerated differentiation induced by ectopic GATA-1/ER appears to be attributable to both the activation of differentiation-specific genes and the simultaneous suppression of genes involved in erythroid self-renewal.

MATERIALS AND METHODS

Tissue culture

Normal SCF-dependent erythroid progenitor cells were prepared by cultivating bone marrow cells from 3- to 7-day-old SPAFAS chicks in modified CFU-E medium (Radke et al., 1982; Hayman et al., 1993) supplemented with 100 ng/ml of recombinant avian stem cell factor (SCF; Bartunek et al., 1996). Homogeneous mass cultures of these progenitors were obtained as described earlier (Hayman et al., 1993).

For transformation of fibroblasts with GATA-1/ER-expressing retroviral constructs, chick embryo fibroblasts (CEF) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal calf serum (FCS) and 2% chicken serum (referred to as standard growth medium). CEF were transfected with 10 µg of DNA of the retroviral vector pSFVCV (Fuerstenberg et al., 1990) containing the GATA-1/ER cDNA together with 1 µg RCAN helper virus DNA, as described before (Briegel et al., 1993). After G418 selection, these virus-releasing CEF were trypsinized and used in further experiments.

To generate primary, GATA-1/ER-expressing erythroid progenitor clones, virus-producing GATA-1/ER CEF and *ts-v-sea* transformed CEF were mixed, treated with mitomycin C (1 hour, 5 µg/ml) and used for coinfection of bone marrow cells (Briegel et al., 1993). The outgrowth of normal erythroid progenitors was primed by addition of 100 ng/ml SCF during coinfection. Subsequently, aliquots of the cells were seeded into CFU-E methocel containing 2 mg/ml G418 and neomycin-resistant clones were isolated and expanded in CFU-E medium (Radke et al., 1982).

Differentiation assays

To induce erythroid differentiation, mass cultures of SCF-dependent red blood cell progenitors were incubated in differentiation medium (Zenke et al., 1990) supplemented with 3% anemic chicken serum (as a source for erythropoietin) plus 10 ng/ml insulin at 37°C. *Ts-v-sea* transformed erythroblasts were shifted to 42°C (to inactivate the *ts-v-sea* oncogene product) under identical medium conditions. GATA-1/ER activity was turned on and off by addition of either 10⁻⁶ M β-estradiol and 10⁻⁶ M ICI 164,384 (ICI), respectively (Wakeling and Bowler, 1988a,b). Erythroid differentiation was assayed by counting undifferentiated, partially differentiated and differentiated erythroid cells in cytospin preparations stained with neutral benzidine and by measuring [³H]thymidine incorporation as well as hemoglobin accumulation according to Beug et al. (1995). [³H]thymidine incorporation and hemoglobin measurements were normalized to cell numbers. Cell numbers and cell size were determined by employing the CASY-1 Cell Counter and Analyser System.

Cell fractionation

SCF-dependent erythroid progenitors prior to and 2, 24 and 48 hours post-induction of differentiation were harvested, washed once with PBS and fractionated. Briefly, 1 × 10⁷ cells were lysed in 1 ml lysis

buffer (50 mM Tris-HCl pH 7.5, 1 mM MgCl₂, 0.5% Triton X-100, 10 mM DTT, 1 mM PMSF, 28 µg/ml Trasylol) for 10 minutes on ice. The cytoplasmic and nuclear fractions were separated by centrifugation at 3500 revs/minute in an Eppendorf centrifuge (10 minutes). After recovery of the cytoplasmic supernatants, nuclear pellets were washed once with lysis buffer followed by a wash with 50 mM Tris-HCl pH 7.5, 1 mM MgCl₂ and resuspended in 100 µl of extraction buffer (30 mM Tris-HCl pH 7.5, 0.2 M NaCl, 5 mM MgCl₂, 10% glycerol). Lysis of nuclei was achieved by adding NaCl to a final concentration of 0.4 M and subsequent incubation on ice for 20 minutes. The nuclear extracts obtained were depleted from debris by centrifugation at 15000 revs/minute for 15 minutes. Subsequently, both the cytoplasmic and nuclear extracts were used for western blot analysis and electrophoretic mobility shift assays.

Preparation of polyclonal GATA-1-specific anti-peptide antibody

A 13-mer peptide corresponding to a specific epitope of the chicken GATA-1 protein (Yamamoto et al., 1990; amino acids 1-13) plus a C-terminally attached 3 amino acid spacer: MEFVALGGPDAGS-GGC, was synthesized on an Applied Biosystems 431A peptide synthesizer. Preparation of antigenic peptide-soybean trypsin inhibitor (STI) conjugate and rabbit immunization was performed as described earlier (Briegel et al., 1993). Sera were taken 10 to 14 days after each injection and tested for immunoreactivity against endogenous GATA-1 protein in HD3 erythroblasts (Beug et al., 1982) in western blot analysis. After the fourth boost, high titer serum was obtained. For immunofluorescence studies, the GATA-1 antiserum was purified by CM Affi-Gel blue chromatography (BioRad).

Immunofluorescence and confocal microscopy

SCF-dependent erythroid progenitor cells were taken prior to and 24 hours after differentiation induction, washed in PBS and mounted onto adhesion slides (BioRad). Immunofluorescence was performed as described earlier (Boehmelt et al., 1992), using polyclonal GATA-1-specific anti-peptide antibody (see above) and a c-kit-specific mouse monoclonal antibody (kindly provided by M. Hayman, SUNY, Stony Brook, USA). Secondary antibodies labelled with fluorochromes were FITC- or TRITC-conjugated goat anti-rabbit and goat anti-mouse antibodies (Sigma). Images were taken with a confocal microscope (Zeiss Axiopt fluorescent microscope equipped with a BioRad laser) and processed by Comos software.

RNA analysis

RNA was isolated from either mass cultures of SCF-dependent erythroid progenitor cells or individual *ts-v-sea*-transformed erythroblast clones and analysed by northern blotting as described earlier (Briegel et al., 1993). Probes corresponding to the full-length cDNAs of the chicken GATA-1 or GATA-2 (Yamamoto et al., 1990), to the *v-myb* *EcoRI-XbaI* fragment, the *EcoRI-HindIII* fragment of the chicken β -globin gene and to 18S rRNA standard were used (Dolan et al., 1983; Zenke et al., 1990). Blots were hybridized and washed at moderate stringency, exposed to film and/or evaluated by Phosphor-Imager and ImageQuant software.

Western blot analysis

Total, cytoplasmic and nuclear extracts from SCF-dependent red blood cell progenitors, or total cell extracts from GATA-1/ER-expressing *ts-v-sea*-transformed erythroblasts, respectively, were separated on a 10% SDS-PAGE gel and blotted onto nitrocellulose membranes (BA85, Schleicher & Schuell). After blocking membranes overnight in TBS (25 mM Tris-HCl pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM CaCl₂, 0.5 mM MgCl₂, 0.6 mM Na₂HPO₄) containing 3% BSA, 1 mM EDTA, 0.05% Tween-20 at 37°C in a roller incubator, and two washes with wash buffer (50 mM Tris-HCl pH 8.0, 0.1 M NaCl, 0.1% Tween-20) the first antibody reactions were performed in wash buffer for 1 hour at 37°C. The primary antibodies

were polyclonal GATA-1-specific anti-peptide antibody (see above), monoclonal GATA-1-specific (CG1-1.2.2) or ER-specific (F3; Ali et al., 1993) antibodies (hybridoma cell supernatant). Subsequently, the blots were washed 5 times with wash buffer and incubated with the appropriate second antibodies of the ECL kit (Amersham) in TBS supplemented with 5% non-fat milk powder for 45 minutes at 37°C. The membranes were washed another 5 times with wash buffer, developed in ECL-reagents (Amersham) and exposed to film.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using a ³²P-radiolabelled oligonucleotide containing a single GATA-binding site derived from the mouse α 1-globin gene promoter (M α P; Tsai et al., 1989). In oligonucleotide competition experiments, a 200-fold excess of unlabelled M α P oligonucleotide was added to the gel shift reactions. Mutant M α P (mM α P) oligonucleotide had the core recognition sequence GATA changed to GGCA and was used as a control. The reactions were incubated for 30 minutes on ice. For supershifts, 0.5 µl of polyclonal GATA-1-specific or GATA-2-specific anti-peptide antibodies (Briegel et al., 1993) were added to the reactions after 10 minutes of preincubation. Protein-DNA complexes were then fractionated on a 5% non-denaturing polyacrylamide gel, which was prepared and run in 0.25× TBE buffer.

Immunoprecipitation analysis

Cells were incubated in methionine-free or phosphate-free medium supplemented with 2% dialysed FCS (30 minutes, 4 hours, respectively) and labelled for 6 hours with 250 µCi/ml [³⁵S]methionine or with 1 mCi/ml [³²P]orthophosphate, respectively. Following labelling, cells were washed once with ice-cold PBS and lysed in 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.1 mg/ml PMSF, 1 µg/ml leupeptin, 1 µg/ml Trasylol. After centrifugation (5 minutes, 15000 revs/minute) lysates were precleared with anti-rabbit IgG agarose. Immunoprecipitations were carried out at 4°C using rabbit polyclonal antibodies specific for chicken GATA-1 (see above) and *v-erbA* (Glineur et al. 1990), respectively. Anti-rabbit IgG coupled to agarose beads (Sigma) was used as a secondary antibody and immunoprecipitates were analyzed on 10% gel using SDS-PAGE followed by autoradiography.

RESULTS

GATA-1 cellular localization during terminal red blood cell differentiation

Previous studies have shown that GATA-1 mRNA is abundantly expressed in immature erythroid progenitor cells, and induced to even higher levels as the cells mature (Yamamoto et al., 1990; Leonard et al., 1993). As a first step towards attempting to better understand how GATA-1 activity is regulated in these cells, we first determined steady state GATA-1 protein levels in both undifferentiated primary erythroid cells and after induction of terminal differentiation.

Normal stem cell factor (SCF)-dependent red blood cell progenitors were obtained by cultivating chicken bone marrow cells for 3 days in the presence of recombinant avian SCF (Hayman et al., 1993) and differentiation was induced in a medium containing anemic chick serum (as the source of erythropoietin) and insulin (see Materials and Methods). To discriminate between early and late events, cells were harvested either prior to, or 24 and 48 hours after, differentiation induction. RNA and protein expression was monitored by northern and western blot analysis (Fig. 1). Both GATA-1-specific polyclonal anti-peptide and mouse monoclonal antibodies were used to assay for presence of the factor.

Following induction of differentiation, the erythroid progenitors begin to accumulate hemoglobin and gradually acquire the morphology of normal avian reticulocytes (Fig. 1A). In parallel to the onset of differentiation, a decrease in cell size and proliferative potential is routinely observed (Fig. 1B and data not shown). Under the conditions employed, normal erythroid progenitor cells differentiate into mature erythrocytes after 2 to 3 days in culture (data not shown). As anticipated, β -globin mRNA levels were low in immature cells and increased significantly during differentiation (Fig. 1C). In contrast, GATA-1 mRNA was abundantly expressed in both immature and differentiating cells (Fig. 1C). When examined by western blotting, GATA-1 protein was found to be present in undifferentiated cells and increased further after differentiation (Fig. 1C). To our surprise, multiple GATA-1-specific bands, with slightly different mobilities, were detected using either antibody (Fig. 1C; see also Fig. 4), indicating that different forms of GATA-1 may exist in these cells. Thus, GATA-1 is abundantly expressed in committed erythroid progenitors and is upregulated during terminal differentiation.

We next examined whether functional properties of the GATA-1 protein (such as sequence-specific DNA-binding activity) were specifically altered during red blood cell differentiation. To this end, nuclear extracts from both undifferentiated and differentiated normal progenitors were tested in electrophoretic mobility shift assays (EMSA). Despite the presence of abundant GATA-1 protein (Fig. 1C), no specific complex formation was detected with nuclear extracts isolated from undifferentiated cells. However, a competent DNA-binding complex was detected within 2 hours after differentiation induction, which further increased after longer induction periods (Fig. 2A,B). To ascertain whether or not the GATA factor:DNA complexes contained GATA-1 (or GATA-2, which is also expressed in these cells; Yamamoto et al., 1990; Briegel et al., 1993; Leonard et al., 1993), supershift experiments with GATA-1- and GATA-2-specific anti-peptide antibodies were performed. As shown in Fig. 2B, the mobility of the protein:DNA complex was retarded only in the presence of GATA-1-specific antibody, while addition of GATA-2-specific antibody had no effect. Thus, it is GATA-1 and not another member of the GATA family (or another protein factor) that binds *in vitro* to this DNA element. Furthermore, the GATA-1 protein:DNA complex formed was competed by the addition of a 200-fold excess of the same (unlabeled M α P) oligonucleotide but not with a 200-fold excess of mutant (mM α P) oligonucleotide, indicating that the binding is sequence-specific. Thus, the absence of a GATA-1-specific DNA complex in immature cells suggests either that the GATA-1 protein present in progenitor cells might display low DNA-binding activity or, alternatively, might be absent in the nuclear compartment.

To investigate the subcellular distribution of GATA-1 protein, cytoplasmic and nuclear extracts of SCF-dependent red blood cell pro-

genitors were prepared before and after differentiation induction, and GATA-1 accumulation was assayed by western blotting. In undifferentiated cells, GATA-1 protein was predominantly detected in the cytoplasm, while, as early as 2 hours after differentiation induction, GATA-1 was partitioned primarily into the nucleus (Fig. 2C). In some experiments, however, GATA-1 was found in both the cytoplasmic and nuclear fraction of undifferentiated cells. This could be due to a minor cell population of already partially differentiated cells present in the culture (as also revealed by histological staining; see Fig. 1A and data not shown). Interestingly, while only two GATA-1-specific protein species of slightly different electrophoretic mobility were detected in cytoplasmic extracts, a third form of the protein was found exclusively in nuclear extracts (Fig. 2C and see below).

To extend the conclusions derived from these cell fractionation data, subcellular localization of GATA-1 protein in immature and differentiation-induced red blood cell progenitors was examined by indirect immunofluorescence using a GATA-1-specific antibody. As a control for cytoplasmically expressed protein, cells were co-stained with a monoclonal antibody that recognizes c-kit (both membrane-anchored and soluble SCF/c-Kit-receptor; kindly provided by M. Hayman, SUNY, Stony Brook, NY, USA). The staining patterns were then recorded using confocal fluorescence microscopy.

As shown in Fig. 3, GATA-1 protein was predominantly confined to the cytoplasm of undifferentiated primary erythroid

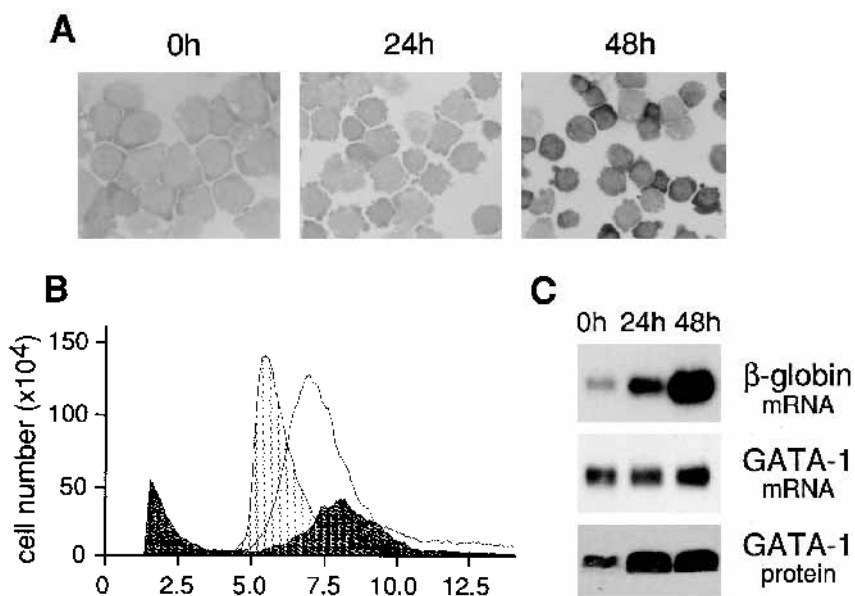


Fig. 1. Differentiation-dependent increase in GATA-1 mRNA and protein expression. (A) Cytospin preparations of SCF-dependent red blood cell progenitors prior to and 24 or 48 hours after differentiation were stained with neutral benzidine and histological dyes (Beug et al., 1982, 1995). To specifically reveal hemoglobin accumulation, photographs were taken under blue light. (B) Cell profiles of the same SCF-dependent erythroid progenitors as in A demonstrate the reduction in cell size during differentiation: undifferentiated cells (dark), differentiated cells at 24 hours (white) and 48 hours (grey) after induction. (C) Total RNA and total cell lysates of the same red cell progenitor cultures as in A were examined by northern or western blot analysis, as indicated (lane 1, cells plus SCF or following differentiation for 24 and 48 hours, lanes 2 and 3, respectively). Northern blots of 9 μ g RNA per sample were hybridized separately to specific probes for β -globin and GATA-1; the western blot shown was reacted with monoclonal antibody specific to GATA-1.

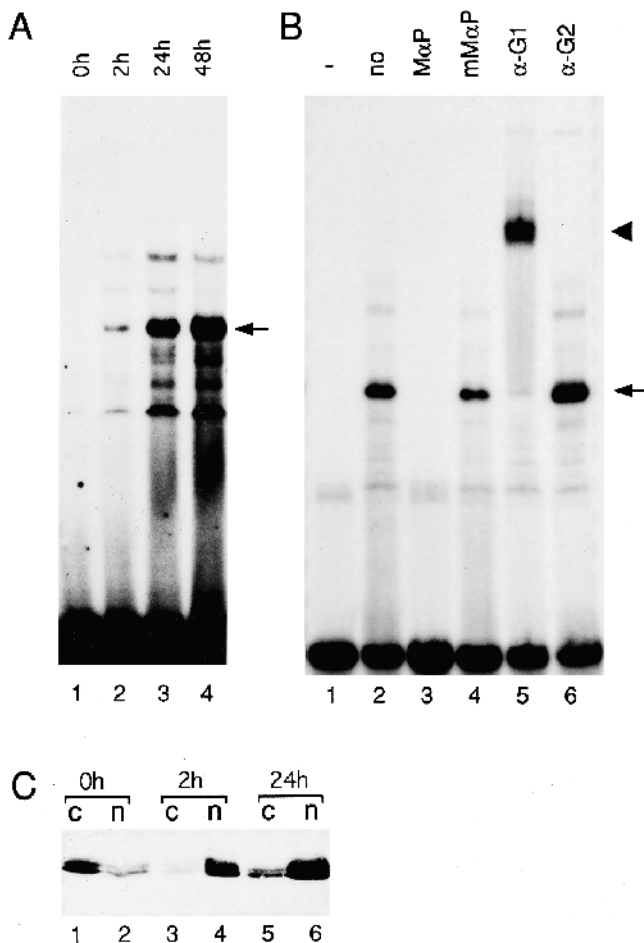


Fig. 2. GATA-1 protein activity is regulated by nuclear translocation during erythroid differentiation. (A) Sequence-specific DNA binding of GATA-1 in nuclear extracts of undifferentiated (0 hour; lane 1) and differentiated (2, 24 and 48 hours; lanes 2 to 3) SCF-dependent progenitors was measured in electrophoretic mobility shift assays (EMSA). Nuclear extracts were normalized for protein content. Complex formation of GATA-1 with a ^{32}P -labelled M α P probe containing a GATA consensus site of the mouse α 1-globin gene promoter (Tsai et al., 1989) is indicated (arrow). Bandshift reactions for a Sp1-like activity (P. B. and M. Z., unpublished) were used to control for integrity of the extracts and equal loading per lane. We also note that upon longer exposure a weak GATA-1-specific band was observed in lane 1. (B) EMSA showing that the molecular complex described in A contains GATA-1. Nuclear extracts of cells from the same experiment as in Figs 1 and 2A (24 hours after differentiation) were incubated with ^{32}P -labelled M α P probe (lanes 2 to 6). In oligonucleotide competition experiments, no competitor (lane 2), unlabelled M α P oligonucleotide or mutant M α P oligonucleotide (lane 3 and 4, respectively) was added to the gel shift reactions. In supershift experiments, polyclonal GATA-1- and GATA-2-specific anti-peptide antibodies (Briegel et al., 1993; lanes 5 and 6, respectively) were added. Specific protein:DNA complexes and the 'supershifted' complex are indicated by an arrow and triangle, respectively. Lane 1 no extract added. (C) Western blot analysis of the same cells as in A and B prior to (0 hour) and 2 or 24 hours after differentiation using the GATA-1-specific monoclonal antibody CG1-1.2.2. The result of cytoplasmic (c; lanes 1, 3, 5) and nuclear (n; lanes 2, 4, 6) extracts is shown. Please note that following differentiation induction GATA-1 is selectively detected in the nuclear compartment. In 3 out of 7 cell fractionation experiments, GATA-1 protein was found in both the cytoplasmic and nuclear fraction of undifferentiated cells, due to the presence of already partially differentiated cells in these cell preparations as revealed by cytological staining (data not shown).

progenitor cells, although some fluorescence within the nuclear compartment was also observed. After differentiation induction for 24 hours, the expression pattern changed dramatically and GATA-1 protein was detected almost exclusively in the nucleus. The differential nuclear accumulation during erythroid maturation was confirmed by superimposing the GATA-1- (red) and c-kit-specific (green) fluorescence, again clearly revealing a prominent GATA-1-specific nuclear signal (Fig. 3). Thus, GATA-1 protein selectively partitions to the nucleus in primary differentiating erythroid cells, while it localizes predominantly in the cytosol in proliferating immature cells. We note, however, that at least some of the GATA-1 contained in the cytoplasmic fraction was capable of binding to DNA in bandshift assays (data not shown).

Nuclear GATA-1 protein is hyperphosphorylated

Previous studies demonstrated that mouse GATA-1 protein is phosphorylated at specific serine residues; however, these studies failed to ascribe any specific function to this modification (Crossley and Orkin, 1994). To investigate whether phosphorylation might be involved in nuclear translocation of GATA-1, we determined the subcellular distribution of phosphorylated GATA-1 protein. Cells were labelled with [^{32}P]orthophosphate and, as a control, with [^{35}S]methionine. Both total and nuclear extracts were then prepared and subjected to immunoprecipitation analysis using a polyclonal GATA-1-specific antibody. As an internal experimental

control, extracts were also treated with an anti-*v-erbA*-specific antibody to selectively detect phosphorylated *v-erbA* which is expressed in these cells. As shown in Fig. 4, total cell extracts contain multiple ^{35}S -labelled GATA-1 protein species, which are phosphorylated to different extents. Nuclear extracts, however, contain only one major, hyperphosphorylated, slowly migrating GATA-1 species, while multiple ^{35}S -labelled GATA-1 proteins were also detected. As expected, the anti-*v-erbA*-specific antibody, used as an experimental control, detects both ^{35}S - and ^{32}P -labelled *v-erbA* protein (Fig. 4). Thus, we conclude that hyperphosphorylated GATA-1 protein is preferentially found in the nuclear extract, suggesting that phosphorylation might be involved in nuclear translocation of the GATA-1 protein.

Hormone-activated GATA-1/ER accelerates erythroid differentiation

To gain further insights into how GATA-1 might trigger red blood cell differentiation, we employed a conditionally active hormone-inducible GATA-1/ER. As previously reported, both nuclear translocation as well as *trans*-activation activity of GATA-1/ER are strictly hormone-dependent. While our initial *trans*-activation experiments were done in QT6 fibroblasts (Briegel et al., 1993), we confirmed that GATA-1/ER acts as a hormone-inducible transcription factor in erythroid cells as well (data not shown). Furthermore, in these cells, estrogen-bound GATA-1/ER activated reporter gene expression as efficiently as wild-type GATA-1, suggesting that, in erythroid

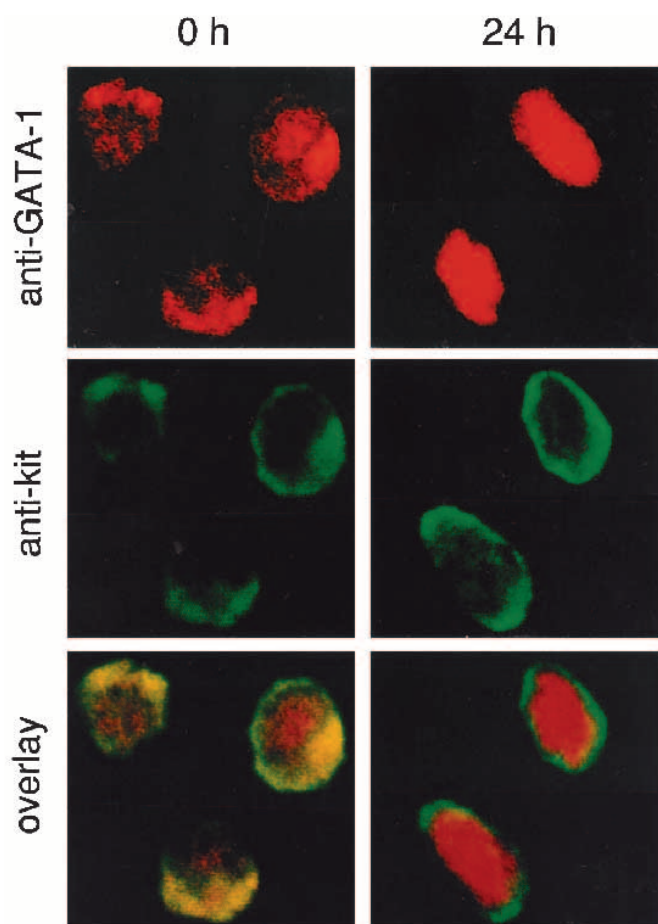


Fig. 3. Nuclear translocation of GATA-1 protein during differentiation of SCF-dependent red cell progenitors. Confocal immunofluorescence analysis of the SCF-dependent erythroid progenitor cells shown in Figs 1 and 2 prior to and 24 hours after differentiation induction. Cells were incubated with polyclonal GATA-1-specific anti-peptide antibody (followed by TRITC-conjugated goat anti-rabbit IgG; red; upper panel), and, as a control, reacted with anti-c-kit monoclonal antibody, which recognizes SCF/c-kit receptor (followed by FITC-conjugated goat anti-mouse IgG; green; middle panel). A superimposition of the two images (lower panel) reveals that, in undifferentiated cells, the majority of GATA-1 protein co-localizes with c-kit in the cytosol (indicated by the yellow color), while it exclusively partitions to the nucleus in differentiated cells.

cells, the estrogen receptor domain of GATA-1/ER does not affect *trans*-activation.

We then used the hybrid protein to examine the effects of ectopic GATA-1/ER expression on differentiation of chicken erythroid progenitor cells. Since we have consistently failed to stably express GATA-1/ER in normal, untransformed red cell progenitor cells (e.g. transforming growth factor (TGF α)-dependent progenitors; Briegel et al., 1993), the conditional GATA-1/ER protein was expressed in primary *ts-v-sea*-transformed erythroblasts, where terminal differentiation can be induced by inactivating the *ts-v-sea* oncogene product at 42°C (Knight et al., 1988). Chicken bone marrow cells were either infected simultaneously with the recombinant GATA-1/ER(neo) and *ts-v-sea* retroviruses, or, as a control, infected with *ts-v-sea* virus alone. Following selection in G418-con-

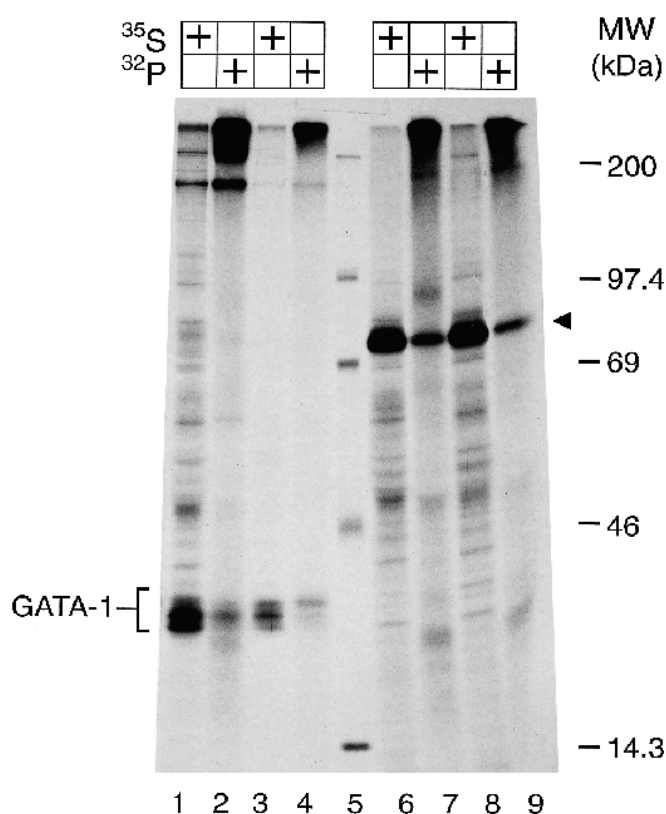
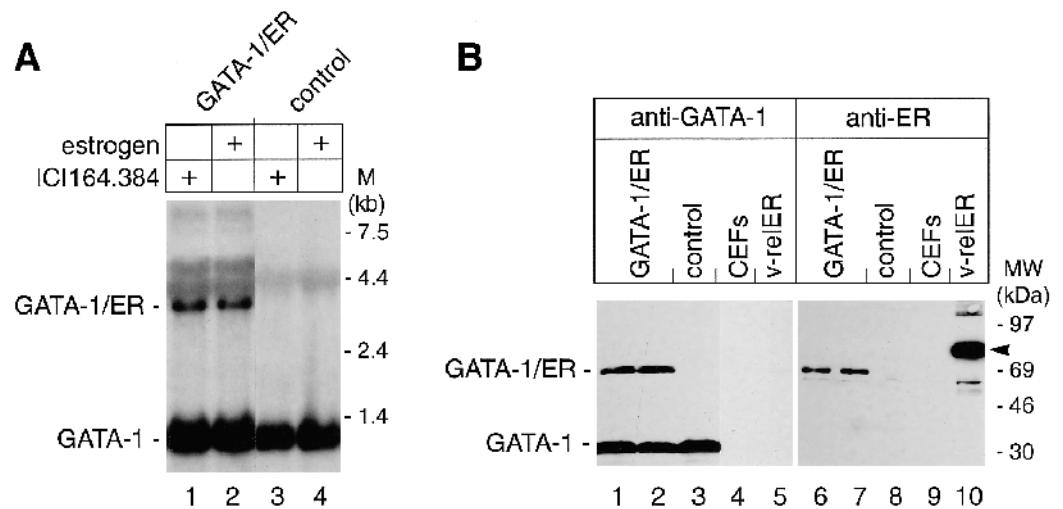


Fig. 4. Nuclear GATA-1 protein is hyperphosphorylated. To detect phosphorylated GATA-1, cells were labelled with [32 P]orthophosphate for 6 hours, and total cell extracts and nuclear extracts were prepared (see Materials and Methods section). Alternatively, cells were labelled with [35 S]methionine as indicated. To control for efficient 32 P-labelling and nuclear localisation, HD3 erythroblasts expressing the *v-erbA* protein were used. Total cell extracts (lanes 1, 2, 6 and 7) and nuclear extracts (lanes 3, 4, 8 and 9) were analysed by immunoprecipitation using GATA-1- and *v-erbA*-specific antibodies (lanes 1-4 and 6-9, respectively). Positions of GATA-1 protein and of *v-erbA* (triangle) are indicated. Lane 5 represents the protein size marker.

taining methocel, individual transformed erythroblast clones were isolated and GATA-1/ER expression was analysed by northern and western blotting (Fig. 5 and data not shown). These *ts-v-sea*-transformed erythroblasts were then induced to differentiate (see Materials and Methods), modulating GATA-1/ER activity by administration of estrogen or its antagonist ICI 164,384 (ICI). Differentiation of the cells was monitored by neutral benzidine staining of cytospin preparations and by quantitatively measuring hemoglobin levels (Beug et al., 1995).

In the initial experiment, 60 G418-resistant GATA-1/ER transformed erythroblast clones were analysed. Following addition of estrogen, 33 clones were found to differentiate with more rapid induction kinetics than 30 equivalent control clones containing the *ts-v-sea* virus alone. The remaining 27 GATA-1/ER clones did not differentiate or differentiated aberrantly due to clonal variation and/or lack of GATA-1/ER expression as observed previously (Briegel et al., 1993; data not shown). Additionally, the majority of control clones were slightly slower to differentiate in the presence of estrogen than without hormone, an effect likely due to the differentiation-retarding

Fig. 5. Expression of endogenous GATA-1 and exogenous GATA-1/ER in *ts-v-sea*-transformed erythroblast clones. (A) A representative GATA-1/ER-expressing *ts-v-sea* erythroblast clone (clone F4, lanes 1 and 2) and a *ts-v-sea* control clone (clone B7; lanes 3 and 4) were grown at 37°C in the presence of estrogen or ICI (lanes 2 and 4 and 1 and 3, respectively), for 1 day. Total RNA was extracted from these cells and examined in northern blot analysis (10 µg RNA per lane) using a GATA-1-specific probe. The GATA-1 (1.3 kb) and GATA-1/ER (3.14 kb, subgenomic) mRNAs are indicated. (B) Protein expression was monitored by western blot analysis using total cell extracts of *ts-v-sea*-transformed erythroblast clones stably expressing GATA-1/ER (clone D5, lanes 1 and 6; clone F4, lanes 2 and 7), or no transgene (clone B7, lanes 3 and 8). As additional controls, chicken embryo fibroblasts (CEF, lanes 4 and 9) and *v-rel/ER*-transformed bone marrow cells (clone 25; Boehmelt et al., 1992, 1995; lanes 5 and 10) were used. The blots were incubated with GATA-1-specific polyclonal anti-peptide antibody, or F3 monoclonal antibody specific to the ER moiety. The positions of endogenous GATA-1 ($31 \times 10^3 M_r$), exogenous GATA-1/ER ($67 \times 10^3 M_r$) and of the *v-rel/ER* proteins (triangle) are indicated.



activity of endogenous ER (Schroeder et al., 1993). Two representative GATA-1/ER+*ts-v-sea* clones (D5 and D10) and two *ts-v-sea* control clones (B7 and B9) were selected for further analysis.

To determine the relative expression levels of retrovirally encoded GATA-1/ER mRNAs (4.8 kb genomic and 3.1 kb subgenomic mRNAs) versus the endogenous GATA-1 mRNA (1.3 kb) in transformed erythroblast clones, northern blot analysis was performed using a GATA-1-specific probe. In all erythroblast clones analysed, virally transduced GATA-1/ER transcripts were expressed at considerably lower levels than endogenous GATA-1 mRNA (Fig. 5A and data not shown). These findings were also reflected at the protein level by employing GATA-1- and ER-specific antibodies. The $67 \times 10^3 M_r$ GATA-1/ER fusion protein was readily detected in GATA-1/ER-expressing erythroblast clones with both the anti-GATA-1 and the anti-ER antibody but was not detected in *ts-v-sea* control clones (Fig. 5B). Endogenous $31 \times 10^3 M_r$ GATA-1 was expressed in both GATA-1/ER-expressing and control *ts-v-sea* cells at similar levels, which were clearly higher than those of the GATA-1/ER protein. As expected, CEF expressed neither GATA-1/ER nor GATA-1, while the anti-ER antibody detected the expected $90 \times 10^3 M_r$ *v-rel/ER* fusion protein in *v-rel/ER*-transformed bone marrow cells (Fig. 5B). Taken together, these results demonstrate that even modest increases in GATA-1/ER protein levels are sufficient to quite effectively accelerate erythroid differentiation.

Further studies more clearly defined the physiological nature of the GATA-1-dependent acceleration of erythroid differentiation. Cell morphology, hemoglobin content and cell proliferation in differentiating cells were each examined. The differences in differentiation induction that were attributable to GATA-1/ER were found to peak after 2 days. As anticipated, maturation of control *ts-v-sea* erythroid progenitor cells was slightly delayed in the presence of estrogen. In contrast, activated GATA-1/ER efficiently accelerated differentiation in

GATA-1/ER-expressing clones, despite this inhibitory effect: estrogen-treated clones contained about 65% fully differentiated erythrocytes, while the parallel ICI-treated cultures contained only 30% (Fig. 6A,B). To underscore this conclusion, we measured hemoglobin levels 2 and 3 days after differentiation induction (Beug et al., 1995). Cells expressing the estrogen-activated GATA-1/ER contained significantly higher hemoglobin levels in comparison to the ICI-treated cells (Fig. 6C). In contrast, control clones contained considerably less hemoglobin in the presence of estrogen rather than ICI (Fig. 6C). Interestingly, the hemoglobin levels per cell expressed in differentiated GATA-1/ER+*ts-v-sea* cells (plus estrogen) were much higher than usually found in differentiated *ts-v-sea* control cells (Fig. 6C; and data not shown). However, elevated hemoglobin expression in these cells did not correlate with specific morphological changes normally expected for mature cells: the strongly hemoglobinized GATA-1/ER cells were larger than mature control cells and retained a rounded cell shape and an incompletely condensed nucleus (Fig. 6A).

As a third parameter of differentiation, [3 H]thymidine incorporation was measured to assay for differentiation-induced withdrawal from the cell cycle (Beug et al., 1995). GATA-1/ER-expressing erythroblast clones showed reduced proliferation rates in the presence of estrogen (data not shown), confirming the notion that hormone-activated GATA-1/ER accelerates differentiation. In contrast, estrogen-treated *ts-v-sea* control clones displayed the expected, slightly increased levels of [3 H]thymidine incorporation due to activation of the endogenous ER.

In conclusion, these observations argue strongly that GATA-1 promotes terminal erythroid differentiation and concurrently suppresses progenitor cell proliferation. However, while GATA-1/ER efficiently induced elevated hemoglobin levels, it appears to be incapable of establishing a complete erythroid differentiation program, indicating that other regulators of erythroid cell differentiation are also involved.

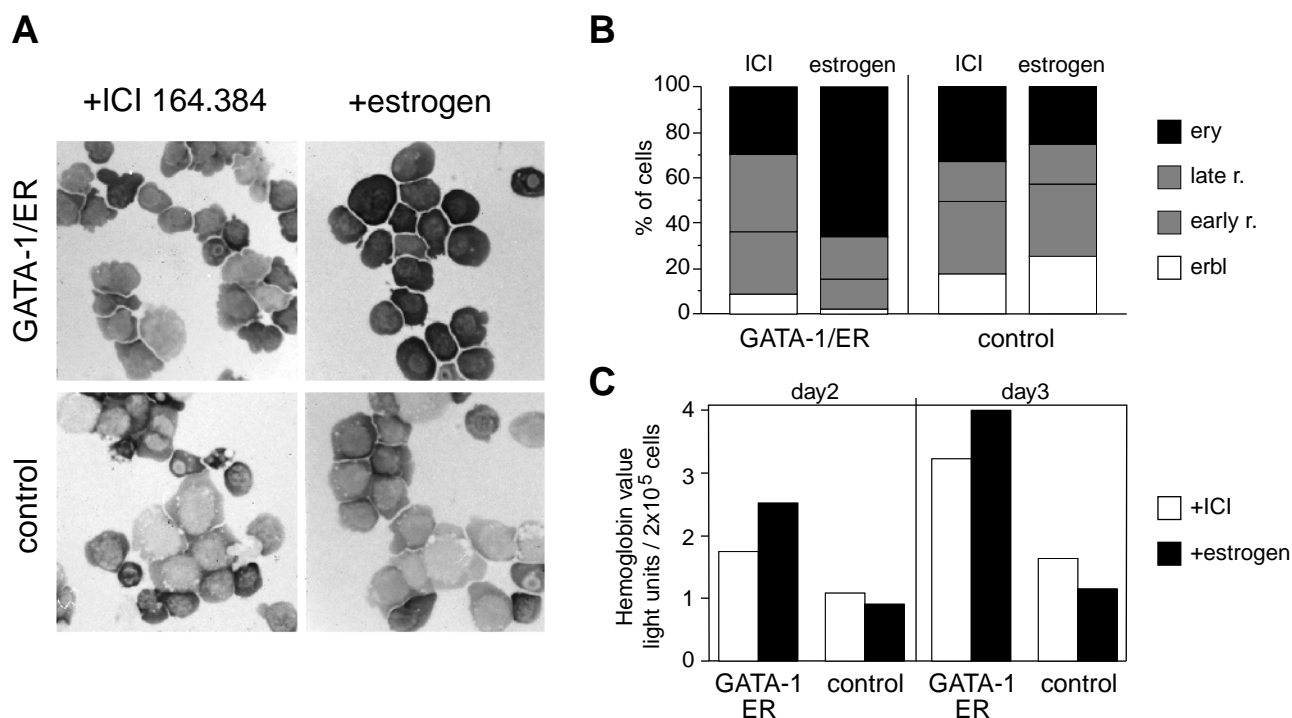


Fig. 6. GATA-1/ER accelerates maturation of *ts-v-sea*-transformed erythroid progenitors in a hormone-regulated fashion. (A) *Ts-v-sea*-transformed erythroblast clones stably expressing GATA-1/ER (clone D5) or *ts-v-sea* control clones (clone B9) were induced to differentiate in the presence of estrogen or ICI 164,384. After 2 days of differentiation, cells were subjected to cyto centrifugation and staining as in Fig. 1A. (B) Aliquots of the cells shown in A were evaluated for their stage of differentiation. At least 500 cells were counted and the results were plotted in histograms. Erythrocytes (ery), solid; late reticulocytes (late r.), hatched; early reticulocytes (early r.), stippled; erythroblasts (erbl), open. (C) 2 and 3 days after differentiation in the presence of estrogen or ICI, erythroblasts from the same cultures as in A were analysed for their hemoglobin content in a photometric assay (Beug et al., 1995). Extinctions read at 495 nm were normalized to cell numbers. The GATA-1/ER-specific increase in hemoglobin in response to estrogen was highly reproducible and observed for all clones analysed. There was, however, some clonal variation in the magnitude of this response (data not shown).

GATA-1/ER represses GATA-2 and *c-myb* expression in a hormone-dependent manner

We finally wished to determine whether the GATA-1/ER-induced acceleration of erythroid differentiation might correlate with specific changes in other aspects of erythroid cell gene expression. Since erythroblasts expressing a hormone-activated GATA-1/ER ceased proliferation, we were particularly interested in whether or not GATA-1/ER might affect genes thought or known to be involved in proliferation of erythroid cells, for example, GATA-2 and *c-myb*. Both GATA-2 and *c-myb* are abundantly expressed in erythroid precursor cells and suppressed during terminal differentiation. Moreover, both proteins have been implicated in the self-renewal of erythroid progenitor cells (Lüscher and Eisenman, 1990; Yamamoto et al., 1990; Briegel et al., 1993).

GATA-1/ER+*ts-v-sea* erythroblasts and *ts-v-sea* control clones were induced to differentiate in the presence of estrogen or ICI, RNA was prepared and subjected to northern blot analysis. As expected, the overall GATA-2 and *c-myb* mRNA levels decreased during differentiation in both GATA-1/ER and control cells (Fig. 7A). Additionally, in accord with the observation that an activated GATA-1/ER accelerates differentiation, GATA-2 and *c-myb* expression levels were significantly reduced in the clones expressing active GATA-1/ER (Fig. 7A,B) while, in the control clones, estrogen caused a delay in the GATA-2 and *c-myb* decline. Expression of two

GATA-1 target genes, β -globin and GATA-1 itself, was upregulated by hormone-activated GATA-1/ER (Fig. 7B), consistent with previous observations (Hannon et al., 1991; Tsai et al., 1991). Thus, GATA-1 appears to promote maturation of erythroid progenitors not only by inducing genes which are thought to define terminal erythroid differentiation, but also, through either direct or indirect mechanisms, by suppressing genes such as GATA-2 and *c-myb* which are involved in self-renewal of these cells.

DISCUSSION

Gene inactivation experiments have clearly defined a pivotal role for GATA-1 in erythropoiesis (Pevny et al., 1991). However, such genetic studies cannot reveal how GATA-1 might function in a mechanistic sense. In this investigation, we have employed a strategy based on conditionally forced expression of the GATA-1 transcription factor to explore the curious observation that, while GATA-1 is abundantly expressed in erythroid progenitors, it does not execute terminal differentiation due to its presence alone. We show that at least one function controlling GATA-1 activity appears to be its translocation to the nucleus when erythroid differentiation commences. To further define this activity, we attempted to mimic this post-translational regulatory mechanism by expression in erythroid progenitor cells of a con-

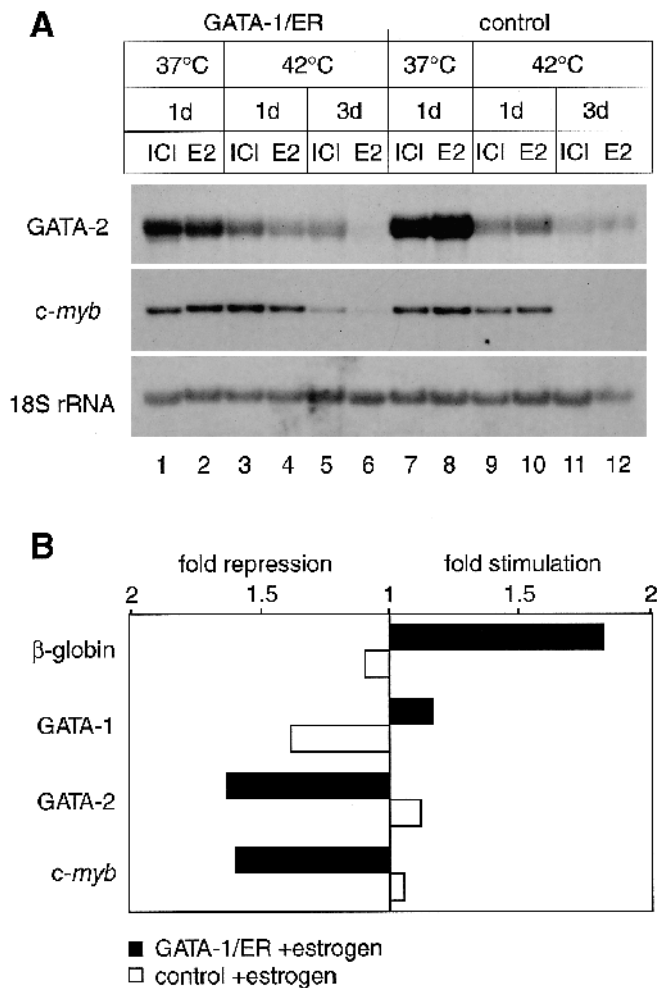


Fig. 7. Hormone-activated GATA-1/ER suppresses GATA-2 and *c-myb* gene expression. (A) Northern blot analysis of *ts-v-sea*-transformed erythroid progenitors stably expressing GATA-1/ER (clone F4; lanes 1 to 6) and of control *ts-v-sea* cells expressing no transgene (clone B7; lanes 7 to 12). A GATA-1/ER+*ts-v-sea* clone (clone F4) that displayed the GATA-1/ER-specific phenotype but with slower differentiation kinetics (due to normal clonal variation) was chosen. Total RNAs (10 μ g/lane) of undifferentiated (37°C; lanes 1 and 2, 7 and 8) and differentiating *ts-v-sea* cells (42°C; lanes 3 to 6, 9 to 12) that were treated with ICI or estrogen (E2), were analysed. Blots, separately hybridized with probes specific to GATA-2, *c-myb* and to 18S rRNA standard, are shown. (B) The northern blots in A were also hybridized to probes specific to GATA-1 and β -globin, quantitated by PhosphorImager and normalized to standard. Stimulation or repression of β -globin, GATA-1, GATA-2 and *c-myb* mRNA expression (at day 1 of differentiation) were determined. Whereas β -globin and GATA-1 become upregulated by hormone-activated GATA-1/ER, GATA-2 and *c-myb* are downmodulated. Please note that the estrogen-specific effects observed for the GATA-1/ER+*ts-v-sea* cells are the combined result of GATA-1/ER activity and the antagonistic effect of endogenous ER.

expression in erythroid progenitor cells of a conditional GATA-1/ER fusion protein (Briegel et al., 1993), whose nuclear translocation and transcriptional activity are controlled by estrogen. We demonstrate that the hormone-activated GATA-1/ER accelerates terminal differentiation of erythroid progenitors and is accompanied by the induction of differentiation-specific genes simul-

taneously with the direct or indirect suppression of genes involved in the proliferation of immature cells.

How is GATA-1 regulated during erythroid differentiation?

The available expression data of GATA-1 in immature, self-renewing erythroid progenitors initially presented an enigma. In these cells, GATA-1 mRNA is abundantly expressed, while at the same time the expression of the β -globin gene, a presumptive direct target of GATA-1 activity, is relatively low (Evans and Felsenfeld, 1989; Tsai et al., 1989; Gallarda et al., 1989; Martin et al., 1990; Yamamoto et al., 1990; Daylot et al., 1993; Leonard et al., 1993). Moreover, gene targeting and rescue experiments (Pevny et al., 1991; Simon et al., 1992) demonstrated a role for GATA-1 in the differentiation of erythroid progenitors rather than in regulation of their self-renewal. These apparently disparate observations prompted us to investigate whether activity of the GATA-1 factor itself might be regulated during terminal erythroid differentiation.

The results presented here reveal a potentially crucial control mechanism for GATA-1 activity. Self-renewing avian erythroid progenitor cells, in which normal erythropoiesis is reproduced in tissue culture (Hayman et al., 1993; Beug et al., 1994, 1995), express high levels of both GATA-1 mRNA and protein. However, at this immature stage, the vast majority of transcription factor GATA-1 is restricted to the cytoplasm. Most importantly, upon differentiation induction, essentially all GATA-1 protein accumulates in the nucleus and avidly binds to DNA. These observations therefore suggest that GATA-1 activity is principally regulated post-transcriptionally by a mechanism whereby translocation of pre-existing GATA-1 from the cytoplasm into the nucleus is the primary response when committed erythroid progenitor cells are triggered to undergo terminal differentiation.

Intriguingly, a highly conserved and inducible phosphorylation site (Serine 310) has been mapped within the intimate proximity of a nuclear localization signal (NLS) in murine GATA-1 (Crossley and Orkin, 1994). In this paper, we provide initial evidence that, in chicken erythroid cells, a hyperphosphorylated GATA-1 species is indeed preferentially localised in the nucleus, indicating that phosphorylation in response to differentiation signals might be involved in nuclear transition of GATA-1. Such an idea would be in keeping with reports where phosphorylation of amino acid residues neighbouring NLS has been found to increase nuclear uptake of various DNA binding proteins (reviewed in Whiteside and Goodbourn, 1993).

We note, however, that the differentiation-coupled nuclear transition of GATA-1 is only observed in primary erythroid cells and is largely lost in cell lines (data not shown). This observation is in keeping with reports where GATA-1 localisation in erythroid cell lines was investigated and found to be constitutively nuclear (Nicolas et al., 1991; Crossley and Orkin, 1994 and references therein). Nonetheless, it remains enigmatic why in such cell lines nuclear GATA-1 is apparently inactive in inducing terminal differentiation. Since differentiation of (for example) MEL cells is induced by chemicals, GATA-1 regulation in such cell systems might be different from that in the primary erythroid cells examined here. A modest increase in nuclear GATA-1 was observed in the erythropoietin-responsive cell line J2E after exposure to erythropoietin (Taxman and Wojchowski, 1995).

One recently published report focussed on a rather different

feature of localization of the GATA-1 protein than the one that we describe here. In those studies, it was shown that GATA-1, GATA-2 and GATA-3 often localize into punctate structures within the nucleus of hematopoietic cell types that normally express those factors (Elefanty et al., 1996). Two differences in the observations seem most prominent: first, we fail to detect similar condensed bodies of GATA-1 staining in the nuclei of normal progenitor cells, or their differentiated products, and second, Elefanty et al. (1996) failed to note the conspicuous cytoplasmic fluorescence in early progenitors that we describe here (Fig. 3). Two possibilities may explain the differences between those studies and the present analysis: first, it may be that the differences are simply species specific, and what is true for GATA-1 function in murine erythropoiesis is simply not true for the same functions of this protein in avian erythroid cells. This explanation seems implausible given the rich and well-documented parallels in signalling pathways, responses to growth and differentiation factors and erythroid biosynthetic mechanisms that are used in common by avians and mammals. We feel that the more likely explanations are twofold: first, quite different conditions were employed in the immunocytochemical methods used to fix and stain the cells in the two studies, and thus we fail to detect the subnuclear punctate bodies using our fixation conditions. Second, the populations of cells examined by Elefanty et al. (1996) do not represent erythroid progenitors at comparable early stages of differentiation to those reported here, thus explaining their failure to detect any significant cytoplasmic localization of the factor.

Finally, the data shown here suggest a model in which regulation of the intracellular distribution of pre-existing lineage-specific transcription factors might represent a paradigm for rapid induction of differentiation. In keeping with such an idea, the muscle-specific transcriptional regulator MyoD induces myogenesis in *Xenopus* embryos only after translocation (Rupp et al., 1994). One complication in those studies is, however, that nuclear translocation was measured only for exogenous MyoD while, in the present studies, we have examined nuclear translocation of endogenous GATA-1 protein.

A rather straightforward model can account for both the observations presented here and for others in the literature. In this model, rapid recruitment of pre-existing GATA-1 might represent the initiation step in establishing terminal red cell differentiation. Consistent with this notion, nuclear translocation of GATA-1 occurs within a few hours after differentiation induction. As the second step in such a process, an efficient upregulation of GATA-1-specific target genes, such as those for GATA-1 itself and the erythropoietin receptor, occurs (Hannon et al., 1991; Tsai et al., 1991; Chiba et al., 1991; Zon et al., 1991a), thereby leading to an amplification of the primary differentiation signal. Since this induction results in a significant increase in total GATA-1 protein and site-specific DNA binding activity after 16 to 24 hours of differentiation, a provocative possibility is that a specific threshold level of GATA-1 protein is ultimately required to execute the terminal steps in erythroid differentiation.

How does GATA-1 control erythroid differentiation?

To address the role of GATA-1 in regulating late steps in red cell differentiation, we sought to perturb the normal regulatory environment by introducing a conditional GATA-1/ER chimera into primary avian erythroid progenitors which are

capable of undergoing normal terminal differentiation in culture (Briegel et al., 1993). Hormone-inducible transcription factor-ER fusion proteins have proved to be extremely useful for such functional studies (for review see Picard, 1993). In this context, the GATA-1/ER fusion protein was found to selectively partition to the nucleus in its hormone-activated state, while it was predominantly cytoplasmic in the absence of ligand. Therefore, the hormone-inducible GATA-1/ER chimera represents an exquisite vehicle by which we might investigate GATA-1 nuclear translocation and transcriptional activation that is uncoupled from endogenous control mechanisms. Such an approach enabled us to determine the contribution of this fusion protein, even while acting within the context of the normal erythroid differentiation program.

To our surprise, we found that the conditional GATA-1/ER allele, when expressed in *ts-v-sea*-transformed erythroid precursors, profoundly accelerated red blood cell maturation, in spite of the fact that GATA-1/ER was expressed at significantly lower levels than was the endogenous GATA-1 factor. This pronounced phenotype was observed despite that estrogen normally imparts a differentiation-retarding effect on *ts-v-sea*-transformed erythroblasts due to the activity of the endogenous ER (Schroeder et al., 1993). No adverse effects of estrogen were observed in these cells, unlike results reported for MEL cells containing overexpressed human ER (Blobel and Orkin, 1996).

An obvious question was therefore: how was GATA-1/ER able to modify the erythroid differentiation program so distinctly, even though it is clearly expressed at lower levels than endogenous GATA-1? One possibility is that GATA-1/ER contains the ER protein domain, and may therefore efficiently bypass steps required for activation of the endogenous GATA-1. Another is that the GATA-1- and ER-specific *trans*-activation domains may cooperate (Briegel et al., 1993), generating a much stronger *trans*-activation signal for erythroid genes than that elicited from endogenous GATA-1. However, transient transfection experiments in erythroid cells demonstrated that the hormone-activated GATA-1/ER is as efficient as the unmodified GATA-1 in *trans*-activating a GATA-1-dependent reporter gene, but not more (data not shown). Thus, the ER moiety appears not to contribute to the transcriptional activity of GATA-1/ER.

The potential function of GATA-1 as a positive regulator of terminal erythroid differentiation is consistent with its proposed central role in erythropoiesis derived from ES cells harbouring a targeted disruption of the GATA-1 gene (Pevny et al., 1991; Simon et al., 1992). A few additional, unexpected features of hormone-activated GATA-1/ER cells are, however, worth mentioning. First, mature GATA-1/ER-expressing erythrocytes were phenotypically different from mature cells obtained from control *ts-v-sea* clones, in that they were larger and retained a rounded cell shape with incompletely condensed nuclei. Second, in keeping with a less reduced cell size, quantitative analysis of hemoglobin expression revealed elevated hemoglobin levels per cell in hormone-activated GATA-1/ER clones as compared to the control clones. Third, the hormone-activated GATA-1/ER might impose a growth-suppressive effect on both proliferating and differentiating GATA-1/ER+*ts-v-sea* erythroblasts. This is suggested by our observation that, in several independent colony assays, a hormone-activated GATA-1/ER reduced the number of GATA-1/ER+*ts-v-sea*-transformed erythroblast colonies

(data not shown). Furthermore, if inactive GATA-1/ER were to also exert a residual growth-inhibitory effect, this would explain the fact that all GATA-1/ER clones that could be expanded and were successfully used in further experiments expressed rather low levels of exogenous GATA-1/ER.

The observations presented here document several new insights into GATA-1 function. First, GATA-1 appears to exert a strong differentiation-promoting effect, which is, however, apparently insufficient to induce the entire erythroid differentiation program. Previous *in vitro* studies which showed that functional erythroid transcription requires the action of GATA-1 in concert with other transcription factors, like NF-E2, Sp-1 or ELKF (Walters and Martin, 1992; Miller and Bieker, 1993) support this contention. Second, GATA-1 not only induces terminal differentiation, but simultaneously suppresses cell growth by directly or indirectly reducing transcription of two genes presumed to be regulated by GATA-1, GATA-2 and *c-myb* (Weiss et al., 1994; P. B. and M. Z., unpublished). As reported previously, GATA-2 blocks erythroid differentiation and promotes self-renewal of erythroid progenitors (Briegel et al., 1993) suggesting that direct or indirect suppression of GATA-2 activity in these cells is a prerequisite for terminal erythroid differentiation. Thus, downregulation of GATA-2 by GATA-1 might represent a key event required for terminal maturation. This concept is consistent with studies showing that ES cells in which the GATA-1 gene has been ablated by gene targeting can still complete erythropoiesis through the proerythroblast stage, but cannot execute terminal differentiation (Weiss et al., 1994). Additionally, these GATA-1 (*-/-*) ES cells express dramatically elevated levels of both GATA-2 and *c-myb*.

The data presented here also suggest an antagonistic relationship between GATA-1 and the proto-oncogene *c-myb*. *c-myb* is abundantly expressed in immature erythroid progenitors and is downregulated during normal red cell differentiation (Lüscher and Eisenman, 1990). Most strikingly, the phenotypes of mice in which the GATA-1 and *c-myb* loci had been disrupted by homologous recombination are very similar: in both cases, the animals exhibit profound defects in erythropoiesis (Pevny et al., 1991; Mucenski et al., 1991). Finally, GATA-1 has been implicated in both transcriptional activation as well as repression of specific genes (Whyatt et al., 1993). All these observations are in accord with the key observations presented here: GATA-1 is a positive regulator of erythrocyte-specific genes but, at the same time, may be required to silence genes that would prevent execution of the erythroid differentiation program, i.e. GATA-2 and/or *c-myb*.

We thank Dr A. E. Wakeling for ICI 164.384, Dr Lin Gu for monoclonal anti-GATA-1 antibody CG1-1.2.2, Dr M. Hayman for monoclonal anti-c-kit antibody, and Drs P. Chambon and D. Metzger for monoclonal antibody F3. We also thank Dr Christian Plank for peptide synthesis, Dr Gotthold Schaffner for oligonucleotide synthesis, N. Howells for rabbit immunization, H. Tkadletz for photography and I. Gallagher for typing the manuscript. This work was supported by the Institute of Molecular Pathology (IMP), Vienna, Austria, when K. B., P. B. and M. Z. were located at IMP, and in part by the Fonds zur Förderung der wissenschaftlichen Forschung (FWF) to M. Z., and by NIH grants HL 24415 and GM 28896 (to J. D. E.).

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