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GATA transcription factors associate with a novel class of nuclear bodies in erythroblasts and megakaryocytes

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The nuclear distribution of GATA transcription factors in murine haemopoietic cells was examined by indirect immunofluorescence. Specific bright foci of GATA-1 fluorescence were observed in erythroleukaemia cells and primary murine erythroblasts and megakaryocytes, in addition to diffuse nucleoplasmic localization. These foci, which were preferentially found adjacent to nucleoli or at the nuclear periphery, did not represent sites of active transcription or binding of GATA-1 to consensus sites in the β-globin loci. Immunoelectron microscopy demonstrated the presence of intensely labelled structures likely to represent the GATA-1 foci seen by immunofluorescence. The GATA-1 nuclear bodies differed from previously described nuclear structures and there was no co-localization with nuclear antigens involved in RNA processing or other ubiquitous (Sp1, c-Jun and TBP) or haemopoietic (NF-E2) transcription factors. Interestingly, GATA-2 and GATA-3 proteins also localized to the same nuclear bodies in cell lines co-expressing GATA-1 and -2 or GATA-1 and -3 gene products. This pattern of distribution is, thus far, unique to the GATA transcription factors and suggests a protein–protein interaction with other components of the nuclear bodies via the GATA zinc finger domain.

Keywords: GATA proteins/nuclear bodies/transcription factor

Introduction

The GATA nuclear transcription factors comprise a family of proteins which bind the DNA sequence (A/T)GATA-(A/G) via a highly conserved zinc finger domain (Wall *et al.*, 1988; Evans and Felsenfeld, 1989; Tsai *et al.*, 1989; Orkin, 1992). Currently six members have been identified in avians, with homologues in mammals and amphibians (Laverriere *et al.*, 1994). GATA binding proteins have also been cloned from fungi, yeast, flies and worms (Kudla *et al.*, 1990; Cunningham and Cooper, 1991; Spieth *et al.*, 1991; Abel *et al.*, 1993), attesting to the evolutionary conservation of this class of genes. Through their expression profiles the GATA proteins may be distinguished as haemopoietic (GATA-1 to -3) and non-haemopoietic (GATA-4 to -6), a functional grouping which mirrors their structural similarities. Cells of the erythroid, megakaryocytic and mast cell lineages are the major sites of GATA-1 expression, but the protein is also expressed at low levels in multipotential cells and in the Sertoli cells of the testis (Crotta *et al.*, 1990; Martin *et al.*, 1990; Romeo *et al.*, 1990; Yamamoto *et al.*, 1990; Sposi *et al.*, 1992; Ito *et al.*, 1993; Weiss and Orkin, 1995). Whilst GATA-2 shares expression in megakaryocytes and mast cells, it is also found in populations enriched for haemopoietic stem and progenitor cells, endothelial cells and embryonic brain (Wilson *et al.*, 1990; Yamamoto *et al.*, 1990; Lee *et al.*, 1991; Dorfman *et al.*, 1992). In the chicken GATA-3 is expressed in erythroid cells, but mammalian expression is mainly restricted to T lymphocytes and the embryonic nervous system (Yamamoto *et al.*, 1990; Ho *et al.*, 1991; Joulin *et al.*, 1991; Ko *et al.*, 1991). Consistent with these patterns of expression, functionally important GATA binding sites are present in the *cis* regulatory elements of many haemopoietically restricted genes in erythroblasts, megakaryocytes, mast cells and T cells (Weiss and Orkin, 1995). The non-haemopoietic GATA transcription factors (GATA-4 to -6) are expressed in the heart, intestine and diverse tissues such as ovary, liver, lungs or spleen (Arceci *et al.*, 1993; Kelley *et al.*, 1993; Laverriere *et al.*, 1994).

Our understanding of the normal role of haemopoietic GATA proteins is largely based on gene ablation studies. ES cells mutant at the GATA-1 locus fail to contribute to the erythroid lineage of chimeric mice (Pevny *et al.*, 1991) and detailed *in vitro* analyses demonstrate an absence of erythropoietin-responsive primitive erythroid precursors and a block in differentiation of definitive erythroid precursors (defined by their response to the combination of kit ligand and erythropoietin) at the proerythroblast stage (Weiss *et al.*, 1994). Interestingly, formation of other haemopoietic lineages, including mast cells and megakaryocytes (which express both GATA-1 and GATA-2) is not affected (Pevny *et al.*, 1995). Expression of heterologous GATA binding proteins, or even the zinc fingers alone, under the control of GATA-1 regulatory sequences is able to rescue the phenotype *in vitro*, suggesting a degree of functional redundancy within the family (Blobel *et al.*, 1995). Mice deficient in GATA-2 die at embryonic day 10–11 and display widespread defects in haemopoiesis (Tsai *et al.*, 1994). These findings are consistent with a role for GATA-2 at an earlier stage in haemopoiesis than GATA-1. Finally, GATA-3-deficient mice also die at embryonic day 11–12 with a failure of fetal haemopoiesis and defects in the nervous system (Pandolfi *et al.*, 1995).

Murine erythroleukaemia (MEL) cells represent neoplastic erythroid progenitors derived from the spleens of susceptible mice infected with the Friend virus complex (Friend and Pogo, 1985). They proliferate in the absence of exogenously added growth factors and undergo inducible erythroid differentiation upon exposure to various chemicals, most notably dimethylsulfoxide (DMSO) and hexamethylene bisacetamide (HMBA) (Friend *et al.*, 1971). Induction faithfully reproduces many of the features of *in vivo* erythroid differentiation, including haemoglobin synthesis, cessation of division and morphological changes that culminate in enucleation. Thus MEL cells represent a good *in vitro* model in which genes influencing erythroid differentiation may be analysed.

As part of our ongoing investigation into the role of GATA-1 and related transcription factors in haemopoiesis we have examined the distribution of GATA proteins within the nucleus of MEL cells and primary murine haemopoietic cells. In addition to the anticipated diffuse nucleoplasmic localization, we show that GATA-1 localizes to novel nuclear bodies, distinct from previously described structures, within erythroleukaemic cells and also primary murine erythroblasts and megakaryocytes. A similar localization was demonstrated for GATA-2 and GATA-3 in cell lines co-expressing GATA-1 and -2 or GATA-1 and -3 gene products. If the interaction with nuclear bodies involves the conserved GATA zinc finger region, the DNA binding domain may also play a role in protein–protein interactions.

Results

Focal accumulations of GATA-1 protein in MEL cells

Staining of C88 MEL cells (Deisseroth and Hendrick, 1978) with a GATA-1-specific monoclonal antibody (N6) (Ito *et al.*, 1993) demonstrated an unexpected finding: one or two discrete foci of intense fluorescence were present in most cells, in addition to the anticipated pattern of diffuse granular nucleoplasmic fluorescence which was excluded from the nucleoli (Figure 1A). Up to six GATA-1 foci/cell were observed when different MEL cell lines were stained (Figure 1B and data not shown), although the modal number of foci remained between one and three. In the occasional multinucleate erythroblast (see Figure 1C for an example) there was a clear correlation between nuclear ploidy and the number of foci. Close examination revealed that the GATA-1 foci were not randomly distributed, but were preferentially located adjacent to nucleoli or at the nuclear periphery. This is readily appreciated in the single optical section through the multinucleate erythroblast shown in Figure 1D, where several foci clustering around a nucleolus are arrowed and foci at the nuclear periphery are indicated with an arrowhead. Infrequently foci were present side by side in pairs (arrowed in Figure 1E), as if they had undergone 'replication'.

Epitope-tagged GATA-1 co-localizes with endogenous GATA-1

To ensure that our findings did not represent an antibody staining artefact we constructed GATA-1 expression vectors in which sequences from the human *myc* gene or

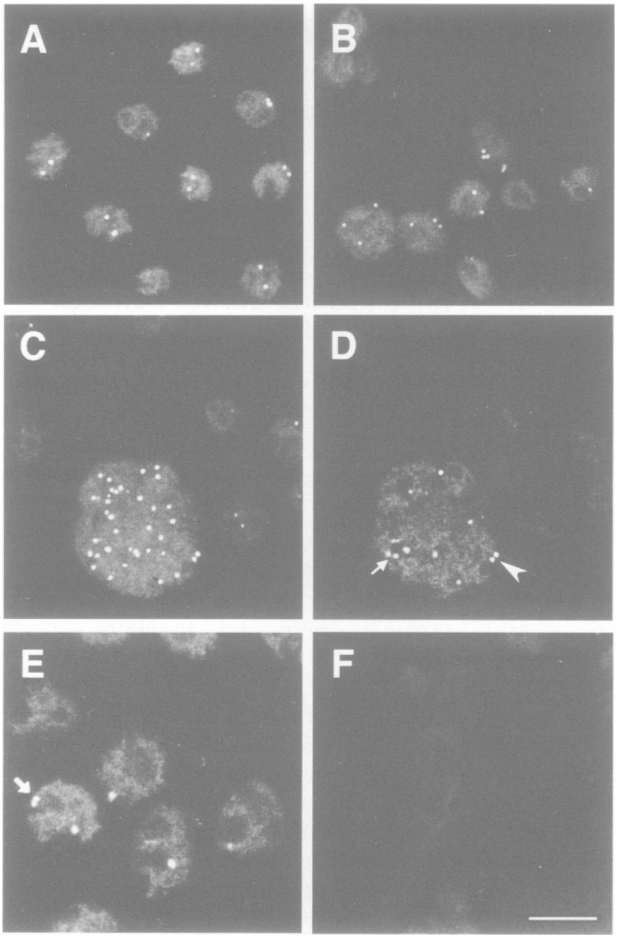

Fig. 1. Confocal images of immunofluorescent staining of MEL cell lines with anti-GATA-1 antibody demonstrating (**A**) 1–2 foci/cell in C88 cells and (**B**) up to 5 foci/cell in Buf707 cells. (**C**) A multinucleate erythroblast in a Buf707 culture displays numerous foci commensurate with its ploidy, whilst a single optical section through the same cell (**D**) highlights the propensity for foci to cluster around nucleoli (arrow) or at the nuclear periphery (arrowhead). (**E**) Some foci were 'paired' (arrowed), as if they had undergone replication. (**F**) Anti-GATA-1 staining of B lymphoid cell line A20L is shown as a negative control. Scale bar, 10 μm (A–D and F) or 5 μm (E).

the influenza virus haemagglutinin gene were fused in-frame to the N-terminal of a murine GATA-1 minigene (Figure 2A). These genes were then incorporated into the human β-globin locus control region (βLCR) expression vector (Collis *et al.*, 1990; Needham *et al.*, 1992) and used to generate stable transfectants of C88 MEL cells which were then cloned in soft agar. The βLCR conferred copy number-dependent high level expression on the linked gene when the MEL cells were induced to undergo erythroid differentiation with DMSO.

Nuclear extracts made from parental and transfected MEL clones and from the T cell line EL-4 were immunoblotted and probed with anti-GATA-1 antibody or antibodies specific for the epitope tags. As shown in Figure 2B, the anti-GATA-1 antibody specifically recognized endogenous GATA-1 in both uninduced (Figure 2B, lanes 2 and 4) and induced cells (lanes 3 and 5). Note that GATA-3 in extracts of the T cell line EL-4 (lane 1) was not detected. Epitope-tagged GATA-1 was readily distinguished from endogenous GATA-1 by its slightly slower electrophoretic mobility (lane 5). Equal quantities

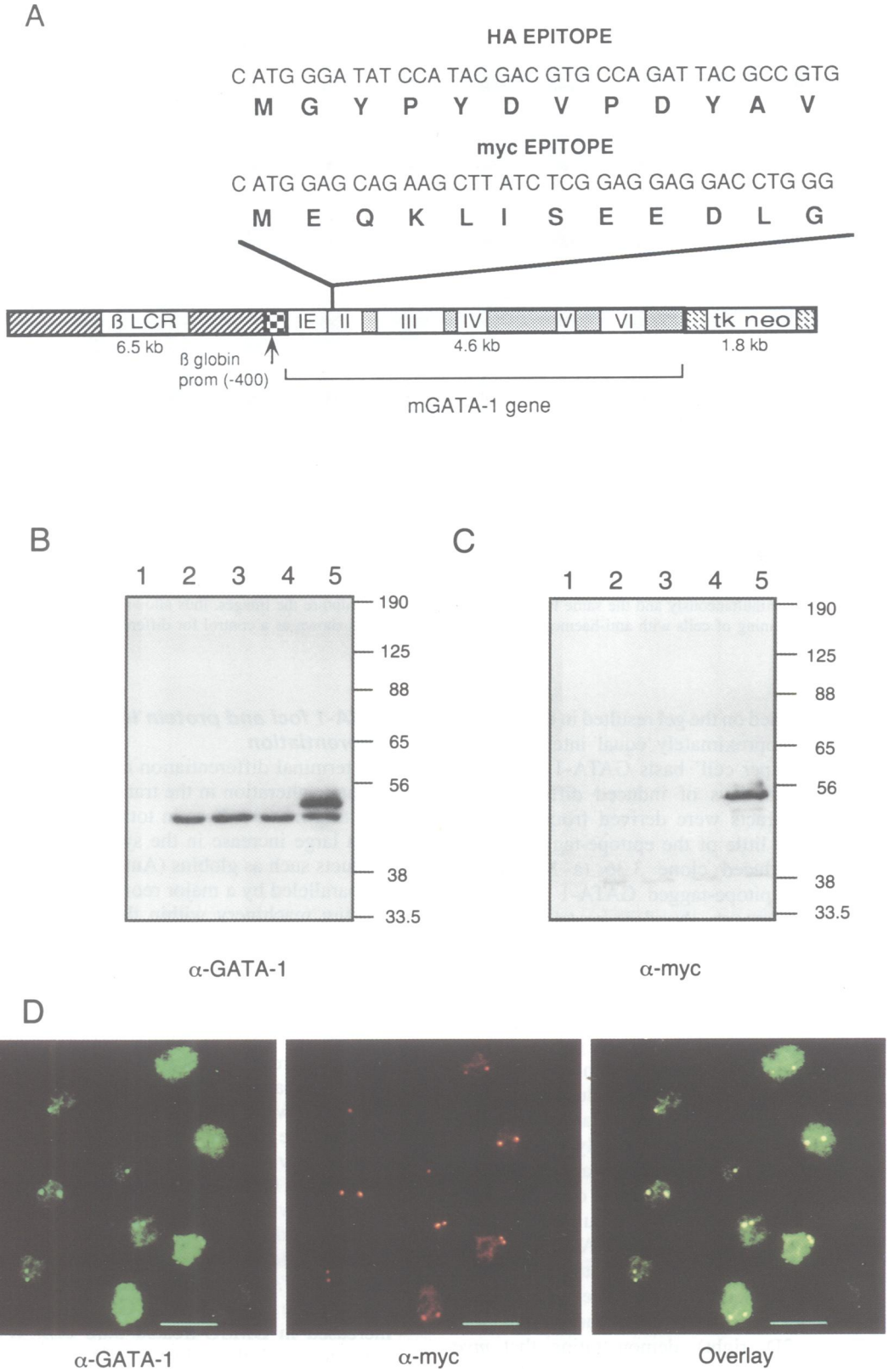

Fig. 2. (**A**) Inducible GATA-1 expression vectors composed of the human βLCR and –400 β-globin promoter driving a murine GATA-1 minigene (mGATA-1) carrying an N-terminal epitope for influenza virus haemagglutinin (HA) or human c-*myc* (myc). Amino acids encoded by the epitope tags are indicated in bold type and the GATA-1 exons are shown in Roman numerals, beginning with the haematopoietic cell-specific untranslated exon IE. A G418 antibiotic resistance gene under the control of the thymidine kinase promoter (*tk neo*) is carried on the same plasmid. (**B** and **C**) Western blots of 30 μg nuclear protein from EL-4 thymoma cells (lane 1), uninduced and induced C88 MEL cells (lanes 2 and 3) and uninduced and induced 3.36 *myc*–GATA cells which carry the *myc* epitope-tagged GATA-1 vector (lanes 4 and 5). The mobilities of molecular weight markers (in kDa) are indicated at the right of each gel. The membrane was probed (B) with anti-GATA-1 antibody or (C) with anti-*myc* antibody. (**D**) Indirect immunofluorescence of DMSO-induced 3.36 *myc*–GATA cells double stained with anti-GATA-1 (left) and anti-*myc* (centre) antibodies. The overlayed image demonstrating confocality of the GATA-1 foci is shown on the right. Scale bar, 10 μm.

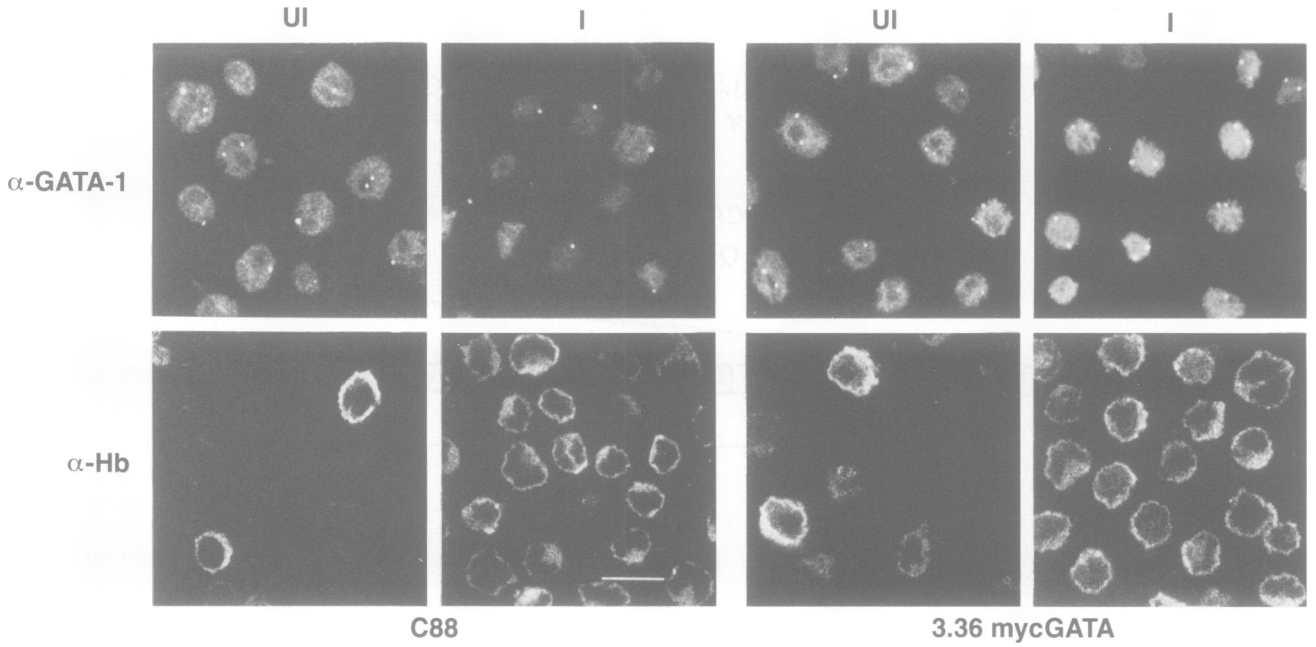

Fig. 3. Expression of GATA-1 (α-GATA-1) in uninduced (UI) and DMSO-induced (I) C88 cells (left) and 3.36 *myc*–GATA cells (right). All samples were stained and analysed simultaneously and the same laser settings were used to capture the images, thus allowing comparison of the relative fluorescence intensities. Staining of cells with anti-haemoglobin antibodies (α-Hb) is shown as a control for differentiation induction. Scale bar, 10 μm.

of nuclear extracts loaded on the gel resulted in endogenous GATA-1 signals of approximately equal intensity in all lanes, although on a 'per cell' basis GATA-1 expression decreased following 4 days of induced differentiation, since the induced extracts were derived from twice the number of cells. Very little of the epitope-tagged protein was present in uninduced clone 3.36 (a MEL clone harbouring the *myc* epitope-tagged GATA-1 gene), but, following DMSO treatment, abundant protein was produced (Figure 2C, lanes 4 and 5). Identical results were obtained with MEL clones harbouring inducible haemagglutinin-tagged GATA-1 (data not shown). There was no evidence for down-regulation of endogenous GATA-1 protein following induction of the tagged gene.

We next examined GATA-1 expression in clone 3.36 cells by immunofluorescence. When uninduced cells were double stained with antibodies to native and *myc*-tagged GATA-1 endogenous GATA-1 was readily detectable in all cells, but the tagged protein was only apparent in rare cells, consistent with the low frequency of spontaneous differentiating MEL cells observed in culture (data not shown). Following induction both anti-GATA-1 and anti-*myc* antibodies showed the same focal pattern of fluorescence (Figure 2D, left and centre). The foci detected by both antibodies co-localized when the images were overlayed (Figure 2D, right), demonstrating that *myc*-tagged GATA-1 protein was present in all the foci targetted by the endogenous protein. The same findings were documented with the haemagglutinin-tagged GATA-1 gene (data not shown).

Thus GATA-1-associated nuclear foci were observed with three independent antibodies and the epitope-tagged and endogenous proteins co-localized, establishing that this finding was neither spurious nor artefactual.

GATA-1 foci and protein level decrease with differentiation

The terminal differentiation of MEL cells is marked by a dramatic alteration in the transcriptional status of the cell, resulting in a decrease in total RNA and protein per cell, but a large increase in the synthesis of erythroid-specific products such as globins (Antoniou, 1991). These changes are paralleled by a major reorganization in the pre-mRNA splicing machinery within the nucleus (Antoniou *et al.*, 1993). Therefore, we examined C88 MEL and clone 3.36 cells by immunofluorescence (Figure 3) during differentiation to investigate the decrease in GATA-1 protein per cell predicted by Western blotting (Figure 2B and C). All samples were stained simultaneously and the same laser settings were used to capture and process the images, to allow comparison of the relative fluorescence intensities. The cells were stained for haemoglobin expression to monitor the degree of erythroid induction. Following treatment with DMSO we observed a decrease in the intensity of GATA-1 staining in untransfected C88 cells, consistent with the immunoblot analysis shown in Figure 2, though there was considerable cell-to-cell variation (Figure 3, left). Interestingly, the number of cells containing GATA-1 foci decreased from 98 to 77% (Table I). In contrast, the overall level of GATA-1 immunofluorescence increased in DMSO-treated 3.36 cells (Figure 3, right), consistent with the induction of tagged GATA-1 regulated by the βLCR. In these cells there was no decrease in the percentage of cells with GATA foci (Table I), suggesting that visualization of the foci depended upon high levels of GATA-1, not the stage of MEL cell differentiation.

GATA-1 foci are present throughout the cell cycle

The high frequency of foci in unsynchronized cells (>95%) and in terminally differentiated cultures (>75%)

Table I. GATA-1 foci in cell lines and primary cell cultures

Cells (*n*)	Foci (%)
C88 MEL	
Uninduced (*n* = 200)	98
Induced (*n* = 200)	77
3.36 *myc*GATA	
Uninduced (*n* = 200)	97
Induced (*n* = 200)	96
Primary haemopoietic cells	
Primitive (day 10.5 embryonic blood) erythroblasts (*n* = 412)	86
Definitive (day 12.5 fetal liver) erythroblasts (*n* = 213)	77
Megakaryocytes (*n* = 30)	73

Prevalence of GATA-1 foci in MEL cells and primary haemopoietic cells, showing the percentage of cells with foci in each category. The total number of cells counted (*n*) in several experiments is indicated.

suggested they remain present throughout the G_0, G_1, S and G_2 phases of the cell cycle. When mitotic cells were examined foci could be identified in ~80% of cases. In the example shown (Figure 4) a telophase cell double stained with antibodies to GATA-1 (in green) and to the mitotic spindle protein α-tubulin (in red) clearly displays GATA-1 foci, physically distinct from the mitotic spindle, in both forming daughter nuclei. GATA-1 staining was invariably weak in mitotic cells (which do not have an intact nuclear membrane), presumably because much of the soluble nuclear protein was lost during the permeabilization and fixation steps.

GATA-1 foci are seen in murine primary erythroblasts and megakaryocytes

To determine whether the GATA-1 foci observed in MEL cells reflected the staining pattern of primary haemopoietic cells, nucleated erythroblasts at different developmental time points were stained for GATA-1 expression. Bright foci of GATA-1 fluorescence were observed in cells from embryonic day 10.5 yolk sac and blood (Figure 5A and B) (examples of primitive erythropoiesis) and fetal liver erythroblasts from days 11.5 to 14.5 (Figure 5C–E) (representing definitive erythropoiesis). The proportion of cells with GATA-1 foci was lower than in uninduced cultures of MEL cells (Table I), perhaps because most of the cells were more differentiated and expressed relatively lower levels of GATA-1. Furthermore, most cells only contained one bright GATA-1 focus, in contrast to cells from the MEL cell lines, most of which contained two or more foci.

We determined whether mast cells and megakaryocytes also displayed GATA-1 foci. Only weak GATA-1 fluorescence was observed in primary mast cells grown from murine bone marrow and GATA-1 foci were not detected (Figure 5F). Conversely, prominent GATA-1 foci were present in primary megakaryocytes, identified in fetal liver samples of days 12.5–14.5 by their polyploidy and histochemical staining for acetylcholinesterase (see Figure 5G–I for examples). Therefore, our experiments demonstrated that GATA-1 foci were present in primary haemopoietic cells and are not restricted to leukaemic cell lines.

GATA-1 foci are not sites of active transcription and do not represent DNA binding

We reasoned that foci detected by immunofluorescence may reflect protein binding to regions of DNA bearing a high density of GATA binding sites, the most likely region being the β-globin locus (deBoer *et al.*, 1988; Wall *et al.*, 1988; Grosveld *et al.*, 1993). Furthermore, GATA-1 binding might be associated with active transcription of genes from the loci. If so, such an association should be more pronounced during terminal erythroid differentiation, when transcription of GATA-1-dependent globin genes is markedly induced.

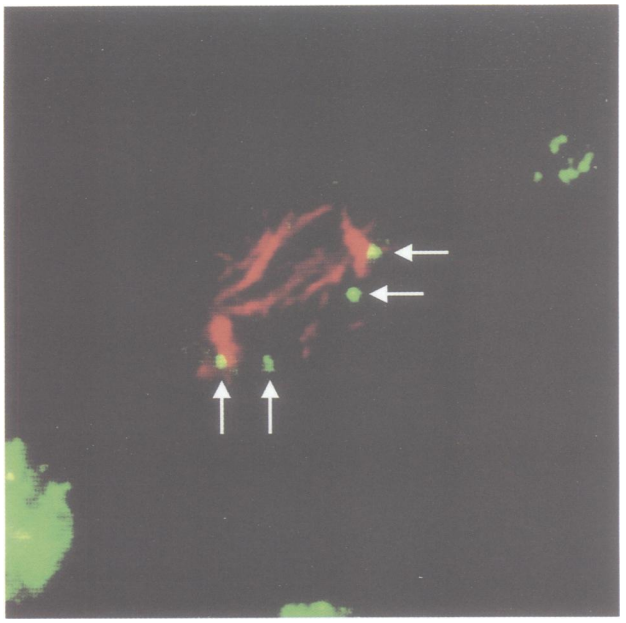

Fig. 4. Telophase cell in a C88 MEL cell culture double stained with antibodies to GATA-1, demonstrating two green foci in both daughter cells (arrowed) and α-tubulin (showing the mitotic spindle) in red. The yellowing of one GATA-1 focus in each daughter nucleus results from the overlay of tubulin staining present in adjacent optical sections and does not represent true co-localization. Note the low level of GATA-1 staining in this mitotic cell (see text). Magnification ×5000.

To address these points nascent RNA transcripts in uninduced and induced MEL cells were labelled *in vitro* with bromouridine triphosphate (BrUTP) and the cells stained with antibodies specific for BrUTP and GATA-1. In the representative experiment shown in Figure 6A GATA-1 fluorescence of doubly stained cells is depicted in green in the left panels and the sites of transcription are seen as a granular red nucleoplasmic fluorescence in the central panels. When the images were overlayed (right) no co-localization of nascent transcripts with the bright GATA-1 foci could be detected. This finding was anticipated in undifferentiated MEL cells, because globin transcription is at a low level, even though the GATA sites in the β-globin locus are occupied (Ikuta and Kan, 1991; Strauss and Orkin, 1992). The more significant observation was that enhanced globin transcription following induction did not lead to foci of transcription which co-localized with the GATA foci.

Since we may have failed to detect co-localizing sites of transcription because the foci of fluorescence produced were too faint, we formally excluded the possibility that the GATA foci represented binding to the β-globin loci by imaging MEL cells after sequential hybridization with a DNA probe for the murine β-major globin gene followed by staining with anti-GATA-1 antibody (Figure 6B). In this experiment the GATA-1 foci (arrowed, in green) were

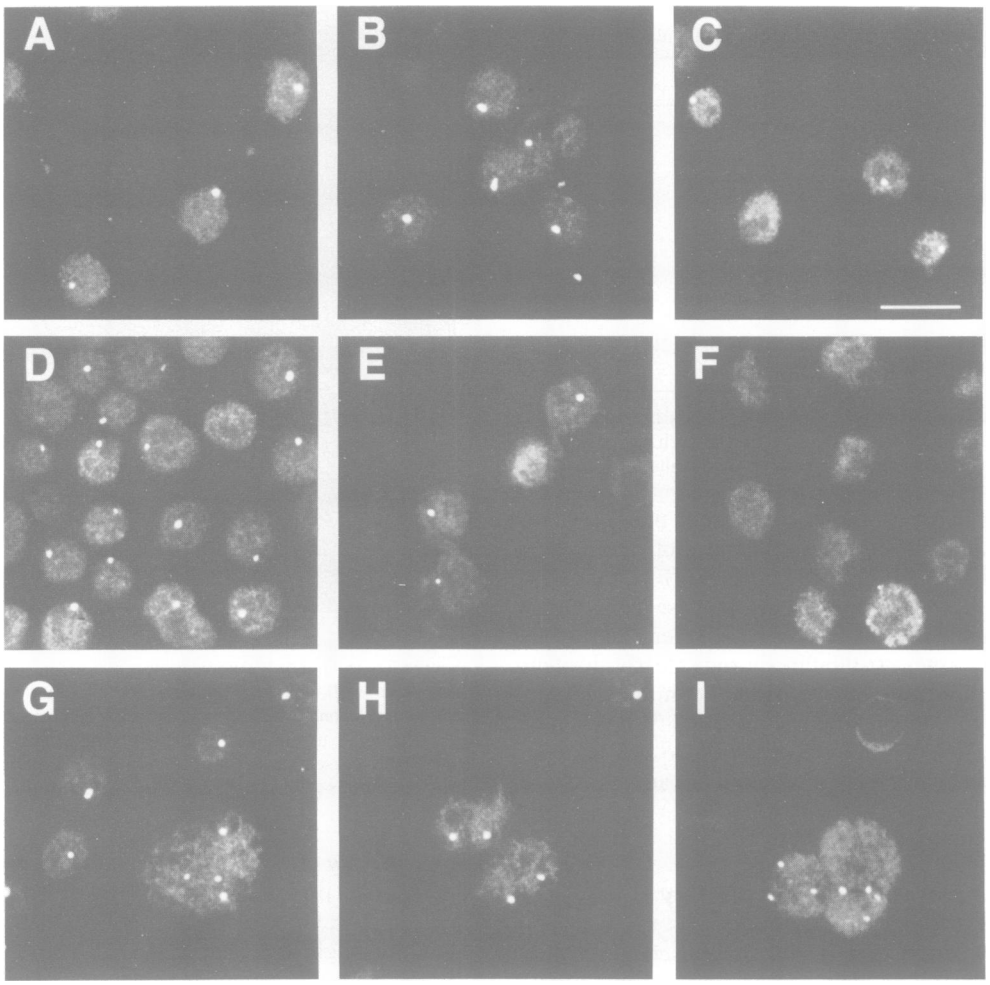

Fig. 5. Anti-GATA-1 immunofluorescence of primitive erythroblasts from (**A**) day 10.5 yolk sac and (**B**) day 10.5 embryonic blood, (**C–E**) definitive erythroblasts from day 11.5, 12.5 and 14.5 fetal liver, (**F**) bone marrow-derived mast cells and (**G–I**) megakaryocytes from day 12.5 and 14.5 fetal liver. Scale bar, 10 μm.

quite distinct from the β-globin genes (arrowhead, in red). The same failure of co-localization was observed when the experiment was repeated wih a MEL cell line carrying multiple (>10) tandemly integrated copies of a human β-globin gene under βLCR control (data not shown). In this case, despite the apposition of >100 functional GATA binding sites within a 150 kb stretch of DNA, there was no co-localization between the transgene locus and the GATA-1 foci. Additional circumstantial evidence against the hypothesis that the GATA-1 foci represented binding of the protein to nucleic acid was provided by our failure to document an increase in the number of foci in MEL cells following transfection with βLCR-driven GATA-1 constructs in multiple cell lines, a failure of GATA-1 foci to co-localize with DNA in MEL cells double stained with anti-GATA-1 and antibodies to DNA and resistance of the foci to RNase and DNase treatment (data not shown).

The foci represent nuclear bodies containing GATA-1 transcription factor

Electron microscopy was performed on MEL cells to determine whether the foci seen by immunofluorescence specifically localized to a nuclear structure. Cells were labelled using anti-GATA-1 antibodies and a secondary antibody coupled to 5 nm colloidal gold particles. Numerous small clusters of immunogold particles dispersed through the nucleoplasm (Figure 7, small arrows) probably corresponded to the diffuse nucleoplasmic staining pattern detected by immunofluorescence. Occasionally intensely labelled round homogeneous 'nuclear bodies' (Bouteille *et al.*, 1974) 0.2–0.4 μm in diameter were observed (Figure 7, large arrows). These were likely to correspond to the bright foci seen by fluorescence microscopy. The uniform, compact nature of these GATA-1-containing structures distinguished them from the ring-like class of nuclear bodies which contain the PML protein (see Discussion). Consistent with the immunofluorescence observations, GATA-1 nuclear bodies were frequently found near the nuclear periphery, in close association with chromatin (Figure 7, arrowheads) or close to nucleoli (data not shown).

GATA-1 foci are distinct from coiled bodies and do not contain splicing factors

In their number, discrete nature and proximity to the nucleolus the GATA-1-associated structures were reminiscent of coiled bodies, subnuclear domains containing the small nuclear ribonucleoproteins (snRNPs) involved in

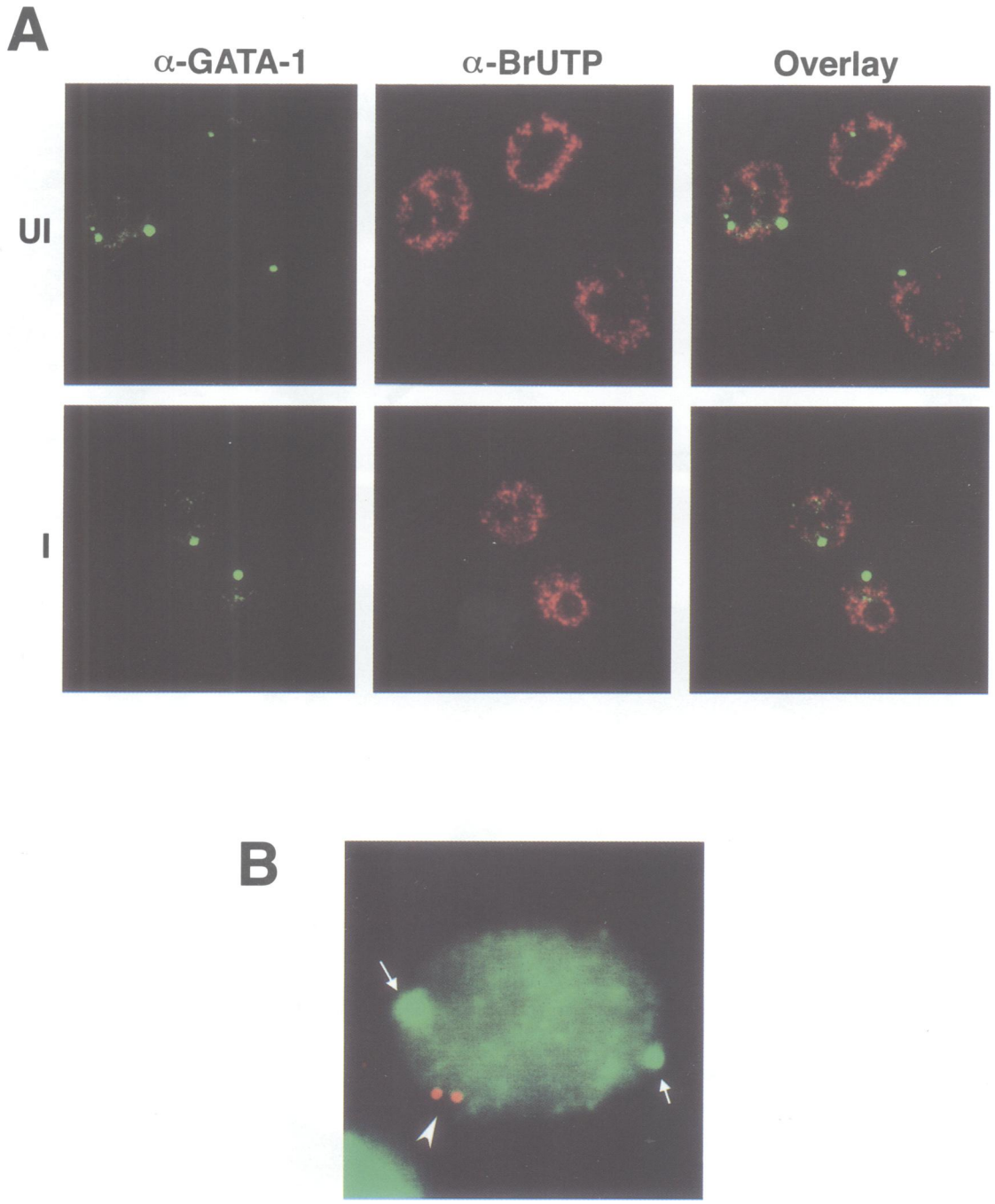

Fig. 6. (**A**) Single optical sections of uninduced (UI) and DMSO-induced (I) C88 MEL cells double stained with antibodies to GATA-1 (α-GATA-1) on the left in green, anti-bromouridine (α-BrUTP) in the centre in red and the overlayed images on the right. Magnification ×2000. (**B**) Detection of the murine β-globin gene loci by *in situ* DNA hybridization (in red, arrowhead) and immunofluorescent localization of GATA-1 staining with anti-GATA-1 antibody (in green), demonstrating that the GATA-1 foci (arrows) are clearly separate from the β-globin loci. Magnification ×5000.

pre-messenger RNA splicing (Lamond and Carmo-Fonseca, 1993a). This prompted us to examine whether GATA-1 associated with coiled bodies or other proteins involved in RNA processing. Double staining of C88 MEL cells with anti-GATA-1 and antibodies to the major coiled body protein, p80 coilin, demonstrated that the GATA-1 nuclear bodies were distinct structures which did not co-localize with coiled bodies either before or after induced differentiation (Figure 8). Similarly, GATA-1 did not co-localize with fibrillarin, a 34 kDa protein expressed in the nucleolus and in coiled bodies (Reimer *et al.*, 1987; data not shown). Specific antisera which recognized the snRNP splicing factors gave nuclear staining broadly coincident with the general GATA-1 staining and accumulated within coiled bodies, but did not specifically detect the GATA-1 nuclear bodies. Finally, the GATA-1 foci were not related to interchromatin granules, structures intimately associated with splicing factors (Lamond and Carmo-Fonseca, 1993b; Spector, 1993; data not shown).

All haemopoietic GATA transcription factors localize to nuclear bodies

A series of double staining experiments was performed in MEL cells to determine whether other transcription factors

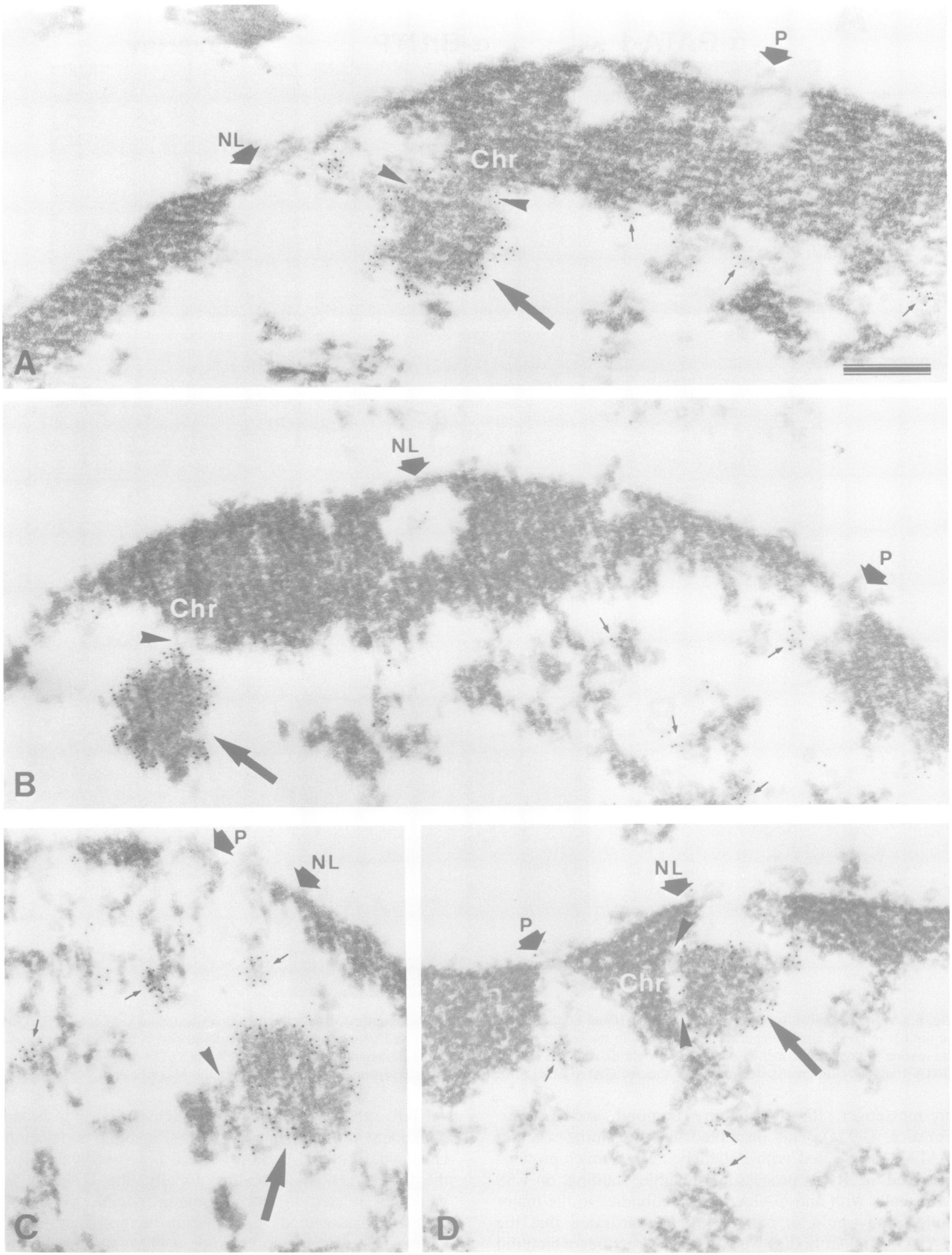

Fig. 7. (**A–D**) Electron microscopy of MEL cells immunogold labelled with anti-GATA-1 antibody. Nuclear bodies are intensely stained (large arrows; note that gold particles are restricted to the periphery of the body because labelling was performed using a pre-embedding procedure, which limits access of the particles to the interior of the compact structures) and localized near the nuclear periphery in close association with chromatin (arrowheads). Scattered small clusters of gold particles are present throughout the nucleoplasm (small arows). NL, nuclear lamina; P, nuclear pore complex; Chr, chromatin. Scale bar, 0.25 μm.

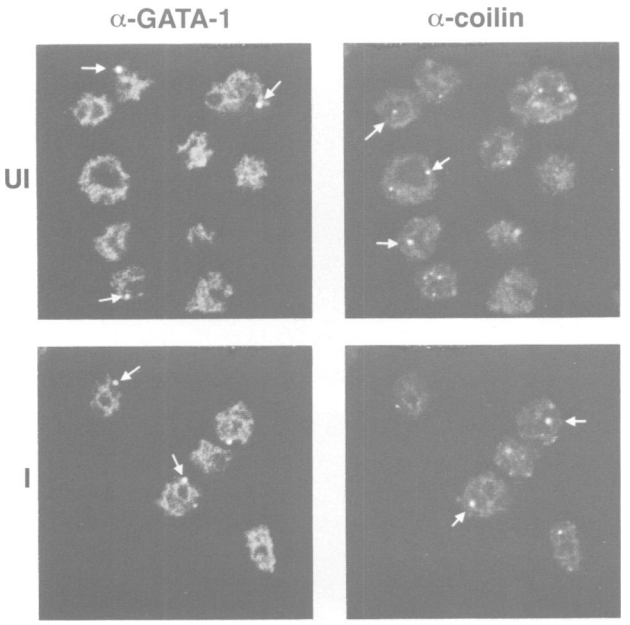

Fig. 8. Single optical sections of uninduced (UI) and DMSO-induced (I) C88 MEL cells double stained with antibodies to GATA-1 (α-GATA-1) on the left and to p80 coilin (α-coilin) on the right. Magnification ×1000.

were associated with nuclear bodies. Although regional variation in the intensity of immunofluorescence within the nucleus was sometimes evident, in no instances were well-defined nuclear bodies observed using antibodies to the TATA binding protein (TBP), the ubiquitously expressed transcription factors Sp1, c-Jun and USF and the haemopoietic-restricted factor NF-E2 (data not shown).

To determine whether other haemopoietically expressed GATA proteins localized to nuclear bodies we examined the GATA-1 ts Wehi-Epo cell line, a factor-dependent adult murine erythroid cell line which expresses both GATA-1 and GATA-2 (Cairns *et al.*, 1994). Immunofluorescent staining with antibodies to GATA-1 demonstrated an average of 3–6 nuclear bodies in 50–80% of cells. Double staining with anti-GATA-1 and a specific monoclonal anti-GATA-2 antibody revealed that 30% of the population were also GATA-2-positive and both GATA proteins localized to the nuclear bodies in these cells (see examples in Figure 9A–F). In most instances anti-GATA-1 fluorescence was stronger than anti-GATA-2, though in the small percentage (~1%) of cells which stained brightly for GATA-2, GATA-1 immunofluorescence was often weak or even absent (see Figure 9D–F for an example). One interpretation of this data is that cells expressing higher levels of GATA-2 may be immature precursors and that as differentiation proceeds GATA-1 increases and GATA-2 is reciprocally down-regulated.

We extended this observation by introducing GATA-2 and GATA-3 expression vectors into MEL cells and examining stably transfected pools for GATA protein expression by immunofluorescence. In all cases the transfected GATA proteins (detected by specific anti-GATA-2 and anti-GATA-3 monoclonal antibodies) localized to the same nuclear bodies detected by antibodies to the endogenous murine GATA-1 (several examples are arrowed in Figure 9G–L). It was interesting to note that high levels of transfected GATA-2 or GATA-3 were frequently associated with lower expression of endogenous GATA-1 (evident as an overall 'green' hue to some cells in the overlayed images in Figure 9I and L), similar to the reciprocal staining intensities seen in the GATA-1 ts Wehi-Epo cell line.

The demonstration that murine GATA-1, murine and human GATA-2 and human GATA-3 proteins localize to the same nuclear structures strongly suggests interaction via a shared domain. Since the most conserved region among the proteins encompasses the DNA binding domain, the possibility of a non-DNA binding role for the GATA zinc fingers is raised (see Discussion).

Discussion

We have demonstrated a unique distribution of GATA-1 protein within the nuclei of murine erythroblasts and megakaryocytes; as well as a nucleoplasmic immunofluorescence staining pattern with nucleolar exclusion, one to six discrete foci of intense staining adjacent to nucleoli or at the nuclear periphery were also observed. Epitope-tagged GATA-1 proteins, detected by independent antibodies, gave the same immunofluorescence pattern as the endogenous GATA-1 protein. The presence of these structures in primary haemopoietic cells (albeit it in lower numbers per cell) excluded the possibility that they were solely a transformation-related phenomenon and justified the subsequent use of MEL cells to study the foci in more detail.

Our initial hypothesis was that these bright foci of GATA-1 fluorescence represented binding of GATA-1 protein to DNA within the globin loci. However, we were unable to demonstrate an association between the GATA-1 foci and sites of transcription or with murine or human β-globin loci, even though the latter were present in multiple tandemly integrated copies in a MEL cell line and thus contained an array of >100 GATA binding sites. While it has not been possible to exclude an association with another undefined region of DNA, the nuclease resistance of the foci and the absence of co-localization between the foci and DNA in cells stained with anti-DNA antibodies argues against this.

Immunoelectron microscopy studies showed the GATA-associated foci to be discrete, electron dense structures located adjacent to nucleoli or at the nuclear periphery. They may thus be classified as nuclear bodies, a generic term coined to describe subnuclear structures 0.3–1.5 μm in diameter which can be detected by transmission electron microscopy in most cell types (Bouteille *et al.*, 1974; Padykula and Clark, 1981). In many cases nuclear bodies are associated with nucleoli and may be present as paired structures, two features in common with our findings. Additional features, such as presence in primary cells (i.e. normal erythroblasts and megakaryocytes), a correlation with nuclear ploidy and an increase in number in transformed cells, have also been described for other nuclear bodies (see Brasch and Ochs, 1992, and references therein). It is now realized that nuclear bodies are a heterogeneous group, some of which can be classified by the presence of specific proteins.

Most relevant to our studies are coiled bodies, the 'nuclear accessory bodies' first described in silver stained

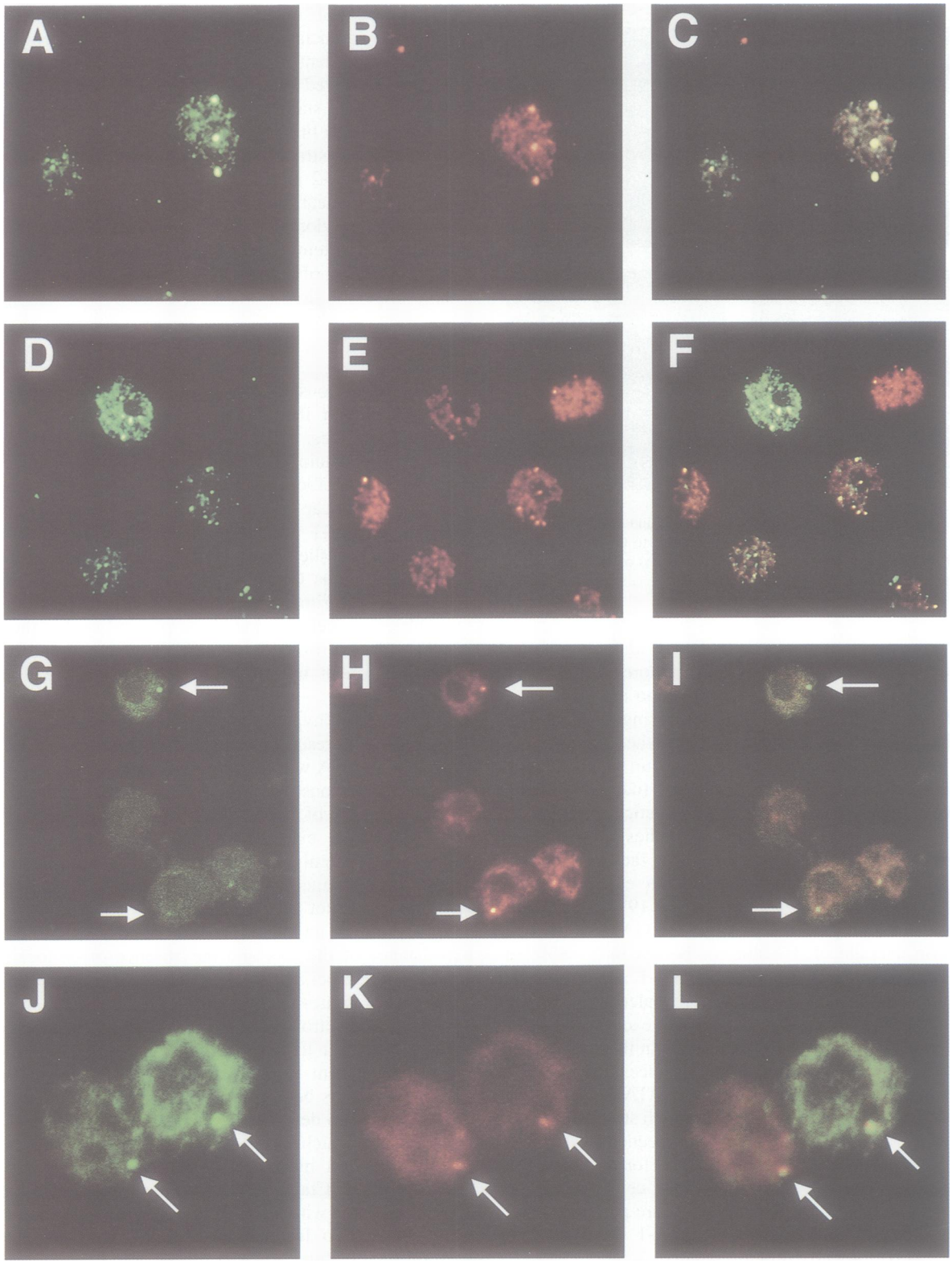

Fig. 9. Expression patterns of GATA-1 and GATA-2 in GATA-1 ts Wehi-Epo cells (**A–F**). For two fields of doubly stained cells anti-GATA-2 staining in green (A and D), anti-GATA-1 staining in red (B and E) and the overlayed images (C and F) are shown. Immunofluorescence of MEL cells transfected with hGATA-2 (**G–I**), showing anti-GATA-2 in green (G), anti-GATA-1 in red (H) and the overlayed image (I). Immunofluorescence of MEL cells transfected with hGATA-3 (**J–L**), showing anti-GATA-3 in green, anti-GATA-1 in red and the overlayed image (L). Examples of GATA nuclear bodies are arrowed. Magnification: (A–F), ×1000; (G–I), ×1500; (J–L), ×3000.

neurons by Ramon-y-Cajal (Lamond and Carmo-Fonseca, 1993a). Coiled bodies share some characteristics with GATA nuclear bodies, as both are present in low numbers in the nucleus, often in close proximity to the nucleolus. However, there are some important differences. First, whilst coiled bodies are dynamic structures which dis-

assemble during mitotis and are not visible again until late G_1 (Andrade *et al.*, 1993; Carmo-Fonseca *et al.*, 1993), GATA bodies are retained in all phases of the cell cycle. Secondly, none of the components identified in coiled bodies, namely p80 coilin (a protein specifically detected by sera from some patients with autoimmune disease), small nuclear ribonucleoproteins (snRNPs) and fibrillarin, were detected in GATA nuclear bodies.

The most recently characterized nuclear body is associated with the PML protein, the fusion partner for retinoic acid receptor α in acute promyelocytic leukaemia. Typically, between five and 30 of these spheroid or doughnut-shaped nuclear bodies are dispersed throughout the cell nucleus (Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994). Two protein components within these structures have been identified thus far, PML itself and Sp100, a nuclear protein with homology to several transcription factors (Szostecki *et al.*, 1990; Ascoli and Maul, 1991; Stuurman *et al.*, 1992; Xie *et al.*, 1993). Near its N-terminus PML has a RING finger domain which co-ordinates two zinc atoms in a $-C_3HC_4-$ structure similar to DNA binding zinc finger proteins (Sánchez-García and Rabbitts, 1994). The requirement for an intact RING finger to target PML to nuclear bodies (Kastner *et al.*, 1992; Borden *et al.*, 1995) may indicate, by analogy, that the GATA-1 zinc finger can also be involved in protein–protein interactions, even though the NMR solution structures of the PML RING finger domain (Borden *et al.*, 1995) and the chicken GATA-1 C-terminal zinc finger (Omichinski *et al.*, 1993) are not very similar. Our preliminary immunofluorescence data indicate that GATA nuclear bodies are distinct from PML bodies and the structures do not co-localize (M.Carmo-Fonseca *et al.*, unpublished results).

It is known that viral infections can perturb normal nuclear architecture. For example, the SV40 T antigen and the retinoblastoma protein co-localize by immunofluorescence to numerous punctate nuclear foci in COS cells (Jiang *et al.*, 1991) and retinoblastoma and EBNA-5 proteins form similar structures in Epstein–Barr virus-immortalized lymphoblastoid cell lines (Jiang *et al.*, 1991). In adenovirus-infected HeLa cells specific nucleolar proteins and snRNP antigens are found in multiple intranuclear foci which coincide with sites of adenovirus DNA replication (Walton *et al.*, 1989). Finally, the Herpes simplex virus immediate early protein Vmw110 (itself a RING finger protein) has recently been shown to alter the distribution of PML-containing nuclear bodies (Everett and Maul, 1994). Not only do these patterns of immunofluorescence differ from those seen for GATA-1, but the demonstration of GATA-1 foci in primary haemopoietic cells argues against an aetiological role for viruses in the formation of the GATA nuclear bodies.

The major region of homology between GATA-1, -2 and -3 (Tsai *et al.*, 1989; Ko *et al.*, 1991; Lee *et al.*, 1991; Dorfman *et al.*, 1992) encompasses the zinc finger domain, with human GATA-2 and GATA-3 sequences 84 and 82% identical to murine GATA-1 between amino acids 200 and 315. In contrast, the GATA proteins show only 16–23% identity across their N- and C-terminal regions. Our experimental data demonstrating that murine GATA-1, murine and human GATA-2 and human GATA-3 localize to the same nuclear structures in MEL cells raises the possibility of a non-DNA binding role for the zinc fingers. However, because of difficulties in expressing GATA-1 cDNA constructs in MEL cells we have so far been unable to test our hypothesis directly.

Utilization of a zinc binding domain for protein–protein interactions is not without precedent. For example, the zinc finger domains of steroid/nuclear receptors take part in dimerization as well as DNA binding (Luisi *et al.*, 1991; Rastinejad *et al.*, 1995) and direct interactions have been demonstrated between the zinc fingers of YY1 and Sp1 (Lee *et al.*, 1993; Seto *et al.*, 1993), the zinc finger of E1A and TBP (Geisberg *et al.*, 1994), the zinc finger-like region of *raf* and an annexin-like domain of 14-3-3 proteins (Freed *et al.*, 1994; Irie *et al.*, 1994) and the zinc finger domain of the yeast Gal4 protein and the bZIP domain of c-Jun (Sollerbrant *et al.*, 1995). Furthermore, the metal binding domains of the LIM protein family, structurally very similar to the GATA zinc fingers (Pérez-Alvarado *et al.*, 1994), appear to be exclusively involved in protein–protein interaction (Feuerstein *et al.*, 1994; Schmeichel and Beckerle, 1994; Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994). Particularly relevant to haemopoiesis, the LIM domains of *rbtn 1* and *rbtn 2* form complexes with the basic helix–loop–helix regions of *scl*, *tal2* or *lyl1* (Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994). In the light of these data it is interesting that the GATA bodies were not targeted by antibodies to the leucine zipper proteins c-Jun or NF-E2, nor was there an association with all zinc finger proteins, since Sp1 was not visibly enriched in the GATA nuclear bodies.

It is noteworthy that the rescue of haemopoietic differentiation in GATA-1-negative embryonic stem cells *in vitro* is possible with the zinc finger domain of GATA-1 alone (Blobel *et al.*, 1995). One interpretation is that the metal binding domain of GATA-1 has a protein interactive role in addition to its DNA binding. In a similar vein, 416B myeloid cells exhibit megakaryocytic differentiation following expression of the C-terminal zinc finger of GATA-1 alone (Visvader *et al.*, 1995).

Whilst this manuscript was in preparation Crossley *et al.* (1995) demonstrated GATA-1 homodimerization *in vitro* requiring the C-terminal zinc finger and showed that the same region mediated heterotypic interactions between GATA-1 and GATA-2 or GATA-3. Also, GATA-1 was shown to interact functionally with the Krüppel family zinc finger proteins Sp1 and EKLF in a zinc finger-dependent fashion (Merika and Orkin, 1995). In both studies the authors demonstrated that this interaction was not compromised by GATA-1 mutations which abolished DNA binding. These observations contrast with the findings of Yang and Evans (1995), who used a chimeric GATA-1 protein (in which the DNA binding domain of the bacterial repressor protein LexA replaced the C-terminal zinc finger) to suggest that GATA-1 homodimerization and GATA-1/GATA-2 (but not GATA-1/GATA-5) heterodimerization were possible *in vitro* in the absence of the GATA-1 C-terminal zinc finger.

Although our studies do not directly address these apparently conflicting data, the intense focal accumulations of GATA-1 protein and its physical co-localization with GATA-2 and GATA-3 in the GATA nuclear bodies would be consistent with homo- or heterotypic interactions occurring between haemopoietic GATA family members

in vivo and we would favour the hypothesis that interaction primarily occurs via the shared DNA binding domain. Of course we cannot exclude the possibility that the GATA proteins in these structures exclusively interact with heterologous proteins.

There remain many puzzling aspects to the GATA nuclear bodies. We have been unable to detect GATA nuclear bodies in primary mast cells or in an immortalized mast cell line (data not shown). Nor are the structures seen in GATA-1-transfected 416B cells, despite the induction of significant megakaryocytic differentiation (Visvader *et al.*, 1992; data not shown). One explanation for these observations is that the formation of GATA nuclear bodies requires additional lineage-restricted proteins not present in mast cells or in 416B myeloid cells, even after GATA-1-induced differentiation. This model would predict that GATA-1 expression alone in a non-erythroid/megakaryocytic cell would be insufficient to allow formation of the GATA nuclear bodies. To test this hypothesis anti-GATA-1 antibody staining of somatic cell hybrids between MEL cells and B lymphoid cell lines (Murrell and Green, 1995) was compared with the staining pattern seen following GATA-1 transfection into the B lymphoid fusion partner alone (A.G.Elefanty, A.M.Murrell and A.R.Green, unpublished results). In the somatic cell hybrids, which expressed a range of erythroid proteins, GATA nuclear bodies were clearly present. Although most of the lymphoblasts expressing GATA-1 in transient transfections displayed only diffuse nuclear fluorescence, a small subset (~10%) did show focally bright staining similar to GATA nuclear bodies. The significance of this finding is currently unclear.

A major unanswered question relates to the role the GATA nuclear bodies may play in erythroid and megakaryocytic development and differentiation. Despite considerable analysis, the function of two other well-defined nuclear bodies, coiled bodies and PML bodies, still remains elusive. Coiled bodies are postulated to play a role in spliceosome assembly or recycling or in the processing of snRNA transcribed from the excised introns of other genes (Lamond and Carmo-Fonseca, 1993). The absence of antigens associated with RNA processing argues against a transcription-related role for the GATA nuclear bodies. PML localization to nuclear bodies is disrupted in acute promyelocytic leukaemia, whilst differentiation induced by retinoic acid leads to normalization of the staining pattern (Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994). We have yet to define an analogous situation in which there is perturbation of the GATA nuclear bodies. Hence, at this stage the function of these nuclear bodies remains obscure and characterization of other constituent proteins will be necessary to shed light on this issue.

Materials and methods

Cell culture

The APRT$^-$ MEL cell line C88 (Deisseroth and Hendrick, 1978) was maintained, transfected and induced to undergo erythroid differentiation following addition of 2% DMSO to the culture medium as previously described (Antoniou, 1991). Buf707 MEL cells, a thymidine kinase-deficient subclone of clone 707 (Friend *et al.*, 1971) and the murine B cell line A20L (Kim *et al.*, 1979), both supplied by Dr A.R.Green, and the murine thymoma cell line EL-4 (Gorer, 1950) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), with the addition of 2×10^{-5} M 2-mercaptoethanol to the latter. GATA ts Wehi-Epo cells (Cairns *et al.*, 1994), a gift from Dr S.Ottolenghi, were maintained in 10% FCS in RPMI with 10% WEHI-3 conditioned medium (as a source of IL-3) and 0.5 U/ml erythropoietin (Boehringer Mannheim Biochemica, Mannheim, Germany) at 32°C.

Fetal erythroblasts were harvested from embryonic circulation, yolk sac and fetal liver between days 10.5 and 14.5 of gestation. The morning of discovery of the vaginal plug was considered day 0.5. Megakaryocytes were detected in fetal liver cell populations by their staining for acetylcholinesterase as described (Metcalf, 1984). Mast cells were expanded from adult bone marrow by culturing cells in DMEM with 10% FCS and 10% WEHI-3 conditioned medium for several weeks. Purity of the cultures was verified by staining with May–Grünwald–Giemsa.

Transfections were performed by electroporating 3×10^7 MEL cells with 50 μg *Pvu*I-linearized plasmids using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, VA) set at 960 μF, 250 V as described (Antoniou, 1991). Following selection in 1 mg/ml G418, clonal cell lines were established by expanding single colonies grown in 0.3% agar as described (Metcalf, 1984).

Plasmids

To generate the epitope-tagged *myc*–GATA-1 and HA–GATA-1 inducible vectors, oligonucleotides encoding the human C-terminal *myc* peptide recognized by the monoclonal antibody 9E10 (Evan *et al.*, 1985) (sense strand, 5′-CATGGAGCAGAAGCTTATCTCGGAGGAGGACCTGGG-3′) or the influenza virus haemagglutinin peptide recognized by the monoclonal antibody 12C5 (Field *et al.*, 1988) (sense strand, 5′-CATGGGATATCCATACGACGTGCCAGATTACGCCGTG-3′) were cloned into the unique *Nco*I site of the murine GATA-1 cDNA (Tsai *et al.*, 1989) in Bluescript (Stratagene Cloning Systems, La Jolla, CA) and verified by sequencing across the region. For the HA–GATA-1 construct the *Nco*I site was first blunted with mung bean nuclease (Sambrook *et al.*, 1989). The epitope-tagged GATA-1 minigenes were generated by replacing the GATA-1 cDNA downstream of the *Stu*I site in the second exon with a 4.3 kb genomic fragment of murine GATA-1 (a gift from Dr D.Whyatt), which included the remaining exons, introns and polyadenylation sequences. To complete the plasmids the modified GATA-1 genes were cloned as 4.6 kb *Not*I–*Asp*718 fragments into the 11 kb *Not*I–*Asp*718 fragment of a human β-globin microlocus expression vector (pEV3) containing the human β-globin promoter (Needham *et al.*, 1992) under the control of the human βLCR (Collis *et al.*, 1990).

The plasmids GATA-2/pEF-MC1neo and GATA-3/pEF-MC1neo, encoding human GATA-2 and GATA-3 cDNAs respectively, were a gift from Dr Jane Visvader and have been described previously (Visvader and Adams, 1993).

Immunofluorescence and in situ hybridization

Staining of cell lines and primary haemopoietic cells was as previously described (Antoniou *et al.*, 1993), except that pre-extraction with 0.5% Triton X-100 for 30–60 s at 4°C prior to fixation in 3.7% paraformaldehyde in CSK buffer (10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 2 mM EGTA) was required to ensure uniform penetration of antibodies into the nucleus. For day 14.5 fetal liver erythroblasts optimal staining was achieved by fixing cells in 3.7% paraformaldehyde in CSK buffer and then permeabilizing in 0.2% SDS buffer as described (Carmo-Fonseca *et al.*, 1992).

The following monoclonal antibodies were used: anti-mouse GATA-1 (clone N-6) (Ito *et al.*, 1993); anti-GATA-2 (clone CG2–96) and anti-GATA-3 (clone HG3-31) (both from Santa Cruz Biotechnology Inc., Santa Cruz, CA); anti-*myc* (clone 9E10) (Evan *et al.*, 1985); anti-haemagglutinin (clone 12C5) (Field *et al.*, 1988); anti-fibrillarin (clone 72B9) (Reimer *et al.*, 1987); anti-interchromatin granule (clone 3C5) (Turner and Franchi, 1987); anti-α-tubulin (clone B-5-1-2) (Sigma Chemical Co., St Louis, MO); anti-DNA and anti-bromodeoxyuridine (both from Boehringer Mannheim Biochemica, Mannheim, Germany). Polyclonal rabbit antisera to p80 coilin (a gift from Dr K.Bohmann), Sp1 (serum 2892-E) (a gift from Dr S.Jackson), p45 NF-E2 (a gift from Dr N.Andrews) (Andrews *et al.*, 1993) and c-Jun (a gift from Dr S.Philipsen), human autoimmune serum (Kung) recognizing the Sm epitope (a gift from Dr K.Weis) and affinity-purified goat anti-mouse haemoglobin (Kirkegaard and Perry Laboratories Inc., Gaithersburg, VA) were used. Species-specific antibodies coupled to FITC or Texas red were used in the second stage of staining, except for anti-GATA-2 staining, when incubation with biotinylated anti-rat antibodies (Vector Laboratories Inc., Burlingame, CA) followed by FITC-conjugated avidin was sometimes used to enhance the signal.

For *in situ* hybridization a 7 kb *Eco*RI fragment of genomic DNA containing the murine β-major globin gene (Tilghman *et al.*, 1977) was labelled with digoxigenin-11-UTP (Boehringer Mannheim Biochemica, Mannheim, Germany) and *in situ* hybridization was performed as described (Zirbel *et al.*, 1993).

Samples were examined using a Bio-Rad MRC 600 confocal microscope (Bio-Rad Laboratories, Richmond, VA), the EMBL compact confocal microscope (Stelzer *et al.*, 1992) and a Leica confocal laser scaning microscope (Leica Lasertechnik, Heidelberg, Germany).

Electron microscopy

Immunoelectron microscopy of C88 MEL cells was performed using a pre-embedding technique as described (Ferreira *et al.*, 1994). The cells were sequentially incubated with rat anti-GATA-1 monoclonal antibody [diluted 1:200 in 0.1% gelatin, 1% bovine serum albumin (BSA), 0.15 M NaCl, 0.1 M Tris–HCl, pH 7.4], rabbit anti-rat IgG [Dako A/S, Glostrup, Denmark; diluted 1:50 in phosphate-buffered saline (PBS), 0.05% Tween 20] and goat anti-rabbit IgG coupled to 5 nm gold particles (Amersham, Little Chalfont, UK; diluted 1:10 in 0.1% gelatin, 1% BSA, 0.15 M NaCl, 0.1 M Tris–HCl, pH 7.4). Following immunogold labelling the cells were post-fixed, dehydrated and embedded in Epon as described (Ferreira *et al.*, 1994). Ultrathin sections were observed with a Jeol 100CXII electron microscope (JEOL USA, Peabody, CA) operated at 80 keV.

In vitro transcription

Nascent RNA transcripts were labelled with BrUTP by a modification of the method of Wansink *et al.* (1993). MEL cells were washed in PBS and then allowed to adhere to poly(L-lysine)-coated (Sigma Chemical Co.) glass coverslips before permeabilization with glycerol/0.05% Triton X-100 containing 1 mM phenylmethylsulfonyl fluoride (PMSF) for 3 min at room temperature. The coverslips were incubated with transcription buffer [100 mM KCl, 50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 0.5 mM EGTA, 25% glycerol, 25 μM *S*-adenosyl-L-methionine (Boehringer Mannheim Biochemica), 5 U/ml RNasin (Promega Corporation, USA), 1 mM PMSF, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP and 0.2 mM BrUTP (Sigma Chemical Co.)] for 15 min at room temperature, the cells re-permeabilized for 1 min with PBS/0.5% Triton X-100 containing 5 U/ml RNasin and 1 mM dithiothreitol (DTT) and the reaction stopped by fixing the cells in 3.7% paraformaldehyde/CSK/1 mM DTT. After three washes in PBS/RNasin/DTT immunofluorescent staining to detect GATA-1 protein and BrUTP incorporated into RNA transcripts was performed. RNasin at 25 U/ml and 1 mM DTT were included in all the antibody solutions and 5 U/ml RNasin and 1 mM DTT were included in all the washes.

Protein extracts and Western blotting

MEL cell extracts enriched in nuclear proteins were produced as described by Dale *et al.* (1989). Briefly, ~5×10^7 cells were harvested, washed in PBS and then snap frozen at −70°C. The cell membranes were lysed by thawing into low salt extraction buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF, 5% glycerol) and then pelleting the nuclei. After one more wash the nuclei were resuspended in the same buffer, the salt concentration was adjusted to 0.35 M NaCl and the nuclear proteins were extracted on ice for 30 min. Nuclear debris was pelletted at 14 000 *g* at 4°C for 25 min and the supernatant stored at −70°C.

For Western blotting 30 μg protein were electrophoresed through a 10% SDS–polyacrylamide gel, transferred to nitrocellulose (Hybond C-Extra, Amersham, Little Chalfont, UK), incubated overnight at 4°C with anti-GATA-1 or anti-*myc* antibodies at 2 μg/ml followed by horseradish peroxidase-coupled secondary antibodies and detected by ECL according to the manufacturer's instructions (Amersham).

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