Regulation of J6 gene expression by transcription factor GATA-4

Malgorzata BIELINSKA and David B. WILSON*

Edward Mallinrodt Departments of Pediatrics and Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, MO 63110, U.S.A.

Retinoic acid-induced differentiation of mouse F9 embryonal carcinoma cells into primitive endoderm is accompanied by increased transcription of the gene for J6, a heat shock protein implicated in collagen biosynthesis. In this paper we present evidence that transcription factor GATA-4, a retinoic acid-inducible GATA-binding protein, is involved in the regulation of J6 gene expression in F9 cells. Northern-blot analysis indicates that transcripts encoding GATA-4 and J6 increase in parallel during retinoic acid-induced differentiation of F9 cells. Gel-shift experiments and antibody binding studies demonstrate that: (1)

GATA-4 is the major GATA-binding protein activity in differentiated F9 cells, and (2) GATA-4 binds to consensus GATA motifs in the retinoic acid-responsive portion of the J6 promoter. Co-transfection studies using NIH 3T3 cells show that GATA-4 is a potent *trans*-activator of the J6 promoter. These lines of evidence suggest that expression of J6 in F9 cells is regulated by GATA-4. We speculate that transcription factor GATA-4 may also control other genes involved in extracellular matrix formation in the yolk sac.

INTRODUCTION

The GATA-binding proteins are a group of structurally related transcription factors that regulate gene expression and differentiation in a variety of cell types [1]. Members of this family of transcription factors recognize a consensus DNA sequence known as the 'GATA' motif [2–5], which is an essential *cis* element in the promoters or enhancers of numerous genes. All GATA-binding proteins contain specialized Cys-Cys finger domains that bind bivalent cations such as Fe²⁺ or Zn²⁺ [6], interact with DNA [7], and influence nuclear localization of these factors [8]. The solution structure of a GATA-binding finger domain complexed with DNA has been determined by NMR [7].

The importance and versatility of the GATA-binding finger are underscored by conservation of this domain through evolution. Single-cell organisms, including *Saccharomyces*, *Aspergillus* and *Neurospora*, contain GATA-binding proteins that regulate nitrogen metabolism [9,10]. The nematode *Caenorhabditis elegans* has an embryonically expressed GATA-binding protein of unknown function [11] and the silkworm *Bombyx mori* has a GATA-binding protein that regulates chorion gene transcription [12]. *Drosophila* express GATA-binding proteins involved in fat body formation [13], neural development [14] and dorsoventral axis gene expression [15].

GATA-binding proteins in vertebrates serve a different set of functions than in lower organisms. The founding member of the vertebrate GATA-binding-protein family, GATA-1, is expressed in erythroid cells, megakaryocytes and other haematopoietic cells [16–25]. GATA-1 regulates expression of genes critical for erythroid and megakaryocytic differentiation including globins, haem biosynthetic enzymes and the erythropoietin receptor [1,25–35]. A second member of the vertebrate GATA-binding-protein family, GATA-2, is expressed in bone-marrow cells and a host of other cell types [36–38]. This factor has been implicated in endothelial gene expression [36,39] as well as in haematopoiesis [40–42]. GATA-3 is expressed in brain and T-lymphocytes, where it is postulated to control expression of T-cell receptor genes [43,44].

We [45] and others [46–48] have identified a new member of the GATA-binding-protein family, termed GATA-4. In adult

animals, GATA-4 is expressed in heart, gut epithelium, gonads and, to a lesser extent, liver [45–48]. During early fetal development, GATA-4 expression is also seen in yolk sac endoderm and cells associated with heart development [45,46,49]. This pattern of expression distinguishes GATA-4 from other members of the GATA-binding protein family and suggests that this factor has a unique function in the organism. *In vitro* promoter studies suggest that GATA-4 may regulate genes critical for myocardial function, including cardiac α -myosin heavy chain [50], cardiac troponin C [51] and brain-type natriuretic peptide [48,52]. The function of GATA-4 in other tissues is less clear. Retinoic acid (RA)-induced differentiation of mouse F9 embryonal carcinoma cells into parietal or visceral yolk sac endoderm is accompanied by increased expression of GATA-4, suggesting that this transcription factor may regulate genes involved in differentiation of embryonal carcinoma cells, such as genes required for extracellular matrix formation [45].

One of the genes induced during F9 cell differentiation is a heat shock protein known as J6 [53,54], which is the mouse orthologue of chicken HSP47 [55] and rat gp46 [56]. J6 shows sequence similarity to the extended serpin family, though the molecule has no known protease inhibitor activity [54]. J6 protein localizes to endoplasmic reticulum, where it is presumed to function as a molecular chaperone for procollagen [56]. J6 mRNA is preferentially expressed in adult heart, yolk sac endoderm and gonads [53], a pattern of expression reminiscent of the distribution of GATA-4 [45,48]. The 5'-regulatory region of the mouse J6 gene has been analysed and the RA-responsive region of the J6 gene has been shown to reside in nucleotides –1050 → –738 of the promoter [57]. Remarkably, this portion of the promoter contains a cluster of four consensus GATA motifs that are recognized by a DNA-binding protein present in differentiated, but not undifferentiated, F9 cells [58]. Given the similarities in expression patterns of GATA-4 and J6, GATA-4 is a logical candidate for regulation of the J6 gene in F9 cells. In this paper we present several lines of evidence, including RNA analysis, gel-shift assays, antibody binding and *trans*-activation studies, which suggest that GATA-4 binds and activates the J6 promoter in F9 cells.

Abbreviations used: dbcAMP, dibutyryl cyclic AMP; FITC, fluorescein isothiocyanate; GH, growth hormone; RA, retinoic acid.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Cell culture

NIH 3T3, COS-1 and F9 cells were maintained as described previously [45]. F9 cells were differentiated into parietal endoderm with RA (3×10^{-7} M) and dibutyryl cyclic AMP (dbcAMP) (1 mM) [59]. NIH 3T3 cells and F9 cells were transfected with plasmid DNA using calcium phosphate precipitation [60], while COS cells were transfected with DEAE-dextran [20].

Gel-shift analysis

Nuclear extracts were prepared [61] from undifferentiated F9 cells, differentiated F9 cells, or COS cells transfected with the eukaryotic expression plasmids pMT₂ [20], pMT₂-GATA-2 [37], pRSV-GATA-3 [38], or pMT₂-GATA-4 [45]. Protein assays on the nuclear extracts were determined using Bradford reagent (Bio-Rad). Gel-shift experiments were performed as described previously [45]. Double-stranded oligonucleotide probes corresponding to portions of the J6 promoter [57] were prepared [39] and end-labelled using [γ -³²P]ATP [62]. The sequences and designations of these oligonucleotide probes are given below (consensus GATA sites are underlined): probe -917, spanning nucleotides -924 → -909, GAAGCTATCTCAGTG; probe -875, spanning nucleotides -882 → -867, CCTGAGATAGTGAGA; probe -842, spanning nucleotides -850 → -835, ATGGAGATAGGGAGG; and probe -823, spanning nucleotides -830 → -815, GAAGGAGATAGCAGG.

Supershifts were performed as described previously [45] using affinity-purified rabbit anti-(GATA-4) IgG [49]. Preimmune serum or IgG was used as a negative control.

RNA analysis

RNA was isolated by the method of Chomczynski and Sacchi [63]. Northern-blot analysis was performed using ³²P-labelled DNA probes for GATA-4 or actin [39,45]. A J6 serpin cDNA probe spanning nucleotides 247–642, which reside in a single exon [57], was prepared via PCR of mouse genomic DNA with synthetic oligonucleotide primers to published sequence. The PCR product was cloned into the T-A cloning vector pCRII (Invitrogen) and then sequenced to confirm its identity and orientation.

Western-blot analysis

Samples of F9 cell nuclear extract (80 µg) were subjected to SDS/PAGE and then electrophoretically transferred to nitrocellulose as described [64]. Western blotting was performed [64] using 5 µg/ml of affinity-purified rabbit anti-(GST-GATA-4) antibody as the primary, a 1:10000 dilution of horseradish peroxidase-conjugated anti-(rabbit IgG) antibody as the secondary and an ECL detection system (BRL).

Trans-activation studies

A portion of the J6 promoter spanning nucleotides (-1050 → -475) was isolated using PCR of mouse genomic DNA with synthetic oligonucleotide primers to the published sequence [57]. This sequence was subcloned in the forward orientation into the *Hind*III site of the commercially available reporter plasmid ptkGH (Allegro). The resultant vector was termed p(-1050 → -475)J6tkGH. Co-transfection studies on NIH 3T3 cells were performed in 6-well Petri dishes. Each well of cells (approximately 30% confluent) was transfected with 1 µg of reporter plasmid [p(-1050 → -475)J6tkGH or ptkGH] and 2 µg of activator

plasmid (pMT₂, pMT₂-GATA-1, or pMT₂-GATA-4) using the calcium phosphate technique [60]. Conditioned cell culture medium was harvested at 48 h and assayed for growth hormone (GH) using a commercially available radioimmunoassay (Allegro).

Indirect immunofluorescence

Undifferentiated F9 cells, RA/dbcAMP-differentiated F9 cells, or COS cells transfected with pMT₂-mouse GATA-1 [20], pMT₂-human GATA-2 [37], pRSV-human GATA-3 [5], or pMT₂-GATA-4 [45] were plated on plastic coverslips, fixed in 4% paraformaldehyde in PBS and subjected to indirect immunofluorescent staining [65]. The primary antibody was affinity-purified rabbit anti-(mouse GATA-4) [49] or preimmune IgG at a concentration of 2 µg/ml, and the secondary antibody was affinity-purified, fluorescein isothiocyanate (FITC)-conjugated goat anti-(rabbit IgG) antibody (Boehringer-Mannheim) at a dilution of 1:120. COS cells transfected with pMT₂-GATA-2 and stained with rabbit anti-(human GATA-2) antibody were used as a positive control.

RESULTS

Expression of GATA-4 and J6 during F9 cell differentiation

Previous studies have shown that transcripts for J6 [53] and GATA-4 [45] increase during RA-induced differentiation of F9

Figure 1 Expression of (a) GATA-4 and J6 mRNA and (b) Western-blot analysis of GATA-4 protein expression during differentiation of F9 cells with RA/dbcAMP

(a) RNA was harvested from F9 cells differentiated for various lengths of time with RA/dbcAMP. Equal amounts of RNA (30 µg) were subjected to Northern-blot analysis using radiolabelled probes for GATA-4, J6 and β -actin. Migration of 28 S and 18 S rRNA are indicated. (b) Equal amounts of F9 cell nuclear extract (80 µg) were subjected to Western-blot analysis with anti-(GATA-4) antibody. The migrations of molecular-mass standards and GATA-4 are indicated.

Figure 2 Indirect immunofluorescence of F9 cells and transfected COS cells using anti-(GATA-4) antibody

Cells were fixed, permeabilized and then incubated with affinity-purified rabbit anti-(mouse GATA-4) IgG, followed by FITC-conjugated goat anti-(rabbit IgG). (A) Undifferentiated F9 cells, which show no staining with anti-(GATA-4) IgG. Bar = 5 μ m. (B) F9 cells differentiated for 4 days with RA/dbcAMP, showing primarily nuclear localization of GATA-4 antigen. Bar = 5 μ m. (C) High magnification view of COS cells transiently transfected with pMT₂-GATA-4. (D) Same field as (C) viewed under fluorescence. One of the COS cells in this field expresses GATA-4 that localizes to the nucleus. The non-transfected COS cells in this field serve as negative controls. Bars = 5 μ m. (E) Low magnification phase-contrast view of COS cells transfected with expression plasmid pMT₂-GATA-1. (F) Same field as (E) viewed under fluorescence. No nuclear staining is present in any of the cells in this field nor any other field examined. Bars = 12 μ m. (G) Positive control of COS cells transfected with the expression plasmid pMT₂-GATA-2 and then stained with rabbit anti-(GATA-2) antibody followed by FITC-labelled goat anti-(rabbit IgG). Bar = 12 μ m.

cells into parietal endoderm. To test the hypothesis that GATA-4 regulates J6 expression in differentiating F9 cells, we first compared the time courses of induction of GATA-4 and J6 mRNAs in cells treated with RA plus dbcAMP. The two transcripts exhibited similar patterns of induction. In undifferentiated F9 cells, neither GATA-4 nor J6 message was detectable by Northern-blot analysis (Figure 1a). After 1 or 2 days of treatment with RA/dbcAMP, both transcripts appeared and then increased in parallel. We also measured GATA-4 protein in differentiating F9 cells using Western blotting and found that GATA-4 protein levels follow GATA-4 RNA levels (Figure 1b). GATA-4 protein first appeared after 1 day of treatment with RA/dbcAMP and plateaued between 2 and 3 days of treatment. Genes induced during RA differentiation fall into two groups: early (or 'primary') response genes and late (or 'secondary') response genes. Transcription of early response genes (e.g. Hox-1.3) is mediated through the direct interaction of RA receptors with *cis* elements in promoters or enhancers,

whereas transcription of late response genes is indirect, mediated in part by the protein products of early response genes [66]. Both GATA-4 and J6 exhibit a pattern of induction characteristic of late RA response genes. The close association between expression of these two genes suggests the possibility that transcription factor GATA-4 regulates expression of J6.

Characterization of rabbit antibody specific for GATA-4

In an earlier study we described the preparation of a rabbit polyclonal antiserum that recognizes recombinant GATA-4 in gel-shift assays [45]. To facilitate the experiments described below, we assessed the specificity of this GATA-4 antibody by performing indirect immunofluorescence experiments. Affinity-purified anti-(GATA-4) IgG stained the nuclei (and to a lesser extent the perinuclear area) of RA/dbcAMP-differentiated F9 cells (Figure 2B). In contrast, this antibody did not stain undifferentiated F9 cells (Figure 2A), indicating that it does not

Figure 3 Anti-(GATA-4) antibody does not cross-react with GATA-1, -2, or -3 in gel-shift assays

Nuclear extract was prepared from COS cells transfected with vector alone or with expression plasmids encoding GATA-1, -2, -3, or -4 and used in gel-shift assays with a radiolabelled cDNA probe containing a single consensus GATA motif [37]. An excess of unlabelled probe was added in lanes 1, 4, 7, 10 and 13. Preimmune IgG was added in lanes 2, 5, 8, 11 and 14. Anti-(GATA-4) IgG was added in lanes 3, 6, 9, 12 and 15. Note that the normal migration of the GATA-4-DNA complex is disrupted by addition of anti-(GATA-4) IgG; some of the complex is now supershifted to the top of the gel. Abbreviations: C, competitor DNA; P, preimmune IgG; I, immune IgG.

react non-specifically with other mouse cell proteins. To show that the antibody does not cross-react with other GATA-binding proteins, we performed indirect immunofluorescence on COS cells transfected with expression plasmids encoding GATA-1, -2, -3, and -4. Nuclear staining was seen with COS cells expressing GATA-4 (Figures 2C and 2D), whereas no immunofluorescent staining was observed with COS cells expressing mouse GATA-1 (Figures 2E and 2F), human GATA-2 (results not shown), or mouse GATA-3 (results not shown). The specificity of the anti-(GATA-4) antibody was further assessed using gel-shift assays. A radiolabelled DNA probe containing a single consensus GATA motif was incubated with nuclear extract from COS cells transfected with expression plasmids encoding GATA-1, -2, -3, or -4 (Figure 3). Gel-shift complexes were observed with each of the recombinant GATA-binding proteins (lanes 5, 6, 8, 9, 11, 12, 14 and 15). Addition of excess unlabelled probe to the incubation mixtures inhibited formation of the radiolabelled complexes (lanes 4, 7, 10 and 13). Incubation with anti-(GATA-4) IgG (lane 15), but not preimmune IgG (lane 14), disrupted the formation and/or migration of the GATA-4 complex, supershifting a portion of the GATA-4-DNA complex to the top of the gel. In contrast, the anti-(GATA-4) IgG neither blocked nor supershifted the complexes between DNA and GATA-1, -2, or -3 (lanes 6, 9 and 12), confirming that this antibody does not cross-react with other GATA-binding proteins in supershift assays.

GATA-4 binds to GATA motifs within the J6 promoter

Four consensus GATA motifs [(A/T)GATA(A/G)] are clustered within the RA-responsive region of the J6 promoter (nucleotides -1050 → -738). These GATA sites are located at nucleotides -917, -875, -842, and -823. To determine whether GATA-4 can bind each of these sites, we performed gel-shift analyses. Short radiolabelled oligonucleotide probes, corresponding to

Figure 4 Recombinant GATA-4 binds to multiple GATA sites in the J6 promoter

Nuclear extract was prepared from COS cells transfected with either the expression vector pMT₂ (lanes 1, 6 and 11) or pMT₂-GATA-4 (all other lanes) and used in gel-shift assays with radiolabelled short, double-stranded, oligonucleotide probes encompassing GATA sites at -842 (lanes 1-5), -917 (lanes 6-10), and -823 (lanes 11-15). An excess of unlabelled probe was added in lanes 3, 8 and 13. Anti-(GATA-4) IgG was added in lanes 5, 10 and 15. Preimmune IgG was added in lanes 4, 9 and 14. Note that the normal migration of the GATA-4-DNA complex is disrupted by addition of anti-(GATA-4) IgG; some of the complex is now supershifted to the top of the gel.

each of the four sites, were prepared and used in gel-shift assays with nuclear extract from COS cells transfected with the GATA-4 expression plasmid. Nuclear extract from COS cells mock-transfected with vector alone was used as a negative control. Results of gel-shift analyses with three of the four probes (-917, -842, and -823) are shown in Figure 4. It was difficult to interpret gel-shift results with the fourth probe (-875) because of the presence of both specific and non-specific DNA-protein complexes migrating near the GATA-4-DNA complex (results not shown). Incubation of probes -917, -842, and -823 with nuclear extract from COS cells transfected with the expression vector pMT₂-GATA-4 resulted in formation of a radiolabelled GATA-4-DNA complex (lanes 2, 7 and 12). These complexes were not formed when nuclear extract from mock-transfected COS cells was incubated with the probe (lanes 1, 6 and 11). Addition of excess unlabelled probe inhibited the formation of the radiolabelled complexes (lanes 3, 8 and 13). Incubation with anti-(GATA-4) IgG (lanes 5, 10 and 15) but not preimmune IgG (lanes 4, 9 and 14) disrupted the formation and/or migration of the DNA-GATA-4 complexes, supershifting a portion of each complex to the top of the gel. These studies indicate that recombinant GATA-4 is capable of binding multiple GATA sites within the RA-responsive region of the promoter.

GATA-4 is the major GATA-binding protein activity in differentiated F9 cells

We next examined binding of proteins present in nuclear extracts from undifferentiated and RA/dbcAMP-differentiated F9 cells to the oligonucleotide probes described above. Protein-DNA complexes were not observed when extracts from undifferentiated cells were incubated with any of the probes (Figure 5, lanes 1, 6 and 11). In contrast, extracts from differentiated F9 cells bound to probes -917, -842 and -823, as shown in Figure 5 (lanes 2, 7 and 12). As was the case with transfected COS nuclear extract, the -875 probe bound to multiple proteins in both undifferentiated and differentiated F9 nuclear extract that complicated interpretation of the results (data not shown). Addition of anti-(GATA-4) antibody to the incubation blocked or supershifted essentially all of the GATA-binding activity present in the differentiated F9 cell nuclear extracts (Figure 5, lanes 5, 10 and

Figure 5 Gel-shift analysis of nuclear extract from undifferentiated and differentiated F9 cells

Gel shifts were performed using nuclear extract from undifferentiated F9 cells (lanes 1, 6 and 11) or RA/dbcAMP-differentiated F9 cells (all other lanes) and radiolabelled probes corresponding to GATA motifs located at the following positions in the J6 promoter: —842 (lanes 1–5), —823 (lanes 6–10), or —917 (lanes 11–15). An excess of unlabelled probe was added in lanes 3, 8 and 13. Anti-(GATA-4) IgG was added in lanes 5, 10 and 14. Preimmune IgG was added in lanes 4, 9 and 15.

Figure 6 Comparison of the gel-shift complexes obtained with recombinant GATA-4 and nuclear extract from differentiated F9 cells

Gel-shift analysis was performed using nuclear extract from pMT₂-transfected COS cells (lane 1), pMT₂-GATA-4-transfected COS cells (lanes 2–5), or RA/dbcAMP-differentiated F9 cells (lanes 6–9) and the radiolabelled probe containing the GATA motif at —842 in the J6 promoter. An excess of unlabelled probe was added in lanes 3 and 6. Anti-(GATA-4) IgG was added in lanes 4 and 8. Preimmune IgG was added in lanes 5 and 9. Note that the GATA-4–DNA complex formed with recombinant GATA-4 co-migrates with the complex formed with differentiated F9 cell nuclear extract. The protein–DNA complexes formed by both recombinant GATA-4 and differentiated F9 nuclear extract are supershifted with anti-(GATA-4) antibody. A faint gel-shift band is present in the mock-transfected extract in lane 1, presumably due to small amounts of endogenous (monkey) GATA-2 in COS cells.

14). No effect was seen with preimmune IgG (Figure 5, lanes 4, 9 and 15). Comparison of the binding of nuclear extract from GATA-4-transfected COS cells and differentiated F9 cells to the GATA-containing probes showed that the protein–DNA complexes formed were indistinguishable in electrophoretic mobility (Figure 6, lanes 2 and 6) and pattern of supershift with antibody (Figure 6, lanes 4 and 8). The above data confirm that GATA-4 is the major, if not only, GATA-binding protein present in differentiated F9 nuclear extract.

Table 1 *Trans*-activation of the J6 promoter by GATA-4 in NIH 3T3 cells

NIH 3T3 cells were co-transfected with 2 μ g of an activator plasmid (pMT₂, pMT₂-GATA-1, or pMT₂-GATA-4) and 1 μ g of a reporter plasmid [p(–1050 → –475)J6tkGH or ptkGH]. After 2 days, GH expression was measured as described in the Materials and methods section. Results are the mean $\pm$ S.E.M. of triplicate determinations. This experiment was repeated on four occasions with similar results.

Activator	Reporter	GH expression (ng/ml)
pMT ₂	ptkGH	0.7 $\pm$ 0.3
pMT ₂	p(–1050 → –475)J6tkGH	0.3 $\pm$ 0.1
pMT ₂ -GATA-1	ptkGH	0.4 $\pm$ 0.1
pMT ₂ -GATA-1	p(–1050 → –475)J6tkGH	1.2 $\pm$ 0.2
pMT ₂ -GATA-4	ptkGH	2.8 $\pm$ 1.1
pMT ₂ -GATA-4	p(–1050 → –475)J6tkGH	19 $\pm$ 2.8

Trans-activation of the J6 promoter by GATA-4

Having shown that GATA-4 binds *in vitro* to GATA motifs in the J6 promoter, we next assessed the ability of GATA-4 to *trans*-activate the J6 promoter. We utilized co-transfection assays in NIH 3T3 cells, a heterologous cell line with low endogenous GATA-binding-protein activity [18]. A reporter gene construct containing the RA-responsive portion of the J6 promoter was prepared. This construct, termed p(–1050 → –475)J6tkGH, contains nucleotides –1050 → –475 of the J6 gene upstream of a thymidine kinase (tk) minimal promoter driving human GH reporter. The reporter plasmids were co-transfected with pMT₂-GATA-4 or pMT₂-GATA-1 as a control [45]. Results of these co-transfection experiments are shown in Table 1. GATA-4 was a potent *trans*-activator of the J6 promoter, increasing expression more than 20-fold. In contrast, GATA-1 only modestly activated the J6 promoter. The findings in this assay system imply that GATA-4 can activate the J6 gene in heterologous cells.

DISCUSSION

Several lines of evidence argue that GATA-4 regulates expression of the J6 gene in differentiating F9 cells. The time courses of induction of GATA-4 and J6 during RA-induced differentiation of F9 cells are similar, suggesting that J6 expression is tied to GATA-4 expression. GATA-4 binds to multiple GATA motifs within the RA-responsive region of the J6 promoter. Antibody blocking/supershift experiments show that GATA-4 is the major, if not only, GATA-binding-protein activity in differentiated F9 cells. Finally, *trans*-activation studies in heterologous cells indicate that GATA-4 can activate the J6 promoter.

While GATA-4 appears to be necessary for J6 expression in F9 cells, it is probably not sufficient. Transient transfection of undifferentiated F9 cells with GATA-4 results in only modest activation of J6 promoter constructs compared with differentiation with RA/dbcAMP (results not shown). We speculate that additional positive and/or negative regulatory factors control J6 expression in undifferentiated and differentiated F9 cells. The nature of these other putative regulatory factors is currently unknown.

Since GATA-4 regulates J6 expression in F9 cells, this transcription factor may also control J6 expression in other cell and tissue types. The tissue distributions of GATA-4 and J6 RNA are similar in a variety of species, with the major sites of expression being yolk sac, heart, gut epithelium and gonads [45,46,48,53]. As with F9 cells, we suspect that expression of GATA-4 in a

particular tissue is not sufficient to induce J6 RNA expression. Using whole mount *in situ* hybridization, we have found that the patterns of expression of J6 RNA and GATA-4 RNA during embryonic mouse development overlap but are not identical. Both GATA-4 and J6 are highly expressed in yolk sac endoderm between 7 and 10 days *post coitum* (p.c.) ([49]; M. Bielinska and D. B. Wilson, unpublished work). At 9 days p.c., though, GATA-4 is readily detectable in the embryonic heart [49] while little if any J6 message is identifiable in this organ at this stage of development (M. Bielinska and D. B. Wilson, unpublished work). To date, J6 expression has not been demonstrated in a tissue that does not express detectable GATA-4. However, it is theoretically possible that another GATA-binding protein substitutes for GATA-4 as an activator of J6 in some cell types. Alternatively, GATA-independent mechanisms of J6 induction may exist in some cells.

The time course of induction of J6 RNA following treatment of F9 cells with RA/dbcAMP indicates that this gene is a late response gene, the induction of which depends on another RA-inducible gene product (or products). Although the J6 promoter contains consensus RA response elements, deletion experiments in heterologous cells suggest that these elements are not essential for promoter function [57,58], consistent with a mechanism of indirect induction of the J6 gene by RA. The data in this paper suggest that at least one of the RA-inducible genes involved in J6 expression is GATA-4.

It is currently not known which of the four GATA motifs within the RA-responsive region of the J6 promoter is essential for GATA-4 binding and promoter function. In addition to the four GATA sites in the RA-responsive region, the proximal J6 promoter contains at least one other consensus GATA site that may also influence gene expression. The multiplicity of GATA sites in the J6 promoter and an inability to use homologous (i.e. F9) cells for *trans*-activation assays, makes delineation of essential elements in this promoter difficult.

Besides J6, several other genes involved in extracellular matrix synthesis are induced during differentiation of F9 cells into parietal endoderm, including collagen IV(α 1) and laminin B1 [66]. Despite the current lack of data, it is tempting to speculate that GATA-4 may regulate other late RA response genes that are involved in extracellular matrix formation. Since J6 is a heat shock protein [55] it is also conceivable that GATA-4 regulates expression of other heat shock genes. Future studies, including targeted mutagenesis of the GATA-4 gene in ES cells, should help to test these speculations as well as provide formal proof that GATA-4 regulates the J6 promoter *in vivo*.

We thank John Scandrett for technical assistance, Julie Trausch-Azar for advice on immunofluorescence, and Markku Heikinheimo for help with *in situ* analysis. This work was supported by HL52134 from the NIH, a Basil O'Connor Starter Scholar Award (to D.B.W.), Grant 91-52 from the McDonnell Foundation, an American Heart Association Grant-in-Aid, and an award from Monsanto/Searle. This work was performed while D.B.W. was a Pfizer Scholar in cardiovascular medicine.

REFERENCES

- Orkin, S. H. (1992) *Blood* **80**, 575–581
- Evans, T., Reitman, M. and Felsenfeld, G. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5976–5980
- Plumb, M., Frampton, H., Wainwright, M., Walker, M., Macleod, K., Goodwin, G. and Harrison, P. (1989) *Nucleic Acids Res.* **17**, 73–92
- Merika, M. and Orkin, S. H. (1993) *Mol. Cell. Biol.* **13**, 3999–4010
- Ko, L. J., Yamamoto, M., Leonard, M. W., George, K. M., Ting, P. and Engel, J. D. (1991) *Mol. Cell. Biol.* **11**, 2778–2874
- Omichinski, J. G., Trainor, C., Evans, T., Gronenborn, A. M., Clore, G. M. and Felsenfeld, G. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1676–1680
- Omichinski, J. G., Clore, G. M., Schaad, O., Felsenfeld, G., Trainor, C., Appella, E., Stahl, S. J. and Gronenborn, A. M. (1993) *Science* **261**, 438–446
- Yang, Z., Gu, L., Romeo, P.-H., Bories, D., Motohashi, H., Yamamoto, M. and Engel, J. D. (1994) *Mol. Cell. Biol.* **14**, 2201–2212
- Kudla, B., Caddick, M. X., Langdon, T., Martinez-Rossi, N. M., Bennett, C. F., Sibley, S., Davies, R. W. and Arst, H. N., Jr. (1990) *EMBO J.* **9**, 1355–1364
- Fu, Y.-H. and Marzluf, G. A. (1990) *Mol. Cell. Biol.* **10**, 1056–1065
- Spieth, J., Shim, Y. H., Lea, K., Conrad, R. and Blumenthal, T. (1991) *Mol. Cell. Biol.* **11**, 4651–4659
- Drevet, J. R., Skeiky, Y. A. W. and Iatrou, K. (1994) *J. Biol. Chem.* **269**, 10660–10667
- Abel, T., Michelson, A. M. and Maniatis, T. (1993) *Development* **119**, 623–633
- Ramain, P., Heitzler, P., Haenlin, M. and Simpson, P. (1993) *Development* **119**, 1277–1291
- Winick, J., Abel, T., Leonard, M. W., Michelson, A. M., Chardon-Loriaux, I., Holmgren, R. A., Maniatis, T. and Engel, J. D. (1993) *Development* **119**, 1035–1065
- Evans, T. and Felsenfeld, G. (1989) *Cell* **58**, 877–885
- Martin, D. I. K., Zon, L. I., Mutter, G. and Orkin, S. H. (1990) *Nature (London)* **344**, 444–447
- Martin, D. I. K. and Orkin, S. H. (1990) *Genes Dev.* **4**, 1886–1898
- Traninor, C. D., Evans, T., Felsenfeld, G. and Boguski, M. S. (1990) *Nature (London)* **343**, 92–96
- Tsai, S., Martin, D. I. K., Zon, L. I., D'Andrea, A. D., Wong, G. G. and Orkin, S. H. (1989) *Nature (London)* **339**, 448–451
- Zon, L. I., Gurish, M. F., Stevens, R. L., Mather, C., Reynolds, D. S., Austen, K. F. and Orkin, S. H. (1991) *J. Biol. Chem.* **266**, 22948–22953
- Ito, E., Toki, T., Ishihara, H., Ohtani, H., Gu, L., Yokoyama, M., Engel, J. D. and Yamamoto, M. (1993) *Nature (London)* **362**, 466–468
- Zon, L. I., Yamaguchi, Y., Yee, K., Albee, E. A., Kimura, A., Bennett, J. C., Orkin, S. H. and Ackerman, S. J. (1993) *Blood* **81**, 3234–3241
- Mignotte, V., Wall, L., de Boer, E., Grosveld, F. and Romeo, P.-H. (1989) *Nucleic Acid Res.* **17**, 37–54
- Weiss, M. J., Keller, G. and Orkin, S. H. (1994) *Genes Dev.* **8**, 1184–1197
- Evans, T. and Felsenfeld, G. (1991) *Mol. Cell. Biol.* **11**, 843–853
- Strauss, E. C., Andrews, N. C., Higgs, D. R. and Orkin, S. H. (1992) *Mol. Cell. Biol.* **12**, 2135–2142
- Chiba, T., Ikawa, Y. and Todokoro, K. (1991) *Nucleic Acids Res.* **19**, 3843–3848
- Zon, L. I., Yousoufian, H., Mather, C., Lodish, H. and Orkin, S. H. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10638–10641
- Max-Audit, I., Eleouet, J.-F. and Romeo, P.-H. (1993) *J. Biol. Chem.* **268**, 5431–5437
- Visvader, J. E., Efeant, A. G., Strasser, A. and Adams, J. M. (1992) *EMBO J.* **11**, 4557–4564
- Visvader, J. and Adams, J. M. (1993) *Blood* **82**, 1493–1501
- Simon, M. C. (1993) *Proc. Soc. Exp. Biol. Med.* **202**, 115–121
- Pevny, L., Simon, M. C., Robertson, E., Klein, W. H., Tsai, S., D'Agati, V., Orkin, S. H. and Costantini, F. (1991) *Nature (London)* **349**, 257–260
- Simon, M. C., Pevny, L., Wiles, M. V., Keller, G., Costantini, F. and Orkin, S. H. (1992) *Nature Gen.* **1**, 92–98
- Lee, M.-E., Temizer, D. H., Clifford, J. A. and Quertermous, T. (1991) *J. Biol. Chem.* **266**, 16188–16192
- Dorfman, D. M., Wilson, D. B., Bruns, G. A. P. and Orkin, S. H. (1992) *J. Biol. Chem.* **267**, 1279–1285
- Yamamoto, M., Ko, L. J., Leonard, M. W., Beug, H., Orkin, S. H. and Engel, J. D. (1990) *Genes Dev.* **4**, 1650–1662
- Wilson, D. B., Dorfman, D. M. and Orkin, S. H. (1990) *Mol. Cell. Biol.* **10**, 4854–4862
- Leonard, M. W., Lim, K.-C. and Engel, J. D. (1993) *Development* **119**, 519–531
- Leonard, M., Brice, M., Engel, J. D. and Papyannopoulou, T. (1993) *Blood* **82**, 1071–1079
- Briegleb, K., Lim, K.-C., Plank, C., Beug, H., Engel, J. D. and Zenke, M. (1993) *Genes Dev.* **7**, 1097–1109
- Ho, I.-C., Vorhees, P., Marin, N., Oakley, B. K., Tsai, S.-F., Orkin, S. H. and Leiden, J. M. (1991) *EMBO J.* **10**, 1187–1192
- Joulin, V., Bories, D., Eleouet, J.-F., Labastie, M.-C., Chretien, S., Mattaei, M. G. and Romeo, P. H. (1991) *EMBO J.* **10**, 1809–1816
- Arcaci, R. J., King, A. A. J., Simon, M. C., Orkin, S. H. and Wilson, D. B. (1993) *Mol. Cell. Biol.* **13**, 2235–2246
- Laverriere, A. C., MacNeill, C., Mueller, C., Poelmann, R. E., Burch, J. B. and Evans, T. (1994) *J. Biol. Chem.* **269**, 23177–23184
- Tamura, S., Wang, X.-H., Maeda, M. and Futai, M. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10876–10880
- Grépin, C., Dagnino, L., Robitaille, L., Haberstroh, L., Antakly, T. and Nemer, M. (1994) *Mol. Cell. Biol.* **14**, 3115–3129
- Heikinheimo, M., Scandrett, J. M. and Wilson, D. B. (1994) *Dev. Biol.* **164**, 361–373

-
- 50 Molketin, J. D., Kalvakolanu, D. V. and Markham, B. E. (1994) *Mol. Cell. Biol.* **14**, 4947–4957
- 51 Ip, H. S., Wilson, D. B., Heikinheimo, M., Tang, Z., Ting, C.-N., Simon, M. C., Leiden, J. M. and Parmacek, M. S. (1994) *Mol. Cell. Biol.* **14**, 7517–7526
- 52 Thuerauf, D. J., Hanford, D. S. and Glembofski, C. C. (1994) *J. Biol. Chem.* **269**, 17772–17775
- 53 Wang, S.-Y., LaRosa, G. J. and Gudas, L. J. (1985) *Dev. Biol.* **107**, 75–86
- 54 Wang, S.-Y. and Gudas, L. J. (1990) *J. Biol. Chem.* **265**, 15818–15822
- 55 Hirayoshi, K., Dudo, H., Takechi, H., Nakai, A., Iwamatsu, A., Yamada, K. M. and Nagata, K. (1991) *Mol. Cell. Biol.* **11**, 4036–4044
- 56 Clarke, E. P., Cates, G. A., Ball, E. H. and Sanwal, B. D. (1991) *J. Biol. Chem.* **266**, 17230–17235
- 57 Wang, S.-Y. (1992) *J. Biol. Chem.* **267**, 15362–15366
- 58 Wang, S.-Y. (1994) *J. Biol. Chem.* **269**, 607–613
- 59 Darrow, A. L., Rickles, R. J. and Strickland, S. (1990) *Methods Enzymol.* **190**, 110–117
- 60 Chen, C. and Okayama, H. (1987) *Mol. Cell. Biol.* **7**, 2745–2752
- 61 Andrews, N. C. and Faller, D. V. (1991) *Nucleic Acids Res.* **19**, 2499
- 62 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- 63 Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- 64 Harlow, E. and Lane, D. (1988) *Antibodies: a Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 65 Trausch, J. S., Grenfell, S. J., Handley-Gearhart, P. M., Ciechanover, A. and Schwartz, A. L. (1993) *Am. J. Physiol.* **264**, C93–C102
- 66 Gudas, L. J. (1990) *Methods Enzymol.* **190**, 131–140
-

Received 12 August 1994/16 November 1994; accepted 23 November 1994