GATA-1 DNA Binding Activity Is Down-regulated in Late S Phase in Erythroid Cells*

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The relationship between cell division and differentiation is still unclear. In the hematopoietic system, it has long been thought that differentiation requires prior or concomitant cell division (1), but direct evidence for a mechanistic relationship has been hard to obtain. One potential mechanism involves the effect of DNA replication on repressive chromatin and stable transcription complexes. The passage of a replication fork through a chromatin template is known to transiently disrupt both histone octamers and complexes of transacting factors required for programming gene expression (2, 3). Subsequently, as nucleosomes reform behind the replication fork, transcription factors have a window of opportunity to bind and either reprogram gene expression or reassemble committed genes (2, 4, 5).

Evidence in support of a role for DNA replication in gene activation for a variety of viral and cellular genes has been provided by the activating effect of an origin of replication in transient transfection assays (6–9). Furthermore, in the normal cellular context, a strong correlation exists between the replication timing of a gene and its state of transcriptional activity. Specifically, replication early in S phase is associated with expression for a large number of tissue-specific genes in a variety of different cell types (10–13). A well studied example is the β globin locus, which is replicated early in erythroid cells where it is transcribed but late in fibroblasts where it is not (13, 14). The major determinant of erythroid expression of this locus is the locus control region, a set of nuclease hypersensitive sites located several kilobases upstream of the first gene in the cluster. The locus control region is required both for transcription and early replication of the β globin locus (15), further maintaining the correlation between replication timing and tissue-specific gene activation, leading to a model where early replication removes repressive chromatin and allows activating transcription factors to bind (4, 16). Transcription factor availability is clearly an important component of such a model. If the overall nuclear concentration of critical factors remained constant in the cell cycle, titration of transcription factors by early replicating genes would limit their availability for binding to later replicating genes with the same control sequences, as has been suggested for the early replicating somatic 5 S RNA genes of Xenopus (4, 17, 18). Alternatively, sequestration of transcription factors to prevent activation of genes replicating in late S phase would be further enhanced by a concomitant decrease in factor DNA binding activity. These considerations have led to the suggestion that fluctuations in the levels of critical transacting factors during S phase may amplify the effect on chromatin remodeling conferred by early replication (6).

We have set out to test this model, using an erythroid cell line that contains both a transcriptionally poised β globin gene that replicates during the first half of S phase (14, 19) and GATA-1, a lineage-restricted transcription factor crucial for erythroid differentiation (20, 21) that has potential binding sites in the regulatory sequences of all erythroid genes (22) and is expressed at increasingly higher levels during erythroid maturation (23). We find that the availability of GATA-1 but not ubiquitous factors does indeed vary in the cell cycle in a manner likely to amplify the effects of replication timing. GATA-1 therefore represents a candidate molecule for linking erythroid transcription and early replication. These data can also be linked with recent observations that GATA-1 may be directly involved in control of the cell cycle and the choice among proliferation, differentiation, and apoptosis (24–26).

EXPERIMENTAL PROCEDURES

Cell Culture and Cell Cycle Fractionation—MEL1 cells (27) were grown in RPMI 1640 supplemented with 2 mM glutamine and 10% fetal

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calf serum in 37°C incubators containing 5% CO2. Asynchronous log phase cells were separated into different stages of the cell cycle by centrifugal elutriation, as follows. 107–109 cells were pelleted, washed in phosphate-buffered saline, resuspended at a concentration of approximately 107 cells/ml, and injected into the chamber of a Beckman JE-5.0 elutriator rotor spinning at 3000 rpm in ice-cold phosphate-buffered saline pumped at 21 ml/min. The pump rate was increased in 10–15 steps up to 65 ml/min, and elutriated fractions were collected onto ice. Fractions were then pelleted, resuspended in 5 ml of phosphate-buffered saline, and analyzed by Coulter counter to determine cell concentration and size distribution. Aliquots of 5 × 106 cells were removed, stained with propidium iodide (28), and analyzed by FACS to determine the quality of the elutriation. Adjacent fractions that consisted of essentially identical cell cycle profiles were pooled, and nuclear proteins were then extracted.

**Cell Extracts—**Elutriated cells were pelleted and then taken up at constant cell concentration in ice-cold 20 mM Hepes, pH 7.0, 2 mM MgCl2, containing 0.05% Triton X-100, and a mixture of protease (5 mM benzamidine, 5 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml aprotinin, 5 μg/ml bestatin), kinase (0.5 mM EDTA), and phosphatase (1 mM β-glycerophosphate, 0.2 mM levamisole, 0.25 mM NaF, 20 mM sodium vanadate) inhibitors. After incubation on ice for 15 min, lysed cells were pelleted at 6500 rpm in a microfuge for 2 min at 4°C, and the pelleted nuclei were resuspended in 2 volumes of 10 mM Hepes, pH 7.0, 2 mM MgCl2, containing 350 mM NaCl plus inhibitors. After 15 min on ice, nuclear debris was removed by centrifugation at 14000 rpm for 10 min. Glycerol was added to the supernatants to 25%, and aliquots were flash frozen and stored at −70°C.

**Electrophoretic Mobility Shift Assays—**Nuclear extracts (0.2–4 μl) were mixed with 7.5 μl of 4 × buffer (20 mM Hepes, pH 7.9, 2 mM MgCl2, 50 mM NaCl, 4% w/v Ficoll), 1 μg of poly(dIdC)poly(dIdC) nonspecific competitor DNA, and specific competitor DNAs where appropriate. Specific 32P-end labeled oligonucleotides (GATA-1, GACAATCTGATAAGGATTC(29); Oct1, AATTCACTGGTTCCCAATGATTTGCATGCTCTGATTTTCGTGGTGCTTTCCGCAATAGTGATCTCATCTCAG TG; Sp1, GTAAGGGCCCGCCCCAAC GAGG (30); typically 20 fmol/μl) were then added to a final volume of 30 μl, and the mixture was incubated on ice for 30 min. Binding reactions were then loaded onto a 4% agarose, 50 mM NaCl, 0.5 × TBE gel that had been pre-electrophoresed at 200 V until the current across it was 11 mA. After 2 h electrophoresis at 4°C, gels were dried down and autoradiographed. Laser densitometry was performed with a Molecular Dynamics Computing Densitometer.

The cell cycle position of the peak in GATA-1 DNA binding activity was estimated by using the quantitative FACS data (see, for example, Fig. 1a, middle panel and 4n), representing DNA content at the beginning and end of the cell cycle, were defined as the positions of the peaks in fractions 1 and 7, respectively. The n value for the fraction with the highest GATA-1 DNA binding activity was determined relative to these. Peak values from four separate elutriations were used to derive a mean cell cycle position for GATA-1 DNA binding activity.

**Western Blotting—**Cell equivalents of cell cycle-fractionated extracts were separated by polyacrylamide gel electrophoresis on 10% SDS-polyacrylamide gel electrophoresis gels and semi-dry électroblotted onto Hybond C+ nitrocellulose (Amersham). GATA-1 protein was immunodetected with anti-mouse GATA-1 antibody (a kind gift of J. D. Engel) after blocking with 5% milk powder in Tris-buffered saline containing 0.05% Tween 20, followed by binding with anti-rabbit IgG horseradish peroxidase and treatment with ECL (Amersham).

**Preparation of Total RNA and Northern Blotting—**Total RNA was extracted from elutriated cells by the method of Chomczynski and Sacchi (31), separated on formaldehyde-agarose gels, alkaline blotted onto Hybond N+ membranes (Amersham), and probed with a 32P random hexamer-labeled GATA-1 XhoI fragment.

**RESULTS AND DISCUSSION**

To perform an analysis of GATA-1 DNA binding activity through the cell cycle, asynchronous MEL cells (27) were fractionated by centrifugal elutriation. This technique separates bulk suspension cells by size and, because cell volume increases through the cell cycle, results in fractionation of cells according to their position in the cell cycle. The cell cycle composition of the fractionation was assessed by FACS analysis of propidium iodide-stained cells taken from each fraction (a typical example is shown in Fig. 1a). The percentages of cells in each phase of the cell cycle for a typical elutriation are summarized in Fig. 1b (cells are defined as G1, S, or G2/M depending on their DNA content (2n, 2–4n, and 4n, respectively)).

We assessed GATA-1 DNA binding activity by electrophoretic mobility shift assay (EMSA). Nuclear proteins, salt extracted from equal numbers of cells through the cell cycle, were incubated with an oligonucleotide containing a strong GATA-1 binding site from the mouse α-globin promoter (29) and electrophoresed (Fig. 2a, upper panel, lanes 1–7). Nuclear protein from asynchronous MEL cells was also analyzed (lane 8), and the identity of the GATA-1 band shift was confirmed by comparing the effects of specific (self-competition, lane 9) and nonspecific (an oligonucleotide containing a mutated GATA-1 site, lane 10) oligonucleotide competitors. Clearly, there is variation in GATA-1 DNA binding activity per cell through the cell cycle, with low levels in G1 (lanes 1 and 2), an increase to a peak of activity in S phase (lane 5), followed by a decrease in late S/G2 (lanes 6 and 7). Comparison of the FACS profiles for these fractions shows that GATA-1 DNA binding activity is highest in mid-S phase cells (Fig. 1a, fraction 5) compared with early S (Fig. 1a, fraction 4) and late S (Fig. 1a, fraction 6). Quantitation of the GATA-1 band intensities indicates that there is a 4-fold increase in GATA-1 DNA binding activity between early G1 and mid-S phase and an 11-fold decrease between mid-S and G2/M (Fig. 2b).

Analysis of GATA-1 DNA binding activity from several different elutriations revealed a consistent peak in mid-S phase. The position of this peak was quantitated by referring to the FACS analysis of each elutriation (see, for example, Fig. 1a). 2n and 4n, representing DNA content at the beginning and end of the cell cycle, were defined as the positions of the G1 and G2/M peaks in fractions 1 and 7, respectively. The n value for the GATA-1 binding peak in each case was calculated relative to these. Data from four separate elutriations yielded a mean value of 2.98 + 0.04 (standard deviation) with a standard deviation of 0.04. Thus, GATA-1 binding activity reproducibly peaks in mid-S phase. The drop in GATA-1 DNA binding from mid-S to G2/M was also reproducible, averaging 7-fold in the four elutriations.

To determine whether the variation in GATA-1 DNA binding activity was particular to GATA-1 in this cell line or whether it was a general feature shared with other transcription factors, we determined the DNA binding activity of Oct1 and Sp1, two transcription factors whose cell cycle-regulated DNA binding activity has been characterized previously. Oct1 DNA binding activity has been studied in HeLa cells, and it does not appear to vary relative to bulk protein through the cell cycle except during mitosis, when it loses DNA binding activity (32, 33). We therefore determined Oct1 DNA binding activity in equal numbers of MEL cells by EMSA, using an oligonucleotide containing the octamer motif from the mouse IgH chain gene enhancer (Fig. 2a, middle panel). In contrast to the result seen with GATA-1, Oct1 DNA binding activity does not appear to peak in S phase but instead is highest in the last elutriated fraction, which contains a mixture of G2 and M cells (Fig. 2a, middle panel, lanes 1–7). As cell volume increases through the cell cycle, we conclude that the nuclear concentration of Oct1 competent for DNA binding remains broadly the same, consistent with the published data (32, 33). A second ubiquitous transcription factor, Sp1, has also been shown not to vary in DNA binding activity through the cell cycle in various cell types (34). We therefore sought to determine its activity in MEL cells. An oligonucleotide containing a binding site for Sp1 gave a similar result to Oct1, with the amount of Sp1 DNA binding activity increasing progressively through the cell cycle (Fig. 2a, lower panel). Thus, for two ubiquitously expressed transcription factors, it appears that there is an increase in DNA binding activity concomitant with increasing nuclear volume through the cell cycle, likely maintaining a broadly constant concentra-
tion of nuclear protein. In contrast, although GATA-1 DNA binding activity increases in a similar way through to S phase, it contrasts with the other two transcription factors by exhibiting a sharp decrease in activity in late S/G2. GATA-1 revealed a similar profile when we took into account the doubling in nuclear chromatin protein during the cell cycle. Fig. 2b is a plot of the data in Fig. 2a showing the change in DNA binding activities of GATA-1, Oct-1, and Sp1 for equal amounts of nuclear protein through the cell cycle. The DNA binding activities of all three factors increase between G1 and early S. However, GATA-1 DNA binding activity decreases in G2/M (to 9% of peak value), while Oct1 and Sp1 remain high in G2/M (93 and 147% of peak values, respectively).

We next asked whether the S phase peak in GATA-1 DNA binding activity reflected a change in the amount of GATA-1 protein through the cell cycle or whether GATA-1 DNA binding activity was being regulated by some post-translational mechanism, such as phosphorylation. Nuclear protein from equal numbers of cell cycle-fractionated cells was separated by polyacrylamide gel electrophoresis, Western blotted, and probed with an antibody specific for GATA-1 (Fig. 3b). Variation in the amount of GATA-1 protein through the cell cycle can be seen (lanes 1–7), with a peak in mid-S phase (lane 5), in the same fraction where DNA binding activity is at its highest (Fig. 3a, lane 5). Furthermore, the magnitude of the changes in GATA-1 protein and DNA binding activity through the cell cycle is broadly similar. Quantitation of band intensities indicates that between early G1 and mid-S the amount of GATA-1 protein increases 5-fold while DNA binding activity increases 4-fold. Similarly, between mid-S and G2/M the amount of GATA-1 protein decreases 8-fold and DNA binding activity 11-fold.

Taken together, these data indicate that the variation in
GATA-1 DNA binding activity through the cell cycle is primarily due to variations in the total amount of GATA-1 protein and that post-translational mechanisms make at most a minor contribution to the change in GATA-1 DNA binding activity.

The varying amounts of nuclear GATA-1 protein through the cell cycle might come about by changes in protein stability, subcellular localization, or gene expression. However, we have assayed GATA-1 DNA binding activity in both nuclear and cytoplasmic extracts and found similar S phase peaks with low levels in G1 and G2/M. In particular, we do not detect high levels of GATA-1 DNA binding activity in the cytoplasm in G2/M, which would be expected if the drop in nuclear GATA-1 DNA binding activity we observe in this phase of the cell cycle was due to shuttling between compartments. To investigate the possibility that the variation in the amount of GATA-1 protein was due to changing levels of gene expression, total RNA was extracted from cell cycle-fractionated cells, separated on an agarose gel, Northern blotted, and then probed for GATA-1 message (Fig. 3).

FIG. 2. GATA-1 DNA binding activity peaks in mid-S phase. a, cell cycle analysis of GATA-1 DNA binding activity. Nuclear protein from equal numbers of elutriated MEL cells was analyzed for binding to a double-stranded end-labeled oligonucleotide containing a strong GATA-1 site by EMSA followed by autoradiography (upper panel, lanes 1–7), as described under “Experimental Procedures.” The identity of GATA-1 was confirmed by observing the effects of 50-fold excess unlabeled GATA-1 oligonucleotide self-competition (lane 9) versus competition with an oligonucleotide mutated in the GATA-1 binding site (lane 10). The GATA-1 DNA binding activity of asynchronous MEL cells was also tested (lane 8). Oct1 (middle panel, lanes 1–7) and Sp1 (lower panel, lanes 1–7) DNA binding activities were also measured, and binding specificities were confirmed by self-competition. b, quantitation of transcription factor DNA binding activity per μg of protein through the cell cycle. Band intensities in the above EMSAs were quantitated by laser densitometry and were normalized to the amount of protein per cell through the cell cycle; they are expressed here as a fraction of the peak of GATA-1 binding.

FIG. 3. GATA-1 DNA binding activity, protein and mRNA peak in mid-S phase. a, EMSA of GATA-1 DNA binding through the cell cycle (see Fig. 2 for details). b, the steady state levels of GATA-1 protein peak in mid-S phase. Nuclear protein from elutriated MEL cells was electrophoresed, blotted, and probed with a GATA-1-specific antibody (lanes 1–7). Asynchronous MEL cells were run alongside the cell cycle-fractionated samples (lane 8). c, the steady state level of GATA-1 mRNA peaks in mid-S phase. Total RNA extracted from elutriated MEL cells was size fractionated, Northern blotted, and probed with a GATA-1-specific DNA fragment (upper panel, lanes 1–7). Total RNA from asynchronous cells was included as a control (lane 8). The stained agarose gel is included as a loading control (lower panel).
early replicating activated genes is sufficient to prevent the activation of later replicating genes, as has been suggested for the differential effect of transcription factor IIIA on the expression of somatic and oocyte S RNA genes (4, 17). Instead, we suggest that the decrease in GATA-1 activity that occurs during late S phase is required to prevent activation of non-erythroid genes subject to activation by other GATA family members (Fig. 4, right side). By ensuring that GATA factor availability and target site accessibility occur in the same temporal compartment of S phase, subsets of genes with closely related control sequences can be activated independently.

Finally, another interpretation of these data has been prompted by several recent papers suggesting a role for GATA-1 in control of the cell cycle. Ectopic expression of GATA-1 in both hematopoietic and non-hematopoietic cell lines can lead to alterations in the length of segments of the cell cycle (24). In particular, high levels of ectopic GATA-1 cause S phase to lengthen in NIH3T3 cells, suggesting that the reduction in GATA-1 DNA binding activity we see here may be necessary for the correctly timed exit from S phase. GATA-1 also appears to function as an erythroid survival factor, since differentiation of GATA-1 negative ES cells along the erythroid lineage, or abrogation of GATA-1 activity in MEL cells, leads to premature apoptosis (25, 26). Thus, the high GATA-1 DNA binding activity we see in S phase may be necessary to prevent apoptosis. The emerging picture is that the choice among proliferation, differentiation, and apoptosis is affected by the level of GATA-1. Our data demonstrating a peak of GATA-1 DNA binding activity in mid-S, downstream of the G1/S cell cycle checkpoints, support the idea that GATA-1 has a cell cycle role in erythroid cells.

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