Neurokinin-B Transcription in Erythroid Cells

DIRECT ACTIVATION BY THE HEMATOPOIETIC TRANSCRIPTION FACTOR GATA-1*

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The GATA family of transcription factors establishes genetic networks that control developmental processes including hematopoiesis, vasculogenesis, and cardiogenesis. We found that GATA-1 strongly activates transcription of the Tac-2 gene, which encodes proneurokinin-B, a precursor of neurokinin-B (NK-B). Neurokinins function through G protein-coupled transmembrane receptors to mediate diverse physiological responses including pain perception and the control of vascular tone. Whereas an elevated level of NK-B was implicated in pregnancy-associated preeclampsia (Page, N. M., Woods, R. J., Gardiner, S. M., Lomthaisong, K., Gladwell, R. T., Butlin, D. J., Manyonda, I. T., and Lowry, P. J. (2000) Nature 405, 797–800), the regulation of NK-B synthesis and function are poorly understood. Tac-2 was expressed in normal murine erythroid cells and was induced upon ex vivo erythropoiesis. An estrogen receptor fusion to GATA-1 (ER-GATA-1) and endogenous GATA-1 both occupied a region of Tac-2 intron-7, which contains two conserved GATA motifs. Genetic complementation analysis in GATA-1-null G1E cells revealed that endogenous GATA-2 occupied the same region of intron-7, and expression of ER-GATA-1 displaced GATA-2 and activated Tac-2 transcription. Erythroid cells did not express neurokinin receptors, whereas aortic and yolk sac endothelial cells differentially expressed neurokinin receptor subtypes. Since NK-B induced cAMP accumulation in yolk sac endothelial cells, these results suggest a new mode of vascular regulation in which GATA-1 controls NK-B synthesis in erythroid cells.

The development of blood cells from hematopoietic stem cells is critically dependent upon integration of the activities of diverse transcription factors. Of particular importance are three members of the GATA family of transcription factors that regulate hematopoiesis via both unique and overlapping activities (1). GATA-1 is required for definitive or adult erythropoiesis, while GATA-2 regulates aspects of lymphopoiesis and central nervous system development (12–16). Understanding how GATA factors regulate development requires elucidation of genetic circuits that control expression of GATA factor target genes and the GATA factors themselves.

The C-terminal zinc finger of dual zinc finger GATA factors recognizes a simple DNA motif (WGATAR) (17–20) that is distributed abundantly throughout genomes. In contrast, the N-terminal zinc finger mediates interactions with the friend of GATA-1 (FOG-1)1 regulator (21, 22) and stabilizes DNA binding at certain GATA motifs (17, 23). Despite the high frequency of WGATAR in genomes, analyses of GATA-1 and GATA-2 binding to chromatin by quantitative ChIP analysis have revealed an exquisite specificity of chromatin occupancy. For example, the β-globin locus contains greater than 250 GATA motifs, but GATA-1 only occupies motifs within hypersensitive sites 1–4 of the β-globin LCR and within a subset of the β-globin promoters (24, 25). Despite the more than 80 GATA motifs within the GATA-2 locus, GATA-1 only occupies motifs within a restricted upstream region (26, 27). Thus, despite there being abundant GATA motifs within chromatin, including motifs that are conserved from mouse to humans, the vast majority of these motifs are not occupied by GATA factors in cells. ChIP analysis (27) has also revealed GATA-1 occupancy at sites previously implicated as being GATA-1 targets: the aminolevulinate synthase-2 promoter, hypersensitive site 1 of the GATA-1 locus, and hypersensitive site −26 of the α-globin locus (28–32).

The determinants of GATA factor recognition of chromatin are unknown, but presumably neighboring cis-elements, intrinsic features of GATA motifs, and the chromatin environment are crucial. Considering the lack of knowledge of these determinants, one cannot predict whether a given GATA motif will be occupied in vivo. Thus, establishing the ensemble of GATA factor target genes that control hematopoiesis cannot be accomplished solely by delineating the distribution of conserved GATA motifs.

1 The abbreviations used are: FOG-1, friend of GATA-1; CREB, cyclic AMP-responsive element-binding protein; ChIP, chromatin immunoprecipitation; ER-GATA-1, estrogen receptor hormone binding domain fusion to GATA-1; FBS, fetal bovine serum; G1E, GATA-1 null proerythroblast-like cell line; IBMX, 3-isobutyl-1-methylxanthine; MAE, mouse aortic endothelial; MEL, mouse erythroleukemia; NK, neurokinin; NK-B, neurokinin-B; PBS, phosphate-buffered saline; RT, reverse transcriptase; YSEC, yolk sac endothelial cell(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Genetic complementation analysis with a GATA-1 fusion to the estrogen receptor hormone binding domain in G1E cells has proven to be a powerful approach for defining GATA-1 target genes (27, 33). The G1E cell line was derived from GATA-1-null embryonic stem cells, and G1E cells recapitulate the phenotype of adult bone marrow proerythroblasts (34). Oligonucleotide array analysis in this system revealed that a similar number of genes is activated and repressed by ER-GATA-1 (33). Although GATA-1 was known to confer activation and repression in a context-dependent manner, it was surprising that a similar number of genes was activated and repressed, since few GATA-1-repressed genes were known. However, since gene-profiling analysis identifies both direct and indirect target genes, it is crucial to use other approaches, such as ChIP, to determine whether a given gene is a direct target.

Insights into the mechanism of GATA-1-mediated repression have arisen from the observation that GATA-1 and GATA-2 have reciprocal expression patterns during hematopoiesis (1). Consistent with this expression pattern, targeted deletion of GATA-1 resulted in up-regulation of GATA-2 (3, 34). These results suggested that GATA-1 represses GATA-2 transcription. Since a mutant of GATA-1 impaired in binding FOG-1 was inefficient in repressing GATA-2 (22), FOG-1 functions in the repression mechanism. We provided evidence for a direct repression mechanism in which GATA-1 displaces GATA-2 in a FOG-1-dependent manner from an upstream region of the GATA-2 locus (26, 27). This GATA switch was temporally coupled with expulsion of the histone acetyltransferase CREB-binding protein from GATA-2 regulatory elements and broad deacetylation throughout the GATA-2 locus. We hypothesized that GATA-1 binding instigates a bimodal repression mechanism consisting of the GATA switch coupled with domain-wide deacetylation.

To assess whether the bimodal repression mechanism is used by GATA-1 to repress other loci, we considered whether GATA-2 occupies the Nab2 (nerve growth factor-activated factor-binding protein 2) locus and whether GATA-1 displaces GATA-2. Nab2 was identified by gene profiling analysis as a GATA-1-repressed gene (33). Nab2 encodes a corepressor for the early growth regulator family of transcription factors, which mediate mitogen-induced cell proliferation (35, 36). Whereas extensive ChIP analysis throughout the Nab2 locus failed to identify sites occupied by GATA-1 and GATA-2, GATA-1 and GATA-2 occupancy was detected at intron-7 of the nearby Tac-2 gene, which encodes a NK-B protein (37, 38). These results are discussed with respect to the underlying transcriptional mechanism and the consequences of GATA factor-mediated control of Tac-2 transcription.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—G1E cells were maintained in Iscove’s modified Dulbecco’s medium containing 2% penicillin/streptomycin (Invitrogen), 2 units/ml erythropoietin, 120 nM monothioglycerol (Sigma), 0.6% conditioned medium from a Kit ligand-producing Chinese hamster ovary cell transcription.

**Primers for Quantitative ChIP Assay**—Primers were as follows: Stat-6 intron-7 (A), TGCCTTAGTTTCCCCATCTGA (forward) and CA-

insulin growth supplement (Cascade Biologics) and 1% antibiotic/antimycotic. D200 medium (Cascade Biologics) supplemented with low serum conditions in which signals were in the linear range.

During acute shaping, the reticulocyte-rich fraction was added to 4 ml of a 1:5 mixture of murine FOG-1 was described previously (21). Affinity-purified rabbit anti-rat IgG (H+L) (Jackson ImmunoResearch) was used as the secondary antibody for the GATA-1 ChIP analysis. Preimmune sera served as controls for all antibodies.

**Primers for Quantitative ChIP Assay**—Primers were as follows: Stat-6 exon-6/intron-6, CTCCGTCATACATTGCTCGT (forward) and CCCCTACAAGCAGTCTAGCTAA (reverse); Stat-6 intron-15/exon-16, GAAATGAGCGGAGGAGACAC (forward) and CAGGTCTTTGGCAGTGAAAG (reverse);

**Primary Erythroid Cell Isolation**—Bone marrow cells were flushed from the hind limbs of young female C57BL/6J (Jackson Laboratory, Bar Harbor, ME) mice into PBS (BioWhittaker, Walkersville, MD) containing 2% heat-inactivated FBS (HyClone, Logan, UT). The cells were collected by centrifugation and resuspended at a concentration of 2 x 10^6 cells/ml in PBS containing 2% FBS. The cells were incubated with phycoerythrin-conjugated TER119 or the appropriate isotype control antibodies (PharMingen, San Diego, CA) for 20 min at 4 °C with regular mixing at the concentrations recommended by the supplier. The cells were washed twice in PBS prior to fluorescence-activated cell sorting on a Vantage SE instrument (BD Biosciences) with a 488-nm argon laser. After sorting, TER119+ cells (~20% of bone marrow cells) and TER119− cells were collected in PBS and concentrated by centrifugation. RNA was isolated with Trizol (Invitrogen). For in vitro human erythroid assays, adult human erythroblasts were cultured from peripheral blood CD34+ cells as previously described (42). Total RNA was isolated from these cells on culture days 0, 2, 4, 6, 8, 10, 12, and 14 using Trizol. The isolated RNA was further processed by DNase I treatment (Qiagen, Valencia, CA).

Adult human reticulocytes were isolated from 20 ml of peripheral blood in heparin following a process of leukodepletion. Leukocytes were concentrated by centrifugation at 3500 rpm for 30 min at 4 °C, forming a disc (buffy coat) at the plasma-red cell interface. Both the plasma and buffy coat were removed, and the cells were washed three times with 10 ml of ice-cold PBS. At the third wash, reticulocytes were concentrated by centrifugation at 3500 rpm for 30 min at 4 °C. The supernatant was removed, and the top 700-μl reticulocyte-rich fraction was added to 4 ml of ice-cold PBS. This red cell suspension was layered on top of a column consisting of ~4.5 ml of 2 parts cellulose (C-8002; Sigma) and 1 part signacell type 50 microcrystallin cellulose (S-5504; Sigma) in normal saline. The red cells were eluted by gently centrifuging the column at 1000 rpm at 4 °C for 1 min. This leukodepleted eluate (~2.5 ml) was washed three times in 10 ml of ice-cold PBS. Finally, the reticulocyte-enriched eluted cells were recovered by centrifugation and resuspended in 2 ml of ice-cold PBS. RNA was extracted with TRI reagent (T9424; Sigma).

**Antibodies**—Rat anti-GATA-1 (N-6, sc-265) and rabbit anti-GATA-2 (H-116, sc-9008) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and a rabbit polyclonal antibody against GATA-2 (1) was purchased from Abcam (Cambridge, UK). GATA-1 cDNA was a generous gift from Dr. F. Begue (Dartmouth College, Hanover, NH). GATA-2 cDNA was a generous gift from Dr. J. Smith (Dartmouth College, Hanover, NH). GATA-1 and GATA-2 cDNA were subcloned into the Bluescript vector (Stratagene, La Jolla, CA). GATA-1 and GATA-2 cDNA were subcloned into the Bluescript vector (Stratagene, La Jolla, CA). GATA-1 and GATA-2 cDNA were subcloned into the Bluescript vector (Stratagene, La Jolla, CA). GATA-1 and GATA-2 cDNA were subcloned into the Bluescript vector (Stratagene, La Jolla, CA). GATA-1 and GATA-2 cDNA were subcloned into the Bluescript vector (Stratagene, La Jolla, CA). GATA-1 and GATA-2 cDNA were subcloned into the Bluescript vector (Stratagene, La Jolla, CA). GATA-1 and GATA-2 cDNA were subcloned into the Bluescript vector (Stratagene, La Jolla, CA). GATA-1 and GATA-2 cDNA were subcloned into the Bluescript vector (Stratagene, La Jolla, CA).
random and oligo(dT) primers at 68 °C for 10 min. This was followed by incubation with Moloney murine leukemia virus reverse transcriptase (Invitrogen) combined with 10 mM dithiobisreitol, RNAsin (Promega), and 0.5 mM dNTPs at 42 °C for 1 h. cDNA synthesis reactions were diluted to a final volume of 100 μl and heat-inactivated at 98 °C for 10 min. Reactions (25 μl) contained 2.5 mM of cDNA, 12.5 μM of SYBR Green Master Mix (Applied Biosystems, CA), and the appropriate primers. Product accumulation was monitored by SYBR Green fluorescence. Control reactions lacking RT yielded very low signals. Relative expression levels were determined from standard curve of serial dilutions of G1E cDNA samples, and measurements were made under conditions in which signals were in the linear range.

**Real Time RT-PCR Primers—** Real time RT-PCR primers were as follows: mouse *GAPDH*, TGGCCCATGGTTGTGAG (forward) and TGGTGGTCATGAGAACTTCT (reverse); mouse *Stat-6*, CATAAGGGGCAAGGAGGAC (forward) and CATAAGGGGCAAGGAGGAC (reverse); mouse *Tac-2*, TGGTGGTCATGAGAACTTCT (forward) and TGGTGGTCATGAGAACTTCT (reverse); mouse *Myo-1a*, ATTGGTAGGAGAACCCCG (forward) and ATTGGTAGGAGAACCCCG (reverse); mouse *GATA-1*, GACGGAGGCAAGGAGGAC (forward) and GACGGAGGCAAGGAGGAC (reverse); mouse *Nab2*, GCCAGTGTCAATTATACG (forward) and GCCAGTGTCAATTATACG (reverse); mouse *Nab1*, CACGGTTCATCGACGGGT (forward) and CACGGTTCATCGACGGGT (reverse); human *HPRT*, TGGGAAACAGTACACCAGGATG (forward) and TGGGAAACAGTACACCAGGATG (reverse); human *Tac-3*, TAAGGAGCCAGAGGAGGAGGT (forward) and TATATGGACAGGAGGAGGAGGT (reverse); human *GATA-1*, TGCCTGCTTCTGTGGGT (forward) and TGCCTGCTTCTGTGGGT (reverse); human *GATA-2*, CCCTGCTTCTGTGGGT (forward) and CCCTGCTTCTGTGGGT (reverse); human *Acctg-GTCCCTATCATGGACAGGACACATCCCA (forward) and GAAAGCAAGCCGGAATACTCT (reverse); mouse *Nab3*, CCAGAGGGCAAGGAGGAC (forward) and CCAGAGGGCAAGGAGGAC (reverse); mouse *Nab2*, CACTCTTCTGGCTG (forward) and GAAAGCAAGCCGGAATCCA (reverse); mouse *Tac-1*, TAAGGAGCCAGAGGAGGAGGT (forward) and TAAGGAGCCAGAGGAGGAGGT (reverse); mouse *Tac-2*, TGGTGGTCATGAGAACTTCT (forward) and TGGTGGTCATGAGAACTTCT (reverse); mouse *Nab2*, CACTCTTCTGGCTG (forward) and GAAAGCAAGCCGGAATCCA (reverse); mouse *Tac-1*, TAAGGAGCCAGAGGAGGAGGT (forward) and TAAGGAGCCAGAGGAGGAGGT (reverse).

**Quantitation of cAMP Levels—** MAE and YSEC cells (1 x 10^6) were resuspended in growth medium containing either 250 μM IBMX (Calbiochem), a phosphodiesterase inhibitor, IBMX + 10 μM forskolin (Sigma), or IBMX + 50 μM NK-B (Sigma) for 1 h at 37 °C. The reaction was terminated by aspiration of the medium. Cells were washed with PBS and were lysed in 500 μl of HCl (0.1 N, 10 min). Cell lysates (100 μl) were assayed for cAMP using a Direct cAMP enzyme-linked immunoassorbent assay kit (Assay Designs, Inc., Ann Arbor, MI) after acetylation as recommended by the manufacturer. In brief, the cAMP assay utilizes a 96-well microtiter plate in which anti-cAMP antibody is immobilized. Cell lysates or cAMP standards are added in the presence of alkaline phosphatase-conjugated cAMP, and after a colorimetric reaction with phosphatase substrate, absorbance is measured at 405 nm under conditions of linearity.

**RESULTS AND DISCUSSION**

**Tac-2 Transcription Is GATA-1-dependent and Is Induced upon Erythroid Differentiation—** A previous microarray analysis revealed that the murine *Nab2* gene (Fig. 1A) is repressed upon tamoxifen treatment of GATA-1-null G1E cells stably expressing ER-GATA-1 (G1E-ER-GATA-1) (33). Based on our previous analysis of the mechanism by which GATA-1 represses *GATA-2* transcription (26, 27), we aimed to compare the mechanism of GATA-1-mediated repression of *GATA-2* and *Nab2* to uncover principles for how GATA factors repress transcription. Quantitative RT-PCR analysis showed that ER-GATA-1 repressed *Nab2* by ~4-fold (Fig. 1B), consistent with the microarray data (33). Since the 3'-end of *Stat-6* abuts the 3'-end of *Nab2* (Fig. 1A) (44), we tested the possibility that ER-GATA-1 might activate *Stat-6* transcription, thereby creating transcriptional interference (45) or anti-sense transcripts (46) that repress *Nab2*. However, *Stat-6* mRNA was detected in G1E cells, and activation of ER-GATA-1 did not affect *Stat-6* mRNA levels. We also tested whether the downstream genes, *Myo-1a*, *Q8K2N3*, and *Tac-2* were regulated by ER-GATA-1. *Q8K2N3* and *Myo-1a* were expressed but were unaffected by ER-GATA-1 activation, whereas *Tac-2* mRNA was highly induced. Thus, ER-GATA-1 reciprocally regulates two nearby genes within an endogenous chromosomal region. Interestingly, *Tac-2* encodes pro-NK-B, which is processed to yield NK-B (47). NK-B is expressed in neuronal tissues (48), the placenta (49, 50), and the uterus (51) but has not been studied in the hematopoietic system.

To determine whether *Tac-2* is expressed in other erythroid cells, quantitative RT-PCR was used to measure *Tac-2* mRNA in erythroid cell lines and in primary murine and human erythroid cells. *Tac-2* expression was induced by tamoxifen in G1E-ER-GATA-1 but not G1E cells (Fig. 2A), indicating that ER-GATA-1 mediated the induction. *Tac-2* was expressed in
uninduced MEL cells, and expression was induced 2-fold upon Me2SO-induced erythroid maturation (Fig. 2A). GATA-1 expression is similar in uninduced and induced MEL cells. Tac-2 was also expressed in primary murine Ter119+ erythroid cells isolated from bone marrow (Fig. 2A). The major adult -globin gene, major, and Tac-2 shared a similar pattern of expression, being repressed in G1E cells, induced by tamoxifen in G1E-ER-GATA-1 cells, induced upon MEL cell maturation, and expressed in Ter119+ cells (Fig. 2A).

Tamoxifen-mediated differentiation of G1E-ER-GATA-1 cells and MEL cell maturation mimic the later stages of definitive erythropoiesis (34, 52). To assess whether Tac-2 was differentially expressed over a broader developmental continuum, we analyzed expression of the human Tac-2 ortholog, TAC-3, during erythropoiesis ex vivo. Primary human erythroid progenitors from peripheral blood were differentiated for 14 days under conditions that recapitulate erythropoiesis in vivo (42). GATA-2 mRNA decreased as GATA-1 mRNA increased (Fig. 2B). GATA-1 mRNA was induced maximally by day 10 (Fig. 2B). Human TAC-3 mRNA increased by day 8 and was highly uninduced MEL cells, and expression was induced -2 fold upon Me2SO-induced erythroid maturation (Fig. 2A). GATA-1 expression is similar in uninduced and induced MEL cells. Tac-2 was also expressed in primary murine Ter119+ erythroid cells isolated from bone marrow (Fig. 2A). The major adult -globin gene, major, and Tac-2 shared a similar pattern of expression, being repressed in G1E cells, induced by tamoxifen in G1E-ER-GATA-1 cells, induced upon MEL cell maturation, and expressed in Ter119+ cells (Fig. 2A).

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induced by day 14 (Fig. 2B). Similar to the G1E-ER-GATA-1 results, Nab2 mRNA levels declined as TAC-3 mRNA levels increased. The patterns of TAC-3 and β-globin expression were similar, albeit not identical, since β-globin mRNA was maximal by day 12, whereas TAC-3 mRNA was maximal by day 14.

At day 14, the majority of erythroid cells generated in the ex vivo system are orthochromatic normoblasts, which have not yet undergone enucleation (42). Upon extravasation through the marrow sinusoids in vivo, the normoblasts enucleate, yielding peripheral reticulocytes. Since reticulocytes are competent to carry out protein synthesis, we asked whether TAC-3 transcripts are present in purified human reticulocytes. TAC-3, β-globin, and HPRT transcripts were detected in reticulocytes from seven normal patients, whereas Nab2 transcripts were undetectable (Fig. 2C). Thus, similar to the Tac-2 results in the murine system, TAC-3 is expressed in human erythroid cells and is highly regulated during human erythropoiesis.

Since ER-GATA-1 regulates Nab2 and Tac-2 within the same chromosomal region, it is possible that Nab2 and Tac-2 are coordinately regulated. To test this hypothesis, G1E-ER-GATA-1 cells were treated with tamoxifen for increasing amounts of time, and Nab2 and Tac-2 mRNA levels were quantitated. Tac-2 activation (Fig. 3A) and Nab2 repression (Fig. 3B) were apparent by 4 h and were maximal by 24 h. These changes in mRNA levels were more rapid than the induction of βmajor transcripts (Fig. 3C), which is known to a direct transcripational target of GATA-1. The correlation between Tac-2 activation and Nab2 repression (Fig. 3D) supports a mechanism in which these two genes are coordinately regulated in a reciprocal manner in the G1E-ER-GATA-1 system.

Both Nab2 and Tac-2 are members of protein families in which the individual members have both unique and shared activities. Like Nab2, Nab1 is an early growth regulator corepressor (53) but is expressed differently from Nab2 in certain scenarios. For example, nerve growth factor treatment of PC12 pheochromocytoma cells and serum stimulation of fibroblasts can induce Nab2 but not Nab1 expression (35). In other scenarios, Nab1 and Nab2 are co-regulated.3 Tac-2 is highly related to Tac-1, which encodes preprotachykinin-1, the precursor to Substance P that mediates pain perception (47). To assess the specificity of GATA-1-mediated repression of Nab2 and activation of Tac-2, we tested whether ER-GATA-1 regulates Nab1 and Tac-1 expression. Nab1 mRNA was detected in G1E-ER-GATA-1 cells but was unaffected by tamoxifen treatment (Fig. 4A). Tac-1 mRNA was undetectable in G1E-ER-

\[3 \text{ J. Svaren, unpublished data.}\]
GATA-1 cells, and tamoxifen did not induce Tac-1 expression (Fig. 4B). In contrast, Tac-1 was highly expressed in the mouse brain (Fig. 4B).

GATA-1 Occupancy of a Conserved Region within Intron-7 of Tac-2—Based on the abundance of high affinity GATA motifs within genomes, the mere presence of a GATA motif does not imply functional significance. Functional insights can be derived from evaluating the evolutionary conservation of GATA motifs, but even a conserved motif does not equate to a functional motif. Fourteen conserved GATA motifs exist within the region upstream of Stat-6 to downstream of Tac-2 (Fig. 5A). Each of these motifs (Fig. 5B) would bind GATA-1 with high affinity in vitro, based upon the established DNA binding specificity of GATA-1 (18, 19). However, analyses of GATA-1 occupancy at the β-globin (24, 26) and GATA-2 (24, 26) loci have revealed that the majority of GATA motifs are not occupied by GATA-1 in cells.

Quantitative ChIP analysis was conducted to assess whether ER-GATA-1 occupies the Nab2-Tac-2 chromosomal region in G1E-ER-GATA-1 cells. Analysis of 15 regions spanning all of the conserved GATA motifs and the Nab2 and Tac-2 promoters revealed that ER-GATA-1 occupies a single region within intron-7 of Tac-2 (Fig. 5C). Endogenous GATA-1 in Me2SO-induced MEL cells also occupies this intron-7 region, but not the Tac-2 promoter, which contains a GATA motif (Fig. 5D). Thus, GATA-1 selects exquisitely among the GATA motifs within the Nab2-Tac-2 chromosomal region. The highly selective occupancy of Tac-2 intron-7 strongly suggests that GATA-1 activates Tac-2 transcription via interaction with this site.

Our previous analyses of GATA factor interactions with chromatin indicated that a subset of the sites occupied by GATA-1 in erythroid cells were occupied by GATA-2 in GATA-1-null G1E cells (26, 27) and in a FOG-1-null hematopoietic precursor cell line that expresses both GATA-1 and GATA-2 (27, 55). A distinct subset of sites, including regions of the β-globin locus was occupied by GATA-1, but GATA-2 occupancy was either not detected or was very low.² GATA-2 and FOG-1 co-localized at chromatin sites, and only in the presence of FOG-1 was GATA-1 able to efficiently induce a GATA switch in which GATA-2 was displaced (27). Occupancy of the βmajor promoter by a GATA-1 mutant exhibiting reduced FOG-1 binding was

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Fig. 6. Rapid ER-GATA-1-mediated displacement of GATA-2 from the Tac-2 intron-7 region. A, G1E-ER-GATA-1 cells were incubated for 0, 0.5, or 4 h with 1 μM tamoxifen. Quantitative ChIP analysis was used to measure ER-GATA-1 and GATA-2 occupancy at Tac-2 intron-7. Samples were analyzed relative to a standard curve generated from input chromatin. The plots depict relative levels of binding (mean ± S.E., three independent experiments). B, FOG-1 occupancy at Tac-2 intron-7 region and the Tac-2 promoter. Quantitative ChIP analysis was used to measure FOG-1 occupancy in untreated and tamoxifen-treated (24 h) G1E-ER-GATA-1 cells (mean, two independent experiments).

Fig. 7. Neurokinin receptor expression and function in G1E-ER-GATA-1 and endothelial cell lines. Quantitative real time RT-PCR analysis of neurokinin receptor subtypes NK1 (A), NK2 (B), and NK3 (C) mRNA levels in untreated and tamoxifen-treated (24 h) G1E-ER-GATA-1 cells, MAE cells, and YSEC. The relative levels of NK1, NK2, and NK3 mRNAs were normalized by the levels of GAPDH transcripts. The plots depict the mRNA/GAPDH mRNA ratios (mean, two independent experiments) in which the ratios obtained for the −RT condition were designated as 1. Note that the untreated and tamoxifen-treated (24 h) G1E-ER-GATA-1 cells do not express the three NK receptors. D, MAE and YSEC cells were treated with 250 μM IBMX with or without 10 μM forskolin or 50 μM NK-B for 1 h. cAMP levels were quantitated in cell lysates by a competitive immunoassay (mean ± S.E., three independent experiments).
impaired (56), providing further evidence that FOG-1 facilitates GATA-1 occupancy at certain chromosomal sites. Thus, we term FOG-1 a “chromatin occupancy facilitator” or COF coregulator (27).

Quantitative ChIP analysis was used to test whether GATA-2 associates with intron-7 of Tac-2 in untreated G1E-ER-GATA-1 cells. GATA-2 occupancy was detected under conditions of very little ER-GATA-1 occupancy (Fig. 6A). Tamoxifen-mediated activation of ER-GATA-1 resulted in the loss of GATA-2 occupancy concomitant with increased ER-GATA-1 occupancy. Reduced GATA-2 occupancy was detected 30 min after tamoxifen treatment, and the switch was nearly complete by 4 h. Based on the previous analysis of the kinetics of GATA-1-mediated repression of GATA-2 (26), these data strongly suggest that the loss of GATA-2 occupancy from intron-7 results from GATA-1-mediated displacement of GATA-2 from the intron-7 region rather than repression of GATA-2, which would lead to insufficient levels of GATA-2 for intron-7 occupancy.

G1E-ER-GATA-1 cells express FOG-1, and, as noted above, FOG-1 co-localizes with GATA-2 at a subset of the chromatin sites bound by GATA-1 later in erythropoiesis (27). To test whether FOG-1 is a component of the endogenous Tac-2 intron-7 complex when either GATA-1 or GATA-2 is bound, quantitative ChIP analysis was conducted with uninduced and tamoxifen-treated G1E-ER-GATA-1 cells. FOG-1 occupancy was detected at intron-7 of the locus in both the transcriptionally inactive and active states (Fig. 6B), similar to our previous findings with the GATA-2 locus (27). Thus, the intron-7 region is occupied by a GATA-2-FOG-1 complex in the inactive state, and the GATA switch, which correlates with transcriptional activation, results in either the replacement of GATA-1 by GATA-2 with FOG-1 retention or the substitution of a GATA-2-FOG-1 complex with a GATA-1-FOG-1 complex.

An Erythroid Cell-derived Neurokinin: Implications for Vascular Regulation—Whereas neurokinins were classically studied in the nervous system, recent studies have expanded the repertoire of sites of neurokinin synthesis and function (47, 50). However, whether erythroid cells are competent to generate neurokinins had not been investigated. We describe herein that erythroid cell lines and primary erythroid cells express Tac-2 in a highly regulated manner during erythropoiesis. Furthermore, GATA-1 and GATA-2 bind directly to intron-7 of Tac-2 in living cells, and GATA-1 rapidly activates Tac-2 transcription. The mechanistic analyses in the G1E system provided strong evidence that Tac-2 is a direct target of GATA-1 and GATA-2. These findings raise the question of what might be the function of erythroid cell-derived NK-B.

Since nucleated definitive erythroid cell precursors exist pre-
dоминантову в костном мозге, NK-B выделенный из эритропоцитов предшественников мог бы функционировать в пределах костного мозга. Для того чтобы определить функции NK-B, мы проанализировали в индуцированной или неиндуцированной G1E-ER-GATA-1 клетке. NK-B имеет наиболее высокую активность для NK3, но также активирует и NK1 и NK2 (47). NK рецептор активирует реверсивную стимуляцию адениловой циклазы и циклической AMP-генерации, а также активацию тирозин-киназ 

\[\text{AMP} \rightarrow \text{cAMP} \rightarrow \text{PKA} \rightarrow \text{CREB} \rightarrow \text{NFAT} \]

Это позволяет предположить, что связывание GATA-1 с этим эпителием может привести к регуляции гена. Принимая эти данные во внимание, NK-B (NK3) способен активироваться через NK-B зависимый путь, и это может быть важно для развития иммунологических реакций к гуморальным пре-в клеткам NK-B, NK-B и NK рецепторы и оценить, насколько NK-B/NK рецептор система контролирует функции в костном мозге. 

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