T to C Substitution at −175 or −173 of the γ-Globin Promoter Affects GATA-1 and Oct-1 Binding in Vitro Differently but Can Independently Reproduce the Hereditary Persistence of Fetal Hemoglobin Phenotype in Transgenic Mice*

Received for publication, October 6, 2004, and in revised form, December 20, 2004 Published, JBC Papers in Press, December 21, 2004, DOI 10.1074/jbc.M411407200

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The T to C substitution at position −175 of the γ-globin gene has been identified in some individuals with non-deletion hereditary persistence of fetal hemoglobin (HPFH). In this study, the HPFH phenotype was reestablished in transgenic mice carrying the μLCRAγ−175T>C construct, which contained a 3.1-kb μLCR cassette linked to a 29-kb fragment from the γ-y globin gene with the natural chromosome arrangement and no the −175 mutation, which provided evidence for this single mutation as the cause of this form of HPFH. The HPFH phenotype was also reproduced in transgenic mice carrying the Aγ- globin gene. In vitro experiments proved that the −175 mutation significantly reduced binding of Oct-1 but not GATA-1, whereas the −173 mutation dramatically decreased binding of GATA-1 but not Oct-1. These results suggest that abrogation of either GATA-1 or Oct-1 binding to this promoter region may result in the HPFH phenotype. An in vitro footprinting assay revealed that either the −175 mutation or the −173 mutation significantly decreased overall protein binding to this promoter region in adult erythrocytes of transgenic mice. We hypothesize that a multiprotein complex containing GATA-1, Oct-1, and other protein factors may contribute to the formation of a repressive chromatin structure that silences γ-globin gene expression in normal adult erythrocytes. Both the −173 and −175 T to C substitutions may disrupt the complex assembly and result in the reactivation of the γ-globin gene in adult erythrocytes.

The human fetal globin genes (Gγ and Aγ) are mainly expressed in fetal liver. The switch from fetal to adult (δ and β) globin gene expression occurs in the perinatal period concomitantly with the establishment of the bone marrow as the main site of erythropoiesis. In some individuals, however, this switching is incomplete, and γ-globin expression persists in adult erythrocytes. Such a condition, found in syndromes of hereditary persistence of fetal hemoglobin (HPFH)\(^1\) and (δβ)\(^0\)-thalassemias, can result from deletions within the β-globin gene cluster (1). However, in some syndromes known as non-deletion HPFH, point mutations in the γ-globin gene promoter seem to be responsible for the continuance of γ-globin expression (1). It has been revealed that most of the point mutations lie in or near the trans-acting factor binding motifs within the promoter and that the mutations usually affect the binding of some trans-acting factors in vitro (2).

Studies in transgenic mice carrying either −117 Aγ HPFH cosmids or −117 Aγ HPFH yeast artificial chromosome showed that transgenic expression could mimic the developmental expression program observed in HPFH, providing powerful proof that the single point mutation was the cause of the Greek form of HPFH (3, 4). Berry et al. (3) speculated that this change of γ-globin expression during development could be correlated with the loss of GATA-1 binding to the γ-promoter region around −117 and deduced that GATA-1 might act as a negative regulator of the γ-globin gene in normal adults. However, earlier in vitro experiments on a black form of HPFH point mutation, a T to C substitution at position −175 of the γ-globin promoter, showed no significant difference in the GATA-1 affinity for the promoter. Nevertheless, this mutation dramatically decreased the affinity of the Oct-1 transcription factor, which binds to an area partially overlapping the GATA-1 binding site (5–9).

To test whether the −175 T to C substitution might be the cause of the black form of HPFH, we introduced two β-globin minilocus constructs into mice. The first was μLCRAγψββ, which contained a 3.1-kb μLCR cassette linked to a 29-kb fragment from the Aγ-β globin gene with the natural chromosome arrangement. The second was μLCRAγ−175T>C, which was similar to μLCRAγψββ except for a T to C substitution at position −175 of the Aγ promoter. Normal developmental expression of γ- and β-globin genes was observed in transgenic mouse lines with μLCRAγψββ, whereas persistent expression of the γ-globin gene in adults of transgenic mouse lines with μLCRAγ−175T>C was noted, suggesting that this single mutation is responsible for the HPFH phenotype.

To further investigate the relationship between GATA-1 or Oct-1 binding and the developmental regulation of the γ-globin gene, we substituted the −175 T to C Aγ-globin gene with a

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\* This work was supported by Grants 39893320 and 30393110 from the National Science Foundation of China (to J.-W. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\(^1\) The abbreviations used are: HPFH, hereditary persistence of fetal hemoglobin; LCR, locus control region; MEL, mouse erythroleukemia; DMS, dimethylsulfate; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; LM-PCR, ligation-mediated PCR.
−173 T to C Aγ-globin gene in the construct. The −173 T base has been reported to be involved in the conserved GATA-1 binding motif (5–9). By electrophoretic mobility shift assays (EMSAs), we found that the −173 mutation decreased GATA-1 binding dramatically but reduced Oct-1 binding only slightly. Despite these differences in transcription factor binding ability, the HFPFH phenotype was reestablished in mice carrying either the GATA-1 or Oct-1 to bind this promoter region. We then tested the protein binding status within this promoter region in adult erythrocytes and that abrogation of binding by either disrupted protein binding status within this promoter region in adult erythrocytes and that abrogation of binding by either disrupted

**Experimental Procedures**

**Cosmid Constructs**—The plasmid pμLCR, containing a 3.1-kb cassette consisting of the intact core sequences of HS1–4, is a gift from Dr. Q. Li. The cosmid construct pμLCR was released as a HindIII fragment from pμLCR and inserted into pBluescript and linearized with BssHII. A XhoI-NotI fragment, which contains the 3.1-kb μLCR, the promoter, and a 5’ part of the Aγ-globin gene, was released from pμLCR and replaced by either the GATA-1 or Oct-1 to bind this promoter region. The GATA-1 or Oct-1 binding to the promoter region in adult erythrocytes and the α-globin gene expression in normal adult erythrocytes and that abrogation of binding by either disrupted the repressor complex and resulted in HFPFH.

**DNA Purification for Microinjection**—The 35-kb KpnI-NotI DNA fragments were identified by PCR and Southern blot analysis of tail skin DNA. Transgenic founder mice were bred with nontransgenic KM mates to establish transgenic lines. The structures of the integrated constructs were analyzed by Southern blot hybridization, using the γ- and the β-globin gene and the μLCR as the probes. Copy numbers were determined by comparing the intensity of hybridization bands from the transgenic lines with that of the human genomic DNA by PhosphorImager analysis.

**Globin Gene Expression Analysis**—For each mouse line, transgenic males were mated with nontransgenic KM females to generate timed pregnancies. The morning when the mating plug was observed was designated day 0.5. RNA samples were prepared from the yolk sac of mouse embryos on day 11.5; from fetal livers on days 13.5, 16.5, and 18.5; and from the blood of newborn pups as well as adult animals (9). Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Human and murine globin RNAs were analyzed by quantitative RNase protection assays (14). Four plasmids containing the pT7 promoter and a fragment of each of the four globin genes were used as probe templates: 1) pT7Aγ, made by cloning a Ncol-PvuII fragment of the human Aγ-globin gene into pBluescript and linearized with BseMI, giving a protected fragment of 170 nucleotides derived from exon 2 of the human Aγ mRNA; 2) pT7Bβ, made by inserting a BamHII-BglII fragment of the human β-globin gene into pBluescript and linearized with SphI, giving a protected fragment of 205 nucleotides derived from exon 2 of the human β mRNA; 3) pT7Mε, made by inserting a PstI-BamHI fragment, corresponding to the 5′ end of mouse ε-globin gene, into pBluescript and linearized with SphI, giving a protected fragment of 128 nucleotides derived from exon 1 of the mouse ε mRNA; and 4) pT7Mγ, made by cloning a mouse γ-globin gene into pBluescript and linearized with BseMI, giving a protected fragment of 151 nucleotides. The RNA probes labeled with α-32P-UTP were obtained by in vitro transcription of the plasmids with pT7 polymerase. 10 μg of total RNA was hybridized overnight at 47 °C with 1 × 106 cpm of each probe, followed by digestion with RNase A and T1 for 30 min at 37 °C using the ribonuclease protection assay kit (Roche Applied Science). The protected fragments were separated on 5%
were performed as described by Kang et al. (15). Electrophoresis was carried out at 25 °C in 4% polyacrylamide gel equipped with GelScan 2.1 software. The 44 bp Apal-AvalII fragments (~201 to ~158) of the γ-globin promoter with the normal sequence or the ~175 or ~175 mutation was used as probe. The probes were labeled with Klenow polymerase and [α-32P]-dCTP (normal probe, 5'-GTCTCCACACTTCTCAATGCGAACATCTGTCTGAAAGGTCTGACGT-3'; ~173 mutation probe, 5'-GTCTCCCCACACTTCTCAATGCGAACATCTGTCTGAAAGGTCTGACGT-3'; and ~175 mutation probe, 5'-GTCTCCCCACACTTCTCAATGCGAACATCTGTCTGAAAGGTCTGACGT-3') by dephosphorylation at 60 °C for 20 min, followed by annealing at 5 °C. The sequences of competitors for GATA-1 and Oct-1 binding were 5'-GTGTTCATATCCTGGAATGACTGAATCGGAAC-3'.

The EMSAs were carried out as described previously (15). Approximately 2 μg of nuclear extracts was added, and the reaction was incubated at 30 °C for 5 min before the addition of labeled probe (1 × 106 cpm, 1 ng). Complete binding reactions were incubated for 1 or 4 h. The sequences of competitors for GATA-1 and Oct-1 binding were 5'-CGAGGCGAAGATATCCTCTTGGAGGA-GT-3' and 5'-GTCTCATGGAATGACTGAATCGGAACATCTGTCTGAAAGGTCTGACGT-3' respectively. Electrophoresis was carried out at 25 °C in 4% polyacrylamide gels, using 0.5× Tris-borate EDTA buffer. The gels were then dried and analyzed by autoradiography. To assess quantitative differences in binding affinity, the intensities of retarded bands were determined by an LKB (Amersham Biosciences) UltroScan XL laser densitometer equipped with GelScan 2.1 software.

ChIP and Ligation-mediated PCR (LM-PCR)—ChIP and LM-PCR were performed as described by Kang et al. (15), with the following modifications. Bone marrow cells (5 × 106) of the transgenic mice were cross-linked by adding formaldehyde to a final concentration of 1% and incubated at room temperature for 10 min. Cells were washed with phosphate-buffered saline and resuspended in ChIP lysis buffer (100 mM Tris, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) with protease inhibitors. After incubation on ice for 10 min, the cells were sonicated to shear DNA to lengths between 200 and 1000 bp on ice. The lysate was transferred to a 15-ml conical tube and diluted with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 16.7 mM NaCl, and protease inhibitors). An aliquot of 500 μg of protein A-Sepharose beads was added to the diluted nuclear lysate and incubated at 4 °C overnight. The beads were pelleted at 2000 rpm for 10 min. The supernatant was divided into three aliquots of 25 μl. Anti-GATA-1 and anti-Oct-1 antibodies (Santa Cruz Biotechnology) were used to add two aliquots, respectively, whereas no antibody was added to the third aliquot, and then the aliquots were incubated at 4 °C overnight while rotating. Dimethylsulfate (DMS) treatment of the immunoprecipitated chromatin fragments was performed using the Maxam and Gilbert guanine-specific sequencing reaction with 0.1% DMS for 45 s at room temperature. An aliquot of the precipitated DNA was also analyzed by PCR with primers specific for the human γ-globin promoter region (forward primer, 5'-GCTTCCCCACACTTCTCAATGCGAACATCTGTCTGAAAGGTCTGACGT-3'; and reverse primer, 5'-GGTTGGGAACTGGAATGACTGAAC-3'). The EMSAs for γ-globin specific sequencing reaction were performed using 0.1% DMS for 45 s at room temperature. An aliquot of the precipitated DNA was also analyzed by PCR with primers specific for the human γ-globin promoter region (forward primer, 5'-GCTTCCCCACACTTCTCAATGCGAACATCTGTCTGAAAGGTCTGACGT-3'; and reverse primer, 5'-GGTTGGGAACTGGAATGACTGAAC-3'). The EMSAs for γ-globin specific sequencing reaction were performed using 0.1% DMS for 45 s at room temperature.

Normal Developmental Expression of the human Aγ- and β-globin and murine κ- and α-globin genes in transgenic mice carrying μ LCRAγψββ. Total RNA isolated from samples on different development days and tissue source was analyzed by Rnase protection assay. d, days post-coitus; y/s, yolk sac; fl, fetal liver; nb bl, blood of newborn pup; ab bl, adult blood. Different transgenic mouse lines are indicated at the top, and the positions and numbers of nucleotides of the protected RNA fragments are indicated at the right.

RESULTS

Normal Developmental Expression of γ-globin Gene Was Observed in Transgenic Mice with μ LCRAγψββ—Two transgenic lines with intact integration of the construct μ LCRAγψββ were established. The levels of human Aγ- and β-globin transcripts as well as endogenous mouse κ- and β-globin transcripts were measured by Rnase protection analysis (Fig. 2; Table I).

In line N1, the highest Aγ-globin mRNA detected was ~17% of mouse α- plus κ-globin mRNA (per transgene copy) on day 13.5, declining significantly to 0.9% in newborn pups and to 0.4% in adult animals. This result suggests active expression of the Aγ-globin gene during the fetal stages and subsequent silence after birth. The human β-globin gene was undetectable at the embryonic stage but became active in the early fetal liver, rising rapidly to maximum levels and remaining there in the adult stage. The pattern of developmental expression of human γ- and β-globin genes in line N5 was very similar to that in line N1. These results indicate that transgenic mice carrying μ LCRAγψββ can mimic the normal developmental expression programs of both the human Aγ- and β-globin genes. This finding is similar to that from a previous study on transgenic mice carrying μ LCRAγψββ, a construct that was the same as μ LCRAψββ except for the use of a 2.6-kb LCR cassette (16).

The HFPF Phenotype Was Reproduced in Transgenic Mice Carrying μ LCRAγψββ—Four transgenic lines with intact integration of the construct μ LCRAγψββ were established. The expression of the human and murine globin mRNAs during mouse development is shown in Fig. 3 and Table II.

The human γ/mouse α + κ mRNA/copy ranged from 8% to 15% in adults, only a moderate decline when compared with the highest Aγ-globin mRNA expression, which ranged from 18% to 29% in the fetal stage. That is, Aγ-globin gene expression in the adult stage was between 28% and 71% of the maximal fetal-stage expression. Although transgene expression varied between lines, the 29–72% reduction of γ-globin gene transcription in adult stage as compared with that in the fetal stage for mice carrying μ LCRAγψββ differed significantly from the 97.7–98.2% reduction occurring in mice carrying μ LCRAψββ. These results demonstrated that the Aγ-globin gene with the ~175 mutation still remained active.
Induced erythroid K562 cells, uninduced and Me2SO-induced erythroid MEL cells, as well as nonerythroid HeLa cells. Two MEL cell lines, MELGM979 (which expresses both adult and embryonic globins) and MEL585 (which expresses adult globin only), were used for nuclear extract preparation. Very similar EMSA profiles were observed between the two lines (data not shown). Two common mobility shifted bands (A and B) were produced when the normal fragment reacted with all the nuclear extracts, with the exception of the HeLa cell extract. (Fig. 4B). Compared with the normal probe, the intensity of band A, produced by the −173 mutation probe, decreased by 19 ± 10%, and the intensity of band B, produced by the same mutation probe, decreased by 85 ± 8%. The competition assay demonstrated that band A resulted from Oct-1 binding and that band B resulted from GATA-1 binding (Fig. 4C). This result indicated that the −173 mutation dramatically decreased the binding affinity of GATA-1 to the mutant promoter but only slightly reduced Oct-1 binding.

EMSA of DNA fragments with the −173 mutation were carried out exhaustively in previous studies (5–9). Our results here showed that when compared with the normal fragment, the binding affinity of GATA-1 to the −173 mutation promoter decreased by 5 ± 2%, whereas the binding affinity of Oct-1 decreased by 93 ± 6% (Fig. 4D). This is consistent with the results of most of the previous studies, i.e., the −173 mutation decreased GATA-1 binding slightly but reduced Oct-1 binding greatly, which was just the opposite of the effects of the −173 mutation.

The HPFH Phenotype Was Also Reproduced in Transgenic Mice Carrying μLCRAγ^{−175}ϕββ—to further investigate whether −173 T to C substitution of the Aγ gene could reestablish the HPFH phenotype in vivo, we produced the construct μLCRAγ^{−175}ϕββ. Three transgenic lines carrying the construct were established, and developmental expression of the globin genes was analyzed (Fig. 5; Table III). The human γ/α′mouse α’ + ς mRNA/copy ranged between 5% and 14% in

| Table I | Table II |
|---------|---------|
| Globin gene expression in transgenic mouse lines carrying μLCRAγ^{−175}ϕββ | Globin gene expression in transgenic mouse lines carrying μLCRAγ^{−175}ϕββ |
| Line | Developmental age | Copy no. | Human γ mRNA/mouse α’ + ς mRNA/copy (%) | Adult γ mRNA/peak fetal γ mRNA | Line | Developmental age | Copy no. | Human γ mRNA/mouse α’ + ς mRNA/copy (%) | Adult γ mRNA/peak fetal γ mRNA |
| d^c 11.5 | d 13.5 | d 16.5 | d 18.5 | Newborn | Adult | d 11.5 | d 13.5 | d 16.5 | d 18.5 | Newborn | Adult | d 11.5 | d 13.5 | d 16.5 | d 18.5 | Newborn | Adult |
| 11.5 ± 3.0 | 17 ± 3.9 | 16 ± 4.3 | 15 ± 2.8 | 0.9 ± 0.5 | 0.4 ± 0.3 | 9 ± 3.1 | 16 ± 2.6 | 15 ± 2.7 | 14 ± 2.0 | 0.6 ± 0.2 | 0.3 ± 0.2 | 11.5 ± 3.0 | 23 ± 1.0 | 29 ± 1.3 | 27 ± 1.6 | 26 ± 8.4 | 8 ± 7.2 | 4 ± 3.0 | 21 ± 4.9 | 19 ± 4.6 | 20 ± 5.0 | 17 ± 5.4 | 15 ± 1.3 | 4 ± 2.5 | 17 ± 4.2 | 18 ± 0.5 | 15 ± 0.7 | 14 ± 0.9 | 12 ± 0.7 | 3 ± 2.1 | 18 ± 4.7 | 17 ± 0.9 | 15 ± 3.1 | 8 ± 0.9 | 8 ± 0.7 |

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*a Copy no., number of transgenes in haploid cell.  
*b Three to four F1 animals were analyzed in each.  
^c d, days post-coitus.  

**FIG. 3. Developmental expression of the globin genes in transgenic mice carrying μLCRAγ^{−175}ϕββ.** Size marker consisted of MspI-digested pBR322 DNA. Total RNA isolated from samples on different developmental days and tissue source was analyzed by RNAse protection assay. d, days post-coitus; y/s, yolk sac; f/l, fetal liver; nb bl, blood of newborn pup; ad bl, adult blood. Different transgenic mouse lines are indicated at the top, and the positions and numbers of nucleotides of the protected RNA fragments are indicated at the right.

During the adult stage and that the point mutation was responsible for the HPFH phenotype.

The −175 and −173 Mutation Affected GATA-1 and Oct-1 Binding Differently in Vitro—The region from −170 to −190 of the Aγ-globin gene promoter contains two GATA-1 binding motifs and one Oct-1 binding motif (Fig. 4A). EMSAs of the normal Apal-AvaII fragment (−201 to −158) of the γ-globin gene promoter and its −173 mutant counterpart were performed using nuclear extracts from uninduced and hemin-
A promoter region. MEL, Me2SO-induced MEL cells, but not from HeLa cells. The positions of bands A and B are indicated at the top, and the numbers of nucleotides of the protected RNA fragments are indicated at the right.

![Figure 4](http://www.jbc.org/)

**FIG. 4.** Binding of GATA-1 and Oct-1 to normal/mutant γ-globin promoter region. A, sequence of the −158 to −201 region in human Aγ-globin promoter. The recognition sequences of GATA-1 are boxed, and the Oct-1 motif is underlined. B, EMSAs of the normal (N) 44-bp Apal-Aval fragment and its −173 T to C counterpart (173), using nuclear extracts (10 μg) from erythroid K562, hemin-induced K562 (+), MEL, Me2SO-induced MEL (+), and nonerythroid HeLa cells. Similar results were obtained when using extracts from K562, K562 (+), MEL, and MEL (+) cells, but not from HeLa cells. The positions of bands A and B are indicated at the left. The marked reduction of band B and slightly weakened band A were seen when the −173 mutant fragment was used as probe. C, competition assays. The nuclear extract was obtained from Me2SO-induced MEL cells. The left lane shows the binding of 10 μg of nuclear extract to 1 ng of the normal fragment without specific competitors. Lanes a–c, with 25, 100, or 250 ng of competitor of Oct-1 binding or GATA-1 binding, respectively. Competition assay demonstrated that mobility-shifted band A resulted from Oct-1 and that mobility-shifted band B resulted from GATA-1 binding. D, EMSAs of the normal Apal-Aval fragment and its −173 T to C counterpart (173) as well as its −173T to C counterpart (173). A slightly decreased band A intensity and a greatly reduced band B intensity were observed when the −173 mutation fragment was used as probe, whereas a greatly decreased band A intensity and a similar band B intensity were noticed when the −173 mutation fragment was used as probe.

**FIG. 5.** Developmental expression of the globin genes in transgenic mice carrying μLCRAγ−173Cγββ. Total RNA isolated from samples on different development days and tissue source was analyzed by RNase protection assay. d, days post-coitus; ys, yolk sac; fl, fetal liver; nb bl, blood of newborn pup; ad bl, adult blood. Different transgenic mouse lines are indicated at the top, and the positions and numbers of nucleotides of the protected RNA fragments are indicated at the right.

| Line | Developmental age | Copy no. | Human γ mRNA/mouse mRNA/peak | Adult γ mRNA/peak | % |
|------|------------------|---------|-------------------------------|-------------------|---|
|      |                  |         |                               |                   |   |
| 307  | d 11.5           | 3 ± 1.5 | 20 ± 0.5                      | 25                |   |
|      | d 13.5           | 20 ± 4.3| 5 ± 0.7                       |                   |   |
|      | d 16.5           | 18 ± 0.5| 13 ± 3.4                      |                   |   |
|      | d 18.5           | 17 ± 2.5| 18 ± 2.4                      |                   |   |
|      | Newborn          | 25 ± 6.7| 18 ± 2.4                      |                   |   |
|      | Adult            | 5 ± 0.7 | 25 ± 6.7                      |                   |   |
| 315  | d 11.5           | 21 ± 12 | 15 ± 4.7                      |                   |   |
|      | d 13.5           | 14 ± 5.3| 14 ± 2.1                      |                   |   |
|      | d 16.5           | 13 ± 3.4| 14 ± 1.2                      |                   |   |
|      | Newborn          | 17 ± 2.6| 16 ± 2.6                      |                   |   |
|      | Adult            | 11 ± 4.4| 13 ± 0.7                      |                   |   |

| Copy no., number of transgenes in haploid cell. | Ad, adult; Tel, telencephalon; Ad bl, blood of newborn pup; F, fetal; P, placenta. 173C mutation significantly decreased Protein Binding to the Aγ promoter region in vivo—Bone marrow cells were collected from a transgenic mouse line carrying μLCRAγ−173Cγββ (line N1), a transgenic line carrying μLCRAγ−175Cγββ (line 518), and a transgenic line carrying μLCRAγ−175Cγββ (line 318), respectively. After obtaining sheared chromosome fragments from the cells, the specific fragments were precipitated with the anti-GATA-1 or anti-Oct-1 antibodies. From an aliquot of the precipitated fragments, the Aγ promoter region was amplified using specific primers. Amplification fragments were explored in all of the samples precipitated with the anti-GATA-1 and anti-Oct-1 antibodies, but not in the samples without antibody precipitation (data not shown). adults, whereas the highest Aγ-globin mRNA expression in the fetal stage of the transgenic animals was between 17% and 25%. The Aγ-globin gene expression in the adult stage was between 25% and 65% of the maximal fetal-stage expression level. Despite expression variation between lines, the results showed that the Aγ-globin gene with the −173 mutation still remained active in the adult stage of mouse development. The −173 mutation resulted in the HPFH phenotype in transgenic mice, similar to the manifestation of the Aγ-globin gene bearing the −175 mutation (Fig. 6).
suggested that the −175 mutation altered the mechanism in which GATA-1 binds to the mutant promoter rather than the affinity of GATA-1 for the promoter. HMG-1 (high-mobility group-1), a ubiquitous and abundant nuclear DNA-binding protein, has also been demonstrated to bind strongly to one of a pair of downstream GATA-1 motifs; the −175 mutation significantly decreased HMG-1 binding (19), but the physiological effect of this decline is unclear.

In most transient expression assays, only a 3- to 4-fold increased expression of the −175 mutant γ-globin gene above that of the wild-type gene was observed in K562 cells, far less than the >100-fold increased expression of the mutant gene in vivo (20–22). Additionally, several putative HPFH mutations such as −117, −196, and −202 mutation, did not result in striking effects on promoter activity in transient K562 and MEL cell assays (7, 23–25). These findings demonstrated that in vitro experiments could not accurately reflect the in vivo conditions.

Because the −117 mutation reproduced the HPFH phenotype in transgenic mice (3, 4) and the −175 mutation affected GATA-1 binding differently from the −117 mutation, it is important to test whether the −175 mutation of γ-globin promoter also reproduces the HPFH phenotype. In a recent study, transgenic mouse lines carrying the μLCR-382Aγ construct with or without the −175 mutation were produced (26). Although a high level of γ-globin expression was observed in the adult animals carrying μLCR-382Aγ with the −175 mutation, only a 1.3-fold higher level of γ-globin expression compared with the μLCR-382Aγ transgene was observed because the μLCR-382Aγ adult transgenic mice expressed high levels of γ-globin themselves. The loss of developmental control of γ-globin gene expression in μLCR-382Aγ was probably due to the removal of a possible silencer sequence between −382 and −730 of the Aγ promoter (27), the lack of the region downstream of the Aγ gene suspected to have a role in Aγ gene silencing (28), or from the absence of β-globin gene competition for interaction with the LCR (10). To exclude these possibilities, we produced constructs containing a 3.1-kb LCR cassette that includes the intact core sequences of the four HS sites, flanked by a 29-kb fragment from the Aγ- to β-globin gene with normal chromosome organization. A similar cosmid construct, containing a 2.6-kb μLCR cassette flanked by the 29-kb fragment, has been demonstrated to confer correct developmental control of the globin genes in transgenic mice (10). In our study, human and endogenous globin mRNAs of mice carrying one or two copies of the transgenes were analyzed. The γ-globin expression displayed normal developmental control in mice carrying μLCRγψβββ, whereas continuance of γ-globin expression was observed in adult mice carrying μLCRγψβββ. These results provided proof that the −175 T to C substitution caused this form of non-deletion HPFH.

Based on the observation that the −117 mutation reproduced the HPFH phenotype in transgenic mice as well as dramatically decreasing GATA-1 binding, Berry et al. (3) proposed GATA-1 might act as a negative regulator of the γ-globin gene in normal adults (3). However, an increased binding of CP1 and CDP and a decreased binding of NF-E3 in the CCAAT region have also been observed (15, 22, 29, 30), making it difficult to evaluate the role of GATA-1 in the developmental regulation of γ-globin gene expression. Establishment of the HPFH phenotype in transgenic mice by the −175 mutation at least suggests that the decreased GATA-1 binding is not necessary for active expression of γ-globin in adults in this form of HPFH. However, it cannot be excluded that GATA-1 binding plays various roles in different regions of the promoter.

To further investigate the relationship between transcrip-
ional factor binding and developmental regulation of γ-globin expression, we introduced a mutation at position −173. Contrary to the −175 mutation, the −173 mutation slightly decreased Oct-1 binding but greatly reduced GATA-1 binding in vitro. Nevertheless, when we introduced the LCRAγ−173phen construct into mice, the HPFH phenotype was also reproduced. Because the −175 mutation greatly decreased Oct-1 affinity for the promoter region, it is reasonable to speculate that this change is responsible for overexpression of the mutant γ-gene in adults. In fact, Oct-1 has been found to possess an intrinsic silencing activity in its alanine-rich C-terminal domain (31).

The role of Oct-1 as a transcriptional repressor has been well studied in promoters of the thyrotropin β subunit gene, pituitary-specific transcription factor pit1/ghf1 gene, von Willibrand factor gene, and B-cell-specific B29 (Igβ) gene (31–34). Recently, a detailed study of the 5′-flanking region of human gonadotropin-releasing hormone receptor gene revealed that the POU domain transcription factor, Oct-1, played an important role in the transcriptional repression of the gonadotropin-releasing hormone receptor gene via binding to an octamer sequence (35). Whether Oct-1 also acts as a transcriptional repressor for γ-globin regulation in adults still remains unclear. However, the fact that the −173 mutation that only slightly decreased the Oct-1 affinity for the promoter region resulted in the HPFH phenotype in transgenic mice reduced the possibility that the change in Oct-1 binding directly increased γ-globin expression of the −175 HPFH.

Because both the −175 and the −173 mutation, which affected binding of GATA-1 and Oct-1 to the promoter region in vitro differently, resulted in HPFH phenotypes, we believe that the continued expression of γ-globin gene in adults is not simply due to the decreased affinity of GATA-1 or Oct-1 alone to the promoter region. Therefore, it is reasonable to assume that Oct-1 and GATA-1 binding are both necessary for silencing of the γ-globin gene in adult erythrocytes.

Finally, we analyzed the protein binding status within this promoter region in adult erythrocytes of transgenic mice carrying normal or mutant γ-globin genes. After the sheared chromosome fragments were precipitated with anti-GATA-1 or anti-Oct-1 antibodies and subjected to PCR amplification using primers specific for the γ-promoter region, the amplification fragments were explored in all samples derived from bone marrow cells of transgenic mice. This means that the ChIP assay alone could not identify changes of GATA-1 and Oct-1 binding status of the upstream GATA-1 binding site (A), where mutations were not involved, could not be detected because only guanine-specific sequencing reactions were performed in this study.
an Oct-1 site within the −233 region and a GATA-1 site within the −117 region in addition to the two GATA-1 sites and the Oct-1 site within the −175 region of the γ-promoter (16), the obtaining of specific chromatin fragments was probably due to the combination of the antibodies with the corresponding proteins bound to the −175 region and/or the alternative sites. Subsequently, a DMS footprint assay was performed. The result showed that both the G residues at −180 and −170 positions of the γ-globin promoter were protected in adult bone marrow cells derived from mice carrying $\mu$LCRAγζβββ construct, suggesting that proteins bind to this promoter region of the chromatin. Whereas in vitro experiments demonstrated that the −175 mutation and the −173 mutation affected Oct-1 and GATA-1 binding differently, our in vivo footprint analysis revealed that both −175 and −173 mutation significantly decreased overall protein binding to the corresponding region of chromatin. Although the in vivo footprint experiment could not confirm which proteins bound to this DNA region of the chromatin in adult erythrocytes of mice with $\mu$LCRAγζβββ, we could assume that GATA-1 and Oct-1 were included because the previous in vitro experiments proved binding of the two transcription factors to this region. Perhaps a multi-protein complex containing GATA-1, Oct-1, and other protein factors bind to this promoter region and contributes to the formation of a repressive chromatin structure, silencing γ-globin gene expression in adult erythrocytes. Both the −175 and −173 mutations may block the formation of such a complex, thereby resulting in the continued expression of the γ-globin gene in adult erythrocytes. Additional experiments are needed to prove and determine the presence and composition of this multi-protein complex in adult erythrocytes.

Acknowledgments—We thank Marc Soares and Wilson Huiyan Luo for critical reading of the manuscript and G. Stamatoyannopoulos and Q. Li for the cosmids $\mu$LCRAγζβββ and plasmid $\mu$LCR constructs.

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T to C Substitution at –175 or –173 of the γ-Globin Promoter Affects GATA-1 and Oct-1 Binding in Vitro Differently but Can Independently Reproduce the Hereditary Persistence of Fetal Hemoglobin Phenotype in Transgenic Mice

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J. Biol. Chem. 2005, 280:7452-7459.
doi: 10.1074/jbc.M411407200 originally published online December 21, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M411407200

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