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NOTES

Point Mutation Associated with Hereditary Persistence of Fetal Hemoglobin Decreases RNA Polymerase III Transcription Upstream of the Affected γ-Globin Gene

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A base substitution in the 5′-flanking region of a human fetal globin gene is associated with abnormal fetal hemoglobin production. It also reduces by 5- to 10-fold in vitro transcription of the gene by RNA polymerase III. We discuss potential links between polymerase III transcription and abnormal hemoglobin production.

The human β-globin gene family includes adjacent embryonic, fetal, and adult genes, which are developmentally regulated by a reciprocal switching mechanism. During the transitions from embryonic to fetal to adult life, expression of one gene decreases as the next increases. However, mutations within the β-like globin gene region can result in continuous expression of fetal genes into adult life, a condition known as hereditary persistence of fetal hemoglobin (HPFH). One class of HPFH mutations involves single base changes at −202, −196, −158, or −117 (the mRNA cap site is +1) of either of the two fetal γ-globin genes (6, 8, 9, 12–14). Several observations suggest that these substitutions cause the HPFH phenotype. (i) In all known cases the mutated gene but not the *cis*-linked, normal fetal gene is affected. (ii) The substitutions are the only nonpolymorphic deviations from the normal sequence over a large region. (iii) There is a strong correlation between the base substitution and HPFH (6, 9).

The mechanism by which these mutations cause elevated fetal and reduced adult globin gene expression in adults is unknown. Three of the mutations are located 100 nucleotides upstream of sequences thought to be important for transcription by RNA polymerase II. Thus, it might be significant that the four HPFH point mutations are located in a region that, in β (adult)- and ε (embryonic)-globin genes, is transcribed by RNA polymerase III (3, 4, 18). The polymerase III transcripts have heterogeneous 5′ termini located 150 to 230 nucleotides upstream of the mRNA cap site, and they contain globin-coding sequences. The quantity of upstream RNA is 10- to 500-fold lower than that of the corresponding mRNA (1, 3, 4, 18, 19).

Upstream transcripts from the γ-globin genes, including some with termini near −210, have been documented (diagram, Fig. 1) (15). To identify the polymerase that transcribes these RNAs, a plasmid containing the Aγ-globin gene (Fig. 1) was transcribed in vitro in a K562 whole-cell extract as described previously (3, 20). The 5′ termini and the abundance of the upstream RNAs were analyzed by S1 mapping. The probe was a 979-base-pair DNA fragment of the 5′-flanking region of the Aγ-globin gene 5′ labeled in the mRNA complementary strand at −52 (3). Multiple upstream transcripts were detected (Fig. 2). Identical transcripts were observed with a Gγ-globin DNA template (see below), which differs from the Aγ gene by only a single base (−158, C to T) between +25 and −270 (21). Synthesis of the upstream transcripts was unaffected by 1 μg of α-amanitin per ml but was inhibited by 400 μg of α-amanitin per ml (Fig. 2, lanes d and e). Cap-site transcription was inhibited more than 90% by 1 μg of α-amanitin per ml (data not shown). RNA polymerase II is inhibited by 1 μg of α-amanitin per ml, and RNA polymerase I is unaffected by 400 μg of α-amanitin per ml. Thus, the γ-globin upstream RNAs are transcribed in vitro by RNA polymerase III.

The RNAs with 5′ termini at −208 and −143 to −150 were also observed in authentic erythroid cells (Fig. 2, lane f), analogous to the situation with mouse and human β-globin polymerase III transcripts (3, 4, 19). Furthermore, the existence of these RNAs in erythroid cells correlates with fetal globin gene expression, because upstream RNAs were detected in neonatal but not in adult reticulocytes (Fig. 2, lanes f and g). These observations indicate that similar upstream transcripts are generated in vitro and in authentic erythroid cells. For unknown reasons, many of the in vitro-synthesized RNAs were not detected in neonatal reticulocytes (Fig. 2).

In vitro transcription rates of the normal Gγ and the −202 HPFH Gγ genes were analyzed by S1 mapping with probes for both the upstream (polymerase III) and cap-site (polymerase II) RNAs. RNAs upstream of −170, including the −208 RNA that exists in reticulocytes, were transcribed 5-fold (Fig. 3A) to 10-fold (Fig. 4) less efficiently with the HPFH gene than with the normal gene. The levels of the −143 to −150 RNAs (Fig. 4) and of RNAs with termini between −50 and −140 (not shown) were relatively unaffected by the mutation. Similar results were observed in seven independent experiments with different cell extract preparations, different plasmid DNA preparations, linearized DNA templates, and normal or HPFH DNA probes.

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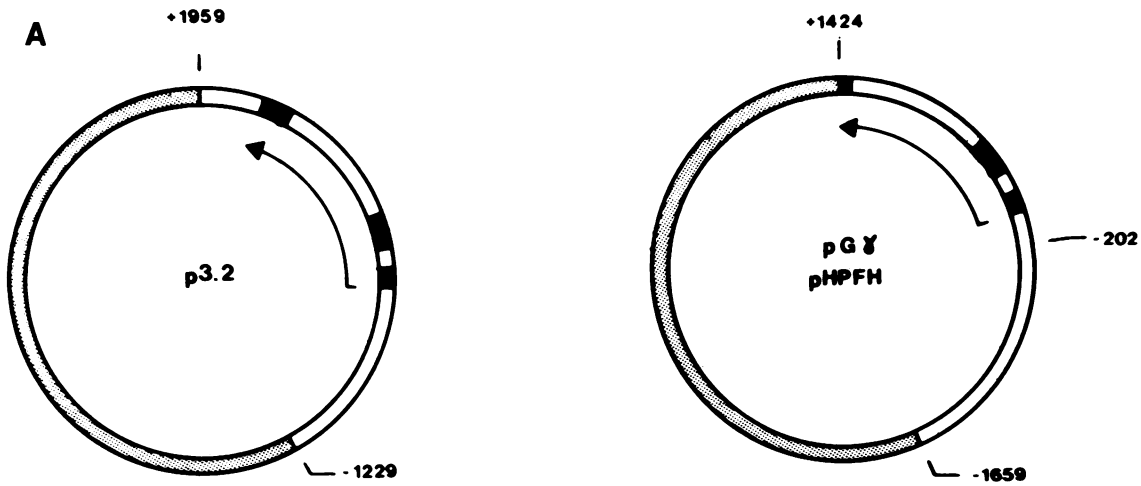
A



B.

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                                                -201
                                          >     [G]        T
CTATAAAAAA AATTAAGCAG CAGTATCCTC TTGGGGGC[C]C CTTCCCCACA

                                    T    -151>>>>>>
CTATCTCAAT GCAAATATCT GTCTGAAACG GTCCCTGGCT AAACTCCACC

                                  A         -101
CATGGGTTGG CCAGCCTTGC CTTGACCAAT AGCCTTGACA AGGCAAACTT

   >>          >  >        >>       >    >      *-51
GACCAATAGT CTTAGAGTAT CCAGTGAGGC CAGGGGCCGG CGGCTGGCTA

                                               -1 >
GGGATGAAGA ATAAAAGGAA GCACCCTTCA GCAGTTCCAC A
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FIG. 1. (A) Plasmid constructs. Filled boxes are globin exons. Open boxes are globin intervening sequences and flanking sequences. Stippled boxes are pBR322 sequences. The direction of globin gene transcription is indicated by arrows. Numbers are base pairs from the globin mRNA cap site (+1). The p3.2 clone of the Aγ-globin gene (left) was obtained from Oliver Smithies. The pGγ and pHPFH plasmids (right) were constructed from a normal genomic Gγ clone, p8.5 (provided by Oliver Smithies), and a genomic HPFH clone, pFC13C7 (provided by Francis Collins and Bernard Forget), respectively. In both clones, the 3,073-base-pair *Bgl*II-*Eco*RI fragment containing the γ-globin promoter was subcloned into pBR322. (B) Sequence of the Gγ-globin upstream region (21). Arrowheads indicate 5′ termini of RNAs that are detected both in human reticulocytes and in cell-free transcription reactions. The −182 to −191 sequences conserved in mammalian nonadult β-like globin genes (16) and the CCAAT and TATAAA sequences are in large letters. Nucleotides altered in different individuals with nondeletion HPFH (7, 8, 12–14) are written above the sequence. The −202 mutation investigated here is boxed. The asterisk indicates the position of the labeled nucleotide in the DNA probe used to detect the upstream RNAs.

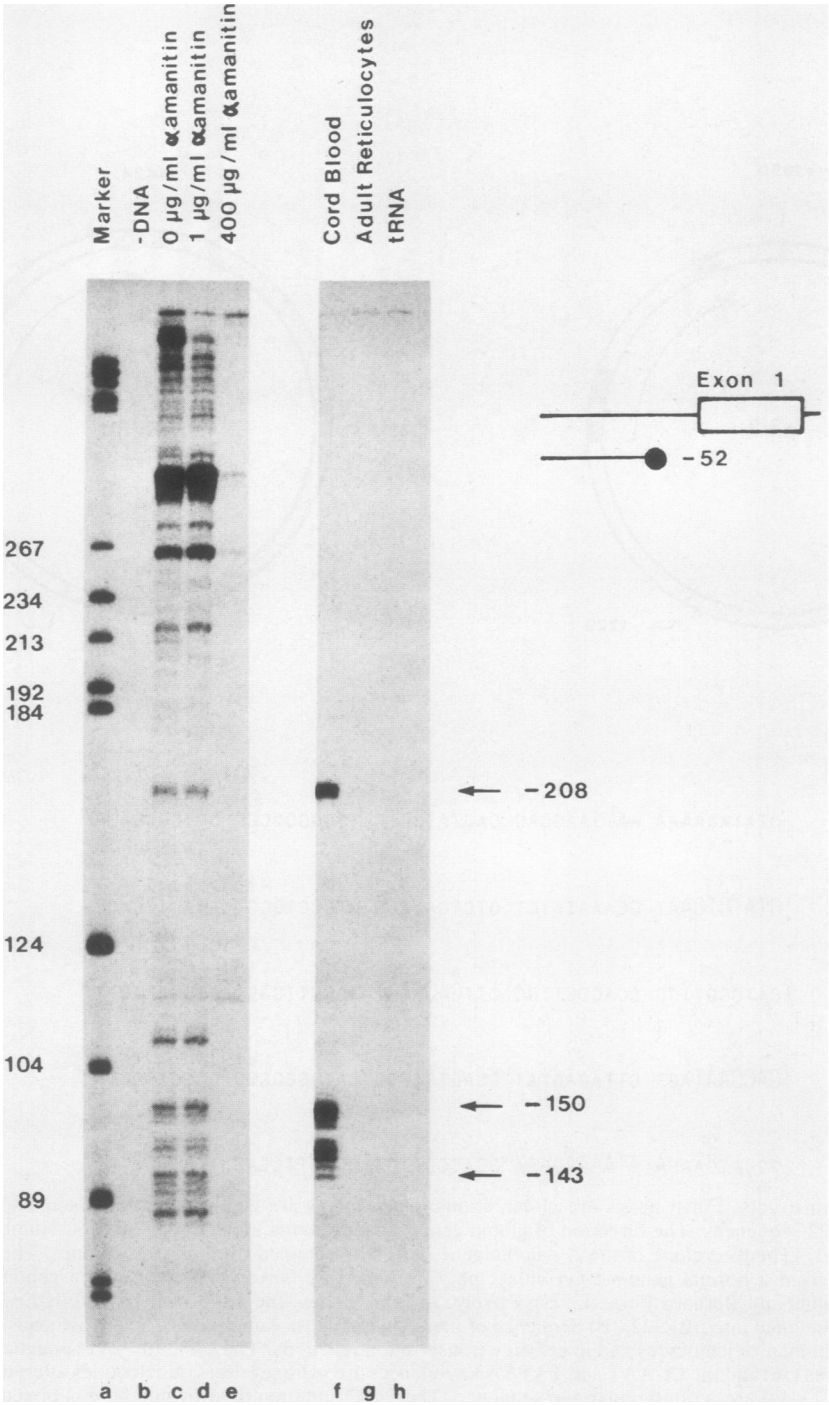

FIG. 2. α-Amanitin sensitivity of normal γ-globin gene upstream transcripts. RNAs were transcribed from the circular Aγ-globin template p3.2 (280 μg/ml) in a whole-cell extract and were analyzed by S1 mapping with a 979-base-pair fragment derived entirely from the 5′-flanking region of the Aγ-globin gene and 5′ end labeled in the mRNA-complementary strand at the −52 *Msp*I site. Lanes: a, *Hae*III-digested pBR322 marker; b, reaction without added DNA template; c, with DNA, without α-amanitin; d, with DNA, with 1 μg of α-amanitin per ml; e, with DNA, with 400 μg of α-amanitin per ml; f, 44 μg of neonatal umbilical cord blood reticulocyte RNA; g, 50 μg of normal peripheral blood reticulocyte RNA (from a non-HPFH adult with hemolytic anemia); h, 45 μg of *Escherichia coli* tRNA. The 8% polyacrylamide sequencing gels were autoradiographed for 7 days at −80°C with a Quanta III (Dupont) intensifying screen. Arrows at right indicate the positions of the 5′ termini of the neonatal reticulocyte upstream RNAs, relative to the cap site (+1). Numbers at left indicate the sizes of the marker DNAs in base pairs. The line drawing at the right shows the labeled position of the DNA probe (filled circle) in the γ-globin 5′-flanking region.

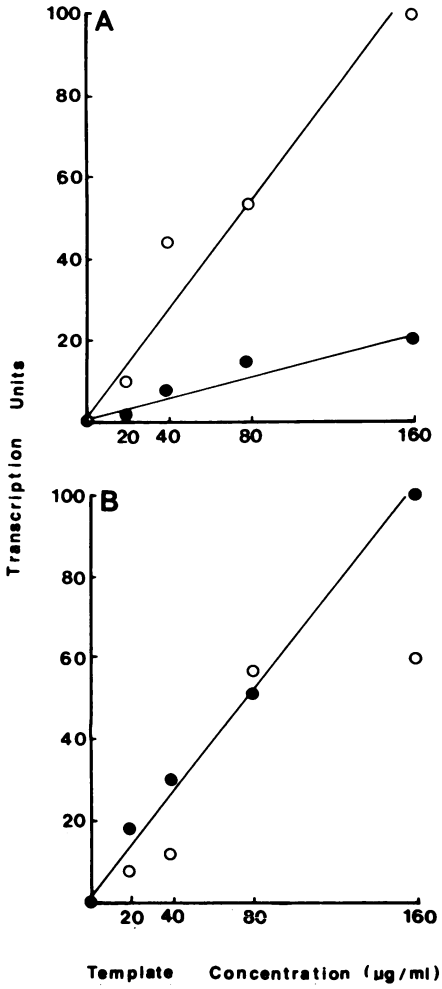

FIG. 3. Transcription of normal and HPFH Gγ-globin gene templates by RNA polymerases II and III. Increasing concentrations of normal and HPFH circular DNA templates (pGγ and pHPFH, Fig. 1) were transcribed in a K562 cell extract. Total DNA concentration was maintained at 280 μg/ml by adding pBR322 DNA. RNAs from 100-μl transcription reactions were analyzed by hybridization and S1 analysis. A 50% portion of each RNA was used for hybridization to a Gγ-globin upstream probe 5′ end labeled at −52, and 1% was used for hybridization to a genomic Gγ-globin mRNA probe 5′ end labeled at +476. Autoradiograms were scanned with a soft laser densitometer. The area under the peaks was determined, and the extent of hybridization was expressed on an arbitrary 1 to 100 scale. (A) Level of −208 polymerase III transcripts detected with a probe from pHPFH labeled at −52 (diagram, Fig. 2). (B) Level of cap-site polymerase II transcripts detected with a probe from pGγ labeled at +476 in exon II. Symbols: ○, pGγ template; ●, pHPFH template. Autoradiography was for 3 days in panel A and 6 h in panel B.

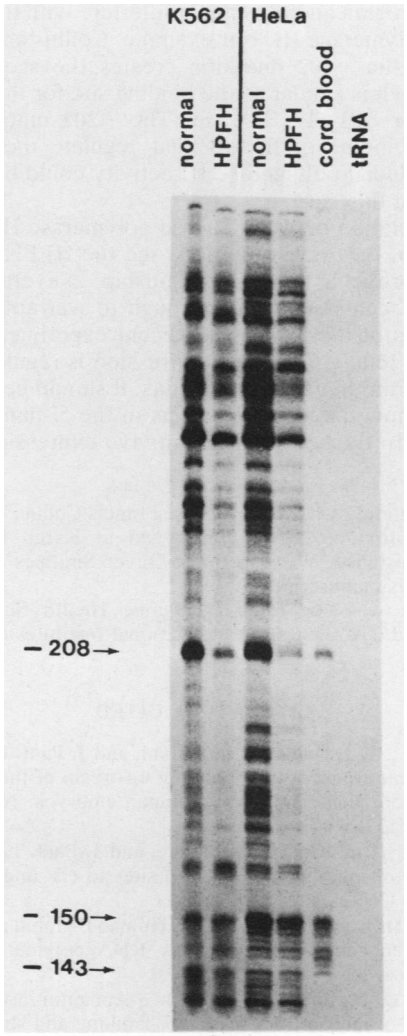

FIG. 4. Transcription of the HPFH gene by RNA polymerase III in erythroid and nonerythroid cell extracts. pGγ or pHPFH (280 μg/ml) was transcribed in either erythroid (K562) or nonerythroid (HeLa) extracts as indicated. Polymerase III RNAs synthesized in 100-μl transcription reactions were analyzed with the −52 probe from pGγ. A 45-μg sample of neonatal umbilical cord blood RNA or *E. coli* tRNA was hybridized where indicated. Numbers at the left indicate the positions of the 5′ termini of some of the RNAs. Autoradiography was for 18 h.

Transcription from the HPFH gene cap site by RNA polymerase II occurs at a similar or slightly higher rate than that from the normal gene (Fig. 3B). This modest difference does not account for the 40-fold increase in Gγ-globin protein levels observed in patients with the −202 base change (8).

To determine whether the difference between normal and HPFH gene transcription resulted from erythroid-specific factors, transcription of the 5′-flanking region of both genes was compared in extracts from erythroid (K562) versus nonerythroid (HeLa) cells. The sizes and autoradiographic intensities of the S1-resistant DNAs were similar for each extract tested, and upstream transcription of the HPFH gene was reduced to approximately the same extent (Fig. 4).

Several models could account for the effect of the −202 mutation on RNA polymerase III transcription and HPFH. (i) Transcription of the 5′-flanking region by polymerase III might reduce the efficiency of transcription from the cap site by polymerase II. If so, a mutation that reduces polymerase III activity might enhance cap-site transcription. The phenomenon of transcriptional interference between tandem polymerase II promoters has been documented (10), and transcription of the 5′-flanking region of transfected human ε-globin genes is inversely related to transcription from the cap site (2, 18). Interference between polymerases II and III was not apparent in our in vitro experiments (Fig. 3B), perhaps because only a small percentage of the globin DNA templates is transcribed. (ii) DNA-binding proteins might have a higher affinity for the HPFH than for the normal

5′-flanking region and thus might interfere with transcription by RNA polymerase III. For example, Collins et al. (8) have noted that the −202 mutation creates the sequence GGGCGC, which is similar to the binding site for the transcriptional factor SP1 (11, 17). (iii) The −202 mutation might affect the binding of factors that regulate the fetal-adult switch. Reduced polymerase III activity could be crucial or incidental in this case.

The correlation between altered polymerase III transcription in vitro, the −202 mutation, and the HPFH phenotype does not prove a causal relationship. Nevertheless, the correlation seems significant enough to warrant more conclusive tests of these models. Recent experiments indicate that human fetal globin gene expression is regulated appropriately in transgenic mice (5). Thus, it should be possible to determine how different mutations in the 5′-flanking region affect in vitro transcription and in vivo expression.

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