BRIEF REPORT

A Gain-of-Function Mutation in *EPO* in Familial Erythrocytosis

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SUMMARY

Familial erythrocytosis with elevated erythropoietin levels is frequently caused by mutations in genes that regulate oxygen-dependent transcription of the gene encoding erythropoietin (*EPO*). We identified a mutation in *EPO* that cosegregated with disease with a logarithm of the odds (LOD) score of 3.3 in a family with autosomal dominant erythrocytosis. This mutation, a single-nucleotide deletion (c.32delG), introduces a frameshift in exon 2 that interrupts translation of the main *EPO* messenger RNA (mRNA) transcript but initiates excess production of erythropoietin from what is normally a noncoding *EPO* mRNA transcribed from an alternative promoter located in intron 1. (Funded by the Gebert Rüf Foundation and others.)

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Inherited forms of primary erythrocytosis with low serum levels of erythropoietin are caused by mutations in the erythropoietin-receptor gene (*EPOR*),[1,2] *SH2B3* (*LNK*),[3,4] or *JAK2*.[5] Inherited forms of secondary erythrocytosis with elevated erythropoietin levels are mainly caused by mutations in genes involved in oxygen-sensing pathways, such as *VHL*,[6] *PHD2* (*EGLN1*),[7] and *HIF2A* (*EPAS1*),[8] or by mutations in *HGB*[9] or *BPGM*.[10] Variants in candidate genes (e.g., *EGLN2*, *EPO*, *HIF3A*, and *OS9*) have been described,[11] but their functional relevance has not been determined. Here, we describe a gain-of-function variant in *EPO* in an extended kindred with familial erythrocytosis, including 10 affected family members in four generations.

METHODS

STUDY FAMILY

Part of the pedigree — including the index patient, in whom erythrocytosis was diagnosed in 1969 (WG02), and three affected family members (WG01, WG08, and WG11) — has been described previously.[12] The study was approved by the Regional Committee for Medical and Health Research Ethics Central, Norway, and by Ethikkommission Beider Basel, Switzerland. All participating family members provided written informed consent to participate in the study.

GENETIC AND MOLECULAR ANALYSES

Linkage analysis, microsatellite mapping, gene editing with the use of CRISPR (clustered regularly interspaced short palindromic repeats), cell-culture assays, 5′ RACE (rapid amplification of complementary DNA ends), and assessment of erythropoietin levels were performed as described in the Supplementary Appendix, available with the full text of this article at NEJM.org.

RESULTS

CLINICAL FINDINGS AND LABORATORY ANALYSIS

The pedigree of the Norwegian family with autosomal dominant erythrocytosis is shown in Figure 1A. The affected family members typically presented with symptoms of headache and dizziness that rapidly disappeared after initiation of phlebotomy.[12] No incidents of venous or arterial thrombosis had occurred in a family member younger than 70 years of age in this pedigree.

The affected family members had hemoglobin levels above 180 g per liter in men or 160 g per liter in women, with the exception of one male family member (WG05), whose hemoglobin level was in the upper normal range, at 167 g per liter. One child (WG20) had a hemoglobin level of 190 g per liter shortly after birth; we do not have data obtained during infancy for the other members of the family. Additional laboratory data are provided in Table S1 in the Supplementary Appendix. Erythropoietin concentrations in serum were elevated in most of the affected family members. When plotted against the linear regression calculated from hemoglobin and serum erythropoietin levels of healthy persons,[13] the erythropoietin level was found to be elevated in relation to the hemoglobin level in each affected family member (Fig. 1B).

GENOMEWIDE LINKAGE ANALYSIS AND DNA SEQUENCING

Sequence analysis of genes that are known to be involved in erythrocytosis yielded negative results. Therefore, we performed genomewide linkage analysis and found a cosegregating region on chromosome 7q22.1 with a LOD score of 3.3 (Fig. 1C, and Fig. S1 in the Supplementary Appendix). Targeted sequencing of all 215 genes within the cosegregating region (Table S2 in the Supplementary Appendix) revealed a heterozygous single-base deletion in exon 2 of *EPO* (chromosome 7: 100,319,199 GG→G) as the only candidate gene mutation (Fig. 1D). This c.32delG mutation (referred to in this report as ΔG) was present in all affected family members (Fig. 1A) and was absent from 138,632 exome and whole-genome sequences reported in the Genome Aggregation Database (gnomAD).[14] Since erythropoietin is the primary regulator of erythropoiesis, the *EPO* ΔG deletion was an excellent candidate for the disease-causing mutation. The deletion creates a frameshift that truncates the erythropoietin signal peptide and generates a novel peptide, terminating after an additional 51 amino acids (Fig. 1E), which would predict a loss of erythropoietin function and is at odds with the erythrocytosis phenotype.

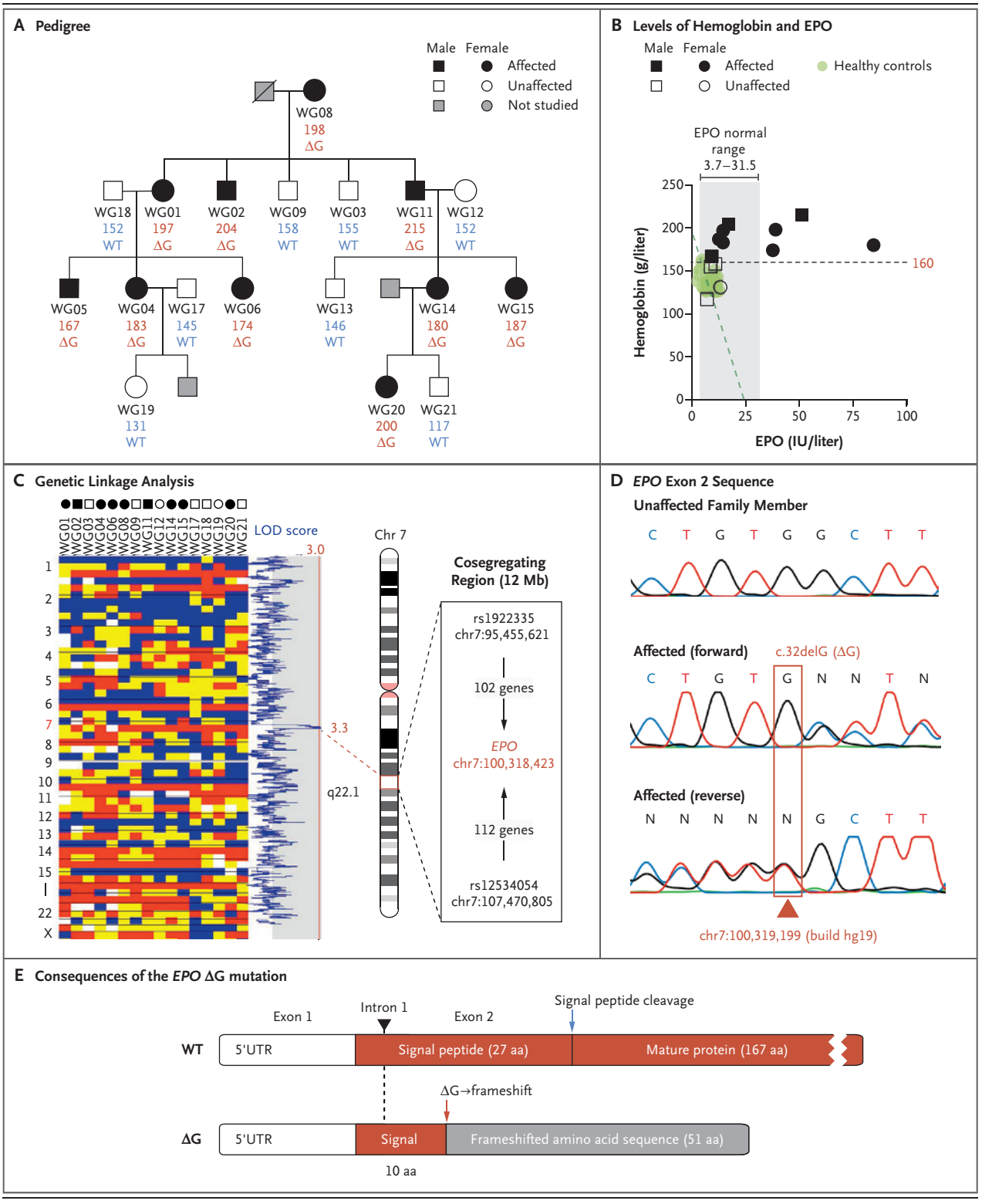
FUNCTIONAL CHARACTERIZATION OF THE MUTATED *EPO*

To determine whether alternative transcripts of *EPO* played a role in the phenotype of these patients, we used CRISPR to introduce the ΔG mutation into *EPO*. Since we had no access to kidney or liver tissues from the patients and were unable to detect *EPO* mRNA in erythroid colonies grown in vitro from a patient's peripheral blood,[15,16] we introduced the mutation into *EPO* in Hep3B cells, a human hepatoma cell line that expresses *EPO*.[17] CRISPR-mediated genome editing (Fig. S2A in the Supplementary Appendix) yielded multiple Hep3B single-cell clones that carried the ΔG mutation in the homozygous state (Fig. 2A, and Fig. S2B in the Supplementary Appendix).

We selected two Hep3B single-cell derived clones (ΔG_1 and ΔG_4) that were homozygous for the *EPO* ΔG mutation and assayed erythropoietin in the supernatants of these clonal cell lines grown in normoxic conditions; the supernatants contained 8 to 10 times as much erythropoietin as the supernatants of parental Hep3B cells or Hep3B cells in which CRISPR failed to modify *EPO* (Fig. 2B). These supernatants were capable of stimulating the growth of an erythropoietin-dependent cell line (BaF3-EpoR), which suggested that the secreted erythropoietin was biologically active (Fig. 2C). The same conclusions were reached with Hep3B cells grown in hypoxic conditions (Fig. S3 in the Supplementary Appendix). We therefore concluded that the ΔG deletion is, in fact, a gain-of-function mutation.

STUDY OF *EPO* MRNA TRANSCRIPTS

To understand how an ostensibly loss-of-function variant of *EPO* produces erythropoietin, we searched for alternative *EPO* mRNAs. In addition to the expected transcripts originating from the physiologic promoter (P1) upstream of exon 1, we discovered two alternative *EPO* mRNA transcripts that originate from an alternative promoter (P2) in intron 1 (Fig. 2D, and Fig. S4A in the Supplementary Appendix). The shorter transcript results from a splicing event that removes 189 nucleotides from the longer intron 1 tran-



script. We detected these two transcripts in parental Hep3B cells as well as in the ΔG clones; the longer P2 transcript was more abundant in the ΔG_1 and ΔG_4 clones than in parental Hep3B cells when grown in either normoxic (Fig. 2E) or hypoxic conditions. P1 and P2 transcripts were also detected in mRNA from normal kidney and liver (Fig. 2F). In kidney, the P1 transcript was

Figure 1 (facing page). Pedigree of the Family with Hereditary Erythrocytosis and Identification of the Mutation in *EPO*, the Gene Encoding Erythropoietin.

Panel A shows a pedigree of the family in this study. Unique patient numbers are placed under the symbols, with numbers below representing hemoglobin levels in grams per liter and the *EPO* sequence status indicated as wild-type (WT) or as having the c.32delG mutation (ΔG). Panel B shows hemoglobin levels plotted against erythropoietin (EPO) levels in serum. The green circles represent hemoglobin and EPO levels of 35 healthy persons,[13] and the green dashed line is the linear regression calculated from these values and represents the expected normal EPO level for a given hemoglobin level. The black dashed line indicates the hemoglobin level of 160 g per liter, which is the upper limit of the normal range among females. The gray shaded area indicates the normal range of EPO levels in serum. Panel C shows mapping of the disease-causing gene locus by the Affymetrix 250K single-nucleotide polymorphism (SNP) array. Red, yellow, and blue boxes indicate genotype calls for each SNP in the array (with alleles at each SNP arbitrarily designated as A and B, red denotes AA, yellow AB, and blue BB). The vertical red line indicates a logarithm of the odds (LOD) score of 3.0. An enlarged view of the cosegregating region on chromosome 7 is shown on the right. The nucleotide positions of the two SNPs that mark the borders of the cosegregating region are also shown. Panel D shows chromatograms of the DNA sequences of the mutated locus. The heterozygous loss of one G nucleotide results in ambiguous sequence reads downstream or upstream of the deleted G in the forward and reverse chromatograms, respectively. The red box indicates the location of the single-base deletion (ΔG). Panel E shows the location of the ΔG mutation and the resulting frameshift in the *EPO* mRNA. The wild-type EPO coding sequence is shown in red, and the sequences encoding the signal peptide and the mature protein are marked. The ΔG mutation induces a frameshift in the *EPO* mRNA sequence that truncates the signal peptide and generates a novel peptide, terminating after additional 51 amino acids (gray box). UTR denotes untranslated region.

predominant, whereas in the liver, the long P2 mRNA was predominant. In Hep3B cells, the presence of the ΔG mutation did not alter the rate of transcription from the P1 and P2 promoters. However, the ΔG P2 mRNAs had a longer half-life than did the wild-type P2 mRNAs, which suggested that increased mRNA stability is responsible for the elevated levels of expression of P2 transcripts observed in Hep3B cells with the ΔG mutation (Fig. 2E). Additional information on the characterization of the P1 and P2 transcripts is provided in Figure S4 in the Supplementary Appendix.

To determine which transcripts are capable of producing erythropoietin, we transfected HEK293 cells with complementary DNAs (cDNAs) representing P1 or P2 transcripts with or without the ΔG mutation and measured erythropoietin in culture supernatants. Cells transfected with ΔG P2 transcripts produced more erythropoietin than cells transfected with wild-type P1 cDNA (Fig. S5A in the Supplementary Appendix). These supernatants also stimulated the growth of erythropoietin-dependent BaF3-EpoR cells (Fig. S5B in the Supplementary Appendix) and supported the growth of erythroid colonies from human peripheral blood (not shown), which showed that ΔG P2 transcripts produce biologically active erythropoietin.

TRANSLATION OF *EPO* MRNA VARIANTS

We performed in vitro transcription–translation experiments and examined the composition of the open reading frames (ORFs) in *EPO* mRNAs to determine why more erythropoietin was produced by the short ΔG P2 cDNA construct than by the long ΔG P2 construct in transfected HEK293 cells (Fig. S5 in the Supplementary Appendix). In vitro translation of the short ΔG P2 transcript produced more erythropoietin than did translation of the long ΔG P2 transcript or the wild-type P1 mRNA because the splicing event eliminates most of the upstream ORFs, which inhibit translation. Since AUG1⋆ (the first start codon in the P2 transcripts; asterisks are used here to highlight start codons located in intron 1) is located within a sequence context that is predicted to be weak at initiating translation (Table S3 in the Supplementary Appendix),[18] ribosomes are likely to skip AUG1⋆ and initiate translation from the next available start codon, which, in the short ΔG P2 transcript, is AUG2, located in exon 2. In vitro translation of the long ΔG P2 mRNA, most likely initiating from upstream AUG3⋆ located in intron 1, produced a larger erythropoietin molecule that was approximately 25 kDa in size. The smaller observed protein product, which is approximately 21.6 kDa in size, is consistent with initiation of translation from AUG2. Removing upstream ORF1 by deleting the 5′ untranslated region (ΔUTR) increased the translational efficiency of the wild-type P1 mRNA, as reported previously.[19] HEK293 cells expressing the ΔG P1 cDNA produced low amounts of erythropoietin. A likely explanation is that ribosomes can skip the physiologic start codon (AUG1) in the ΔG P1 mRNA and initiate translation from AUG2, which connects with the erythropoietin coding sequence through the ΔG

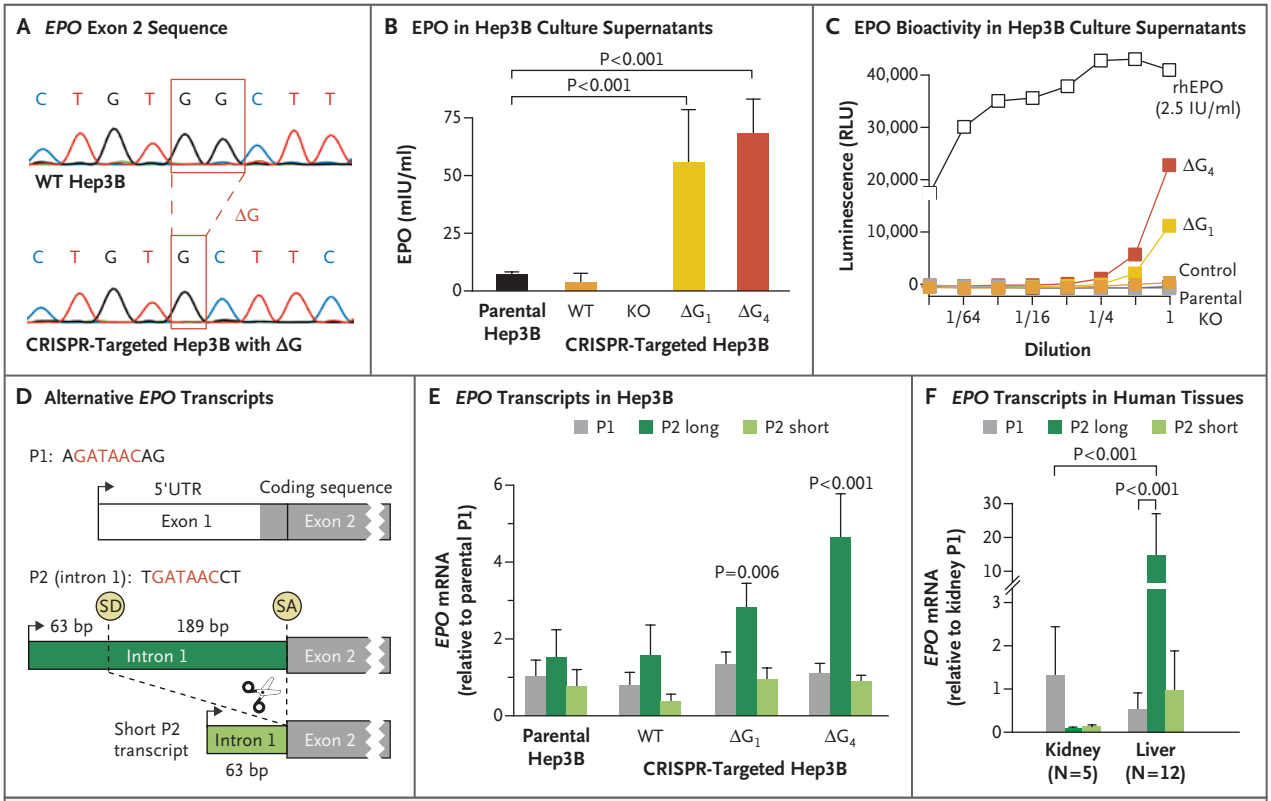


Figure 2. Functional Characterization of the *EPO* ΔG Mutation.

Panel A shows chromatograms of *EPO* exon 2 DNA sequences from WT Hep3B and a CRISPR-modified single-cell clone. The region containing the ΔG deletion is indicated by the red box. Panel B shows concentrations of EPO in culture supernatants from Hep3B cells carrying the ΔG mutation. Parental Hep3B cells, Hep3B cells in which CRISPR failed to modify the *EPO* gene (WT), and Hep3B cells with *EPO* inactivated by a deletion and insertion (KO) are shown for comparison. EPO was measured by means of enzyme-linked immunosorbent assay (ELISA), and EPO concentrations are given as means and standard deviations (T bars) of three biologic replicates. P values were determined by one-way analysis of variance. Panel C shows the biologic activity of EPO produced by parental Hep3B or CRISPR-modified Hep3B cells, measured in culture supernatants by a proliferation assay with the BaF3-EpoR cell line. Serial dilutions of 2.5 IU per milliliter of recombinant human EPO (rhEPO) were used for reference (based on three biologic replicates). Panel D shows the *EPO* transcripts identified by 5′-RACE in Hep3B cells. The P1 transcript starts at exon 1, and its transcription is initiated from *EPO* promoter 1 (P1). A putative promoter 2 (P2) is located in *EPO* intron 1. This promoter generates an alternative *EPO* transcript P2 (dark green) that can be further spliced to produce a short version of the P2 transcript (light green). Splice donor (SD) and splice acceptor (SA) sites are indicated with dashed lines. The GATA motif in the putative promoter is shown in red. The 5′ untranslated region (UTR) and the protein-coding region (gray shading) are shown for the P1 transcript. Panel E shows quantification of *EPO* transcripts in CRISPR-modified and parental Hep3B cell lines by means of quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR). Values are the means and standard deviations (T bars) of four biologic replicates. of mRNA expression relative to expression of the parental P1 transcript, which was defined as 1. (For example, a value of 3 indicates expression 3 times as high as that of the parental P1 transcript, and a value of 0.5 indicates expression half as high.) P values were determined by two-way analysis of variance. Panel F shows quantification by RT-qPCR of *EPO* transcripts in human kidney and liver RNA. Values represent means and standard deviations (T bars) of mRNA expression relative to expression of the P1 transcript in kidney, which was defined as 1. P values were determined by two-way analysis of variance.

frameshift. Thus, erythropoietin protein from all ΔG transcripts appears to be produced by initiating translation from AUG2. Additional information on erythropoietin production by the P1 and P2 transcripts is provided in Figure S5 in the Supplementary Appendix.

Initiation of translation from AUG2 produces an erythropoietin protein with a shortened signal peptide (22 instead of 27 amino acids) and a novel N-terminal, which nevertheless was predicted by computer-based algorithm (SignalP 4.1[20]) to be functional (Fig. S6 in the Supplementary Appendix). To test this prediction, we generated a cDNA construct representing *EPO* ΔG that starts directly with AUG2 and lacks any upstream AUGs. This construct (P2 ΔG ΔUTR) was most active in producing erythropoietin in vitro and in transfected HEK293 cells; it also showed bio-

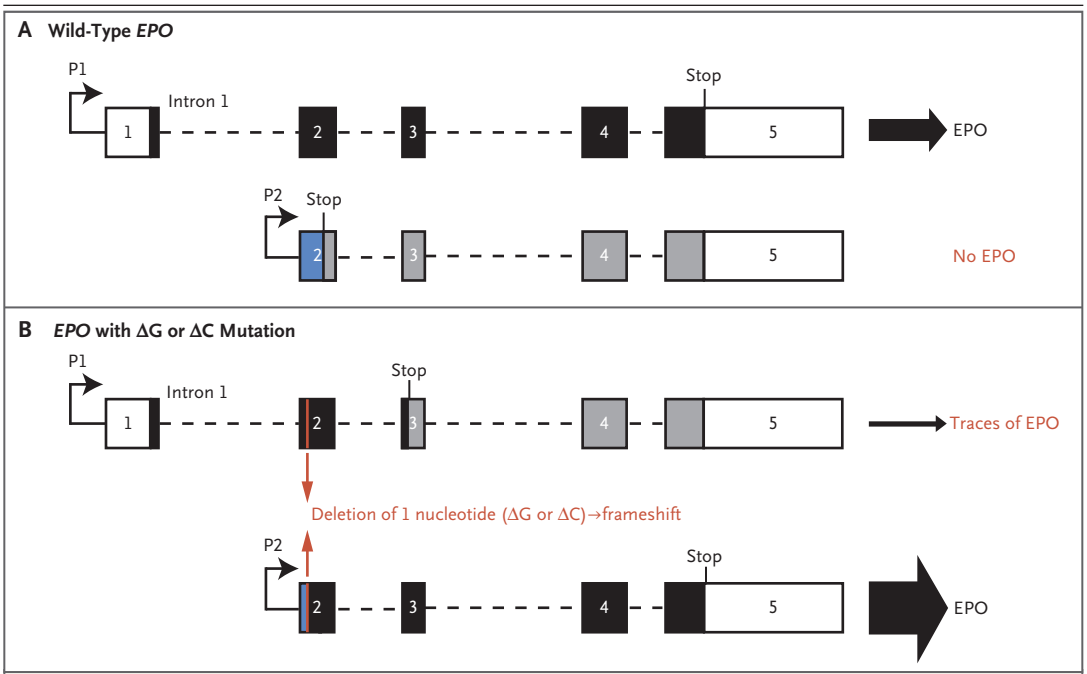


Figure 3. Model and Proposed Mechanism for EPO Overproduction by a Single-Nucleotide Deletion in Exon 2 of *EPO*.

The exons of *EPO* are numbered and drawn as boxes. Open boxes represent 5′ and 3′ UTRs. Panel A shows the wild-type gene producing EPO from P1 transcripts (black boxes), whereas P2 promoter transcripts generate only a short, nonfunctional peptide (blue box). Panel B shows the effects of the frameshift caused by a single-nucleotide deletion in exon 2 of *EPO* (ΔG or ΔC). The mutated P1 transcripts produce only a short nonfunctional peptide terminating in exon 3 (black boxes), and minimal amounts of EPO can be produced if translation is initiated from an alternative AUG2 start site (see Fig. S5A in the Supplementary Appendix). P2 transcripts (blue box) connect through the frameshift to the EPO coding sequence (black boxes) and are responsible for the overproduction of EPO. Gray boxes represent EPO coding sequences that are in a different reading frame.

logic activity in the BaF3-EpoR proliferation assay (Fig. S5A and S5B in the Supplementary Appendix), which indicated that the shortened signal peptide is indeed functional.

SECOND *EPO* ALLELE ASSOCIATED WITH ERYTHROCYTOSIS

A father and daughter with erythrocytosis and a single C nucleotide deletion (ΔC) of unknown significance, which was located in *EPO* exon 2 just 13 nucleotides upstream of ΔG (c.19delC; chromosome 7: 100,319,185 TC→T), were recently described.[11] To test whether *EPO* ΔC has the same effect as *EPO* ΔG, we generated ΔC P2 cDNA constructs, transfected HEK293 cells, and measured erythropoietin protein and erythropoietin bioactivity in cell-culture supernatants. The long P2 and short P2 transcripts with the ΔC mutation also produced high amounts of erythropoietin that was biologically active (Fig. S7 in the Supplementary Appendix), which showed that the *EPO* ΔC mutation causes erythrocytosis through the same mechanism (Fig. 3) as the *EPO* ΔG mutation.

DISCUSSION

We propose that the frameshift mutation ΔG in *EPO* causes erythrocytosis on the basis of genetic linkage with a locus on chromosome 7q21 (LOD score, 3.3), functional data, and our finding of a second, independent allele (ΔC) in a father and daughter with erythrocytosis. Both ΔG and ΔC cause a frameshift and convert a noncoding mRNA transcribed from an alternative (P2) promoter inside *EPO* intron 1 into an mRNA that produces functional erythropoietin protein. This frameshift occurs in the sequence encoding the signal peptide of erythropoietin; the mutant peptide sequence is fully functional. We dissected the relative contribution of individual upstream AUG start codons located in the 5′ untranslated regions of P1 and P2 transcripts and established that erythropoietin protein from both P2 ΔG transcripts appears to be produced by initiating translation from AUG2, located in exon 2.

P2 transcripts were more abundant in normal liver than in kidney, which suggested that the

main source of erythropoietin production in affected family members was the liver. Indeed, P2 transcripts are present in human liver RNAseq data sets from ProteinAtlas[21] and the Illumina Human Body Map (Fig. S8 in the Supplementary Appendix). In a database of capped 5′ ends of mRNAs,[22,23] P2 transcripts were the predominant mRNA species in the liver, whereas P1 transcripts were mainly detected in RNA from kidney (Fig. S9 in the Supplementary Appendix). The physiological function of wild-type P2 transcripts is currently unknown, but it seems likely that they do have a function, because *EPO* intron 1 has a high degree of sequence homology between human and mouse and the region of homologous sequence extends upstream of the P2 promotor (Fig. S10 in the Supplementary Appendix).[24]

Our data indicate that the *EPO* ΔG mutation prematurely terminates translation of *EPO* P1 transcripts, whereas it alters the normally noncoding P2 transcripts such that AUG2 initiates translation of the erythropoietin coding sequence and produces an excess of biologically active erythropoietin. Mutations in *EPO* should be considered in the search for causes of inherited erythrocytosis. More generally, the effect of mutations on multiple mRNA transcripts may explain nonintuitive relationships between phenotype and the ostensible mechanism predicted by the mutation.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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