INTRINSIC REGULATION OF HEMOGLOBIN EXPRESSION BY VARIABLE SUBUNIT INTERFACE STRENGTHS

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Running Title: **Intrinsic Regulation of Hemoglobin Expression**

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SUMMARY

The expression of the six types of human hemoglobin subunits over time is currently considered to be regulated mainly by transcription factors that bind to upstream control regions of the gene (the "extrinsic" component of regulation). Here we describe how subunit pairing and further assembly to tetramers in the liganded state is influenced by the affinity of subunits for one another (the "intrinsic" component of regulation). The adult hemoglobin dimers have the strongest subunit interfaces and the embryonic hemoglobins are the weakest with fetal hemoglobins of intermediate strength, corresponding to the temporal order of their expression. These variable subunit binding strengths and the attenuating effects of acetylation contribute to the differences with which these hemoglobin types form functional O_2-binding tetramers consistent with gene switching.

Extrinsic Regulation of Expression - Hemoglobin is one of the best known proteins regarding its structure/function relationship for binding and delivering O_2 [1, 2]. The adult type is most often studied; results on the embryonic and fetal types are usually interpreted within its framework. The expression of the eight different types of normal human hemoglobins during the embryonic, fetal, and adult stages of life represents a major paradigm of developmental biology which is currently explained by the "switching" on and off of the various globin genes, known as ontogeny. The process is initiated by various transcription factors considered to interact with upstream regulatory regions of the two globin gene clusters [3] that are typified by their sensitivity to digestion by DNA_{ase}. Hence, they are considered to be open areas of chromatin accessible to regulatory factors that control transcription of the downstream globin genes. We refer to this as the extrinsic component for initiation of gene expression. It is shown in Fig. 1 for the transcription factors known as GATA-1 and FOG-1, and the nuclear remodeling complex NuRD, all of which affect the expression of the globin genes, as reviewed in the July 2011 issue of FEBS Journal. Also shown in Fig. 1 is a specific silencing element (BCL11A) of γ-gene expression [5]. Other members of the GATA family of transcription factors are involved in different hematopoietic lineages such as the development of platelets, mast cells and T-cells from stem cells [6].

Intrinsic Regulation of Expression - In the extrinsic component of regulation described above, the hemoglobin subunits themselves have no regulatory role. Although it is well known that the assembled hemoglobin tetramers have different affinities for oxygen, which is crucial during the various stages of pre-natal and post-natal life, there have been fewer studies on their subunit-subunit interactions. In fact, the prevailing view is that all normal human hemoglobins are structurally alike and there is little consideration of their subunit interactions (Fig. 2A) as being significantly different [7, 8]. However, using direct and very sensitive techniques [9, 10] for liganded hemoglobins, we found differences up to 3-orders of magnitude for their tetramer-dimer and dimer-monomer subunit interfaces. These differences are graphically illustrated by molecular sieve chromatography (gel filtration) at concentrations that do not obscure these subunit interface differences, i.e. much lower than their equilibrium constants (see below). Their strikingly different elution patterns represent distinct phenotypes for the embryonic, fetal, and adult hemoglobins. The use of the term "pheno-

types" to describe subunit assembly has been used previously [11] but not in this context. This behavior is the basis for the "intrinsic" component of regulation [12]. The complete regulatory process for human hemoglobin expression is made up of both the intrinsic and extrinsic components together.

Hemoglobin Ontogeny **-** Human hemoglobins comprised of ζ- or α-subunits paired with ε-, γ-, δ-, or β-subunits representing embryonic, fetal, and adult hemoglobins have nearly identical overall structural architecture in their tetrameric states (Fig. 2A). The human hemoglobin subunits are temporally expressed during normal development (Fig. 2B) in a pattern that reflects their gene order, which exist in two separate gene clusters referred to as α-like and β-like on human chromosomes 16 and 11, respectively (Fig. 2C) [13, 14]. There are eight possible combinations of these globin subunits that are formed first as dimer pairs (D/M equilibrium) and then as the functional O_2-binding tetramers (T/D equilibrium) as shown in Eq. 1; their names are given in Fig. 2D.

$$2x + 2y \rightleftharpoons 2\,x\,y \rightleftharpoons x_2y_2 \qquad \text{(Equation 1)}$$

Competing self-assembly reactions involving β- or γ-subunits to form β_4 (HbH) or γ_4 (Hb Bart's) can also occur. These are non-functional tetramers but are diagnostic for certain hemoglobinopathies [13].

Ontogeny is the process whereby certain hemoglobins are present at different times during development, i.e. at the embryonic, fetal, and adult stages. The transition among hemoglobin types over time (Fig. 2B) represents a major paradigm for developmental biology although some of its aspects such as the "switch" from ζ- to α-subunit expression and from ε- to γ-subunit expression during the embryonic stage, and the transition from γ- to β-subunit expression during fetal life are poorly understood [15]. Although differences have been reported in bonding strengths involving subunit interfaces of many mutant hemoglobins in both the liganded (oxy or CO) and unliganded (deoxy) states [16], normal human hemoglobins had not been considered to vary significantly in their

interface properties until our report for liganded hemoglobins [12] to the contrary. Furthermore, although deoxy adult HbA has very strong subunit interfaces [16], some deoxy embryonic hemoglobins [17] and deoxy mutant adult hemoglobins [16] have much weaker interfaces. Therefore, little consideration has been given to post-translational contributions (such as subunit assembly) distinct from transcriptional events in the developmental process.

Most studies on human hemoglobins have been on the adult type with fewer reports on the embryonic or fetal hemoglobins due in part to lack of availability, especially of the embryonic types. This predominance of adult hemoglobin data has thus engendered the notion that all normal human hemoglobins have similar subunit interface properties since their overall structural architectures are so similar (Fig. 2A). However, there are no careful measurements to support this view and we have shown that this widely held belief is erroneous [12]. On the other hand, oxygen affinity differences and responses to allosteric regulation for the various normal hemoglobins, which are conferred by tertiary and quarternary structural changes [18], are well known to be critical for proper development [8]. However, these physiological properties are unlikely to be the reason for the temporal appearance of the various hemoglobins during development.

The embryonic hemoglobins present during normal development are $\zeta_2\gamma_2$ (Hb Portland-1), $\zeta_2\varepsilon_2$ (Hb Gower-1) and $\alpha_2\varepsilon_2$ (Hb Gower-2) (8,19). $\zeta_2\beta_2$ (Hb Portland-2), another embryonic Hb, is found infrequently and mainly in an extreme type of α-thalassemia ("hydrops fetalis") where ζ-chains substitute for α-chains since synthesis of the latter is absent (20). Hence, Hb Portland-2 has not been studied in detail. Its absence in normal embryos has never been adequately explained so we evaluated its properties to determine whether it has any characteristics that might limit its occurrence under normal conditions *in utero*. This effort was initially hindered by the lack of material but the availability of adequate amounts of this and the other embryonic human hemoglobins from transgenic mice transfected with the human globin genes (19) has enabled us to perform a comprehensive study of the subunit binding properties of all the embryonic hemoglobins and a comparison with the fetal and adult types. However, the transgenic embryonic human hemoglobins first required full characterization in order for the findings to be credible.

Embryonic Hb Portland-2 Characterization – Since Hb $\zeta_2\beta_2$ had the weakest subunit interfaces of any Hb that we had studied previously (it even dissociates to free subunits under some conditions (12)), it was essential to prove that its weak subunit interfaces were not the result of some artifact but could account for its absence in the normal embryo. This transgenic Hb showed a single peak on a high resolution FPLC Mono S column and a single band upon isoelectric focusing after its purification as described in [12]. It had the expected amino acid sequence for its ζ- and β-subunits (see below). In order to ensure that the transgenic Hb Portland-2 was properly folded, its circular dichroism pattern was compared with that of human adult HbA [21] measured at the same time and at the same concentration. The results showed that the CD spectra of each are nearly superimposable with only minor differences Hence, human Hb Portland-2 expressed in mice has a native hemoglobin conformation. As described in detail below using comprehensive mass spectrometry, there was faithful expression of the ζ- and β- human globin genes in the mouse and the globin subunits had not incurred any proteolysis.

Mass Spectra of Hb Portland-2 ($\zeta_2\beta_2$) – The most direct approach to establish that the sequence of Hb Portland-2 was correct and intact was to perform comprehensive mass spectrometry on the subunits (22). The three-step mass spectrometric strategy reported previously [23] was utilized. Initially, we used ESI/MS to measure the average molecular masses of both ζ and β chains separately. A typical ESI spectrum is presented in panel A of Fig. 3 with its corresponding deconvoluted spectrum shown in Panel B. The deconvoluted spectrum shows a single component for each hemoglobin chain with differences between the theoretical mass and the experimentally determined mass of less than 15 ppm. Minor peaks, present on the high-mass side of each major peak (panel B), corresponded to sodium, potassium and iron adducts. The two chains from Hb Portland-2 were subsequently analyzed by peptide mapping using both MALDI-TOF/MS and MALDI-QqTOF/MS. Each spectrum was internally calibrated, yielding mass errors of + 10 ppm. MALDI-TOF/MS was utilized to extend sequence coverage to 100% for both chains (data not shown) as well as to verify the nature of the N-terminal residue of the first tryptic peptide from the ζ-chain, ζT[1-7], which was found to be acetylated on Ser-1. These results were in agreement with the mass measurement of the intact chains. Finally, some

tryptic peptides and a few Arg-C peptides, chosen so as to produce 100% coverage of each chain, were sequenced by mass spectrometric fragmentation to verify both polypeptide sequences. Fragmentation analysis showed all peptides to have the expected amino acid sequences (data not shown). We conclude from this analysis that the sequences of the subunits of $\zeta_2\beta_2$ are correct but they join together very weakly; their subunit interface strengths are reciprocally related to the interface strength involving α- and β-subunits, as described below. The tertiary structure of the ζ-subunit compared with the α-subunit is shown in Fig. 3D and shows only very minor folding differences.

Gel Filtration of Nanomolar Concentrations of Hemoglobins Reveals Different Substructures - The molecular weights of the human embryonic, fetal, and adult hemoglobin tetramers are all within a few hundred mass units of 64,000. Each hemoglobin migrates in the same position as a tetrameric species during gel filtration when the concentrations are in the millimolar range, which exceeds their subunit dissociation constants. However, if the concentrations are in the nanomolar range, which is below their tetramer dissociation constants, the elution patterns are radically different from one another (Fig. 4) indicative of significant variability in their subunit interface strengths [24]. The high resolving power of the Superose gel filtration matrix and the absence of excessive peak broadening account for the high degree of precision (0.3%) and reproducibility of the elution patterns shown in Fig. 4. The embryonic hemoglobins depicted in panels A and B show a significant percentage of dimers and monomers but monomers are not detectable for fetal and adult hemoglobins (Panels C and D). Thus, α-subunits have a higher affinity for β-like subunits than do ζ-subunits. Embryonic Hb $\zeta_2\delta_2$ (Hb Portland-3) has been reported only as a band on a gel in α-thalassemia major [20]; it is also considered as a very highly dissociated Hb. Hence, it appears likely that these two hemoglobins are not found normally because their subunit interfaces are so weak that other hemoglobins with stronger subunit interfaces are favored. In general, the gel filtration profiles in Fig. 4 become progressively less complex starting from the embryonic hemoglobins (panels A and B) through the fetal hemoglobins (panel C) and finally for the adult hemoglobins (panel D). These patterns represent the phenotype for each type of Hb.

Role of Subunit Interfaces During Development - The short half-lives *in vivo* of the embryonic hemoglobins are consistent with their very weak subunit interactions shown in Panels A and B of Fig. 4 since monomeric subunits formed from the facile dissociation of weak dimers are unstable and disappear [25]. In contrast, the fetal and adult hemoglobins do not dissociate to monomeric subunits to any measurable degree and hence would have longer durations. Fetal Hb (HbF, $\alpha_2\gamma_2$) is the least dissociated of all the human hemoglobins (Fig. 4, panel C, blue curve) consistent with the rapid onset of its synthesis at 6 weeks [26, 27] due to its very low free energy as calculated from its dissociation constant (28). Subsequently, slow post-translational acetylation at the N-terminus of its γ-subunit (HbF_1, $\alpha_2\gamma_2^{Ac}$) weakens its very strong subunit interactions (Fig. 4C) [28, 29] consistent with the gradual demise in HbF around the time of birth and its gradual replacement by HbA. In addition to this attenuating effect of acetylation, there are polymorphisms at positions 75 and 136 of the two γ-genes [13], making HbF a multi-component system in contrast to HbA, which is a single component (Fig. 4D) without modifications or polymorphisms. In addition, the γ-subunits of HbF undergo unproductive γ_2-homodimer formation which limits the amount of functional αγ heterodimers [30]. The other adult Hb, (HbA_2, $\alpha_2\delta_2$), displays an abnormal equilibrium between dimers and monomers as shown by its wide peak (Fig. 4, panel D, red line) and also by the displacement of its peak width maximum from its tetramer-dimer K_d value [10]. In contrast, HbA formation has a much narrower peak width (Fig. 4, panel D, blue line) whose maximum coincides with its tetramer-dimer K_d value [10, 31] making it the most favorable Hb species.

The Tetramer-Dimer Interfaces of Normal Hemoglobins Have Variable Strengths - The gel filtration patterns in Figure 4 represent a combination of the tetramer-dimer (T/D) equilibrium and the dimer-monomer (D/M) equilibrium (Eq. 1). In order to evaluate their individual contributions, we measured each equilibrium separately using very sensitive methods [9, 10]. The T/D dissociation constants, in contrast to other reports [8, 17], varied by up to 3-orders of magnitude [12]. These constants were converted into Gibbs free energy values as shown in Fig. 5A, which are a measure of tetramer stability (red arrow) and show an undulating pattern similar to the developmental profile in Fig. 2B.

However, there is no apparent correlation of the values themselves to time of expression (Fig. 2B) or to gene order (Fig. 2C). For example, the Gibbs free energy values of embryonic Hb Portland-1 ($\zeta_2\gamma_2$) and of embryonic Hb Gower-2 ($\alpha_2\varepsilon_2$) are equal and lower, respectively, than that of adult HbA ($\alpha_2\beta_2$) (Fig. 2A). However, there is a consistent pattern on the effects of ζ- vs α-subunits on tetramer strength, i.e. the tetramer-dimer interface is weakened by over an order of magnitude for hemoglobins containing ζ-subunits compared to corresponding tetramers containing α-subunits, representing an increase of 2 to 7 kcal/mol (Fig. 5A). To put this change in more familiar terms, a difference of 6 kcal/mol exists between deoxy $\alpha_2\beta_2$ and oxy $\alpha_2\beta_2$ (– 14 kcal/mol and -8 kcal/mol respectively) [16] arising from the more extensive subunit interactions in deoxy $\alpha_2\beta_2$.

The Dimer-Monomer Interface - To measure the D/M step in Equation 1, we used an indirect method employing subunit exchange as described initially by Huehns [32] and modified by us [12]. We found that the embryonic hemoglobin dimers are the weakest, fetal hemoglobins stronger, and the adult hemoglobins are the strongest (progression of strength indicated by the red lines of varying intensity in Fig. 5B). There is no correlation of these results with the hypothesis that subunit net charge has a role [11] since embryonic ε and adult δ subunits have net positive charges whereas fetal γ and adult β subunits have net negative charges. Dimers with α-subunits have increasing strengths that correlate with the gene order of the β-like subunits (ε, γ, δ, β) whereas dimers containing ζ-subunits have strengths of reverse gene order representing a reciprocal relationship. Hence, the red lines in Fig. 5B indicate that the $\alpha\beta$ pair has the strongest subunit interface binding contacts whereas the $\zeta\beta$ pair has the weakest. The red lines in Fig. 5B also have a directionality representing the driving force directing the formation of certain hemoglobins and the exclusion of others. The two weakest hemoglobins, $\zeta_2\beta_2$ and $\zeta_2\delta_2$, (dashed red lines in Fig. 4B), are also the most rare; they are found only in extreme cases of homozygous α-thalassemia ("hydrops fetalis") where synthesis of the α-subunit is absent [20]. The wide range of subunit interface energetics displayed in Fig. 5 has not previously been appreciated and suggests a linkage between their energetic levels and their developmental profile as shown by the dashed and solid red lines of varying intensity (Fig. 5B) (the intrinsic component of regulation) that have been superimposed on the extrinsic component of gene order and the upstream LCR and HS-40 regions.

Subunit Competition Leads To The Most Stable Hemoglobins – The embryonic hemoglobins have such weak subunit interfaces (Panels A and B of Fig. 4) that they can exchange their subunits to form hemoglobins with more stable interfaces [12]. Hence, when $\zeta_2\beta_2$, $\zeta_2\gamma_2$, and $\alpha_2\varepsilon_2$ are mixed *in vitro*, their subunits compete with one another to slowly form the most stable Hb, adult $\alpha_2\beta_2$. Fetal Hb $\alpha_2\gamma_2$ is not formed nor are any of the embryonic hemoglobins. *In vivo* studies using a yeast expression system that does not contain DNAase-sensitive regulatory loci confirm this conclusion in intact cells [29]. Even though the physiological relevance of such subunit exchange is unknown, it conclusively demonstrates that the thermodynamic stability of the human hemoglobins is embryonic < fetal < adult types, which faithfully reflects the normal order of occurrence of these hemoglobins.

Interface Subunit Bonding Strengths Correlate With Transitions In Their Developmental Profile - The results above offer an explanation for the developmental profile in Fig. 2B due simply to the subunit interface properties of the hemoglobins themselves. The three embryonic hemoglobins $\zeta_2\varepsilon_2$, $\zeta_2\gamma_2$ and $\alpha_2\varepsilon_2$ (shown as monomeric ζ, γ and ε in Fig. 2B) disappear rapidly during the prenatal period most likely because they have the weakest dimer-monomer and tetramer-dimer interface strengths (Fig. 4) and the unstable monomers formed disappear. The rapid increase in $\alpha_2\gamma_2$ (HbF) in the early prenatal phase and the much slower increase in $\alpha_2\beta_2$ (HbA) (represented by "β" in Fig. 2B) is consistent with the HbF having a tetramer-dimer free energy lower than that of HbA; both have strong dimer-monomer interfaces (Fig. 5B). Thus, there is an energetic barrier causing a lag in adult $\alpha_2\beta_2$ production (Fig. 2B). The decrease in fetal $\alpha_2\gamma_2$ just prior to birth could be due to the slow post-translational acetylation of γ-subunits to produce $\alpha_2\gamma_2^{Ac}$, which has subunit interface strengths close to that of HbA (28), thus annulling the effect of the low free energy of HbF. HbA_2, $\alpha_2\delta_2$ (shown as δ in Fig. 1B), is present at very low concentrations in adult blood probably because the $\alpha\delta$ dimer is weak as indicated by its inordinately large peak width during gel filtration, suggesting that it exists in a facile equilibrium with its monomers thus limiting its ability to form tetramers efficiently [31]. Thus, the energetic of the subunit interfaces of the human hemoglobins play a major role in their developmental profile. These principles are likely also applicable to other normal as well as abnormal protein assemblies such as those in extensive and interlinked signaling networks [33].

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FOOTNOTES

*The “extrinsic” component for regulation of gene expression describes factors which are external to the genes which they help to transcribe, such as the GATA family for hemoglobin subunit expression (5). It is the model currently invoked almost exclusively; there is no active role for the transcribed gene protein products themselves. The “intrinsic” component of regulation describes how expressed products, e.g. hemoglobin subunits interact with one another to different extents to influence the overall expression and the duration of the interaction. Both extrinsic and intrinsic components likely have a role in regulation.

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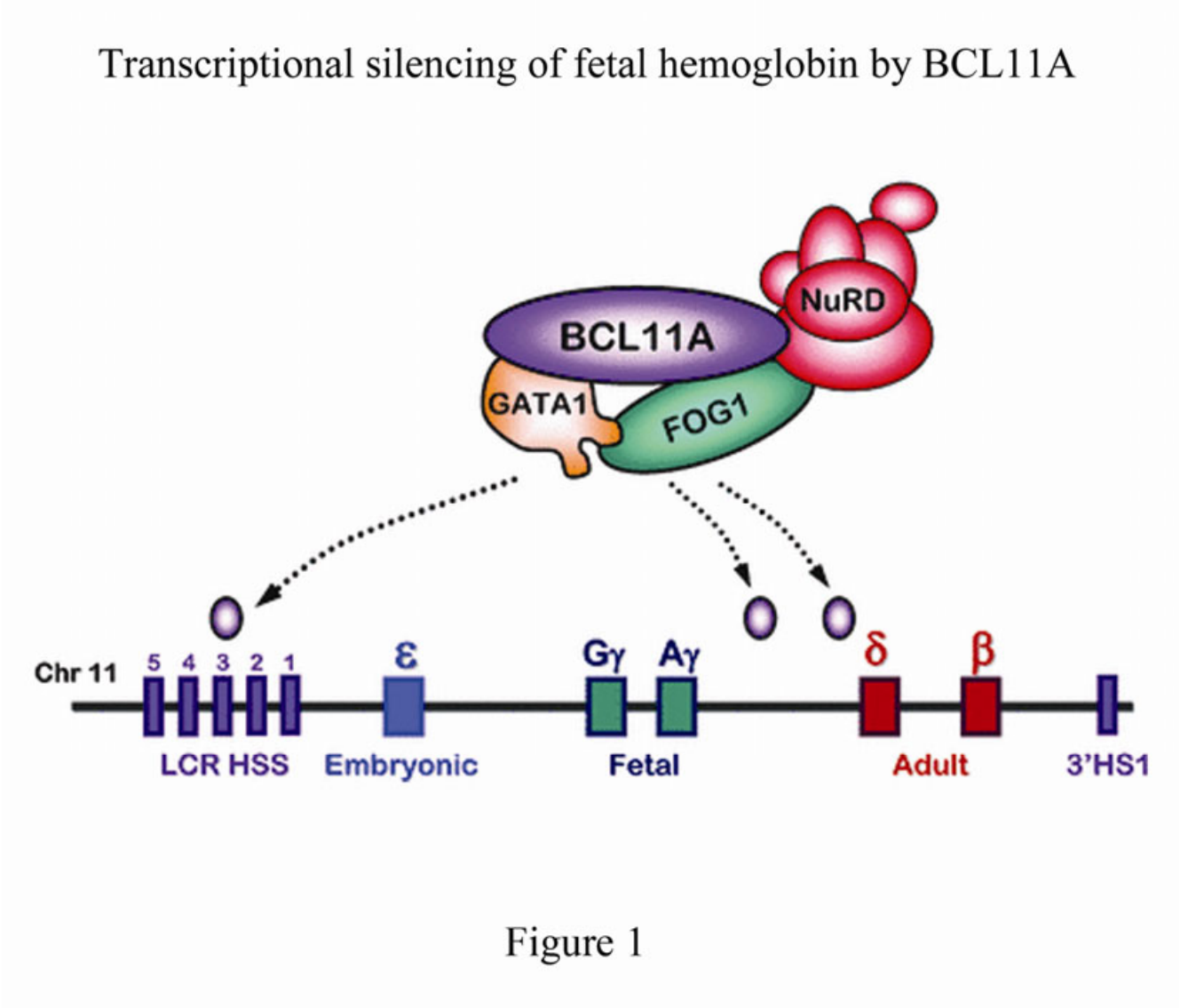

Figure 1

Figure 1: The extrinsic component of hemoglobin gene expression. From Sankaran et al. (2010). Reproduced from "transcriptional silencing of fetal hemoglobin by BCL11A". *Ann. N.Y. Acad. Sci*. **1202**, 64-68 with permission from John Wiley & Sons.

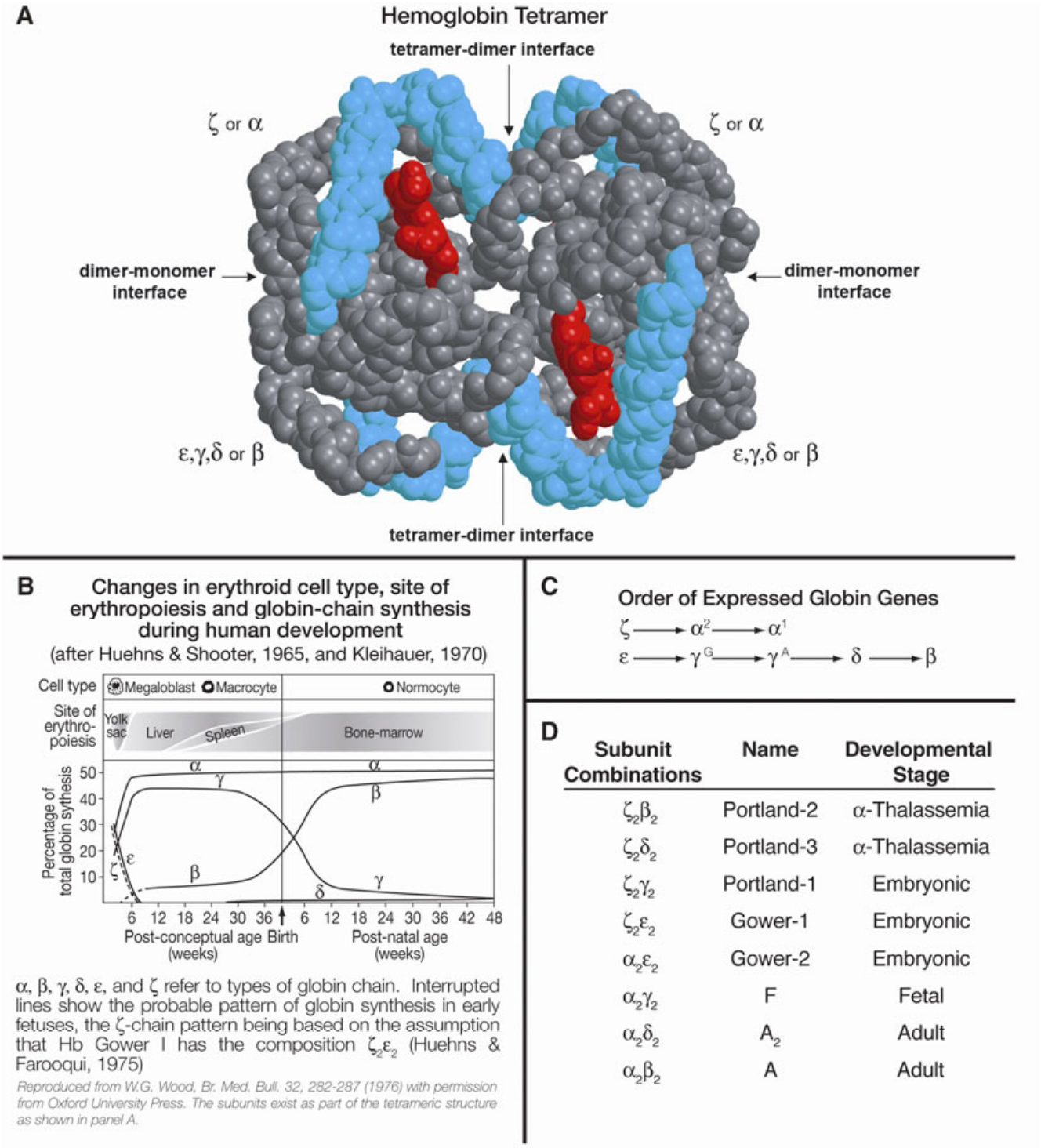

Figure 2

Figure 2: (**A**). Hemoglobin tetramer with the location of the tetramer-dimer and dimer-monomer interfaces indicated by arrows. The cyan color indicates the E and F helices, whereas the rest of each subunit is colored

gray. The heme is colored red. The top subunits can be either ζ or α and the bottom subunits ε, γ, δ, or β. Courtesy of W. Royer.

(**B**). Developmental changes in the expression of Hb types as a function of time. The individual subunits are shown, although they are actually present as tetramers with partner subunits (see panel D).

(**C**). Order of globin subunit expression. Each gene is separated by 10-20 kb of untranscribed DNA.

(**D**). Nomenclature and subunit composition for eight normal human hemoglobins. Those designated as occurring in the α-Thalassemia syndrome are found only in the total absence of α-subunit expression ("hydrops fetalis"). This figure was reproduced from [29] with permission.

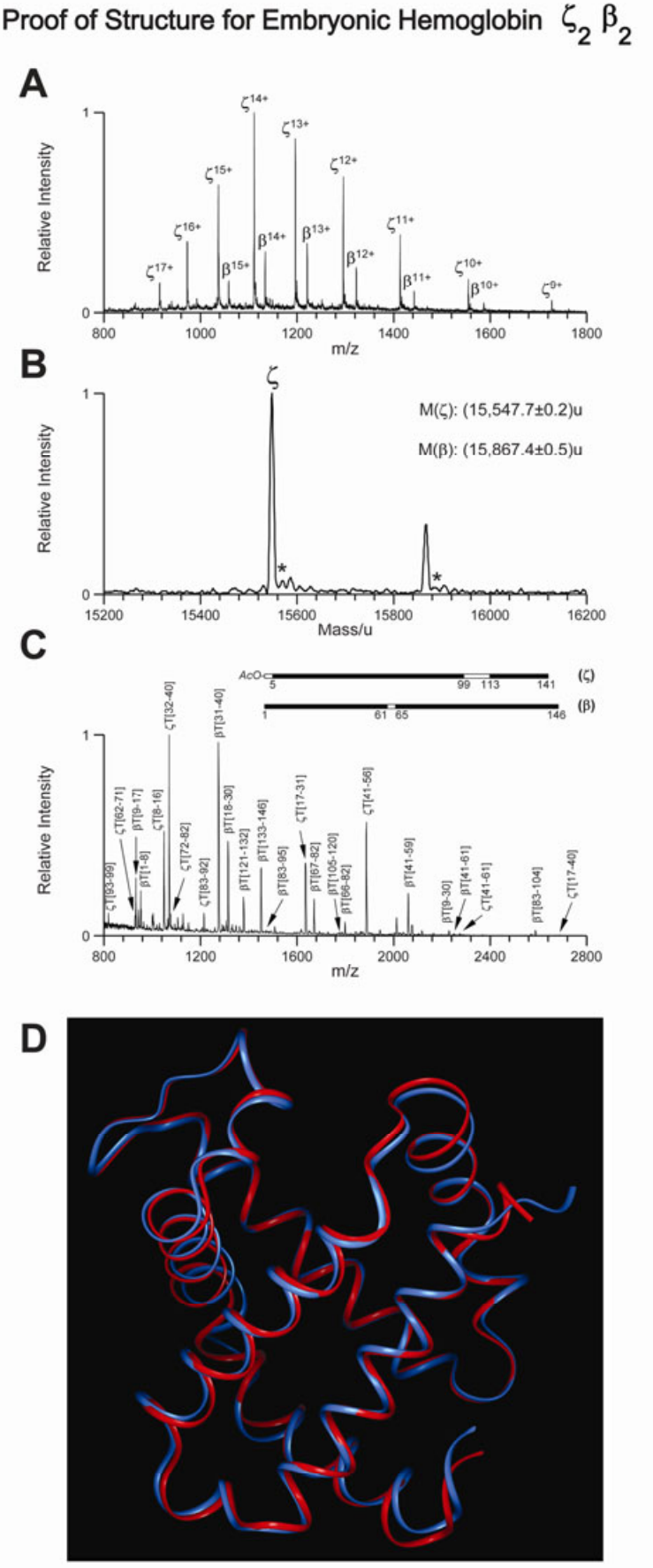
Proof of Structure for Embryonic Hemoglobin ζ2 β2
A
Relative Intensity
m/z
B
Relative Intensity
M(ζ): (15,547.7±0.2)u
M(β): (15,867.4±0.5)u
Mass/u
C
Relative Intensity
m/z
D

Figure 3

Figure 3: (**A**) Mass Spectrometric Analysis.

(**B**). Transformed mass spectrum after deconvolution of the ion envelopes encompassing charges between +16 and +9 (m/z range 950-1750). The experimentally determined values are in agreement with the expected average mass of 15,547.8u for the acetylated form of the ζ chain and of 15,867.2u for the unmodified β chain according to their respective polypeptide sequences. Asterisks denote peaks originating from adduction of metals to each chain.

(**C**). MALDI-Qq-TOF/MS spectrum of a tryptic digest of embryonic hemoglobin Portland-2. Peaks are labeled as ζ- and β- chain peptides followed by 'T' for trypsin and the number of the first and last residues of the peptide in accordance with the primary structure of the globin chain. The filled portion of the horizontal bars represent the sequence coverage obtained for both ζ and β chains for the spectrum shown. Additional results (see text) yielded information covering 100% of both ζ and β chains.

(**D**). Ribbon diagrams of aligned backbones of the PDP structures of the α-subunit of Hb, (blue) [36] and the δ-subunit of Hb Portland-1 (red) Insight II 2000 was used. This figure was reproduced from [12] with permission.

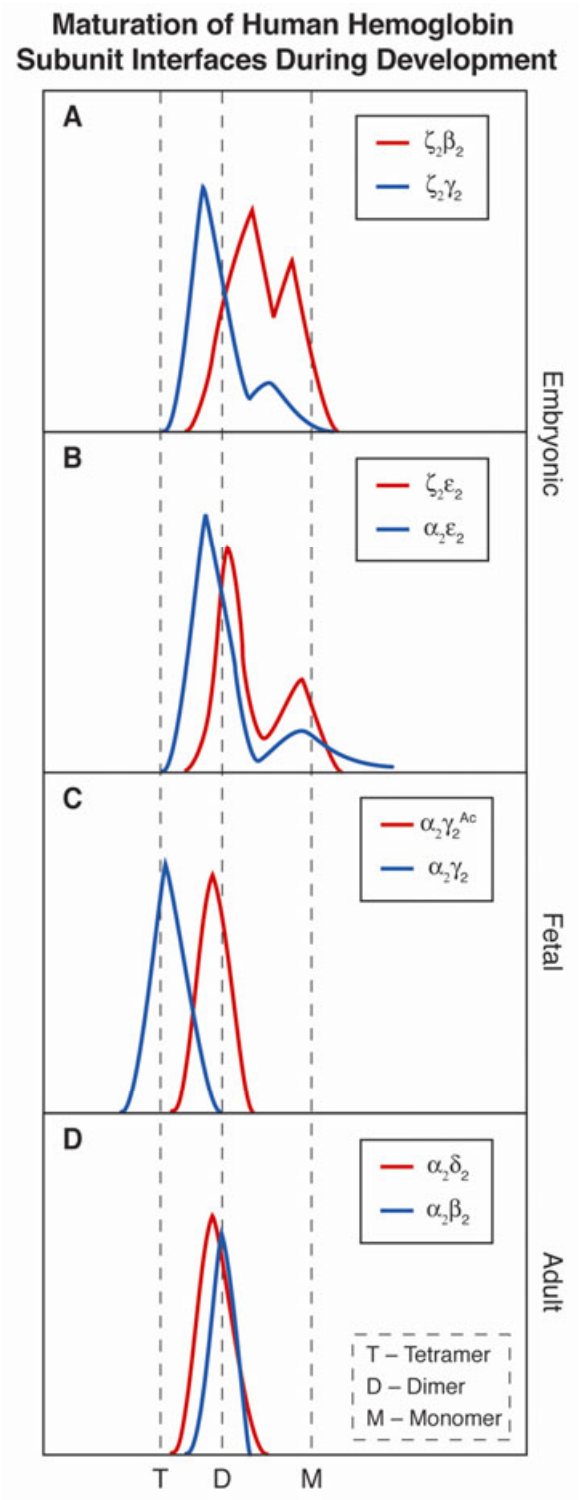

Figure 4

Figure 4: Gel filtration patterns of hemoglobins. Concentrations of 10-100 nM Hb in the liganded form were analyzed. The experimental conditions

are given in [24]. In any given panel, the more dissociated hemoglobin is shown in red. This figure was reproduced from [24] with permission.

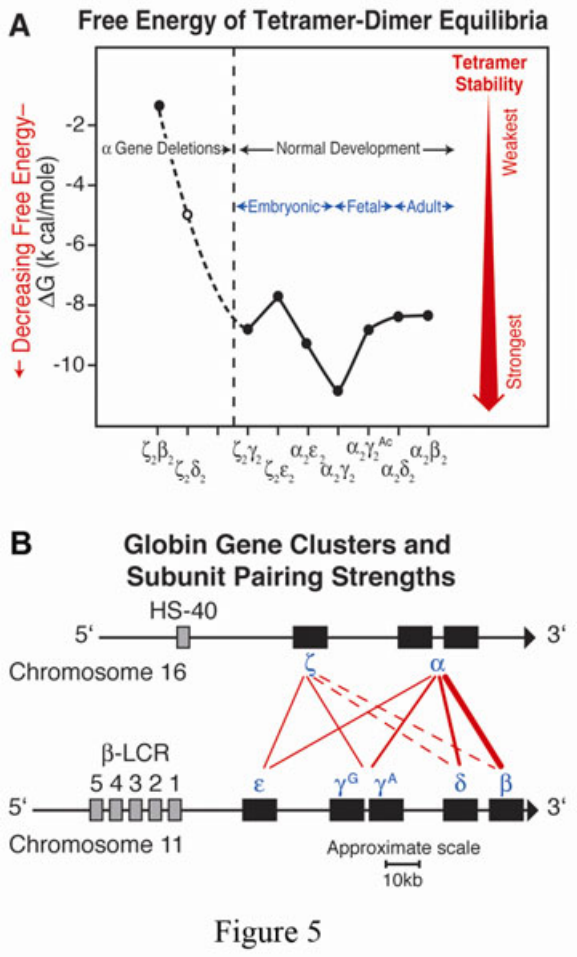

Figure 5

Figure 5:

Panel A – Free energy values for tetramer-dimer equilibria for the normal human hemoglobins calculated from their dissociation constants. This figure was reproduced from [29] with permission.

Panel B – Globin gene clusters and the intrinsic model. The α-like globin genes are located at chromosome 16 and the β-like globin genes are located on chromosome 11. The gray boxes labeled HS-40 on chromosome 16 and β-LCR (5, 4, 3, 2, 1) on chromosome 11 are regions that are sensitive to digestion by nucleases enabling the transcription of the individual globin genes (black boxes) in the order 5' -⟶ 3' to produce the eight globin subunits (blue Greek letters), whose common names and the stages of development at which they are present are shown in the inset (adapted from Higgs et al) (4). The red lines between

the globin subunits represent the strength of subunit interactions of a particular dimer and are from our data. Dashed lines represent the weakest dimer interface strengths whereas continuous lines indicate stronger ones; line widths indicate increasing interface binding strengths. Reproduced from [29] with permission. These red lines define the intrinsic component of regulation.