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Intrinsic regulation of hemoglobin expression by variable subunit interface strengths

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The expression of the six types of human Hb 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 Hb dimers have the strongest subunit interfaces and the embryonic Hbs the weakest, with fetal Hbs being 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 Hb types form functional O_2-binding tetramers consistent with gene switching.

Extrinsic regulation of expression

Hb 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 Hb 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 DNase. 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. This 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 in 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 Hb subunits themselves have no regulatory

Abbreviations
D–M, dimer–monomer; T–D, tetramer–dimer.

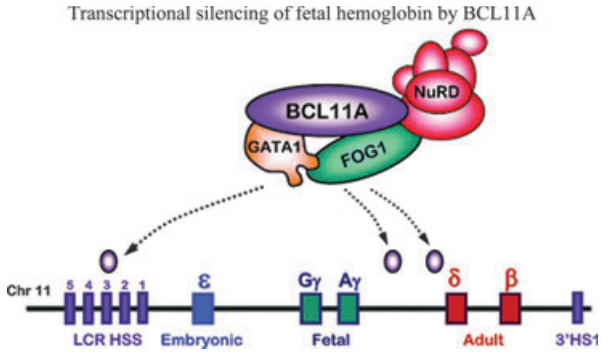

Fig. 1. The extrinsic component of Hb gene expression. Reproduced from Sankaran *et al.* (2010) Transcriptional silencing of fetal hemoglobin by BCL11A. *Ann NY Acad Sci* **1202**, 64–68, with permission from John Wiley & Sons.

role. Although it is well known that the assembled Hb tetramers have different affinities for oxygen, which is crucial during the various stages of prenatal and postnatal life, there have been fewer studies on their subunit–subunit interactions. In fact, the prevailing view is that all normal human Hbs 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 Hbs, we found differences of up to three orders of magnitude for their tetramer–dimer (T–D) and dimer–monomer (D–M) 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 Hbs. The use of the term 'phenotypes' 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 Hb expression is made up of both the intrinsic and extrinsic components together.

Hb ontogeny

Human Hbs composed of ζ-subunits or α-subunits paired with ε-subunits, γ-subunits, δ-subunits or β-subunits representing embryonic, fetal and adult Hbs have nearly identical overall structural architectures in their tetrameric states (Fig. 2A). The human Hb subunits are temporally expressed during normal development (Fig. 2B) in a pattern that reflects the order of their genes, 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, which are formed first as dimer pairs (D–M equilibrium) and then as the functional O_2-binding tetramers (T–D equilibrium), as shown in Eqn (1); their names are given in Fig. 2D.

$$2x + 2y \rightleftharpoons 2xy \rightleftharpoons x_2y_2 \quad (1)$$

Competing self-assembly reactions involving β-subunits 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 Hbs are present at different times during development, i.e. at the embryonic, fetal and adult stages. The transition among Hb types over time (Fig. 2B) represents a major paradigm for developmental biology, although some of its aspects, such as the 'switch' from ζ-subunit to α-subunit expression and from ε-subunit to γ-subunit expression during the embryonic stage, and the transition from γ-subunit to β-subunit expression during fetal life, are poorly understood [3]. Although differences in bonding strengths involving subunit interfaces of many mutant Hbs in both the liganded (HbO_2 or HbCO) and unliganded (deoxy) states [15] have been reported, normal human Hbs had not been considered to vary significantly in their interface properties until our report on liganded Hbs [12] to the contrary. Furthermore, although adult deoxy-HbA has very strong subunit interfaces [15], some embryonic deoxy-Hbs [16] and mutant adult deoxy-Hbs [15] 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 Hbs have been on the adult type; the lower number of reports on the embryonic and fetal Hbs is attributable, in part, to lack of availability, especially of the embryonic types. This predominance of adult Hb data has thus engendered the notion that all normal human Hbs have similar subunit interface properties, because 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 of the various normal Hbs, which are conferred by tertiary and quarternary structural changes [17], 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 Hbs during development.

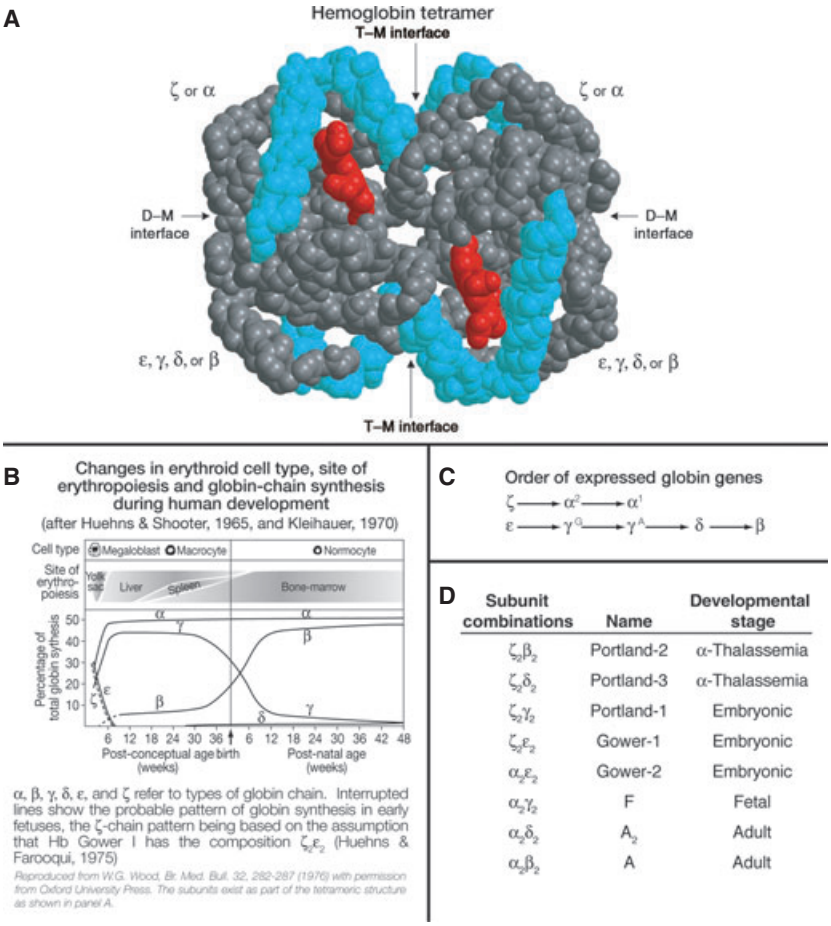

Fig. 2. (A). Hb tetramer with the locations of the T–D and D–M interfaces indicated by arrows. The cyan color indicates the E and F helices; the rest of each subunit is colored gray. The heme is colored red. The top subunits can be either ζ or α, and the bottom subunits ε, γ, δ, or β. Courtesy of W. 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 (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 Hbs. Those designated as occurring in the α-thalassemia syndrome are found only in the total absence of α-subunit expression (hydrops fetalis). (D) is reproduced from [28] with permission.

The embryonic Hbs 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) [8,18]. $\zeta_2\beta_2$ (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, because synthesis of the latter is absent [19]. 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 Hbs from transgenic mice transfected with the human globin genes [18] has enabled us to perform a comprehensive study of the subunit binding properties of all of the embryonic Hbs and a comparison with the fetal and adult types. However, the transgenic embryonic human Hbs first required full characterization in order for the findings to be credible.

Embryonic Hb Portland-2 characterization

As Hb Portland-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 artefact 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 IEF after its purification, as described in [12]. It had the expected amino acid sequence for its ζ-subunits and β-subunits (see below). In order to ensure that the transgenic Hb Portland-2 was properly folded, its CD pattern was compared with that of human adult HbA [20] measured at the same time and at the same concentration. The results showed that the CD spectra were nearly superimposable, with only minor differences. Hence, human Hb Portland-2 expressed in mice has a native Hb conformation. As described in detail below, comprehensive MS showed there was faithful expression of the human ζ-globin and β-globin genes in the mouse, and that the globin subunits had not undergone any proteolysis.

Mass spectra of Hb Portland-2

The most direct approach to establish that the sequence of Hb Portland-2 was correct and intact was to perform comprehensive MS on the subunits [21]. The three-step MS strategy reported previously [22] was utilized. Initially, we used ESI MS to measure the average molecular masses of both ζ-chains and β-chains separately. A typical ESI spectrum is presented in Fig. 3A, and its corresponding deconvoluted spectrum is shown in Fig. 3B. The deconvoluted spectrum shows a single component for each Hb chain, with differences between the theoretical mass and the experimentally determined mass of < 15 p.p.m. Minor peaks, which are present on the high-mass side of each major peak (Fig. 3B), corresponded to sodium, potassium and iron adducts. The two chains from Hb

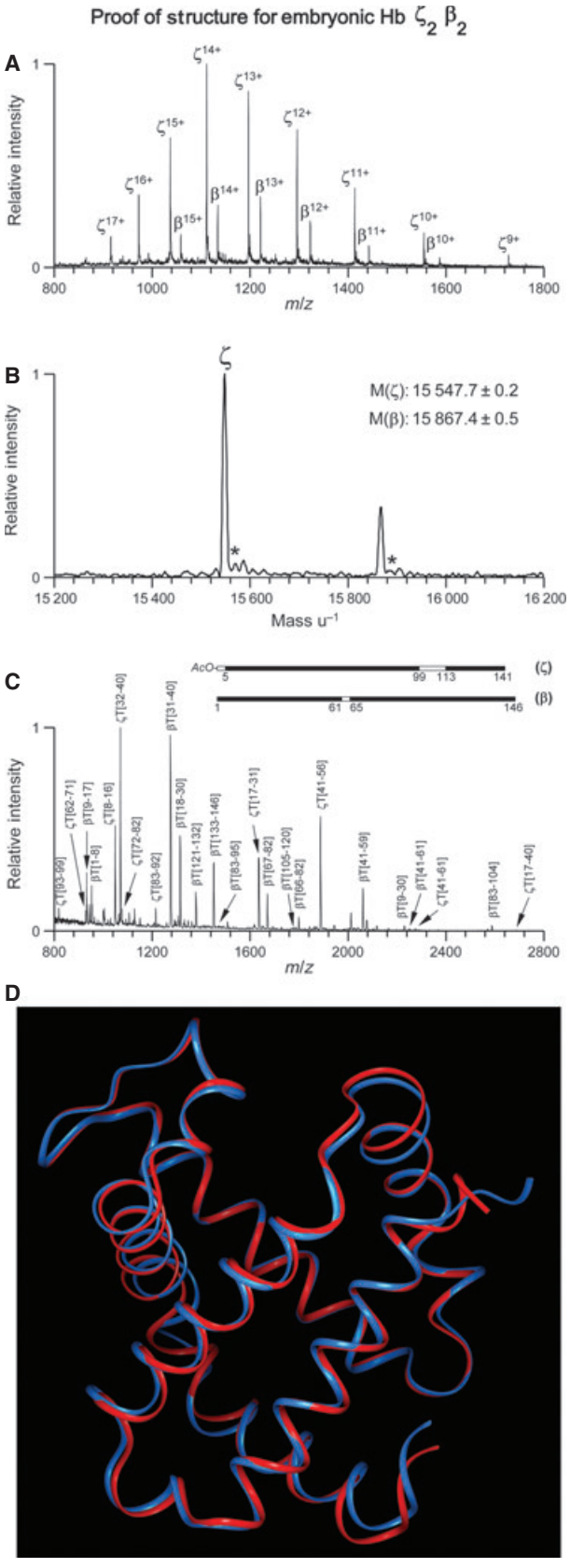

Fig. 3. (A) MS analysis. (B). Transformed mass spectrum after deconvolution of the ion envelopes encompassing charges between +16 and +9 (*m/z* range: 950–1750). The experimentally determined mass values are in agreement with the expected average values of 15 547.8 for the acetylated form of the ζ-chain and of 15 867.2 for the unmodified β-chain, according to their respective polypeptide sequences. Asterisks denote peaks originating from adduction of metals to each chain. (C). MALDI-QqTOF MS spectrum of a tryptic digest of embryonic Hb Portland-2. Peaks are labeled as ζ-chain 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 portions of the horizontal bars represent the sequence coverage obtained for both ζ-chains and β-chains for the spectrum shown. Additional results (see text) yielded information covering 100% of both ζ-chains and β-chains. (D). Ribbon diagrams of aligned backbones of the PDP structures of the α-subunit of Hb (blue) and the δ-subunit of Hb Portland-1 (red). INSIGHT II 2000 was used; reproduced from [12] with permission.

Portland-2 were subsequently analyzed by peptide mapping, with both MALDI-TOF MS and MALDI-QqTOF MS. Each spectrum was internally calibrated, yielding mass errors of ±10 p.p.m. 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 Ser1. 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 MS 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 Hb Portland-2 are correct, but that they join together very weakly; their subunit interface strengths are reciprocally related to the interface strength involving α-subunits and β-subunits, as described below. The tertiary structure of the ζ-subunit as compared with the α-subunit is shown in Fig. 3D, and shows only very minor folding differences.

Gel filtration of nanomolar concentrations of Hbs reveals different substructures

The relative molecular masses of the human embryonic, fetal and adult Hb tetramers are all within a few hundred mass units of 64 000. Each Hb 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 [23]. 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 Hbs depicted in Fig. 4A,B show a significant percentage of dimers and monomers, but monomers are not detectable for fetal and adult Hbs (Fig. 4C,D). Thus, α-subunits have a higher affinity for β-like subunits than do ζ-subunits. Embryonic $\zeta_2\delta_2$ (Hb Portland-3) has been reported only as a band on a gel in α-thalassemia major [19]; it is also considered to be a very highly dissociated Hb. Hence, it appears likely that these two Hbs are not found normally, because their subunit interfaces are so weak that other Hbs with stronger subunit interfaces are favored. In general, the gel filtration profiles in Fig. 4 become progressively less complex from the embryonic Hbs (Fig. 4A,B) through the fetal Hbs (Fig. 4C) and finally to the adult Hbs (Fig. 4D). These patterns represent the phenotype for each type of Hb.

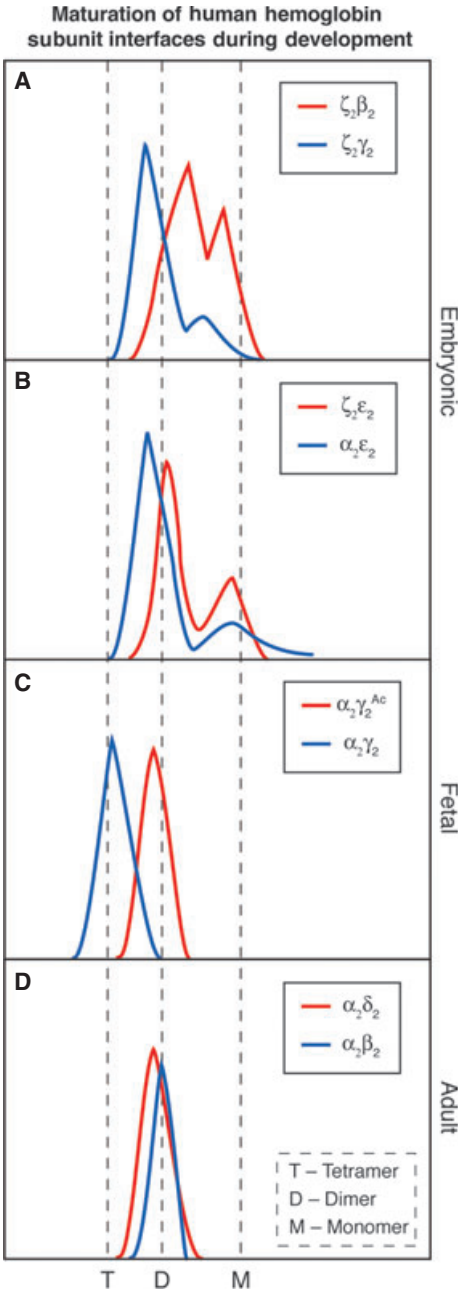

Fig. 4. Gel filtration patterns of Hbs. Concentrations of 10–100 nM Hb in the liganded form were analyzed. The experimental conditions are given in [23]. In any given panel, the more dissociated Hb is shown in red. Reproduced from [23] with permission.

Role of subunit interfaces during development

The short half-lives *in vivo* of the embryonic Hbs are consistent with their very weak subunit interactions shown in Fig. 4A,B, as monomeric subunits formed from the facile dissociation of weak dimers are unstable and disappear [24]. In contrast, the fetal and adult Hbs 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 Hbs (Fig. 4C, blue curve), consistent with the rapid onset of its synthesis at 6 weeks [25,26], owing to its very low free energy as calculated from its dissociation constant [27]. 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) [27,28], 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 multicomponent 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 [29]. 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. 4D, red line) and also by the displacement of its peak width maximum from its T–D K_d value [10]. In contrast, HbA formation has a much narrower peak width (Fig. 4D, blue line) whose maximum coincides with its T–D K_d value [10,30], making it the most favorable Hb species.

The T–D interfaces of normal Hbs have variable strengths

The gel filtration patterns in Fig. 4 represent a combination of the T–D equilibrium and the D–M equilibrium (Eqn 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 [8,16], varied by up to three orders of magnitude [12]. These constants were converted into Gibbs free energy values (Fig. 5A), which constitute a measure of tetramer stability (red arrow), and they show an undulating pattern similar to the developmental profile in Fig. 2B.

However, there is no apparent correlation of the values themselves with time of expression (Fig. 2B) or with gene order (Fig. 2C). For example, the Gibbs free energy values of embryonic Hb Portland-1 and of embryonic Hb Gower-2 are equal and lower, respectively, than that of adult HbA ($\alpha_2\beta_2$) (Fig. 2A). However, there is a consistent pattern for the effects of ζ-subunits versus α-subunits on tetramer strength; that is, the T–D interface is weakened by over an order of magnitude for Hbs containing ζ-subunits as compared with corresponding tetramers containing α-subunits, representing an increase of 2–7 kcal·mol^{-1} (Fig. 5A). To put this change in more familiar terms, a difference

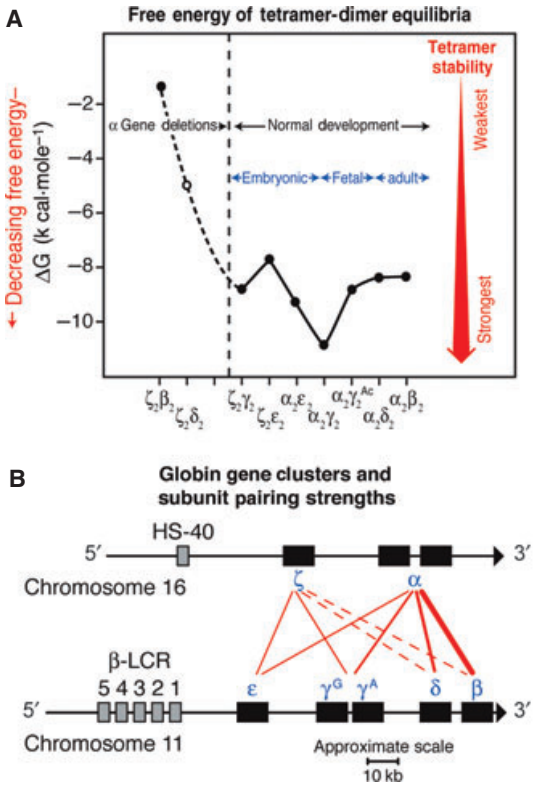

Fig. 5. (A) Free energy values for T–D equilibria for the normal human Hbs calculated from their dissociation constants. Reproduced from [28] with permission. (B) Globin gene clusters and the intrinsic model. The α-like globin genes are located on chromosome 16 and the β-like globin genes are located on chromosome 11. The gray boxes labeled HS-40 on chromosome 16 and β-LCR (5, 4, 3, 2, and 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 Fig. 1D (adapted from [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, and continuous lines indicate stronger ones; line widths indicate increasing interface binding strengths. These red lines define the intrinsic component of regulation. Reproduced from [28] with permission.

of 6 kcal·mol^{-1} exists between deoxy-$\alpha_2\beta_2$ and oxy-$\alpha_2\beta_2$ (−14 and −8 kcal·mol^{-1}, respectively) [15] arising from the more extensive subunit interactions in deoxy-$\alpha_2\beta_2$.

The D–M interface

To measure the D–M step in Eqn (1), we used an indirect method employing subunit exchange as described initially by Huehns [31] and modified by us [12]. We found that the embryonic Hb dimers are the weakest, the fetal Hbs are stronger, and the adult Hbs are the strongest (the progression of strength is 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], as embryonic ε-subunits and adult δ-subunits have net positive charges, whereas fetal γ-subunits and adult β-subunits have net negative charges. Dimers containing α-subunits have increasing strengths that correlate with the gene order of the β-like subunits (ε, γ, δ, and 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 αβ pair has the strongest subunit interface binding contacts, and the ζβ pair has the weakest. The red lines in Fig. 5B also have a directionality representing the driving force directing the formation of certain Hbs and the exclusion of others. The two weakest Hbs, $\zeta_2\beta_2$ 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 [19]. 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.

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The 'extrinsic' component for regulation of gene expression describes factors that are external to the genes that they help to transcribe, such as the GATA family for Hb 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. Hb subunits, interact with one another to different extents to influence the overall expression and the duration of the interaction. Both extrinsic and intrinsic components probably have a role in regulation.

Subunit competition leads to the most stable Hbs

The embryonic Hbs have such weak subunit interfaces (Fig. 4A,B) that they can exchange their subunits to form Hbs 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 $\alpha_2\gamma_2$ is not formed and nor are any of the embryonic Hbs. *In vivo* studies using a yeast expression system that does not contain DNase-sensitive regulatory loci have confirmed this conclusion in intact cells [28]. Even though the physiological relevance of such subunit exchange is unknown, it conclusively demonstrates that the thermodynamic stability of the human Hbs is embryonic < fetal < adult, which faithfully reflects the normal order of occurrence of these Hbs.

Interface subunit bonding strengths correlate with transitions in their developmental profile

The results above offer an explanation for the developmental profile in Fig. 2B based simply on the subunit interface properties of the Hbs 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 D–M and T–D 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 T–D free energy lower than that of HbA; both have strong D–M 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 result from 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 [27], 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 αδ 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 [30]. Thus, the energetics of

the subunit interfaces of the human Hbs play a major role in their developmental profile. These principles are probably also applicable to other normal and abnormal protein assemblies, such as those in extensive and interlinked signaling networks [32].

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