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NEPHROLOGY FORUM

Molecular biology of erythropoietin

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Case presentation

A 42-year-old man presented with hypertension and proteinuria 22 years ago and was found to have adult polycystic kidney disease. Within 8 years he was approaching end-stage renal failure (creatinine clearance, 10 ml/min). The hemoglobin (Hb) was 10.0 g/dl. Hemodialysis was initiated 13 years ago, when his Hb was 6.6 g/dl. Two months later, it had risen spontaneously to 10.0 g/dl. The serum erythropoietin was 20 IU/liter. A left nephrectomy was performed to make space for a renal transplant. The Hb was 9.7 g/dl preoperatively and fell to 8.7 g/dl in the first postoperative month. He had two unsuccessful renal transplants, one 12 years ago and one 10 years ago. Nine years ago, his remaining polycystic kidney became infected and had to be removed. He became dependent on blood transfusions and received a total of 80 units over the subsequent 3 years. Six years ago, he was enrolled in a trial of recombinant human erythropoietin. His Hb was 6.2 g/dl; red cell mass, 6.7 ml/kg (normal, 25–35 ml/kg); serum erythropoietin, 7.3 IU/liter; erythron transferrin uptake (ETU), 24 μmol iron/liter whole blood/day (normal, 60 ± 12); and absolute reticulocytes 23 × 10^9/liter. The white blood cell count was 4.5 × 10^9/liter and platelet count was 171 × 10^9/liter. At a dose of 96 IU/kg given intravenously 3 times weekly, his Hb rose to 11.9 g/dl over 10 weeks; the red cell mass rose to 18.4 ml/kg, the ETU to 116 μmol/liter whole blood/day, and the absolute reticulocytes to 206 × 10^9/liter. The white blood cell and platelet counts were unchanged at 4.2 × 10^9/liter and 168 × 10^9/liter.

Discussion

DR. PETER J. RATCLIFFE (*University Lecturer and Honorary Consultant Physician, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, England*): In addition to providing the means for producing recombinant erythropoietin for clinical use, molecular cloning of the erythropoietin gene [1–5] has provided a new impetus for studying the biology of erythropoietin and the control of erythropoiesis. The use of recombinant erythropoietin in the treatment of anemia of chronic renal failure was discussed in a Nephrology Forum by Dr. J. W. Eschbach [6]; here I would like to focus on some of the recent advances in our understanding of the basic biology of this system.

Erythropoietin is unique among the hemopoietic growth factors in functioning, at least in the adult, as a blood stream hormone. Important insights into the regulation of erythropoietin and erythropoiesis can, therefore, be gained from measurements of the serum erythropoietin level in health and disease. I will begin by commenting on some of these observations. People without renal disease have an approximately inverse linear relationship between hemoglobin and the logarithm of the serum erythropoietin level (Fig. 1), with levels of erythropoietin reaching several hundredfold above baseline at the lowest levels of hemoglobin [7–10]. Many clinical and experimental observations have demonstrated that not only anemia [7–11] but reduced arterial pO_2 [12–14] and increased hemoglobin affinity [13–16] can stimulate erythropoietin production. These observations have led to the proposal that blood oxygen availability rather than hematocrit or red cell mass per se is sensed and suggest that the stimulus for erythropoietin production is lowered tissue oxygenation. Thus a homeostatic response is effected: a reduction in the hematocrit is corrected by hypoxic stimulation of erythropoietin production and hence erythropoiesis [17].

The central importance of these responses in control of erythropoiesis is demonstrated by the patient presented. After the initial period of maintenance dialysis, his hemoglobin stabilized in the range of 8–10 g/dl. His serum erythropoietin was in the normal range, although in the setting of anemia, it was somewhat below the level observed in anemias not associated with renal disease. Just prior to starting dialysis, his anemia was exacerbated; this phenomenon is well recognized [18]. In some cases, overhydration is partly responsible, and in patients with good cardiac function who are receiving antihypertensive medication, the degree of overhydration is easily underestimated. Unidentified uremic toxins could exacerbate the anemia of renal disease [19, 20]. Such toxins might be removed in part when maintenance dialysis begins [18], although significant doubt has been cast on the specificity and hence pathophysiologic relevance of in-vitro demonstrations of uremic erythropoietic inhibitors [6].

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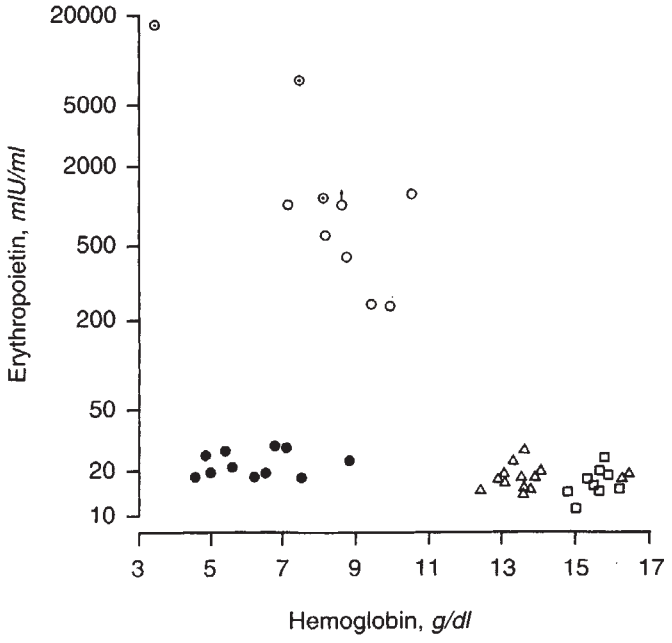

Fig. 1. *Estimates of immunoreactive erythropoietin in serum of normal men (□) and women (△) and of patients with hypoplastic anemia from renal disease (●) and aplastic (○) or Fanconi's (⊙) anemias* (From Ref. 10 with permission).

After hemodialysis was established, the patient maintained a hemoglobin level that was somewhat higher than usual for dialysis patients until he was rendered anephric. Erythropoietin production by the polycystic kidneys was most probably responsible; erythropoietin levels in patients with polycystic kidneys tend to be higher than erythropoietin levels in other dialysis patients [21, 22], and in-situ hybridization studies have localized erythropoietin mRNA in stroma cells of the cyst walls [23].

After the second nephrectomy, the patient's anemia worsened; severely reduced red cell mass, reticulocyte count, and erythron transferrin uptake indicated a severe hypoplastic state. The serum erythropoietin level was very low in relation to what would be anticipated for the degree of anemia. Several important points are illustrated: (*1*) The role of the kidney is central in erythropoietin production. (*2*) Measurable serum erythropoietin after bilateral nephrectomy indicates the existence of non-renal sources. (*3*) Low levels of erythropoietin, similar to those in the normal basal state, appear to have a major effect on erythropoiesis. (*4*) The response to exogenous erythropoietin emphasizes the specific role of erythropoietin in supporting erythropoiesis. When the patient was treated with recombinant erythropoietin, all parameters of erythropoiesis were dramatically increased and, despite the uremia, the patient achieved a normal red cell mass. The slightly increased erythron transferrin uptake probably arose from the need to compensate for a modestly increased red cell turnover.

Erythropoietin therefore appears to be essential for supporting erythropoiesis. In addition to providing a graded response to varying degrees of hypoxia, regulated erythropoietin production probably contributes to the maintenance of hematocrit near the normal range. Several other observations support this view. For instance, rodents immunized against erythropoietin develop fatal anemia [24, 25]. Donation of a unit of blood is associated with a rise in the serum erythropoietin level, albeit to levels that are still within the normal range [26, 27]. Furthermore, patients with primary polycythemia generally have serum erythropoietin levels below the normal value [8, 28–30]; the implication is that a supranormal hematocrit improves tissue oxygenation at the critical site(s) and thus reduces erythropoietin production below normal.

This understanding derived from clinical physiology provides an important background to the molecular analysis of control of erythropoietin production and erythropoiesis. First, erythropoietin is seen to be more than a permissive survival factor; rather, it acts as a growth regulator over an unusually large dynamic range. Second, tissue hypoxia is identified as a major error signal in the feedback loop. Third, in contrast with many hematopoietic growth factors [31], redundancy is limited, and an absolute requirement for erythropoietin exists. Against this background I would like to discuss some recent advances in our understanding of how erythropoietin promotes erythropoiesis, and how the production of erythropoietin is regulated by blood oxygen availability.

The erythropoietin receptor

Stimulation of erythropoiesis by erythropoietin involves interaction with a specific receptor. The availability of recombinant erythropoietin enabled D'Andrea and colleagues to isolate a cDNA clone for the mouse erythropoietin receptor by expression cloning [32]. Cos cells, an SV40 virus-transformed kidney fibroblastoid cell line, were transfected with cDNA prepared from erythropoietin receptor expressing mouse erythroleukemia cells; the transfected cells then were screened for expression of the erythropoietin receptor with radioiodinated erythropoietin. The erythropoietin receptor cDNA was recovered from positive transfectants [32]. Isolation of human clones for the erythropoietin receptor has subsequently been reported [33, 34]. The availability of cloned genes for both erythropoietin and its receptor has allowed detailed studies of this molecular interaction and the consequent stimulation of erythropoiesis. These studies demonstrate important homologies with molecular and functional aspects of other growth control systems.

The mouse erythropoietin receptor cDNA codes for a 507 amino acid polypeptide with a single membrane-spanning domain [32]. The extracellular N-terminal region contains the erythropoietin-binding domain, and the C-terminal intracellular domain is associated with signal transduction [32]. Significant sequence homologies have been found with other growth factor receptors, including hemopoietic cytokine receptors for G-CSF, GM-CSF, Il-2, Il-3, Il-4, Il-5, Il-6, Il-7, and receptors for other growth-related molecules such as growth hormone and prolactin [31, 35–39]. Hence, it is now recognized that the erythropoietin receptor is a member of a large superfamily of receptors. More distant homologies have been recognized with interferon receptors [40] and with the type-III domain of the cell-adhesion protein fibronectin [41]. Bazan proposed that these molecules have evolved from primitive adhesive molecules to their present role as receptors for specific proteins [38].

Recognition of homology in part depends on the existence of characteristic motifs (Fig. 2). For instance, in the N-terminal extracellular ligand-binding domain, two distinctive motifs are

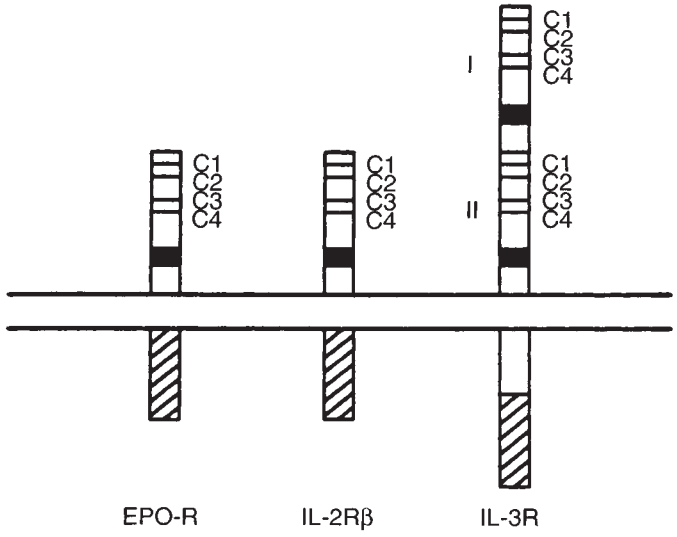

Fig. 2. *Structural and functional homologies among different growth factor receptors.* Homologous domains of the cytokine receptors EPO-R, IL-2Rβ, and IL-3R are aligned. The extracellular domains share a highly similar 20-amino acid sequence, which includes a conserved Trp-Ser-X-Trp-Ser motif (*black box*). The four conserved cysteine residues (C1–C4) are also aligned. The homologous external domain is duplicated in the IL-3R (regions I and II). The cytoplasmic domain of these three receptors contains a conserved region rich in proline, serine, and acidic residues (*cross-hatched bars*) (From Ref. 35).

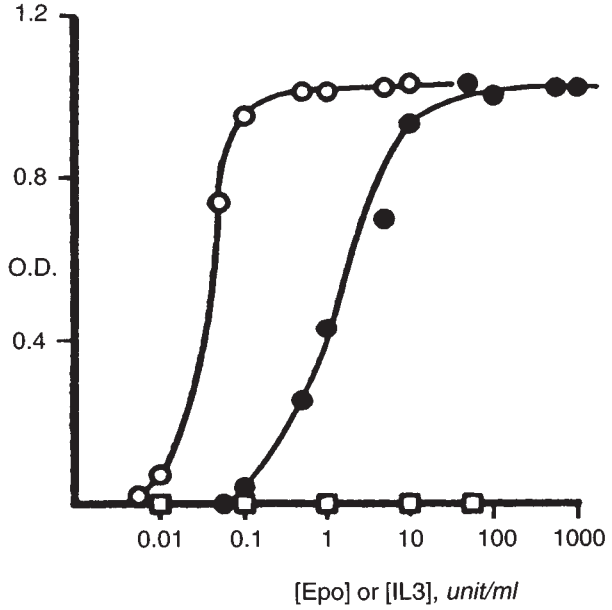

Fig. 3. *Erythropoietin-dependent proliferation of the transfected mouse pro-B cell line LyD9.* O.D. refers to the optical density of the colorimetric MTT (tetrazolium) assay used to measure cell proliferation. Untransfected cells did not proliferate in response to erythropoietin (*squares*). In contrast, transfectants expressing the erythropoietin receptor showed dose-dependent proliferation in response to erythropoietin (*open circles*), which resembled the response to IL-3 (*closed circles*). In further experiments, erythropoietin receptors bearing mutations in the Trp-Ser-X-Trp-Ser motif were found to be inactive in this system (From Ref. 42).

seen: 2 pairs of cysteine residues near the N-terminus and a Trp-Ser-X-Trp-Ser sequence lying just outside the membrane-spanning domain [35]. If these motifs have indeed been conserved during evolution from a common ancestral gene, they probably are of key functional significance. Experimental site-directed mutagenesis has shown this to be the case. Two groups recently reported that mutations in the Trp-Ser-X-Trp-Ser motifs do destroy the function of the erythropoietin receptor [42, 43]. For instance, one study showed that when mutations were made in this motif, the mutant receptor polypeptide was not processed correctly, and it could not bind erythropoietin [43]. Furthermore, when introduced into an erythropoietin receptor with a pre-existing mutation conveying constitutive activation [44], mutations of the Trp-Ser-X-Trp-Ser motif eliminated cellular activation [43].

Homology in the ligand-binding domains of this receptor superfamily raises the question as to whether the ligands themselves contain significant homologies. Bazan postulated that, although important similarities are difficult to discern from the primary amino acid structure, they do exist [39]. The ligands are rich in sequences that are predicted to form α helices and can be modeled to form a bundle of 4 antiparallel helices, a structure that has been demonstrated crystallographically for growth hormone [45]. Site-directed mutagenesis studies and antibody-blocking studies are consistent with such a model for the structure of erythropoietin [46–48], although these studies cannot prove the structure. In the case of growth hormone, the crystal structure of the ligand-receptor complex has recently been obtained [49, 50] and the molecular basis of this type of ligand-receptor interaction soon may be determined precisely.

These receptors also have important similarities in their mechanisms of cell activation. Tyrosine kinase activity is associated with growth control systems, in that most of the known tyrosine kinases are either growth-factor receptors or proto-oncogenes involved in growth regulation [51]. Activation of the erythropoietin receptor is followed rapidly by tyrosine phosphorylation of a set of proteins that includes the receptor itself [52–55]. No enzymatic activities are predicted from analysis of the sequence of the cloned gene for the erythropoietin receptor [35], and this molecule is probably non-covalently associated with other proteins possessing tyrosine kinase activity. This possibility would conform with cross-linking studies that consistently link erythropoietin to a complex that is bigger than that predicted from the sequence of the cloned erythropoietin receptor gene [35, 56–58]. Such a mechanism is similar to that proposed for other members of this receptor family, such as the interleukin-2 receptor [59].

Transfection experiments with the erythropoietin receptor provide a striking demonstration of the receptor's ability to interact with common cell activation mechanisms [42, 60, 61]. For instance, after transfection of the Il-3-dependent pro-B cell line LyD9 with the erythropoietin receptor, the cell line becomes erythropoietin responsive and Il-3 can be substituted by erythropoietin in the culture medium [42] (Fig. 3). Although this phenomenon is demonstrable in other cells, the interactive mechanisms do not appear to be universal; although expression of the receptor in Cos cell transfectants permitted ligand binding, this binding did not alter cell growth [32].

Given functional overlap with growth control mechanisms in other cells, the specific dependence of erythropoiesis on erythropoietin must arise from controlled expression of the erythropoietin receptor and specific liganding by erythropoietin. This dependence is clearly indicated by the parallel development of erythropoietin dependence and expression of the erythropoietin receptor in cultured marrow cells. Thus the appearance of substantial erythropoietin receptor expression in erythroid progenitor cells (BFU-E: burst-forming units-erythroid) correlates with the onset of erythropoietin dependency. Later erythroid progenitors (CFU-E: colony forming units-erythroid), which demonstrate striking erythropoietin-dependent responses, express the erythropoietin receptor most abundantly [57, 62].

One important insight into how expression of the erythropoietin receptor is controlled during erythroid differentiation has come from the identification and molecular cloning of the gene for the erythroid transcription factor GATA-1 [63–65], a nucleoprotein that controls gene transcription in erythroid cells and that is so named because it binds the DNA motif $^{T}/_{A}$ (GATA) $^{A}/_{G}$. This motif was first recognized in control sequences regulating globin gene expression, but it subsequently has been found in many erythroid genes with diverse functions, including the heme synthetic enzyme porphobilinogen deaminase, the membrane protein glycophorin, and the erythropoietin receptor [65]. Proof of the central importance of GATA-1 in erythroid differentiation has been obtained by targeted mutation of the GATA-1 gene. The X-linked GATA-1 gene was disrupted in male (XY) embryonic stem cells, and the cells were introduced into mouse embryos at the blastocyst stage. Examination of the resultant chimeric mice revealed that the mutant stem cells contributed to all differentiated tissues except erythroid tissues. This study thus demonstrated that GATA-1 is essential for erythroid development [66]. Co-transfection experiments have shown that GATA-1 plays a dominant role in direct control of erythropoietin receptor expression through interaction with a GATA motif in the erythropoietin receptor gene promoter [67, 68]. Furthermore, binding of erythropoietin to the erythropoietin receptor up-regulates expression of GATA-1, thus potentially reinforcing the cell's commitment to erythroid differentiation [68].

The interaction between the erythropoietin receptor and GATA-1 provides some insight as to how a tissue-specific transcription factor interacts with a growth factor receptor to control differentiated growth. Clearly much remains unexplained, particularly the early events in progenitor cells, which regulate initial expression of GATA-1. What I would like to emphasize is that erythropoietin plays a central role in growth control of the erythron, using mechanisms that appear to operate in a wide variety of settings.

Regulation of erythropoietin

As I said, an unusual feature of the erythropoietin/erythropoiesis growth control system is that the error signal indicating inadequate growth has been clearly defined. Reduced tissue oxygenation signals the existence of an inadequate erythron, and leads to production of erythropoietin. I would therefore now like to consider the mechanism by which blood oxygen availability regulates serum erythropoietin. To understand this mechanism, we first must consider the sites of erythropoietin production and oxygen sensing; second, what determines tissue oxygenation at these sites; and third, the mechanisms of signal reception and of induction of gene expression.

Changes in serum erythropoietin levels appear to depend largely or entirely on changes in hormone production rate [69–71]. Moreover, in response to stimulation, increases in serum erythropoietin levels are paralleled by changes in the abundance of erythropoietin mRNA. These changes indicate that the level of erythropoietin mRNA is the major determinant of hormone production rate [72–74]. Studies of the organ distribution of erythropoietin mRNA [75, 76] have confirmed the results of the classical organ ablation studies which demonstrated, in adults, that the kidney is the major organ responsible for erythropoietin production [77] but that the liver is capable of significant erythropoietin production as well [13, 78].

More sensitive methods for detection of erythropoietin mRNA also have demonstrated small quantities of erythropoietin mRNA in testis, brain, lung, and spleen of rodents [74, 79]. In anemic or hypoxically stimulated animals, these tissues harbor amounts of erythropoietin mRNA that are insignificant in comparison to the large amounts found in kidney and liver [74], and their physiologic role is uncertain.

Even after bilateral nephrectomy, the patient presented here had detectable circulating erythropoietin. Organ ablation studies in animals have indicated that the highest serum erythropoietin level achievable after bilateral nephrectomy is only 10% to 20% of the level achievable normally, and that the liver is the site of production under these circumstances [13, 77, 78, 80]. A sustained level of this magnitude should be sufficient to correct the anemia of renal disease, yet clearly this does not happen. Using RNase protection assays to obtain a quantitative assessment of the total erythropoietin mRNA, my colleagues and I examined hepatic erythropoietin mRNA regulation in the rat. During very severe stimulation by anemia or hypoxia, hepatic erythropoietin mRNA accounts for 30% to 40% of the total body content; it is possible, therefore, that the organ ablation studies have underestimated hepatic erythropoietin production [73, 74]. This hepatic potential for erythropoietin mRNA synthesis was retained in rats rendered uremic by subtotal nephrectomy, even though, as in humans, hepatic erythropoietin production cannot support a normal hematocrit [79]. One possible explanation for this apparent paradox is that hepatic erythropoietin mRNA is inefficiently translated, but a more interesting explanation might lie in the different sensitivity of the organs to hypoxic stimuli. Examination of the separate responses of hepatic and renal erythropoietin mRNA in the rat to graded degrees of hypoxia or anemia indicate that both organs made some response even to mild stimulation, but that the quantitative characteristics of the response were different: the liver responded less well to mild stimulation than did the kidney [74] (Fig. 4). Thus, while the potential for hepatic erythropoietin production is preserved in severe renal disease, failure of anemia to be corrected might reflect different setting of the hepatic feedback response. A similar unrealized potential for erythropoietin production might be present in diseased kidneys. Disturbance of the normally well-organized vascular anatomy and reduced oxygen consumption arising from reduced transport work in renal failure [81–84] might shift the renal feedback response. Alternatively, local cytokine production [85, 86] might directly reduce erythropoietin gene expression. Studies

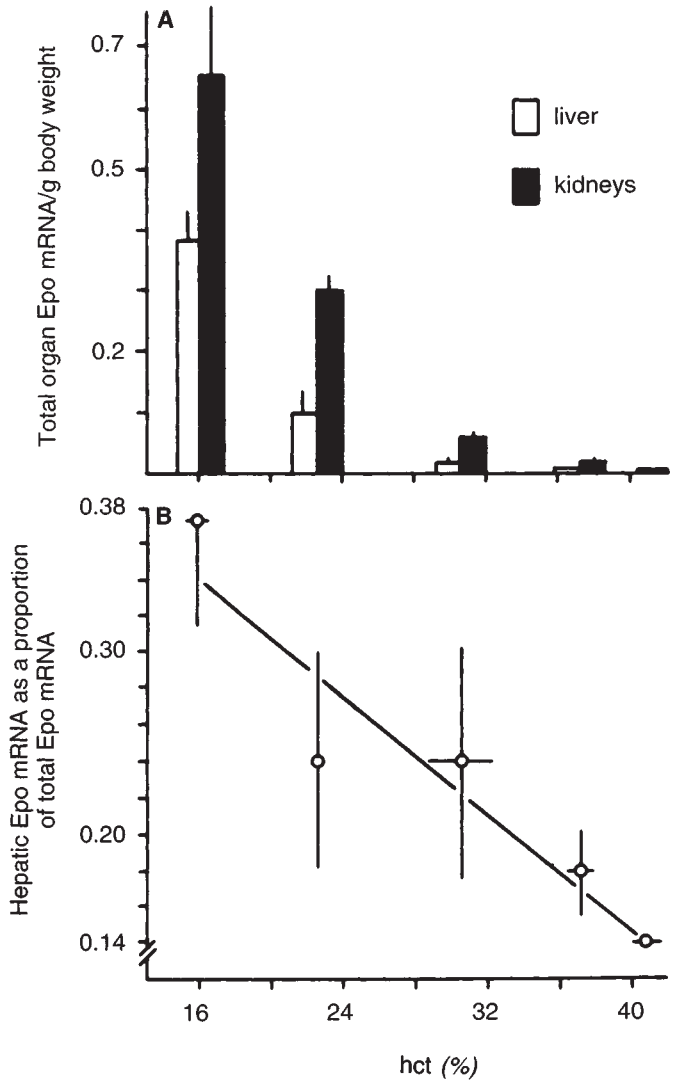

Fig. 4. *Stimulation of erythropoietin mRNA in the liver and kidneys of rats subjected to acute hemorrhage (8 hr) with saline replacement.* **A** Increases in total organ erythropoietin mRNA with progressively more severe anemia. Both organs show an exponential increase in erythropoietin mRNA with reduction in hematocrit. **B** Total hepatic erythropoietin mRNA as a proportion of the total (hepatic plus renal) erythropoietin mRNA. The hepatic contribution to the total is lower in less severe anemia (From Ref. 74).

in dialysis patients subject to intercurrent hypoxic stress demonstrate substantial increases in serum erythropoietin [87, 88] and support the view that a potential for erythropoietin production exists in many such patients, although it is not clear whether the liver or the diseased kidneys are the source.

Cellular site of erythropoietin formation

Using Northern blot analysis of RNA prepared from tubular and glomerular fractions of partially disaggregated kidneys from hypoxic rats, Schuster and colleagues found erythropoietin mRNA in the tubular fraction but not in the glomerular fraction [89]. This result has been confirmed by in-situ hybridization studies of rodent kidneys. One study has concluded that tubular cells contain erythropoietin mRNA [90], and in renal adenocarcinomas that produce erythropoietin, the epithelial cells themselves appear to contain erythropoietin mRNA [91]. Nevertheless, two groups using the higher resolution provided by ^{35}S labeling of DNA and RNA probes have convincingly demonstrated that erythropoietin mRNA is produced by cells lying between the renal tubules [92, 93]. Consistent with this result is the report by Eckardt and colleagues, who detected erythropoietin mRNA in interstitial cells within cyst walls of polycystic kidneys [23].

Two recent studies provide evidence that the source of renal erythropoietin is most probably a population of interstitial fibroblasts. Using a digoxigenin-labeled riboprobe, Bachmann et al observed co-localization of erythropoietin mRNA and immunohistochemical staining of a renal interstitial population with antibodies to 5′ ectonucleotidase [94]. We obtained a similar result using double immunohistochemical labeling of renal tissue from transgenic mice bearing an erythropoietin-SV40 T-antigen reporter transgene (I will return to this later). Although 5′ ectonucleotidase is present on many other cell populations, a fibroblast-like cell population is strongly and specifically positive within the renal interstitium [95, 96]. This fibroblast cell population, therefore, is the likely source of renal erythropoietin.

Of interest in relation to the regulation of erythropoietin production is the distribution of erythropoietin-producing cells within the kidney. In unstimulated rodent kidney, only occasional cells in the deep cortex or outer stripe of outer medulla are positive for erythropoietin mRNA. With increasing anemia, clusters of positive cells increase in number and spread from deeper cortical regions to superficial cortex [97]. In these kidneys, cells appeared to express erythropoietin mRNA in an all-or-none fashion; the major determinant of total renal erythropoietin mRNA was the number of positive cells [97]. Erythropoietin mRNA-producing cells are found within the cortical labyrinth and are rarely found in the medullary rays [98]. Interestingly, interstitial expression of 5′ ectonucleotidase is also confined within the cortical labyrinth. Expression of this antigen, although constitutive in mesangial cells and along the brush border of proximal tubular cells, is induced by anemia in these interstitial cells. As is the case with erythropoietin induction, the expression of 5′ ectonucleotidase is found not only in the deep but also in the superficial layers of the cortex as anemia becomes increasingly severe [99, 100]. Whether the 5′ ectonucleotidase activity plays any role in the regulation of erythropoietin is unknown, however, and differences exist in the temporal and spatial pattern of expression between the two molecules. First, the time course of induction of 5′ ectonucleotidase following anemic stimulation is significantly slower than that for erythropoietin [89, 99]. Second, whereas many erythropoietin mRNA-producing cells are observed in the outer stripe of the outer medulla in anemic rodents [97], interstitial 5′ ectonucleotidase is very weakly expressed in the interstitium of this region [95].

Since the lowest oxygen tension is in the renal medulla [101–103], it is clear that local oxygen tension is not the sole factor determining the distribution of erythropoietin-producing cells. Although it is tempting to speculate that, within the cortical labyrinth, progressive activation from deep to superficial regions reflects intrarenal oxygen gradients in anemia,

proof of this has not been obtained. Furthermore, since the renal cells that produce erythropoietin have not yet been studied in isolation, it is not clear whether they rely on intrinsic or extrinsic sensing mechanisms.

In-situ hybridization studies in mouse liver have identified two cell populations that produce erythropoietin [104, 105]. About 80% of cells detected were a subpopulation of hepatocytes; 20% had a non-epithelial morphology. In transgenic animals that overexpress a human erythropoietin transgene in hepatocytes, the distribution of erythropoietin mRNA was clearly centrilobular [104]; this finding is in keeping with known oxygen gradients within liver.

Intrarenal oxygenation

Experiments in isolated perfused kidneys, which exhibit oxygen-regulated modulation of erythropoietin mRNA levels [106] and of erythropoietin production [107, 108], demonstrate that all the events necessary for detection of hypoxia and production of erythropoietin can operate intrarenally, although not necessarily in the same cell. At first sight, the kidney might appear ill suited to playing a major role in hypoxic sensing. Renal blood flow and oxygen supply greatly exceed metabolic requirements, arteriovenous oxygen extraction being on the order of only 15% under normal circumstances. But it has long been appreciated that the kidney contains poorly oxygenated regions despite its overall high blood flow [109, 110]. The generation of intrarenal hypoxia has been considered in detail in relation to the susceptibility of the kidney to hypoperfusion injury [103] and I will only consider it briefly here. Of major importance is the countercurrent arrangement of renal blood vessels, which allows for the shunting of oxygen directly from arterial to venous limbs [110, 111]. This countercurrent exchange of oxygen is most marked in the renal medulla, where hypoxia is extreme, but it also operates in the renal cortex [112] and creates tissue pO_2 levels still well below those observed in the renal veins [102, 113]. Although the operation of such a system in the organ principally responsible for sensing blood oxygen availability is intriguing, the exact role of this system is unclear.

In another respect, the kidney might appear especially well suited to sensing reduction in blood oxygen content as distinct from changes in organ perfusion. Transport work accounts for the majority of renal oxygen consumption [81–83, 114]. Since more than 95% of the glomerular filtrate is reabsorbed, it follows that renal oxygen consumption should be closely determined by the glomerular filtration rate. Thus, parallel changes in renal blood flow and glomerular filtration rate might not disturb the balance of oxygen consumption and supply, and these changes might enable the kidney to respond to the change in blood oxygen availability arising from anemia without confounding effects from alterations in renal hemodynamics [115]. Some evidence that a reduction in tubular transport work might reduce erythropoietin production has been provided by studies in hypoxic mice of the effect of diuretic agents on erythropoietin production [116]. Whereas agents acting on distal tubular transport had no effect, acetazolamide reduced erythropoietin production. Acetazolamide acts primarily on the proximal tubule; it is thus of interest that the cells producing erythropoietin lie adjacent to proximal tubules [92]. Furthermore, when isolated kidneys are perfused with hyperoncotic bovine serum albumin at concentrations sufficient to oppose hydrostatic forces and prevent glomerular filtration, levels of erythropoietin mRNA are lower than those in filtering perfused kidneys receiving equivalent perfusate oxygen delivery [117]. In neither of these experimental settings, however, were direct measurements of intrarenal oxygen tension made, so it is not clear that improved renal oxygenation was the reason for the reduced erythropoietin production. Although failure of glomerular filtration in renal disease theoretically could reduce oxygen consumption, and hence reduce the hypoxic signals for erythropoietin production, it seems likely that the pathogenesis of erythropoietin deficiency is much more complex. It is striking that the intact isolated perfused kidney produces erythropoietin in a physiologically regulated manner [106–108], but there is as yet no convincing demonstration of erythropoietin production by an anatomically disrupted renal preparation. This raises the possibility that some aspect of intrarenal architecture is of key importance for erythropoietin production.

The oxygen-sensing mechanism

Many attempts have been made to define the nature of the oxygen sensor. Hypoxia cannot be mimicked by the application of inhibitors of mitochondrial respiration. Thus experiments in vivo [118], in hepatoma cells [119], and in isolated perfused kidneys [120] have shown that neither cyanide nor other inhibitors of the respiratory chain stimulate erythropoietin production. Failure of the erythropoietin gene to be activated in these experiments does not reflect nonspecific damage; in all these systems it is possible to observe stimulation by hypoxia in the presence of toxic concentrations of cyanide. These experiments suggest that the sensing system is distinct from cell stress responses triggered by nonspecific damage, and make it less likely that the hypoxic sensing system operates through metabolic derangements such as an alteration of phosphorylation potential and cellular redox potential, which would be expected to characterize both exposure to hypoxia and application of inhibitors of the respiratory chain [121].

Drawing from results of experiments which they performed on hepatoma cells, Goldberg et al proposed that the oxygen sensor is a specific heme protein that can bind reversibly to molecular oxygen [122] (Fig. 5). Their hypothesis is of considerable interest because of the widespread operation of heme and hemoproteins in oxygen-dependent regulatory processes in lower organisms [123–125]. Goldberg and colleagues noted that not only hypoxia, but cobalt, nickel, and manganese could stimulate erythropoietin production in human hepatoma cells and proposed that these ions could substitute for the ferrous ion in a putative heme protein and lock it in the deoxy form. Several experiments were performed that support this hypothesis. The behavior of carbon monoxide was studied because it specifically binds ferrous heme proteins [126]. Exposure to carbon monoxide greatly reduced the erythropoietin response to hypoxia but not to cobalt. This response, which argues against nonspecific toxicity, would be consistent with liganding of carbon monoxide to the ferrous heme protein, but not to cobalt protoporphyrin. Finally, when hepatoma cells were incubated with deferoxamine and 4,6-dioxoheptanoic acid to reduce heme synthesis, erythropoietin production in response to hypoxia, nickel, and cobalt also declined.

Although oxygen gradients exist within cells [127], there is no

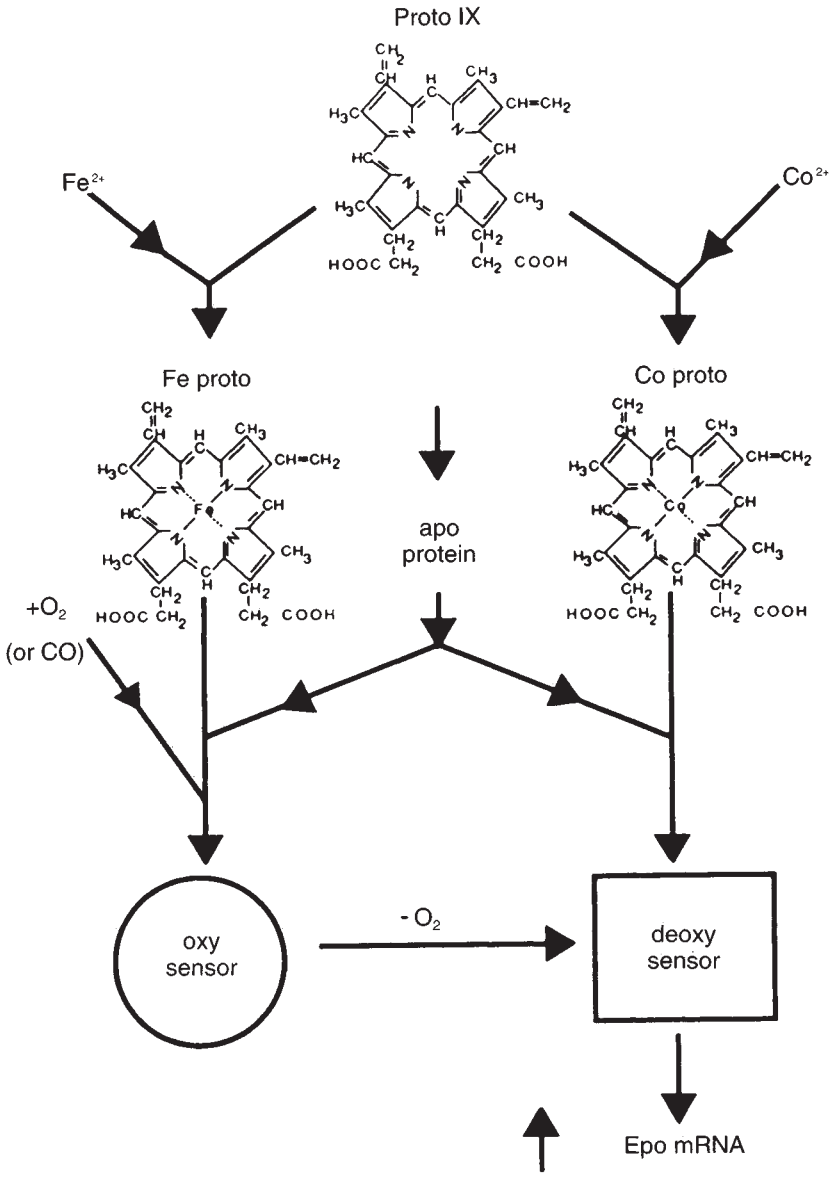

Fig. 5. *Model proposed by Goldberg, Bunn, and colleagues for the oxygen sensor in erythropoietin-producing cells.* It is proposed that the action of cobaltous ions in promoting erythropoietin gene expression arises from substitution for ferrous ions in the porphyrin ring of a putative heme protein, thus mimicking the de-oxy conformation. Carbon monoxide is postulated to reduce the hypoxic induction of gene expression by mimicking oxygen in liganding the heme moiety (From Ref. 191). See Ref. 122 for details.

compelling reason for siting oxygen sensing in any particular cellular location. It is thus plausible that oxygen could interact directly with nuclear proteins to induce DNA binding, or that it could modify proteins bound to DNA at the erythropoietin locus to induce transcription of the erythropoietin gene. The effects of cycloheximide, an inhibitor of protein synthesis, suggest that this is not the case. In hepatoma cells, cycloheximide blocks induction of erythropoietin gene expression [122]. It is difficult to exclude the possibility that some permissive effect is being abrogated by cycloheximide or even that the effect simply arises from toxicity. However, sensitivity of the inducing system to cycloheximide would be compatible with the requirement for new protein synthesis in a cascade of events leading to erythropoietin gene expression.

Coordination of erythropoietin gene expression

The rapid, high-amplitude induction of gene expression by such a fundamental physiologic stimulus as hypoxia has attracted interest in the regulation of erythropoietin as an interesting example of tissue-restricted and inducible gene expression. In the final part of this Forum, I would like to describe some selected experiments that address this issue.

Control of gene expression involves very complex interactions between DNA and nuclear proteins. A first step in understanding these mechanisms is the definition of *cis*-acting elements, sequences that are located on the same DNA molecule in the vicinity of the gene and that regulate gene transcription by coordinating the DNA-protein, protein-protein, and possibly DNA-DNA interactions [for review see 128, 129]. In mRNA, *cis*-acting sequences also interact with proteins to control splicing, transport, degradation, and rate of translation [130, 131]. Studies of *cis*-acting sequences provide the basis for identification and purification of the associated DNA- or RNA-binding proteins that mediate interaction between the signal transduction pathway and the gene [132, 133].

Many approaches are possible for identifying *cis*-acting sequences, but a particular problem in the analysis of erythropoietin gene expression has been posed by uncertainty about the nature of the renal interstitial cell that produces erythropoietin, and by the lack of a tissue culture cell line from this source. Therefore, I would first like to describe experiments in transgenic mice that give clues as to the mechanism of tissue restriction in erythropoietin gene expression [134–136]. Introduction of a test gene into the germ line of mice to create transgenic animals allows the effects of the presence or absence of *cis*-acting elements in the inserted transgene to be studied in vivo. The transgene is fully integrated into chromatin and, if sufficient DNA flanking the gene is included, all physiologic aspects of transcriptional control theoretically can operate [137, 138]. Such a model is of particular use in studying developmental and tissue-specific gene expression, because simpler systems do not always accurately reflect these influences.

Semenza and colleagues have described human erythropoietin gene expression in transgenic mice containing 4 different human erythropoietin transgenes [134–136]; 3 transgenes contained 0.7 kb of 3′ sequence but differed at the 5′ end in containing 0.4 kb, 6 kb, and 14 kb of 5′ flanking sequence. The fourth transgene contained 16.5 kb of 5′ sequence and 2.2 kb of 3′ sequence. The 0.4 kb transgene was widely expressed but was inducible only in liver; the 6 kb transgene was inducibly expressed in liver but was not expressed elsewhere; and the longest two transgenes were expressed in an inducible manner in the liver and kidney. This pattern led the authors to propose the existence of an element between 0.4 kb and 6 kb that represses expression in most tissues, and the existence of an element between 6 and 14 kb that permits or controls renal gene expression.

Such elements are believed to operate by binding tissue-specific patterns of transcription factors that interact with chromatin [for review see 139]. Interaction between bound proteins over large distances probably occurs by bending of DNA to permit direct protein-protein interactions [for review see 128, 140]. Sequences themselves can bind inducible factors or can operate permissively to control access of inducible binding proteins at other sites [139, 141]. Thus, the absolute requirement for a distant 5′ sequence for renal expression but not hepatic gene expression might imply the existence of different mechanisms of gene control in these organs. Or the 5′ sequence simply might permit the operation of local sequences in renal cells that are similar to those that operate in liver cells.

The achievement of erythropoietin tissue-specific gene expression in transgenic mice allows another approach to the identification of the erythropoietin-producing cells. If an identifiable reporter gene is placed behind the erythropoietin promoter, erythropoietin tissue-specific gene expression of the reporter gene might be produced. The use of an oncogene as a reporter might induce tumor formation and aid the setting up of cell lines from specific tissues [142, 143]. One such oncogenic product is the simian virus 40 large tumor antigen (SV40 T-antigen). Using a 16.5 kb mouse erythropoietin construct containing 9 kb of 5′ erythropoietin sequence fused to the SV40 T-antigen, my colleagues and I have produced several lines of transgenic mice that show regulated expression of T-antigen in the nuclei of a renal interstitial cell population (Fig. 6) [144]. Immunohistochemical detection of T-antigen in the nucleus can be combined with detection of cytoplasmic antigens in double labeling studies. These studies demonstrate co-localization with 5′ ectonucleotidase in many of the interstitial cells, providing strong evidence that it is the fibroblast-like renal interstitial cell population that produces erythropoietin.

Definition of oxygen-inducible control elements in tissue culture cells

In the absence of an appropriate renal cell line, two hepatoma cell lines, Hep3B and HepG2, which show oxygen-regulated erythropoietin gene expression [145], have been used widely for tissue culture studies of erythropoietin gene regulation. These experiments, together with studies using nucleoprotein extract from whole kidney and liver, indicate that erythropoietin gene control is probably the subject of several positive and negative interactions mediated through DNA sequences in the promoter [146–149], the 3′ untranslated region [150], and the sequence lying just 3′ to the poly A addition site [151–153]. To provide an example of the definition and functional analysis of one of these elements, I would like to describe the control sequence lying 3′ to the poly A addition site. To date, this is the most clearly defined and powerful *cis*-acting sequence controlling oxygen-regulated erythropoietin gene expression [151–153].

Transient transfection studies in human hepatoma cell lines have been important in the functional definition of this element. In this type of study, recombinant plasmid DNA containing the sequence under investigation is introduced into cells by a variety of techniques that permit DNA macromolecules to cross cell membranes and enter the nucleus, where they can interact with the nucleoproteins controlling gene transcription. Often a reporter gene is fused to the *cis*-acting element under study to provide a stable and easily measured product. Transfer of the regulatory property to the heterologous reporter gene also provides proof of independent operation of the control element [for review see 154].

Beck et al used transient transfection of a shortened human erythropoietin gene in Hep3B cells [151]. By making successive deletions, they demonstrated that a sequence within a 150 bp restriction fragment lying 120 bp 3′ to the human erythropoietin gene appeared to be responsible for mediation of the hypoxia-inducible transcription. The region defined in these studies corresponded with the 3′ portion of a 256 bp region of DNA shown by Semenza and colleagues to operate on the SV40 promoter in transfected Hep3B cells [152]. In a similar analysis of the mouse erythropoietin gene, using both α globin and the ferritin gene as reporters, Pugh et al found the active sequence to be located in an identical position 120 bp 3′ to the mouse erythropoietin gene [153] (Figs. 7A and 7B). This sequence has the classical features of a eukaryotic transcriptional enhancer in operating independently of orientation and distance on a variety of heterologous gene promoters. In addition to transducing hypoxic responsiveness, it is also responsive to cobalt [153], thus mimicking the physiology of erythropoietin gene activation and supporting the view that these stimuli interact on the same signaling mechanism [122].

As expected for a functionally important sequence, a high degree of homology between human and murine sequence was observed. Although the sequence homology extends over at least 140 bp, the minimal functional element required for

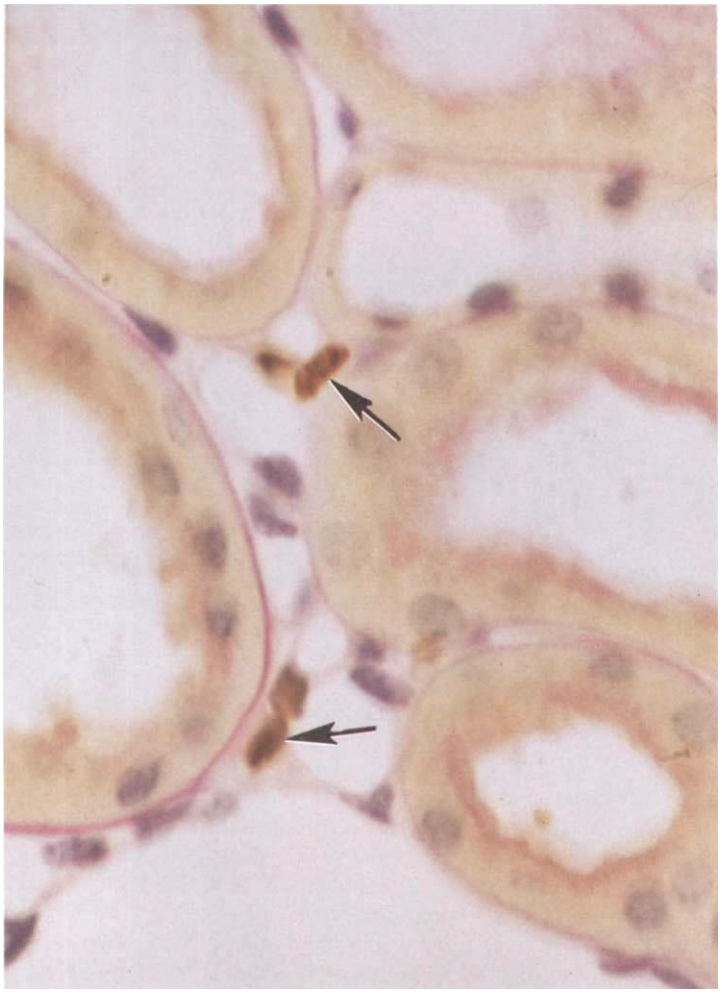

Fig. 6. *Photomicrograph of renal cortex from a transgenic mouse expressing an erythropoietin SV40 T-antigen fusion gene after anemic stimulation.* Erythropoietin sequence was used to direct expression of the viral oncogene T-antigen into the erythropoietin-producing cells. Cells expressing the transgene are demonstrated by immunoperoxidase using a rabbit antiserum against T-antigen (*arrows*). These cells lie within the renal interstitium. Additional double labeling studies (not shown) indicate that they do not bear endothelial or leukocyte markers but that a proportion express 5′ ectonucleotidase (Maxwell PH, Johnson M, Ratcliffe P, unpublished observations). They correspond to the fibroblast-like type-1 renal interstitial cells [192].

enhancer activity in transiently transfected cells is shorter and likely varies with the distance from the promoter on which it is operating. Approximately 60–70 bp were necessary when the enhancer element was placed 1.4 kb from an alpha globin promoter [153], whereas a 43 bp sequence was sufficient for enhancer function when placed close to the erythropoietin promoter or close to a herpes simplex virus thymidine kinase promoter [149]. The requirement for a more extensive DNA-protein complex for operation at greater distances and in chromatin is well established in other systems [139, 155]. It is probable that the more extensive sequence homology in this region indicates the need for more complex DNA-protein interactions for the operation in this sequence in vivo from its position 4 kb 3′ of the erythropoietin gene promoter.

The structure of chromatin is altered at sites of gene expression, reflecting the binding of transcription factors. These structural alterations change the sensitivity of the DNA to chemical modification or cleavage by nucleases and result in regions termed "hypersensitive sites." Thus important evidence that this 3′ enhancer does indeed function in vivo, at least in liver, is provided by the demonstration of DNase I hypersensitivity at this site in nuclei from liver [152]. More detailed analysis of the exact position of the binding proteins in vivo requires selection or amplification of the region of interest. This technique, known as in-vivo footprinting [156], has not yet been applied successfully to this sequence. However, nucleoproteins that bind to this region have been detected by the more commonly used in-vitro methods, in which nucleoprotein extract is bound in vitro to cloned DNA sequences. Bound proteins can be detected by retardation of a labeled DNA probe during electrophoresis. In addition, binding sites can be revealed as areas of protection from chemical modification or digestion by enzymes such as DNase I.

Using nucleoprotein extract prepared from Hep3B or HepG2 cells, an area of protection from DNase I digestion is observed in both human [149, 157] and mouse sequences [158]; this area lies within the minimal enhancer element defined in transfection assays. This protected area contains a direct repeat of a steroid/thyroid receptor-binding element half site. Blanchard and colleagues demonstrated the functional importance of this site by mutagenesis [149]. However, they were unable to demonstrate modulation of hypoxic induction by glucocorticoids, thyroxine, or a variety of other known ligands for members of this hormone receptor gene family. For many members of this receptor family, known as orphan receptors, the ligand is unidentified. The authors have proposed that such

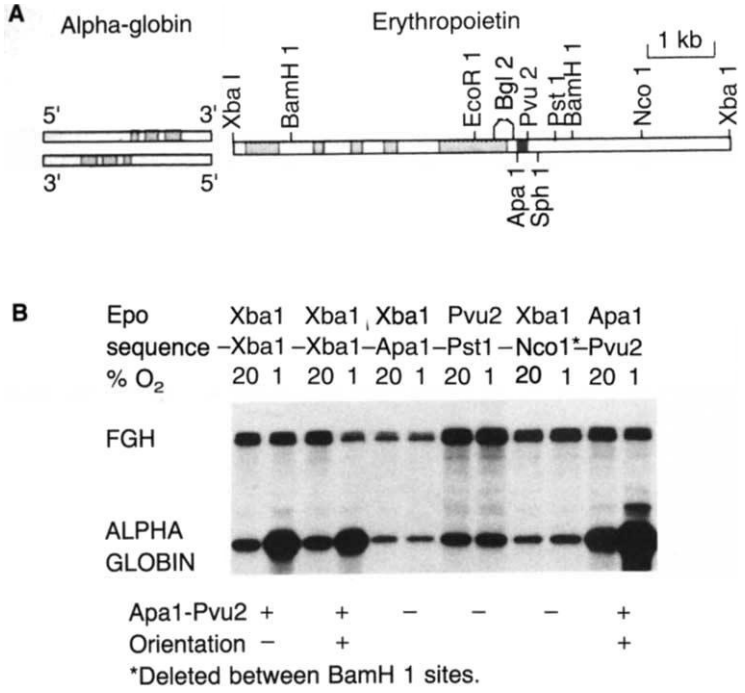

Fig. 7. *Example of experiments demonstrating* cis*-acting sequences that convey oxygen-regulated gene expression in transiently transfected tissue culture cells.* **A** Diagram of human α_1 globin-mouse erythropoietin fusion genes used to test mouse erythropoietin sequences for ability to convey oxygen regulation on the α globin reporter in HepG2 cells. Restriction sites used in making the deletions are indicated. Exons are marked by stippling. **B** RNAse protection assay showing reporter (alpha globin) and control (FGH) plasmid expression after transient transfection into HepG2 cells. Alternate lanes show normoxic and hypoxic expression. The erythropoietin gene fragments in the constructs are indicated above each lane. An Apa1-Pvu2 fragment lying at the 3′ end of the mouse erythropoietin gene (*indicated by the black box in* **A**) is shown to convey hypoxically induced expression independent of distance or orientation and therefore to contain a transcriptional enhancer (From Ref. 153).

a molecule might mediate hypoxic sensing. However, the region identified in these DNase I protection studies does not function as an oxygen-regulated enhancer in isolation, and deletional analysis has demonstrated that a sequence extending approximately 20 nucleotides 5′ to this site is also necessary for this action [149, 153, 157].

Mutational analysis of both the human [157] and mouse sequence [158] is consistent with this region containing two more distinct protein-binding sites. Furthermore, binding of a hypoxic-induced nuclear factor to the most 5′ of these sites has recently been demonstrated by gel retardation assays (Fig. 8) [157]. Thus the functional enhancer provides binding sites for at least three DNA binding proteins. At present it is unclear whether DNA binding proteins at all these sites interact with the hypoxic sensing mechanism or whether the hypoxic modification is confined to one particular transcriptional factor, with other proteins providing cooperative or permissive interactions.

One interesting aspect of this sequence, which has recently been explored by Drs. Maxwell and Pugh working in my laboratory, has been its oxygen-regulated operation when transfected into a wide variety of tissue culture cells. These cells include lines that do not make erythropoietin and are derived from organs such as skin, lung, and ovary, organs that do not contribute importantly to erythropoietin production in vivo (Fig. 9) [159]. It is well known that nucleoprotein interactions with transiently transfected DNA allow the promiscuous expression of tissue-specific genes. However, the demonstration that transfected erythropoietin enhancer activity was inducible by hypoxia in many different cells strongly suggests that a physiologically relevant component of the oxygen-sensing mechanism responsible for regulation of erythropoietin operates widely. The purpose of this widespread oxygen-sensing mechanism is unknown, but it is possible that the same oxygen-sensing system acts on other oxygen-responsive genes in cells that are not specialized for the production of erythropoietin.

For erythropoietin regulation, the 10- to 15-fold induction of transcription by the 3′ enhancer is less than the 300-fold changes in erythropoietin mRNA levels observed in vivo. This observation implies the operation of other control mechanisms. The 3′ sequence could interact with other transcription control elements to achieve much larger increases in the transcriptional rate. Evidence for cooperation of this sort has recently been obtained in Hep3B cells [149]. Transient transfection studies demonstrated that combination of the 3′ enhancer with the erythropoietin promoter produced a cooperative interaction capable of approximately 50-fold induction. Interestingly, sequence similarities were observed between the 3′ enhancer and the minimum inducible promoter.

Modulation of the steady-state levels of mRNA also could be achieved through changes in the rate of degradation of the mRNA superimposed on changes in the rate of gene transcription. Evidence that both mechanisms operate to control erythropoietin mRNA levels has been obtained by comparison of the amplitude of modulation of erythropoietin mRNA with direct measurement of transcriptional rate by nuclear run-on experiments [160]. Using nuclei from Hep3B cells, Goldberg and colleagues estimated that the increase in transcriptional rate during hypoxia was less than the increase in erythropoietin mRNA, implying the occurrence of changes in erythropoietin mRNA stability that are oxygen dependent. To demonstrate this directly, mRNA half-life must be measured under varying conditions of oxygenation. When Hep3B cells were switched from a hypoxic to a normoxic environment, steady-state erythropoietin mRNA decreased by 50% within 1.5 to 2.0 hours. Because new transcription was not blocked, this result represents a maximum estimate of the half-life in normoxic cells.

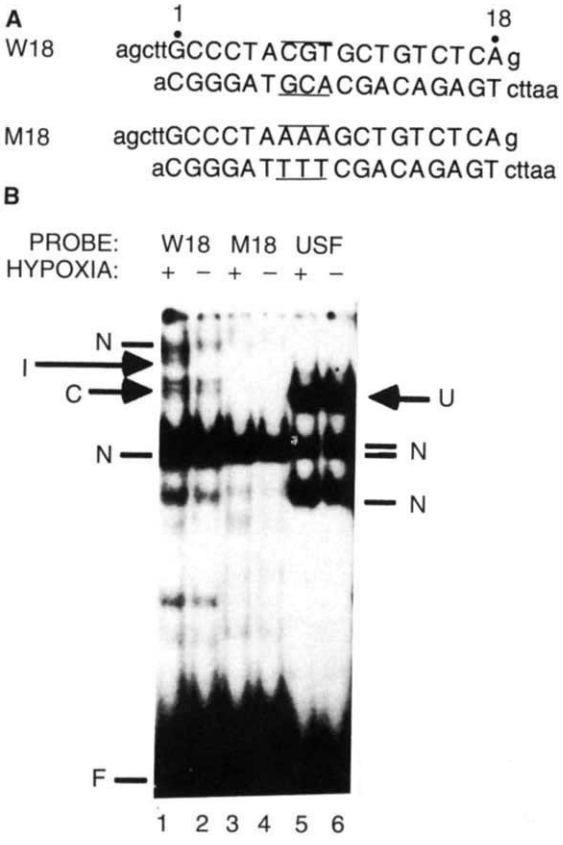

Fig. 8. *Gel-shift assay identifying a DNA-binding activity to the human erythropoietin 3′ enhancer, which is induced by hypoxia.* **A** Nucleotide sequence of wild-type (W18) and mutant (M18) double-stranded oligonucleotide probes. Nucleotides 1 to 18 from the hypoxia-inducible enhancer are shown in uppercase letters, with the 3-nucleotide site of mutation in M18 overlined and underlined. **B** Autoradiograph of a gel-shift assay demonstrating binding of an induced nuclear factor to the wild-type but not mutant probe. Nuclear extracts from Hep3B cells, cultured hypoxically (+) or normoxically (−), were incubated with W18 probe, M18 probe, or a control probe USF. The control probe USF binds a constitutively expressed factor and provides a control for normoxic and hypoxic extract comparison. Several binding activities are observed and are labeled as follows: N, nonspecific; C, constitutive; I, induced; U, USF; F, free probe. Hypoxically inducible binding activity was demonstrated to the wild type but not mutant probe. Specificity of this binding was shown in other experiments by competition with unlabeled mutant and wild-type probes (From Ref. 157).

However, when new transcription was blocked by actinomycin D, the half-life of erythropoietin mRNA surprisingly increased to approximately 8 hours and was, in fact, similar in normoxic and hypoxic cells [160]. This effect of actinomycin D itself on mRNA stability precluded measurement of the effect of oxygenation on mRNA stability by this method, but the finding is itself of interest. Similar observations have been made with a number of other genes [161, 162] and have been interpreted as indicating the existence of a specific ribonuclease that itself turns over rapidly.

An element in the 3′ untranslated region of erythropoietin mRNA, which binds cytoplasmic proteins, has been identified [163]. Although the binding activity of extract from brain was inducible by hypoxia, inducibility was not seen in extracts from liver or kidney, so the precise relation to erythropoietin regulation is unclear. Thus hypoxia likely increases erythropoietin mRNA stability in addition to activating transcription, but this is as yet unproven.

Questions and answers

DR. JOHN T. HARRINGTON (*Chief of Medicine, Newton-Wellesley Hospital, Newton, Massachusetts*): Perhaps I could start by asking about the evolutionary aspects of renal oxygen sensing and erthropoietin production. Where in the phylogenetic tree does the renal tissue oxygen sensor appear?

DR. RATCLIFFE: This is an interesting question. A circulating erythropoietic factor has been demonstrated by bioassay in a large number of species, including birds, amphibians, and fish [164–166]. However, there is no clear proof that these activities are due to an erythropoietin; cross-reactivity with mammalian erythropoietin is low or absent and, to my knowledge, biochemical or genetic analysis has not been performed. In some ways the responses resemble those of mammalian erythropoietin. For instance, in a study in fish, plasma erythropoietic activity was induced by bleeding but suppressed by starvation [166]. Nevertheless, important differences have been observed. Frogs produce an erythropoietic response to bleeding, but apparently not to stimulation with cobaltous ions or hypoxic atmosphere, and they do not become polycythemic during chronic hypoxia [164]. Lack of polycythemia in chronic hypoxia also has been reported in other cold-blooded species, such as turtles [167]. In contrast, birds appear to respond similarly to mammals in that they respond to both hypoxic atmosphere and to bleeding [165]. Whether this could indicate a different mechanism of oxygen sensing and whether the difference might be a fundamental one between cold-blooded and warm-blooded animals is unclear to me.

As to the kidney itself as the site of oxygen sensing, I am not aware of any evolutionary data. It is interesting, however, that in certain fish and larval amphibia, the kidney is a significant site of erythropoiesis with erythropoietic tissue lying between mesonephric tubules [168].

DR. ADRIAN S. WOOLF (*Lecturer, University College, London Medical School, London, England*): Is there any evidence that in the chronically hypoxic normal kidney the erythropoietin-producing cells proliferate?

DR. RATCLIFFE: In acute studies of rats and mice made increasingly anemic, in-situ hybrization studies demonstrate the rapid recruitment of what is almost certainly an existing population of cells within the kidney [97]. I am not aware of any in-situ hybridization studies in chronically anemic rodents that securely answer the question as to whether proliferation of the cells also occurs. Morphometric analysis of severely anemic rats' kidneys, 8 days after induction of anemia by phenylhydralazine and irradiation, has shown an increased interstitial volume, an increase in the abundance of the processes of the interstitial fibroblasts, and increased expression of 5′ ectonucleotidase by these cells [100]. However, mitoses were seldom seen in these cells, even in very severely anemic animals. Although this study did not examine erythropoietin production, the transgenic work from my own group and recent in-situ studies from others [94] indicate that it is this fibroblast-like cell population that produces erythropoietin.

DR. JOHN E. SCOBLE (*Consultant Nephrologist, Dulwich Hospital, London*): You suggested that the cells responsible for

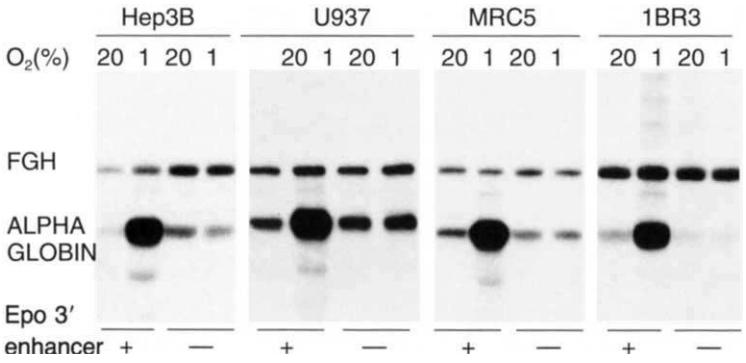

Fig. 9. *Comparison of the activity of the mouse erythropoietin 3′ enhancer in different cell types.* Autoradiograph shows expression of reporter (alpha globin) and control FGH transcripts in transiently transfected human cell lines Hep3B (hepatoma), U937 (monocyte/macrophage), MRC5 (lung fibroblast), and IBR3 (skin fibroblast). In each panel the first pair of lanes shows the response when the reporter plasmid contained the erythropoietin enhancer, and the second pair shows the response when the reporter plasmid did not contain the erythropoietin enhancer. Induction of enhancer activity by hypoxia is observed in all the cell types. Of these cells, only the hepatoma cells express the endogenous erythropoietin gene, suggesting that the oxygen-sensing mechanism might have other functions in the other cells (From Ref. 159).

erythropoietin production are renal fibroblasts, and you commented that renal fibroblasts grown in vitro do not produce erythropoietin. Is it possible that these fibroblasts in vivo are under some form of control by the cells surrounding them?

DR. RATCLIFFE: Yes, that is quite possible. Many unsuccessful attempts have been made to obtain erythropoietin from anatomically disrupted renal preparations. In contrast, in the first 90 to 120 minutes of isolated renal perfusion, we have observed up-regulation of erythropoietin mRNA that is similar to that induced by anemia in vivo [106]. This paradox might be explained if cellular cooperation were required for erythropoietin production. Such a possibility also might provide a mechanism for loss of erythropoietin production in renal diseases, where the intrarenal microenvironment is likely to be severely disrupted.

DR. VICTORIA CATTELL (*Senior Lecturer, Department of Experimental Pathology, St. Mary's Hospital Medical School, London*): It has been suggested that the erythropoietin-producing cells might be peritubular capillary endothelial cells [92, 93]. These cells would be ideally sited for sensing the oxygen content of blood. Also, the peritubular capillary obliteration in chronic renal scarring might explain the loss of erythropoietin production in chronic renal diseases. Can you be sure that the erythropoietin-producing cells are not endothelial? Have you performed any double-labeling immunohistochemical studies with endothelial markers?

DR. RATCLIFFE: Using our Epo-Tag transgenic mice, we attempted double-labeling studies with antiserum to T-antigen and to factor VIII antigen. Unfortunately, whereas large vessels stained well with factor VIII angtigen, interstitial capillary staining was weak and our result was inconclusive. Recently Dr. A. Vecchi and colleagues of the Mario Negri Institute in Milan kindly gave us a rat monoclonal antibody to the murine homologue of CD31. This antibody does stain the interstitial capillary endothelium strongly; double-labeling studies with this antibody indicate that the endothelial cell population is distinct from the cell population expressing T-antigen in the Epo-Tag mice. From this result we infer that erythropoietin itself is not produced by the endothelium. Your remarks are nevertheless very interesting; we cannot exclude the possibility that the endothelium plays a role in oxygen sensing or that other interactions with the endothelium are required for erythropoietin production.

PROF. JOHN WALLS (*Professor of Nephrology, Leicester General Hospital, Leicester, England*): You briefly mentioned demand in connection with the relationship of oxygen supply and blood supply within the kidney. Would you comment on whether this is a sensing system for O_2 concentration or utilization in view of the observation that diuretics alter erythropoietin levels and that clinical states of hypoxia also are associated with increased sodium reabsorption?

DR. RATCLIFFE: You mention an interesting point. I do not know the answer to your question at the molecular level. An oxygen sensor could respond directly to oxygen concentration or to some consequence of oxygen utilization, so that both possibilities are plausible, although the failure of cyanide to mimic hypoxia [118–120] suggests that reduction in mitochondrial oxygen utilization is not the signal. At the level of the cellular microenvironment, I think it is likely that oxygen utilization by adjacent tissues such as the renal tubule is a determinant of tissue oxygen concentration. Determination of whether this is important in regulating erythropoietin production will, I think, have to wait until we know more of the cellular site and molecular mechanism of oxygen sensing in the kidney.

PROF. ANTHONY E. G. RAINE (*Professor of Renal Medicine, St. Bartholomew's Hospital, London*): There are many responses to hypoxia other than those concerned with erythropoietin production, for example, hypoxic vasodilation. Is it known whether the presumed oxygen sensor involved in these situations is the same as that linked to production of erythropoietin?

DR. RATCLIFFE: The evidence for this is by no means conclusive. For instance, expression of platelet-derived growth factor β chain is increased by hypoxia in cultured endothelial cells [169]. As with erythropoietin expression in hepatoma cells, hypoxic induction is reduced by exposure to carbon monoxide. So circumstantial evidence suggests that the mechanism might be similar. Another gene that shows hypoxic induction with some similarities to erythropoietin gene expression is vascular endothelial growth factor [170]. Our demonstration that the erythropoietin 3′ enhancer can interact with an oxygen-sensing system in cell types that include endothelial

cells would also be consistent with the possibility that a similar mechanism might be involved, but we have as yet no direct evidence for this. Another possibility is that a similar oxygen-sensing mechanism might be involved in controlling glycolytic and gluconeogenic capacity in liver [171].

DR. JOHN DONOHOE (*Consultant Nephrologist, Beaumont Hospital, Dublin, Ireland*): You showed data indicating a fairly large potential for erythropoietin production in the liver. Might there be ways of augmenting this extrarenal capacity in renal failure?

DR. RATCLIFFE: Perhaps. It has long been recognized that during regeneration following injury the potential for hepatic erythropoietin production is increased. For instance, in one study in rats, hepatic erythropoietin production in response to hypoxia was increased about threefold during recovery from partial hepatectomy when compared with that observed in sham-hepatectomized animals [172]. The greatest erythropoietin response was observed at the time of maximum cell proliferation. However, I am unaware of any advances in our understanding of this phenomenon that could lead to more clinical methods of augmenting hepatic erythropoietin production.

DR. HARRINGTON: Returning to erythropoiesis itself, perhaps you could expand on what controls the expression of GATA-1. You moved on after calling it the master switch. I always like to know what regulates a master switch.

DR. RATCLIFFE: The control of GATA-1, although currently the subject of active research, is not clearly understood yet. The GATA-1 gene promoter contains GATA-binding sites that are functional in erythroid tissues [173]; some type of autoregulatory response is likely, but that does not answer the question as to what starts it all off.

DR. JOHN S. SAVILL (*Wellcome Senior Fellow, Hammersmith Hospital, London*): What types of molecules are candidates for oxygen sensors? Are they likely to employ iron atoms in a manner analogous to oxygen transport proteins?

DR. RATCLIFFE: That possibility is supported by the experiments by Goldberg, Dunning, and Bunn on Hep3B, which I outlined [122]. They have proposed the existence of a hemoprotein sensor that reversibly ligands molecular oxygen. Such a model is by no means proven, but there are examples of this mechanism in other systems. For instance, fixL, which operates in control of the oxygen-sensitive nitrogen fixation genes of *Rhizobium meliloti*, has recently been cloned, expressed, and shown to be a hemoprotein operating in this way [123].

A great many other types of biochemical interactions with oxygen could potentially provide sensing informaton, however. Iron atoms can operate in a large variety of oxido-reduction reactions. Redox systems, of course, would not necessarily involve iron. For instance, recent studies indicate that the DNA-binding activity of transcription factors such as AP-1 and NF-kB is subject to control by oxido-reduction status, and Toledano and Leonard have proposed that reactions with protein sulfhydryl groups could mediate this change in activity [174]. Unfortunately the list of candidate oxygen sensing molecules is not a short one.

DR. ANAND K. SAGGAR-MALIK (*Research Fellow, St. George's Hospital, London*): Could you comment on what is specific about polycystic kidney disease that allows erythropoietin levels to be maintained compared to other renal diseases? Can this model be used to study the oxygen-sensing mechanism?

DR. RATCLIFFE: That is an interesting question. As I said, within the cyst wall, it appears that stromal cells resembling those identified in rodent kidneys contain erythropoietin mRNA [23]. Why they should be able to function in that environment, but less well in other anatomically disrupted kidneys, I do not know. Interestingly, long-term dialysis patients can develop acquired cystic disease and sometimes increase their hematocrit [175, 176]. This apparently erythropoietin-dependent response [177] suggests that increased erythropoietin formation is not related directly to the genetic defect but is in some way a consequence of cyst formation. Perhaps this is simply a question of local hypoxia arising from vascular distortion, but I suspect it will be more complex and more interesting than that.

DR. CHARLES R. V. TOMSON (*Consultant Nephrologist, St. Bartholomew's Hospital*): Would you comment on the possible influence of the renin-angiotensin system on renal and extrarenal erythropoietin production?

DR. RATCLIFFE: I can comment on some of the published observations, but I cannot explain them. A number of early studies demonstrated that infusion of angiotensin II could increase erythropoietin as measured by bioassay [178, 179], although not all experiments showed this effect [180]. It is tempting to suggest that angiotensin operates by inducing renal hypoxia, but in one of these studies, to which I think you are alluding, the effect was observed in nephrectomized animals. This suggested that extrarenal production of erythropoietin also might be augmented by angiotensin II [181]. More recently it has been observed that angiotensin-converting-enzyme inhibitors can ameliorate post-renal-transplant erythrocytosis [182, 183]. The coincident reduction in serum erythropoietin indicates that the effect most probably is due to inhibition of erythropoietin production [184]. Again, this might simply be due to relief of renal hypoxia by vasodilation. It is nevertheless puzzling that the source of erythropoietin in post-transplant erythrocytosis appears most commonly to be the native kidneys [185], where one might not expect well-preserved vascular responses.

DR. DRAGAN LJUTICE (*ISN Clinical Fellow, Guy's Hospital, London*): Is there any connection between erythropoietin production and circulating hormones other than the renin-angiotensin system, specifically atrial natriuretic factor?

DR. RATCLIFFE: I am aware of published work on this, but I would like to refer your question to Professor Raine, who has worked on ANF for some time.

PROF. RAINE: Yes, a 1990 paper described increased erythropoietin production by human renal carcinoma cells in response to atrial natriuretic factor [186]. Because supraphysiologic concentrations were used, the relevance of this finding to erythropoietin production in vivo is uncertain. We have measured serum erythropoietin in a double-blind study in which we administered ANF or placebo to renal transplant recipients, and we saw no effect of ANF on serum erythropoietin [187].

DR. WOOLF: In view of the fact that a kidney (epithelial) tumor cell line produces erythropoietin, what information is known about erythropoietin production in the developing undifferentiated kidney?

DR. RATCLIFFE: The developing kidney does produce erythropoietin [71], but I know of no studies that have established the

cellular source of erythropoietin in the developing kidney, nor how this cellular source might correlate with the process of differentiation. Of course, erythropoietin production by Wilms' tumor is a well-recognized phenomenon [188], but I am not at all clear as to how this relates to erythropoietin production during normal renal differentiation.

DR. CHRISTOPHER G. WINEARLS (*Consultant Nephrologist, Oxford, England*): You drew our attention to the similarities between erythropoietin and other growth factors. Do they also act by preventing programmed cell death following liganding of the receptor, as Dr. Bondurant recently suggested for erythropoietin?

DR. RATCLIFFE: That is the case, but perhaps I could ask Dr. Savill to comment more specifically.

DR. SAVILL: Yes, apoptosis is activated in many systems after withdrawal of growth factors, and these include other hemopoietic lineages [189]. It may in fact be that separate signals exist for preventing apoptosis and inducing cell proliferation. Some evidence that this is the case with liganding of the erythropoietin receptor and erythropoiesis has recently come from the study of what appear to truncated forms of the erythropoietin receptor [190]. In these experiments, cells transfected with different forms of the erythropoietin receptor were assessed for proliferative responses to erythropoietin. Transfectants carrying the full-length erythropoietin receptor and those carrying the truncated erythropoietin receptor proliferated equally in high concentrations of erythropoietin, but the cells bearing the truncated erythropoietin receptor were more susceptible to apoptosis. No difference between the transfectants in the binding affinity for erythropoietin was observed, and the authors have proposed that a distinct region of the cytoplasmic portion of the erythropoietin receptor could be responsible for signals that prevent apoptosis.

DR. HARRINGTON: Would you speculate on ways in which the new information on the molecular biology of erythropoietin might be applied to clinical medicine?

DR. RATCLIFFE: It is always difficult to predict the application of advances in basic science, but I will try. Obviously we hope that definition of the oxygen-sensing mechanism will have medical applications way beyond the field of erythropoietin itself, but it is far too early to consider what these might be. Perhaps a more immediate possibility lies in further consideration of the molecular interaction of erythropoietin with its receptor. Such an interaction recently has been studied at the crystallographic level for growth hormone and its receptor [49, 50]. This type of study could provide the basis for the design of erythropoietic drugs that could be easier to administer or cheaper to produce than erythropoietin itself.

Note added in proof

The hypoxically inducible DNA binding activity shown in Figure 8 has recently been demonstrated in multiple cell types [193, 194] and supports the functional demonstration of the widespread oxygen-sensing system shown in Figure 9.

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