Blood Cells, Molecules, and Diseases (2001) **27**(5) Sept/Oct: 830–843
doi:10.1006/bcmd.2001.0457, available online at http://www.idealibrary.com on IDEAL®

The Bigger the C-Value, the Larger the Cell: Genome Size and Red Blood Cell Size in Vertebrates

Submitted 07/22/01
(Communicated by J. Hoffman, M.D., 08/28/01)

T. Ryan Gregory[1]

ABSTRACT: Vertebrate genome sizes vary roughly 350-fold and correlate with a variety of cellular and organismal parameters. Most notable among these is the relationship between genome size ("C-value") and red blood cell (RBC) size, which can be identified within and among each of the five vertebrate classes. This relationship, in turn, leads to important associations between genome size and features such as metabolic rate (at least in homeotherms). The present article describes the correlation between genome size and RBC size in vertebrates and discusses some of the cytological, physiological, and evolutionary implications of this relationship.

INTRODUCTION

The term "genome" was coined in 1920 by H. Winkler as a hybrid of "gene" and "chromosome," and was meant to simultaneously signify the full set of chromosomes and all the genes contained therein. Decades later, as light was shed on the structures and functions of both genes and chromosomes, it became increasingly apparent that one could not have it both ways—total chromosome content and number of genes are not interchangeable. By the early 1950s, interest had begun to grow regarding the actual amount of DNA contained within the chromosomes of different species. This was based in large part on the report by Vendrely and Vendrely (1) of "a remarkable constancy in the nuclear DNA content of all the cells in all the individuals within a given animal species" [my translation], which they took as evidence that DNA, and not protein, was the hereditary material. A short time later, Mirsky and Ris (2) undertook the first systematic examination of DNA content variation among animals, and found it to be unrelated to intuitive notions of organismal complexity. As Comings (3) later put it (and rather bluntly at that):

> "Being a little chauvinistic toward our own species, we like to think that man is surely one of the most complicated species on earth and thus needs just about the maximum number of genes. However, the lowly liverwort has 18 times as much DNA as we, and the slimy, dull salamander known as *Amphiuma* has 26 times our complement of DNA. To further add to the insult, the unicellular Euglena has almost as much DNA as man."

Thus, it appeared that C-values—a term coined in 1950 by Swift (4) in reference to the haploid "class" of DNA content, now used interchangeably with "genome size" in diploid animals—though constant because DNA is the stuff of genes, were totally unrelated to gene number. This confusing situation became known as the "C-value paradox" in the early 1970s (5).

Unlike most problems in biology, the C-value paradox had a very simple solution. Namely, not all DNA is genes. In fact, most of the (eukaryotic) DNA in the biosphere consists of repetitive sequences with no protein-coding function (and indeed, no immediately obvious function of any kind). Despite this, the long-outdated term "C-value paradox" continues to enjoy widespread use. As an alternative, it has recently been suggested that the remaining puzzles relating to the origin(s), mechanism(s) of spread and loss, and cytological/phenotypic/evolutionary implications

Correspondence and reprint requests to author. Fax: (519) 767-1656. E-mail: rgregory@uoguelph.ca.
[1] Department of Zoology, University of Guelph, Guelph, Ontario, Canada N1G 2W1.

1079-9796/01 $35.00

Blood Cells, Molecules, and Diseases (2001) **27**(5) Sept/Oct: 830–843
doi:10.1006/bcmd.2001.0457, available online at http://www.idealibrary.com on IDEAL®

of this non-coding DNA be given the more appropriate moniker "the C-value enigma" (6, 7).

The C-value enigma, unlike the earlier "paradox," is a complex puzzle falling under the purview of many biological disciplines, including red blood cell physiology. As part of their pioneering survey of animal genome sizes, Mirsky and Ris (2) reported that "in the nucleated red cells of vertebrates . . . there is an approximately direct relationship between cell mass and DNA content." This observation has been confirmed repeatedly over the past half-century, and the relationship is now known to exist both across vertebrates at large and within each vertebrate class taken individually. The biological implications of this association between DNA content and red blood cell (RBC) size have not always been appreciated, but may in some cases be of substantial import in the context of cellular and organismal physiology. In turn, these physiological effects have likely exerted a significant influence on the evolution of genome size among vertebrates.

GENOME SIZE AND RBC SIZE

Perhaps the simplest demonstration of the relationship between DNA content and cell size is that provided by polyploidy. In this case, entire chromosome sets are duplicated such that differences in DNA content among species are large and easily quantified. Polyploidy is extremely rare in mammals, and non-existent in birds, but cases abound of polyploid fish and amphibians. In these animals, polyploidy has long been known to result in larger epidermal and blood cells (e.g., 8, 9), and today erythrocyte size is often used as a means of identifying polyploid individuals (e.g., 10, 11).

Shifts in haploid genome sizes are usually much more subtle than changes in ploidy levels, but there is nevertheless a great deal of variation among taxa. In vertebrates alone, C-values vary more than 350-fold. (Even more impressive is the genome size range among protists, which exceeds 300,000-fold!). Early comparisons had suggested that in vertebrates at large this variation in genome size is correlated positively with erythrocyte size (e.g., 2, 12–14). However, most of these data came from reptiles, amphibians, and some fish, while the homeothermic classes had been all but ignored until very recently. In the following sections, the relationship between genome size and RBC size is examined for each vertebrate class taken separately, since RBC size has not been quantified in a uniform fashion in all classes, and since the biological implications may vary from group to group.

Fish

Fish represent, by a considerable degree, the dominant vertebrate class. They also display both the smallest (pufferfish *Takifugu rubripes*, 1C $\approx$ 0.4 pg) and largest (marbled lungfish *Protopterus aethiopicus*, 1C $\approx$ 130 pg) genomes among vertebrates. In total, genome sizes are currently known for about 900 fishes—about 3% of described species—which represents the largest dataset for any vertebrate group. Yet it is in fish that the relationship between genome size and erythrocyte size is so far the least well established. Small surveys of fish species have revealed the general trend (e.g., 15–17), and Olmo's (14) classic study found fish to fit well on the overall vertebrate regression, but recent (apparent) counterexamples have placed some doubt on the correlation among fish.

As part of their survey of genome and cell sizes in 23 species of cartilaginous fishes of the Superorder Batoidea (ranging in C-value from 2.5 to 12 pg), Chang *et al.* (18) reported that no relationship existed between nuclear DNA content and RBC size. In this case, cell volume was not reported directly but was instead measured in relative terms compared to chicken erythrocytes, with most values falling between 0.5 and 1.5 chicken units. As has been pointed out elsewhere, some amphibians with genome sizes of 12 pg have *nuclei* larger than entire chicken RBCs (7), such that the conclusions of Chang *et al.* (18) seem highly dubious. Most likely, the lack of relationship in this study merely reflects difficulties of measurement.

In an even more recent study, Lay and Baldwin (19) present data for nuclear and cell volumes for 52 tropical species of teleost fish and conclude that no significant relationship exists between

these two parameters. But once again, this purported counterexample may simply reflect problems with the chosen methodology. Specifically, Lay and Baldwin (19) measured *wet* cell volumes by dividing hematocrit by cell counts, and compared this against *dry* nuclear "volumes" calculated from two-dimensional measurements of Feulgen-stained nuclei. The error inherent in this comparison aside, these authors assumed a three-dimensional ellipsoid shape of nuclei when calculating nuclear volumes from length and width measurements—clearly an unjustified assumption given the profound flattening that occurs when blood smears are dried. Unfortunately, genome sizes are not known for the species studied by Lay and Baldwin (19), so a more direct comparison of the relationship between cell size and DNA content is not yet possible for this group.

What is needed is a detailed analysis—using consistent measures of nucleus, cell, and genome sizes—of the relationship in fish. As a preliminary demonstration based on currently available data, Fig. 1A shows the relationship between genome size and dry cell area in roughly 50 species of fish, while Fig. 1B presents the correlation between dry nucleus area and dry cell area from 32 species. Clearly, the relationship appears to hold very well over a 175-fold range in genome size among a broad sample of agnathans, chondrichthyes, teleosts, and dipnoans. For the time being, those with remaining doubts regarding the association in fish are directed to Fig. 2, which shows Feulgen-stained erythrocytes from the Siamese fighting fish (*Betta splendens*, 1C $\approx$ 0.6 pg) arranged alongside those from the Australian lungfish (*Neoceratodus forsteri*, 1C $\approx$ 50 pg).

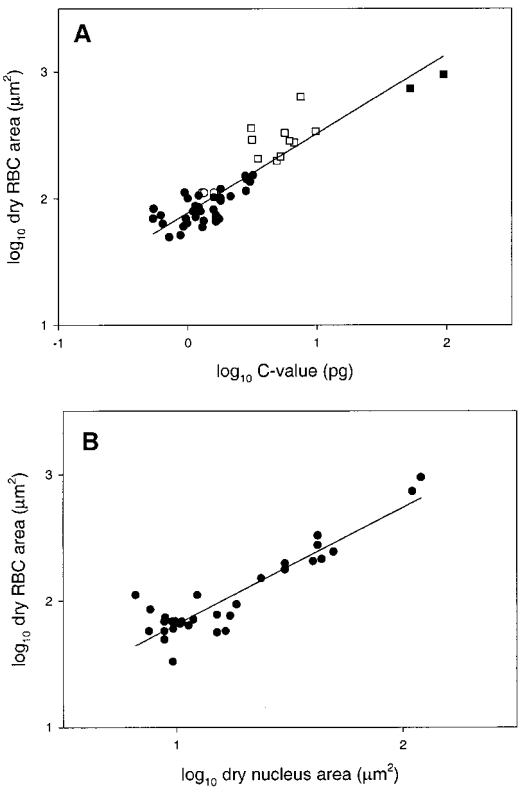

FIG. 1. Preliminary demonstration of the relationship between erythrocyte size (measured as dry elliptical area) and (A) genome size and (B) nucleus size (dry area) in fish. These relationships are highly significant (all $r^2 > 0.82$, all $P < 0.0001$), and extend across agnathans (○), chondrichthyes (□), teleosts (●), and lungfishes (■). Cell and nucleus size data from Refs 27, 47, and 48. Genome size data from Ref. 24.

Amphibians

The genome sizes of amphibians vary enormously in size, from the diminutive C-value of the myobatrachid frog *Limnodynastes ornatus* (1C = 0.95 pg) to the giant genomes of aquatic urodeles of the genus *Necturus* (1C $\approx$ 120 pg). Throughout this range, genome size is strongly associated with cell size—an observation apparent regardless of how cell size is measured, be it as dry volumes (14, 20, 21), dry areas (13), or wet volumes (22). Nucleus size is also positively related to genome size in this group (13, 14, 20, 21).

As variable as they are, the genome sizes of amphibians are not distributed at random. In fact, there is very little overlap between the genome sizes of frogs (Order Anura, 1C = 0.95 to 19 pg) on the one hand and those of salamanders (Order Urodela, 1C = 13 to 120 pg) on the other. Possible reasons for this will be discussed briefly below, but it is worthwhile to note that the relationship with cell size spans this difference and remains significant in amphibians at large (Fig. 3A). Nevertheless, slight variation in the exact nature of the relationship, specifically the slope of the regression, can be detected in frogs versus salamanders (Fig. 3A). Many explanations have been proposed for the large genomes and cell

Blood Cells, Molecules, and Diseases (2001) **27**(5) Sept/Oct: 830–843
doi:10.1006/bcmd.2001.0457, available online at http://www.idealibrary.com on IDEAL®

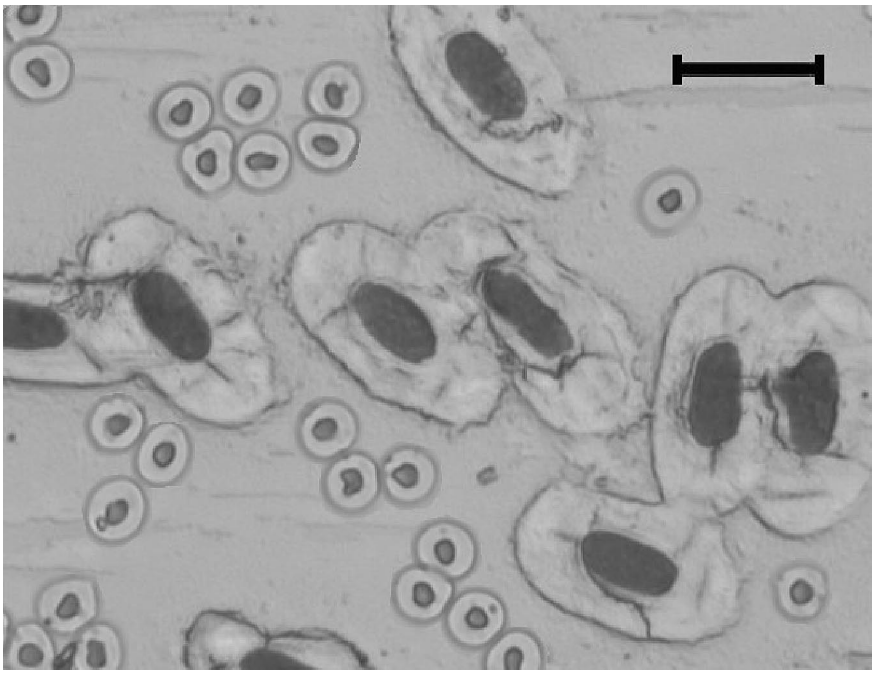

FIG. 2. Photomicrographs of Feulgen-stained erythrocytes from the Siamese fighting fish (*Betta splendens*, 1C ≈ 0.6 pg) and the Australian lungfish (*Neoceratodus forsteri*, 1C ≈ 50 pg), which has a genome roughly 100 times larger. When viewed side-by-side, these cells provide a rather extreme demonstration of the relationship between genome size and erythrocyte size. Photographed at 40× magnification. Scale bar = 20 μm.

sizes of salamanders, but so far these have been unsatisfactory (as discussed below).

Reptiles

Though his large survey included representatives of all five vertebrate classes, 3/4 of the nearly 240 species studied by De Smet (13) were reptiles. Within this class, as with the amphibians, De Smet (13) found a highly significant positive correlation between genome size and dry cellular and nuclear areas. A year later, a similar correlation was reported by Olmo and Odierna (23) based on dry cell volumes (Fig. 3B). Unlike with the amphibians, both De Smet's (13) and Olmo and Odierna's (23) studies showed no difference in the nature of the relationship among orders of reptiles (Fig. 3A vs Fig. 3B). And although snakes and lizards have slightly lower genome sizes on average than turtles (24), overall genome sizes in the group range only about fivefold (1C = 1.1 to 5.4 pg).

Birds

Birds are diametrically opposed to amphibians when it comes to genome size variation. Throughout the entire sample of birds measured to date—including roughly 160 species from 17 different orders—variation in genome size is only about twofold (1C = 1 to 2.1 pg; Ref. 24). Given this extraordinarily narrow range in available data, it is perhaps not surprising that a relationship with cell size has been difficult to establish in this class. In fact, until recently very few authors had even attempted to demonstrate such a relationship in birds. Olmo's (14) classic survey, for example, contained only four bird species. In the few available cases, data from birds were found to fit well on the overall vertebrate line (e.g., 12–14), but within the class the relationship remained unexamined. That is, until a recent analysis of a wide range of bird species revealed that dry erythrocyte area is indeed positively correlated with both nucleus and genome size in birds (25; Fig. 3C). Thus, even in the most narrowly-ranging group of vertebrates with nucleated RBCs, the relationship between genome size and cell size can be identified.

Mammals

Mammals are unique among vertebrates in the possession of non-nucleated erythrocytes. In other

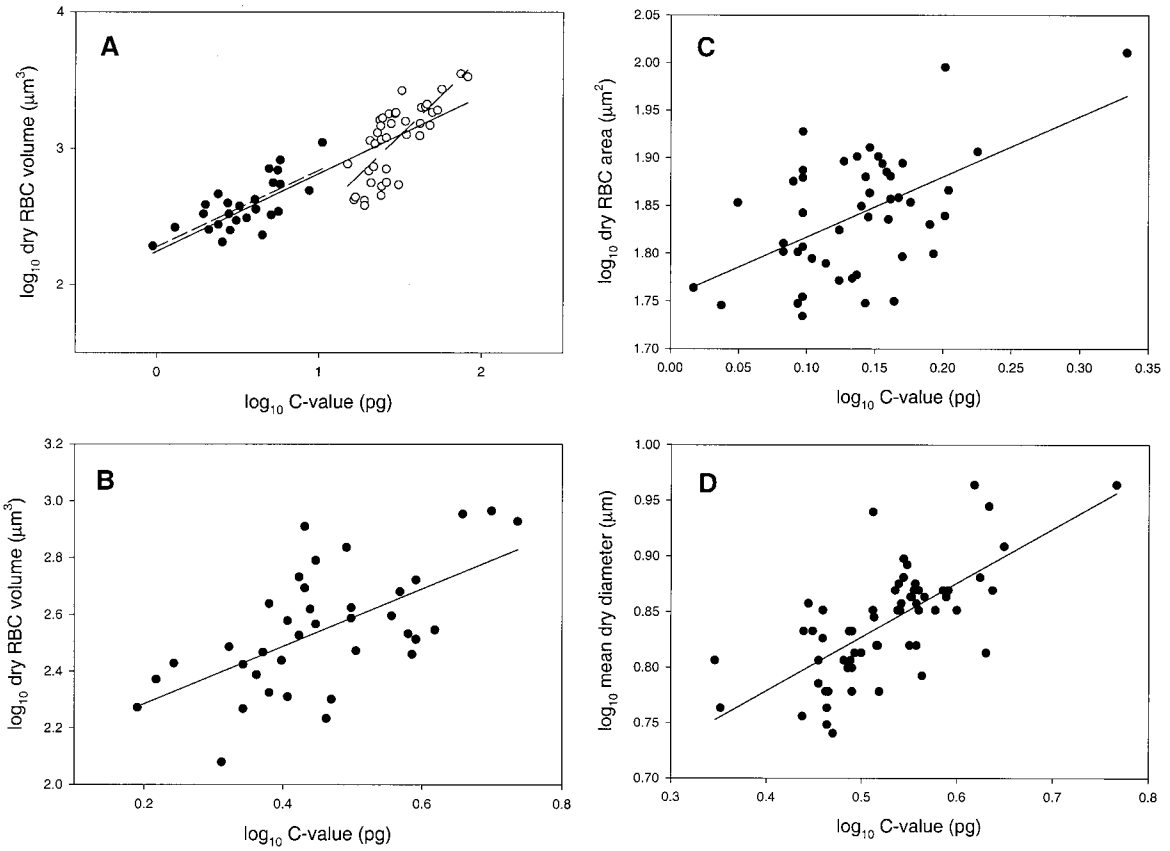

FIG. 3. The relationship between genome size and erythrocyte size as previously shown for vertebrate classes other than fish. (A) Amphibians. The relationship is highly significant for frogs (●) and salamanders (○) taken together (solid line, $r^2 = 0.72$, $P < 0.0001$), but appears somewhat more strongly allometric in urodeles (large-dashed line, $r^2 = 0.58$, $P < 0.0001$) versus anurans (small-dashed line, $r^2 = 0.51$, $P < 0.0001$). RBC size measured as dry cell volume. Data from Refs. 20 and 21. (B) Reptiles. The relationship is highly significant ($r^2 = 0.37$, $P < 0.0001$). RBC size measured as dry cell volume. Data from Ref. 23. (C) Birds. The relationship is highly significant ($r^2 = 0.28$, $P < 0.001$), even though genome sizes in this class range only about twofold. There is a significant negative correlation between genome size and resting metabolic rate in this class. RBC size measured as dry cell area. Data from Ref. 25. (D) Mammals. The relationship is highly significant ($r^2 = 0.48$, $P < 0.0001$) despite the fact that mature mammalian RBCs do not contain nuclei. This analysis does not include members of the order Artiodactyla, which display highly atypical erythrocyte shapes (see Fig. 4, aka "Ruminantia"). There is a significant negative correlation between genome size and basal metabolic rate in this class. RBC size measured as dry cell diameter. Data from Ref. 7, with additional cell size data from Ref. 47 and genome size data from Ref. 24.

words, mature RBCs in mammals are DNA-free. So in some respects (and indeed, under some theories), a relationship between genome size and RBC size would not be expected in this class. The first hint at such a relationship came with the discovery of the only known polyploid mammal, the desert-dwelling red viscacha rat (*Tympanoctomys barrerae*), in 1999 (26). As a result of a polyploidization event, DNA contents in this species were found to be twice as high as in their diploid relatives. The diameters of *T. barrerae's* nucleated cells (e.g., hepatocytes) were larger by a factor of roughly 15–20% compared to related diploids. So, although not strictly proportionate to their larger DNA content, nucleated cells in this mammal are indeed larger as a result of polyploidy. Even more surprising was the fact that enucleate erythrocytes of this species were also about 15% larger than the rodent average (dry diameters approximately 8.3 μm vs 7.2 μm; T. R. Gregory, unpublished).

A more detailed evaluation of the relationship between dry erythrocyte diameter and genome size in nearly 70 species of mammals later

Blood Cells, Molecules, and Diseases (2001) **27**(5) Sept/Oct: 830–843
doi:10.1006/bcmd.2001.0457, available online at http://www.idealibrary.com on IDEAL®

showed that despite their lack of nuclei, the sizes of mammalian RBCs are strongly positively correlated with genome size (6; Fig. 3D). The single exception to this observation occurred with the artiodactyls (even-toed ungulates including cattle, deer, and their relatives), the omission of which further strengthened the observed correlation. Members of this order display all manner of bizarre erythrocyte morphologies, and have clearly evolved a mode of RBC development substantially different from the mammalian norm. For example, the typical mammalian biconcave disk is replaced by small and highly ovaloid cells in llamas and camels (family Camelidae), by tiny spherical cells in mouse deer (family Tragulidae), and by cells which, according to Gulliver's (27) description, "assume fusiform, lanceolate, crescentic, and irregularly polygonal and other angular forms" in red deer and wapiti (family Cervidae). Many of these divergent artiodactyl erythrocyte shapes are featured in Fig. 4 ("Ruminantia" in Gulliver's older terminology).

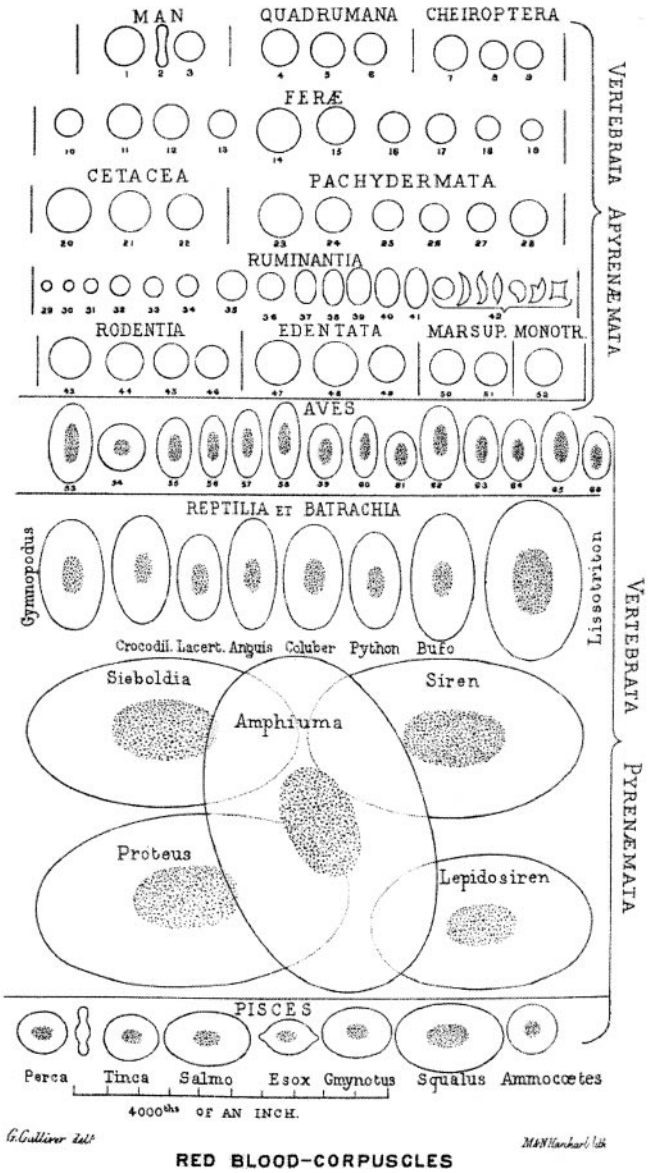

FIG. 4. The nucleocytoplasmic ratio, as demonstrated by the figure published by George Gulliver in 1875 (Ref. 27). Note the immense size range in vertebrate erythrocytes, and the clear association between cell and nucleus size. Note also that mammalian erythrocytes, which do not contain nuclei, are considerably smaller than those of most other vertebrates.

Summary

As is apparent from the brief review given above, the relationship between genome size and erythrocyte size is detectable in each of the vertebrate classes, even in the uniquely enucleate case of mammals. In general, this relationship is visible regardless of the measurement parameters used as a proxy for "size," be it wet or dry cell volume, area, or diameter. However, since these measures are not interchangeable, comparisons among classes can be difficult. Nevertheless, it is possible to draw some conclusions regarding the nature of the correlation as viewed among vertebrates at large.

As noted above, the exact slope of the relationship differs somewhat between frogs and salamanders, with the cells of the latter increasing more slowly in size with increased DNA content. In a broader comparison, De Smet (13) noted that amphibians and reptiles in general have larger cells per unit DNA than birds (based on differences in y-intercepts). It is difficult to evaluate this suggestion in any detail, but this would be consistent with the highly compacted nature of avian erythrocyte nuclei, and the very small cells found throughout the class. The difference that *can* be clearly seen is that between mammals and other vertebrates. Mammalian erythrocytes are smaller in absolute terms even than bird cells, and are very much smaller in relative terms, considering the genome sizes of most mammals (1C = 1.7 to 6.3 pg; 24). So, although mammals are not free from the developmental influence of bulk

DNA, the removal of nuclei from mature erythrocytes does seem to allow them to possess much smaller cells than would be possible had their nuclei persisted. As Cavalier-Smith (28) put it, "mammals can tolerate an average C-value double that of reptiles and birds only because they alone are able to eliminate their erythrocyte nuclei to compensate for their increased volume."

When more consistent cell and genome size datasets become available, it may be possible to compare in detail features of the relationship in and among the various vertebrate classes, such as the strength and slope of the regression, as well as potential differences in y-intercepts. However, it is also important to note that without correction for phylogenetic non-independence, slopes of regressions involving species data can be unreliable (29). Of course, the significance of the relationships themselves are not likely to be affected by phylogenetic associations, given the very broad taxonomic surveys involved and the fact that some studies have corrected for this and found the relationship to persist nonetheless (e.g., 6, 25, 30). As it stands, it is a very safe conclusion that more DNA per genome means larger red blood cells sizes, whether it belongs to a fish, an amphibian, a reptile, a bird, or a mammal.

EXPLAINING THE CORRELATION

The various explanations for the correlation between genome size and cell size each fall into one of two broad categories: mutation pressure theories, or optimal DNA theories. In the first of these, genome sizes are viewed as the product of an ongoing process of DNA accumulation (i.e., an upward mutation pressure) halted only when the replicative costs exceed the tolerance of the "host" cell. Under these theories, it is generally assumed that larger cells can simply tolerate more DNA, thereby making this correlation merely coincidental. The two main examples of this approach include the selfish DNA theory and the junk DNA theory, both of which are of substantial utility in addressing the origin and spread of non-coding DNA, but which falter with regard to the cell size relationship. In the simplest terms, there is no reason to expect large cells to be proportionately more tolerant of non-coding DNA than smaller cells (see Ref. 7 for review).

As an alternative approach, optimal DNA theories postulate some physiological, cytological, or other such role for non-coding DNA. That is to say, a possible function (or at least, an *effect*) of bulk DNA that is independent of its specific nucleotide sequence. In particular, optimal DNA-based explanations for the correlation between genome size and cell size typically center on the relationship between these two parameters and nucleus size. This relationship, for its part, has been known since the last turn of the century as either the "cytonuclear ratio" or the "karyoplasmic ratio" (31–33). Indeed, Fig. 4—first published in 1875—illustrates this relationship rather nicely.

There are currently two main optimal DNA theories. These are the *nucleoskeletal* theory and the *nucleotypic* theory, both of which assume a causative link between DNA content and nucleus size, but which part company when it comes to explaining why cell size should relate to nucleus size (or vice versa).

The Nucleoskeletal Theory

The nucleoskeletal theory was first introduced by Cavalier-Smith in 1978 (Ref. 28). This initial formulation included two causative components, whereby DNA amount directly influenced both nuclear and cell sizes. However, when revised a few years later (34, 35), this causation was limited to properties of the nucleus. Thus, the "nucleoskeleton" is the amount and structural arrangement of DNA within the nucleus that determines nuclear size. But instead of directly affecting cell size, nucleus size is seen as being set adaptively in response to changes in cell size (which itself is determined by genes). In this case larger cells *require* larger nuclei, which in turn require larger DNA contents. As such, the relationship between genome size and cell size, under the nucleoskeletal theory, is a coevolutionary one—cell sizes change first, and genome sizes then play catch-up.

The proposed reasons why larger cells should require larger nuclei have changed significantly during the development of the nucleoskeletal the-

Blood Cells, Molecules, and Diseases (2001) **27**(5) Sept/Oct: 830–843
doi:10.1006/bcmd.2001.0457, available online at http://www.idealibrary.com on IDEAL®

ory, but have always been based on the notion of maintaining balanced growth in larger cells. In its earlier form, the theory suggested that larger nuclei were required for the increased transport of RNA out of the nucleus in response to the greater transcriptional needs of a larger cell. However, this suggestion proved implausible and the theory was subsequently revised. In the most recent incarnation, the nucleoskeletal theory shifts from export to production, and emphasizes the needs of larger cells for greater amounts of nuclear space to meet the higher demands for proteins in larger cells (36, 37). As Cavalier-Smith and Beaton (37) put it:

> "The situation is like that of a car factory aiming for a steady output of cars: engines, wheels, and doors must be made at the same rate; if overall output is to be increased the number of each must be increased by the same proportion. Moreover, if each robot, machine tool, and operative is already working at the maximal rate, one can increase output only by increasing the number of assembly lines. As these take up space, the factory also has to be larger. In a cell the nucleus is the production line for RNA molecules. To produce more per cell cycle one must have more copies of [the] production machinery . . . . Thus nuclei have to be larger in larger cells."

The analogy is certainly an interesting one, but the obvious difficulty lies in the fact that a cell twice as large as another does not necessarily require twice as many copies of every protein, nor does the production of more protein products necessarily require a proportionate increase in nuclear space. Moreover, even in cases where nucleus/genome size and cell size are matched proportionately, there is still a negative relationship between genome size and cell growth/division rate (i.e., "balanced growth" is nevertheless not maintained). Finally, it is not obvious why reductions in cell size should require proportionate reductions in genome size, or why non-growing differentiated cells (like erythrocytes, and especially those without nuclei) should follow this rule. These problems, among others, suggest that while the nucleoskeletal theory provides a useful explanation for the relationship between genome size and *nucleus* size, its explanatory range does not extend to the relationship between either of these parameters and *cell* size (see Ref. 7 for critical review).

The Nucleotypic Theory

In 1964, Barry Commoner (12) suggested that DNA played not one, but two roles in the hereditary process. The first was, of course, the well-known genetic role of coding for proteins. The other role, according to Commoner (12), was a quantitative one in which bulk DNA exerted an influence on the cell's size and metabolism. Specifically, he proposed a "nucleotide sequestration hypothesis" under which large amounts of DNA requiring replication would usurp free nucleotides and make them unavailable for use in metabolic pathways (e.g., as ATP). This in turn would force the cell's metabolic balance to shift from catabolism to anabolism, thereby resulting in lower metabolic rates and larger cells. This hypothesis, the first explicit model relating genome size to cell size, was unfortunately flawed on several keys points (see Refs. 7 and 35 for discussions).

Working with plants, Martin (38) argued a short time later that "increase in DNA content automatically results in increased cell size," and that "selection acts on a variant with increased DNA through its effect on cell size." This clearly stands in stark contrast to the cell-size-first approach of Cavalier-Smith's nucleoskeletal theory. A much more explicit version of the view that DNA directly influences cellular parameters was provided in the early 1970s by Michael Bennett. Again in reference to plants, Bennett (39, 40) developed the concept of the "nucleotype" (a term chosen as an analogy to the "genotype") which he defined as "that condition of the nucleus [most notably, DNA content] that affects the phenotype independently of the informational content of the DNA." Thus, Commoner's (12) second role for DNA had been given a name, and the nucleotypic theory soon became a mainstay among botanists (and to a much lesser extent, zoologists).

Direct evidence for a causal influence of DNA content on cell size has been difficult to come by, but several observations lend support to the nucleotypic approach. For example, hybrid animal species often display genome and cell sizes that are intermediate between those of their parental species, non-coding B chromosomes increase cell size by their very presence, and so on

(for review, see Ref. 7). Perhaps more damaging to the case for the nucleotypic theory is that until very recently, no working model for this effect had been proposed. Commoner's (12) nucleotide sequestration model was implausible, and the only other model was a "division-initiation model" proposed by Cavalier-Smith (35) for the sole purpose of rejecting it in favor of his nucleoskeletal theory.

As Nurse (41) pointed out more than 15 years ago, "cell size is determined by an interaction of the function of specific genes with the total DNA content of the cell," and that "such an interactive system can be best understood in terms of cell cycle controls which coordinate progress through the cell cycle with an increase in mass." The details of these cell cycle controls are still being worked out, but the system is now sufficiently well understood to allow Nurse's suggestion to be properly taken to heart. Most important in the regulation of the cell cycle are the proteins known as cyclin-dependent kinases (CDKs), which are activated by cyclin molecules and play various roles in initiating and regulating DNA replication. And whereas CDKs remain intact for long periods of time, cyclin molecules must be synthesized anew in each cell cycle. As such, any mechanisms that delay the production or accumulation of cyclin molecules can delay the onset of cell division and, because growth continues throughout the cell cycle, result in larger daughter cells. In particular, the recent "gene–nucleus interaction model" (7) has emphasized the role(s) of bulk DNA in influencing the simple space-filling requirements for cyclins in larger nuclei, and possibly even the influx of regulatory proteins (and therefore cyclin gene expression) based on the effects of nuclear surface area to volume ratios and/or the arrangement of chromatin within the nucleus. In addition, larger amounts of DNA may prolong the DNA synthesis phase and/or the timing of replication initiation (7). Altogether, this means that more DNA results in larger and more slowly dividing cells. The important point about such a cell-cycle-based model is that it is readily applicable to all cells, including non-growing differentiated cells like erythrocytes (even ones that ultimately shed their nuclei).

Summary

A general relationship between DNA content (or at least, nucleus size) and cell size has been known for well over a century. However, the explanation for this association remains a subject of debate even today. Some approaches to the question, especially the "coincidental" interpretation of mutation pressure theories, can be dismissed. More complex hypotheses involving natural selection are difficult to disentangle, however, and it may be some time before a conclusive answer becomes available. Short of the direct experimental manipulation of DNA content, it may not be possible to vindicate either of the optimal DNA theories. Nevertheless, it should be borne in mind that the various theories discussed here are in conflict only in terms of explaining this relationship between genome size and cell size. Unlike the selfish and junk DNA theories, both the nucleoskeletal and nucleotypic theories are silent regarding the *origin* of noncoding DNA. Similarly, the nucleotypic theory is totally compatible with the nucleoskeletal theory insofar as the two are discussing the relationship with *nucleus* size. The C-value enigma, which involves all of these issues, will not be solved by the application of a single one-dimensional approach. Instead, a theoretical framework open to many different mechanisms operating on several levels of biological organization will likely be needed if the puzzle is to be resolved.

PHYSIOLOGICAL AND EVOLUTIONARY IMPLICATIONS

There are many ways in which erythrocyte size is of relevance to organismal biology. Larger RBCs contain more hemoglobin (42), but they also require larger blood vessels. Species with large cells also typically have fewer cells (e.g., 42, 43). Blood viscosity, total hemoglobin content, and other such parameters are of obvious significance to organismal physiology, but no other parameter has received more attention in regards to genome size/cell size interactions than erythrocyte surface area to volume (SA:V) ratios.

The significance of erythrocyte SA:V ratios to

Blood Cells, Molecules, and Diseases (2001) **27**(5) Sept/Oct: 830–843
doi:10.1006/bcmd.2001.0457, available online at http://www.idealibrary.com on IDEAL®

organismal metabolism was discussed in some detail by Harvey Smith in 1925 (Ref. 44): "The fact that the mass of [an animal's] body increases as the cube of the linear dimension, while the surface increases as the square, has long been recognized as of importance in biology. . . It is only a step farther to apply the same idea to cell size." Since then, the notion of erythrocyte size directly influencing organismal physiology has been applied unilaterally among the vertebrate classes. Smith (44) himself discussed the association between cell size and metabolic rate in amphibians, and the large cells and genomes of aquatic urodeles and lungfishes have long been interpreted as adaptations for life in hypoxic environments (particularly as related to aestivation) (e.g., 12, 45, 46). The small erythrocytes of birds and mammals have typically been viewed as adaptations for homeothermy, and so on. Various correlations have also been suggested to exist between cell size and "evolutionary advancement" (a nebulous term often apparently including a component of higher metabolism) both within and among vertebrate classes (e.g., 13, 27, 47–49). Not surprisingly, a great deal of emphasis has been placed on these physiological effects in regards to genome size evolution. Both optimal DNA theories argue for the adaptive modulation of cell size as a driving force behind evolutionary shifts in genome size (though causation runs in opposite directions in the nucleoskeletal vs nucleotypic theories). However, as with any other complex biological question, the application of a single explanatory principle to all groups concerned may be ill-advised.

In fish, some authors have suggested an association between cell volume and swimming ability (e.g., 19). If true, then swim performance-related constraints on genome expansion could explain some of the patterns of genome size distribution among fish. However, such a relationship fails to survive closer scrutiny. Notably, some of the smallest genomes (and presumably also cells), occur in poor swimmers like pufferfish, sea horses, and other morphologically very specialized groups, whereas strong swimmers like salmonids have relatively large genomes as a result of ancient polyploidization events. Large genomes (and cells) are also characteristic of cartilaginous fishes, like sharks and rays, some of which are excellent swimmers. Even the speedy skipjack tuna has a genome size *larger* than most bottom-dwelling flounders! Instead, in fish it appears that genome size is associated with some measure of "developmental complexity." That is, small genomes are typical of complex species which deviate significantly in their morphology from the typical "fishy" design (as first discussed by Hinegardner in Refs. 50 and 51). One plausible explanation for this is that the number of steps to be carried out in a complex developmental program like that of a seahorse exceed those of a "fishy" fish like a trout, and that cell division and differentiation must therefore be faster (and genomes smaller) in the former (T. R. Gregory, ms in review). Thus, although a relationship clearly exists between genome size and erythrocyte size in fish (Fig. 1), this may be more of a secondary consequence than a primary target of selection in this class.

The same may be true of amphibians. Genome size is correlated only very weakly (if at all) with metabolic rate in amphibians (52; T. R. Gregory, unpublished). Moreover, aestivation now seems an unlikely explanation for the enormous genomes of aquatic salamanders and lungfishes. For example, some species of aestivating frogs are able to reduce their metabolic rates to a low level comparable to some urodeles (53), despite their almost 40-fold smaller genomes! A large quantity of DNA (and large erythrocyte size) is not required for an aestivating lifestyle, and it is therefore unlikely that such large (and costly) genomes would evolve for this purpose. Once again, the focus may best be placed on development in this group. The most immediate pattern observed among amphibians is that there is almost no overlap between the genome sizes of frogs and salamanders, C-values being much larger in the latter. Notably, metamorphosis—a short, intensive period of cell division and differentiation—is much more severe in frogs than in salamanders. Frogs inhabiting short-lived ephemeral pools have the smallest genomes in the class, while neoteny (loss of metamorphosis) and very large genomes have evolved together independently at least three

times in salamanders (T. R. Gregory, ms in review). Developmental complexity (in this case, the complexity of the developmental process itself) may also present a much more suitable explanation for the genome sizes displayed by amphibians. And although not irrelevant, the physiological effects of their highly varied genome (and cell) sizes may not have played the most prominent role in genome size evolution in these animals as once believed. Still, other not-so-metabolic cell size-related hypotheses are also worthy of consideration. For example, those relating to the need for large blood vessels (and therefore large cells, and thus large genomes) in the early evolution of amphibian and lungfish circulatory systems (54).

Some authors have suggested similar developmental constraints in regards to reptiles, in which the need of eggs laid in the ground for relatively constant temperature limits their development to a short period of time (and would result in selection for rapid cell division and smaller genomes) (55). It is not known whether metabolic parameters are of relevance in this class. In mammals and birds, on the other hand, there is no association between genome size and developmental rate (e.g., 56, 57). In these classes, metabolism seems to be of legitimate (and substantial) importance in constraining genome size evolution. For example, significant negative correlations have been found between genome size and basal metabolic rate in mammals at large, as well as within the rodent order (58). In fact, the relationship is sufficiently strong to suggest that modulations of DNA content may be a means by which mammals can fine-tune metabolic rates via the intermediate of cell size (7, 54). Erythrocyte size also correlates negatively with body temperature in mammals (59), and it has long been noted that bats, with their high metabolic demands of powered flight, display genomes much smaller than the mammalian mean (e.g., 60, 61).

As a result of their specialized volant lifestyle, birds typically have much higher overall metabolic rates than other vertebrates. However, their erythrocytes are nucleated and are therefore typically larger than those of mammals. Rather than ejecting entire nuclei during RBC development, birds appear to have jettisoned (or perhaps never loaded) a great deal of genomic baggage over evolutionary time. Again, avian genomes are smaller than those of other terrestrial vertebrates, and range only twofold throughout the entire class. This in itself has been interpreted as a product of flight-related metabolic constraints, even though a clear correlation between genome size and metabolic rate could previously only be discerned within the passerine order (54, 62). More recently, and with the use of a more taxonomically broad dataset, it has been shown that a negative relationship between genome (and cell) size and metabolic rate similar to that in mammals also exists in birds (25). Moreover, Hughes (63) has shown average genome sizes to group well according to a measure of flight ability among families of birds. The loss of flight has also been associated with an increase in genome size several times during avian evolution (63). Indeed, some of the largest genomes (and erythrocytes) in the class are found among flightless birds like ostriches and emus (25). Of course, these physiological effects are not limited to the influence of erythrocyte size, but this is almost certainly an important component of the constraints on the evolution of mammalian and avian genomes, cells, and physiologies. Again, an appreciation for the interaction of these levels of biological organization will be a key step in deciphering the C-value enigma and in properly understanding the evolution of homeotherms.

SUMMARY AND CONCLUSIONS

The relationship between DNA content and erythrocyte size, though long recognized by some, has not been appreciated by many. Although C-value and cell size are correlated in a strongly positive way in every vertebrate class, the precise features of the relationship—and more importantly, its causes—have remained undetermined for decades. To rectify this, molecular biologists interested in the large-scale characteristics of the vertebrate genome must familiarize themselves with the principles of cell cycle regulation and erythrocyte physiology. At the same time, cell biologists interested in the various factors that

doi:10.1006/bcmd.2001.0457, available online at http://www.idealibrary.com on IDEAL®

influence red blood cell size would undoubtedly benefit from an understanding of the interplay between the nucleotype and the cellular phenotype. In more general terms, the relationship between genome size and red blood cell size has been an important feature of the evolution of vertebrates at large, at times perhaps largely as a secondary effect, at other times in a more directly adaptive sense. In any case, it is clear that a proper appreciation for the complex evolutionary processes responsible for this relationship between C-value and RBC size cannot help but illuminate numerous biological disciplines.

ACKNOWLEDGMENTS

This study was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) Postgraduate and University of Guelph Alumni Doctoral scholarships to T.R.G. and by an NSERC research grant to Paul Hebert. Sincere thanks to Joseph Hoffman for his assistance with the paper.

REFERENCES

1. Vendrely, R., and Vendrely, C. (1948) La teneur du noyau cellulaire en acide désoxyribonucléique à travers les organes, les individus et les espèces animales: Techniques et premiers résultats. *Experientia* **4,** 434–436.
2. Mirsky, A. E., and Ris, H. (1951) The desoxyribonucleic acid content of animal cells and its evolutionary significance. *J. Gen. Physiol.* **34,** 451–462.
3. Comings, D. E. (1972) The structure and function of chromatin. *Adv. Hum. Genet.* **3,** 237–431.
4. Swift, H. (1950) The constancy of desoxyribose nucleic acid in plant nuclei. *Proc. Natl. Acad. Sci. USA* **36,** 643–654.
5. Thomas, C. A. (1971) The genetic organization of chromosomes. *Annu. Rev. Genet.* **5,** 237–256.
6. Gregory, T. R. (2000) Nucleotypic effects without nuclei: Genome size and erythrocyte size in mammals. *Genome* **43,** 895–901.
7. Gregory, T. R. (2001a) Coincidence, coevolution, or causation? DNA content, cell size, and the C-value enigma. *Biol. Rev.* **76,** 65–101.
8. Fankhauser, G. (1955) Role of nucleus and cytoplasm. *In* Analysis of Development (Willier, B. H., Weiss, P. A., and Hamburger, V., Eds.), pp. 126–150. Saunders, Philadelphia.
9. Licht, L. E., and Bogart, J. P. (1987) Comparative size of epidermal cell nuclei from shed skin of diploid, triploid and tetraploid salamanders (Genus *Ambystoma*). *Copeia* **1987,** 284–290.
10. Austin, N. E., and Bogart, J. P. (1982) Erythrocyte area and ploidy determination in the salamanders of the *Ambystoma jeffersonianum* complex. *Copeia* **1982,** 485–488.
11. Garcia-Abiado, M. A. R., Dabrowski, K., Christensen, J. E., and Czesny, S. (1999) Use of erythrocyte measurements to identify triploid saugeyes. *N. Am. J. Aquacult.* **61,** 319–325.
12. Commoner, B. (1964) Roles of deoxyribonucleic acid in inheritance. *Nature* **202,** 960–968.
13. De Smet, W. H. O. (1981) The nuclear Feulgen-DNA content of the vertebrates (especially reptiles), as measured by fluorescence cytophotometry, with notes on the cell and chromosome size. *Acta Zool. Pathol. Antverp.* **76,** 119–167.
14. Olmo, E. (1983) Nucleotype and cell size in vertebrates: A review. *Bas. Appl. Histochem.* **27,** 227–256.
15. Pedersen, R. A. (1971) DNA content, ribosomal gene multiplicity, and cell size in fish. *J. Exp. Zool.* **177,** 65–79.
16. Fontana, F. (1976) Nuclear DNA content and cytometry of erythrocytes of *Huso huso* L., *Acipenser sturio* L., and *Acipenser naccarii* Bonaparte. *Caryologia* **29,** 127–137.
17. Banerjee, S. K., Misra, K. K., Banerjee, S., and Ray-Chaudhuri, S. P. (1988) Chromosome numbers, genome sizes, cell volumes and evolution of snake-head fish (family Channidae). *J. Fish Biol.* **33,** 781–789.
18. Chang, H.-Y., Sang, T.-K., Jan, K.-Y., and Chen, C.-T. (1995) Cellular DNA contents and cell volumes of Batoids. *Copeia* **1995,** 571–576.
19. Lay, P. A., and Baldwin, J. (1999) What determines the size of teleost erythrocytes? Correlations with oxygen transport and nuclear volume. *Fish Physiol. Biochem.* **20,** 31–35.
20. Olmo, E., and Morescalchi, A. (1975) Evolution of the genome and cell sizes in salamanders. *Experientia* **31,** 804–806.
21. Olmo, E., and Morescalchi, A. (1978) Genome and cell size in frogs: A comparison with salamanders. *Experientia* **34,** 44–46.
22. Horner, H. A., and Macgregor, H. C. (1983) C value and cell volume: Their significance in the evolution and development of amphibians. *J. Cell Sci.* **63,** 135–146.
23. Olmo, E., and Odierna, G. (1982) Relationships between DNA content and cell morphometric parameters in reptiles. *Bas. Appl. Histochem.* **26,** 27–34.
24. Gregory, T. R. (2001b) Animal Genome Size Database. http://www.genomesize.com.
25. Gregory, T. R. (2002) A bird's eye view of the C-value enigma: Genome size, cell size, and metabolic rate in the class Aves. *Evolution,* in press.

26. Gallardo, M. H., Bickham, J. W., Honeycutt, R. L., Ojeda, R. A., and Köhler, N. (1999) Discovery of tetraploidy in a mammal. *Nature* **401,** 341.
27. Gulliver, G. (1875) Observations on the sizes and shapes of the red corpuscles of the blood of vertebrates, with drawings of them to a uniform scale, and extended and revised tables of measurements. *Proc. Zool. Soc. London* **1875,** 474–495.
28. Cavalier-Smith, T. (1978) Nuclear volume control by nucleoskeletal DNA, selection for cell volume and cell growth rate, and the solution of the DNA C-value paradox. *J. Cell Sci.* **34,** 247–278.
29. Harvey, P. H., and Pagel, M. D. (1991) The Comparative Method in Evolutionary Biology. Oxford Univ. Press, Oxford.
30. Pagel, M., and Johnstone, R. A. (1992) Variation across species in the size of the nuclear genome supports the junk-DNA explanation for the C-value paradox. *Proc. R. Soc. London B* **249,** 119–124.
31. Strasburger, E. (1893) Über die Wirkungssphäre der Kerne und die Zellgrösse. *Histol. Beitr.* **5,** 97–124.
32. Hertwig, R. (1903) Über Korrelation von Zell- und Kerngrösse und ihre Bedeutung für die geschechtliche Differenzierung und die Teilung der Zelle. *Biol. Centralbl.* **23,** 49–62.
33. Wilson, E. B. (1925) The Cell in Development and Heredity. Macmillan, New York.
34. Cavalier-Smith, T. (1982) Skeletal DNA and the evolution of genome size. *Annu. Rev. Biophys. Bioeng.* **11,** 273–302.
35. Cavalier-Smith, T. (1985) Cell volume and the evolution of eukaryotic genome size. *In* The Evolution of Genome Size (Cavalier-Smith, T., Ed.), pp. 104–184. Wiley, Chichester.
36. Beaton, M. J., and Cavalier-Smith, T. (1999) Eukaryotic non-coding DNA is functional: evidence from the differential scaling of cryptomonad genomes. *Proc. R. Soc. London B* **266,** 2053–2059.
37. Cavalier-Smith, T., and Beaton, M. J. (1999) The skeletal function of non-genic nuclear DNA: New evidence from ancient cell chimaeras. *Genetica* **106,** 3–13.
38. Martin, P. G. (1966) Variation in the amounts of nucleic acids in the cells of different species of higher plants. *Exp. Cell Res.* **44,** 84–94.
39. Bennett, M. D. (1971) The duration of meiosis. *Proc. R. Soc. London B* **178,** 277–299.
40. Bennett, M. D. (1972) Nuclear DNA content and minimum generation time in herbaceous plants. *Proc. R. Soc. London B* **181,** 109–135.
41. Nurse, P. (1985) The genetic control of cell volume. *In* The Evolution of Genome Size (Cavalier-Smith, T., Ed.), pp. 185–196. Wiley, Chichester.
42. Hawkey, C. M., Bennett, P. M., Gascoyne, S. C., Hart, M. G., and Kirkwood, J. K. (1991) Erythrocyte size, number and haemoglobin content in vertebrates. *Br. J. Haematol.* **77,** 392–397.
43. Kuramoto, M. (1981) Relationships between number, size and shape of red blood cells in amphibians. *Comp. Biochem. Physiol.* **69A,** 771–775.
44. Smith, H. M. (1925) Cell size and metabolic activity in amphibia. *Biol. Bull.* **48,** 347–378.
45. Cavalier-Smith, T. (1991) Coevolution of vertebrate genome, cell, and nuclear sizes. *In* Symposium on the Evolution of Terrestrial Vertebrates (Ghiara, G., *et al.*, Eds.), pp. 51–86. Mucchi, Modena.
46. Gregory, T. R., and Hebert, P. D. N. (1999) The modulation of DNA content: Proximate causes and ultimate consequences. *Genome Res.* **9,** 317–324.
47. Cleland, J. B., and Johnston, T. H. (1912) Relative dimensions of the red blood cells of vertebrates, especially of birds. *Emu* **11,** 188–197.
48. Hartman, F. A., and Lessler, M. A. (1964) Erythrocyte measurements in fishes, amphibia, and reptiles. *Biol. Bull.* **126,** 83–88.
49. Saint Girons, M.-C., and Saint Girons, H. (1969) Contribution à la morphologie comparée des érythrocytes chez les reptiles. *Br. J. Herpetol.* **4,** 67–82.
50. Hinegardner, R. (1968) Evolution of cellular DNA content in teleost fishes. *Am. Nat.* **102,** 517–523.
51. Hinegardner, R. (1976) Evolution of genome size. *In* Molecular Evolution (Ayala, F. J., Ed.), pp. 179–199. Sinauer, Sunderland.
52. Licht, L. E., and Lowcock, L. A. (1991) Genome size and metabolic rate in salamanders. *Comp. Biochem. Physiol.* **100B,** 83–92.
53. Pinder, A. W., Storey, K. B., and Ultsch, G. R. (1992) Estivation and hibernation. *In* Environmental Physiology of the Amphibians (Feder, M. E., and Burggren, W. W., Eds.), pp. 250–274. Univ. Chicago Press, Chicago.
54. Snyder, G. K., and Sheafor, B. A. (1999) Red blood cells: Centerpiece in the evolution of the vertebrate circulatory system. *Am. Zool.* **39,** 189–198.
55. Olmo, E. (1991) Genome variations in the transition from amphibians to reptiles. *J. Mol. Evol.* **33,** 68–75.
56. John, B., and Miklos, G. L. G. (1988) The Eukaryote Genome in Evolution and Development. Allen & Unwin, London.
57. Monaghan, P., and Metcalfe, N. B. (2000) Genome size and longevity. *Trends Genet.* **16,** 331–332.
58. Vinogradov, A. E. (1995) Nucleotypic effect in homeotherms: Body mass-corrected basal metabolic rate of mammals is related to genome size. *Evolution* **49,** 1249–1259.
59. Salienko, Y. A. (1995) On the correlation of the set point of body temperature with erythrocyte size. *Hum. Physiol.* **21,** 625–626.
60. Capanna, E., and Manfredi Romanini, M. G. (1971) Nuclear DNA content and morphology of the karyo-

Blood Cells, Molecules, and Diseases (2001) **27**(5) Sept/Oct: 830–843
doi:10.1006/bcmd.2001.0457, available online at http://www.idealibrary.com on IDEAL®

type in certain palearctic Microchiroptera. *Caryologia* **24,** 471–482.

61. Burton, D. W., Bickham, J. W., and Genoways, H. H. (1989) Flow-cytometric analyses of nuclear DNA content in four families of neotropical bats. *Evolution* **43,** 756–765.
62. Vinogradov, A. E. (1997) Nucleotypic effect in homeotherms: Body-mass independent metabolic rate of passerine birds is related to genome size. *Evolution* **51,** 220–225.
63. Hughes, A. L. (1999) Adaptive Evolution of Genes and Genomes. Oxford Univ. Press, Oxford.