cofactors on the DNA binding of NPAS2 and its close relative Clock. When bound to BMAL1, both of these proteins recognize E-box motifs in DNA recognition sequences, but BMAL1 can also bind to DNA on its own (that is, as a homodimer). The authors used purified recombinant proteins and either unphosphorylated cofactors (NAD^+ and its reduced form NADH) or phosphorylated cofactors (NADPH and its reduced form $NADP^+$) at physiological concentrations. They report that reduced and oxidized NAD(P) electron carriers have inverse effects on the proportion of NPAS2:BMAL1 (or Clock:BMAL1) heterodimers to BMAL1:BMAL1 homodimers that bind to DNA. Although the heterodimeric interaction is greatly stimulated by NAD(P)H, it is strongly inhibited by $NAD(P)^+$. As a consequence, only BMAL1 homodimers (whose affinity for DNA is not affected by NAD cofactors) occupy E-box motifs at a low $NAD(P)H/NAD(P)^+$ ratio. Because BMAL1 homodimers are incapable of activating transcription, the susceptibility of the heterodimer to this redox potential establishes a molecular switch for activating the Clock:BMAL1 transcription complex.

Redox electron transfer through NADH and NAD may also provide an elegant way for cryptochromes to inhibit the activity of Clock and NPAS2. Preliminary experiments from the McKnight laboratory suggest that NPAS2 also binds to a heme cofactor (*11*). Conceivably, the interaction of CRYs with NPAS2 provokes electron transfer from NPAS2-associated NAD(P)H to CRY-associated FAD, and finally to the heme cofactor bound by NPAS2. This electron shuttle would convert NAD(P)H into $NAD(P)^+$ while conserving the redox state of CRY-associated FAD. The standard reduction potentials of NAD, FAD, and heme (a measure of the ease with which a molecule can be converted to its reduced form) are in perfect alignment with such a scheme. Depending on the stability of the change, binding of NPAS2 to DNA might be abolished.

The unexpected relationship between circadian clock proteins and redox potential adds a new wrinkle to chronobiology research. If the NAD interaction domains of the Clock and NPAS2 proteins can be narrowed down to a minimal peptide sequence, it may become possible to engineer proteins that cannot bind to NAD(P)H cofactors. The activity of such proteins in cells and intact animals then could be investigated, hopefully providing validation for the clock-redox connection observed in the test tube and enabling fundamental questions to be addressed. For example, is redox potential involved in light- or food-induced phase shifting, and in the operation of the circadian oscillator? The balance between reduced and oxidized NAD cofactors in the cell cytoplasm depends on the intracellular concentrations of fuels (such as glucose), oxygen, and LDH, and this balance is itself subject to daily oscillations (*12, 13*). It is also noteworthy that the NPAS2:BMAL1 heterodimer has as a potential target LDH, an enzyme that influences cellular redox potential and thereby changes the activity of its activator. Such a negative feedback loop could contribute to circadian clock activity, promoting LDH from a routine metabolic enzyme to an integral clock component.

Some years ago, it was proposed that NAD cofactors formed the gears of the circadian clock of *Euglena gracilis* (*14*). In this unicellular phytoflagellate, NAD^+ levels oscillate, and the addition of NAD^+ or $NADP^+$ to the culture medium results in steady-state phase shifts. However, in this system, the ratio of phosphorylated to unphosphorylated cofactors (rather than the ratio of reduced to oxidized NAD cofactors) is believed to participate in the generation of circadian oscillations. It is worth mentioning in this context that NADPH is about three times as efficient as NADH in stimulating the binding of NPAS2 to DNA (although phosphorylated and unphosphorylated NAD electron carriers have the same redox potential). The relevance of these observations to Clock:BMAL1 activity and to general clock activity can also be tested, at least in cultured cells.

The unexpected connection between NAD balance and Clock protein activity discovered by McKnight and colleagues could revolutionize our notions of circadian oscillators and circadian phase signaling to peripheral clocks. It will be fascinating to study the relationship between cellular redox states and circadian timing through future experiments in vivo.

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PERSPECTIVES: EVOLUTION

The Cambrian Explosion Exploded?

Richard Fortey

The beginning of the Cambrian period, some 545 million years ago, saw the sudden appearance in the fossil record of almost all the main types of animals (phyla) that still dominate the biota today. To be sure, there are fossils in older strata, but they are either very small (such as bacteria and algae), or their relationships to the living fauna are highly contentious, as is the case with the famous soft-bodied fossils from the late Precambrian Pound Quartzite, Ediacara, South Australia.

Consequently, it has been concluded that exceptional evolutionary activity over 10 million years or so at the base of the Cambrian generated ancestors of most of the living phyla and maybe many other "failed phyla" besides (*1*). Other paleontologists have questioned whether such rapid evolution is possible and have instead postulated a phylogenetic "fuse"—an extended period of evolutionary genesis that has left little or no fossil record (*2*). So just how explosive was the Cambrian evolutionary "explosion"?

Support for a phylogenetic fuse is provided by the discovery of a true crustacean in early Cambrian strata from Shropshire, England, reported by Siveter *et al.* on page 479 of this issue (*3*). This fossil phosphatocopid "ostracod" is preserved extraordinarily well, with all its delicate limbs cast in calcium phosphate, allowing it to be assigned to the crustaceans with confidence. Very few fossils of this great antiquity reveal so

The author is in the Department of Palaeontology, The Natural History Museum, London SW7 5BD, and Department of Zoology, Oxford University, Oxford OX1 3PS, UK. E-mail: raf@nhm.ac.uk

much detail or can be interpreted with such certainty.

Crustacea are one of the great groups of living arthropods, embracing crabs, shrimps, lobsters, and slaters (*4*). Hitherto, the oldest undoubted crustaceans came from the late Cambrian "orsten" of southern Sweden (*5*) (the alleged crustacean *Canadaspis*, from the mid-Cambrian Burgess Shale, British Columbia, has proved controversial). This allowed some 40 million years from the base of the Cambrian to generate an ancestral crustacean from some primitive arthropod—time enough, indeed. But if crustaceans were already present in the early Cambrian, this pushes back in time the necessary steps in the evolutionary tree of arthropods that led to the crustacean design. It then becomes perfectly plausible that this early radiation happened in the late Precambrian.

This squares with previous critiques, which noted that in the early Cambrian, some arthropods—especially the ubiquitous trilobites—had already differentiated into different kinds with separate geographical distributions. This differential evolution and dispersal, too, must have required a previous history of the group for which there is no fossil record (*6*). Furthermore, cladistic analyses of arthropod phylogeny revealed that trilobites, like eucrustaceans, are fairly advanced "twigs" on the arthropod tree (see the figure). Trilobite-like trace fossils extend to the base of the Cambrian in Newfoundland, and it would be easy to conclude that appropriate trace makers must have appeared still earlier, in the late Precambrian. But fossils of these alleged ancestral arthropods are lacking.

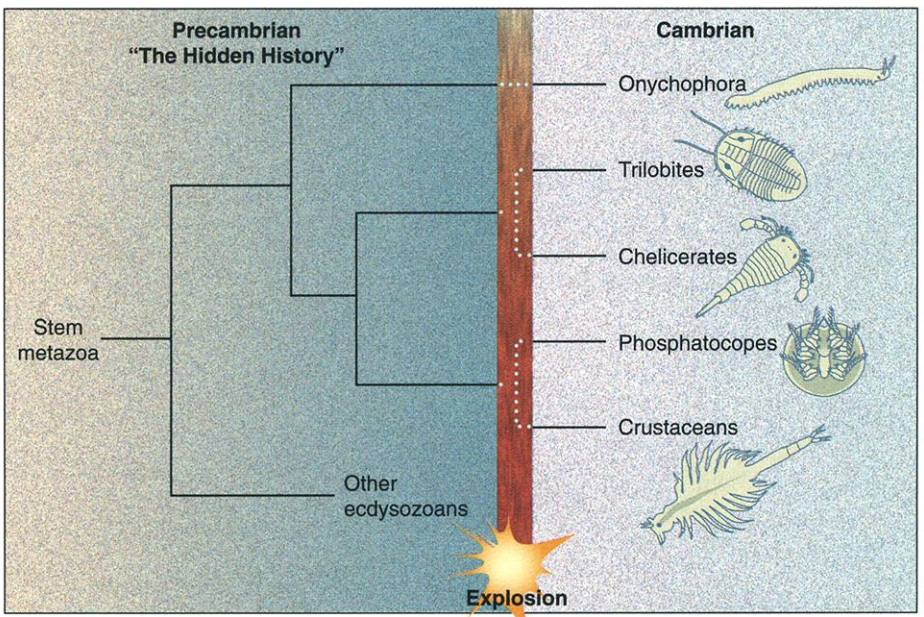
Another, independent test of the divergence times is provided by molecular "clocks." If living groups had indeed diverged from common ancestors in the Precambrian, then a calibration of this event may be preserved in the additive sequence changes accumulated on appropriate, conservative protein coding genes or other molecules such as ribosomal RNA. By comparing sequences from distantly related living animals, which ultimately descended from one or another at one of these deep branching events, an estimate of their divergence times may be obtained (but only if one assumes a clocklike behavior in substitutions).

Several such estimates have now been made. All point to Precambrian divergences (from 700 to more than 1500 million years ago) for branches between phyla, thus allowing plenty of time for the phylogenetic fuse (*7*). The divergence estimates vary widely, however, reflecting both methodological assumptions and choice of genes. They agree only in being Precambrian. Critics (*8*) of this method point to the possibility that there may be a systematic bias in evolutionary rates (a speeding up) at times of "explosion." Divergence times made under the assumption of standard, clocklike behavior would then be greatly overestimated.

In the context of such unresolved controversies, a reliably identified, very early arthropod fossil is of considerable importance. It is now becoming increasingly clear that all arthropods descended from a common ancestor. The crustaceans are probably more closely related to the insects than they are to the millipeds, centipeds, trilobites, and chelicerates (spiders, scorpions, and horseshoe crabs) (*9*). However, all these kinds of animal ultimately descended from some basal arthropod. Given that a crustacean from the early Cambrian has now been found, the fundamental earlier steps in this tree of descent must have already happened at that time. Furthermore, Onychophora (velvet worms) were probably the most closely related group to the arthropods as a whole; this group and the arthropods must have diverged even earlier. And arthropods may well be part of an even larger group, the Ecdysozoa (animals sharing moulting habits), which probably differentiated still earlier again into fundamentally distinctive designs. It begins to look more and more probable that these evolutionary events happened in the Precambrian.

The hunt is on for fossils that might cast light on these remote events. The chances of making such a discovery are slim because the early animals would have lacked skeletons and shells and were almost certainly very small. Nonetheless, phosphatized animal embryos have been discovered in late Precambrian strata (*10*), suggesting that light may yet be cast on the mysterious origins of the animal phyla.

Even if evidence for an earlier origin is discovered, it remains a challenge to explain why so many animals should have increased in size and acquired shells within so short a time at the base of the Cambrian. At the moment, there are almost as many explanations as there are animals caught in this belated "explosion." But it is more than likely that the evolutionary fuse was lit long before the Cambrian.



The early evolutionary history of the arthropods. The new discovery of a crustacean-like fossil (Phophatocope) in the early Cambrian pushes the branching events leading up to it and its relatives further back in time—into the Precambrian. Drawings are not to scale.

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