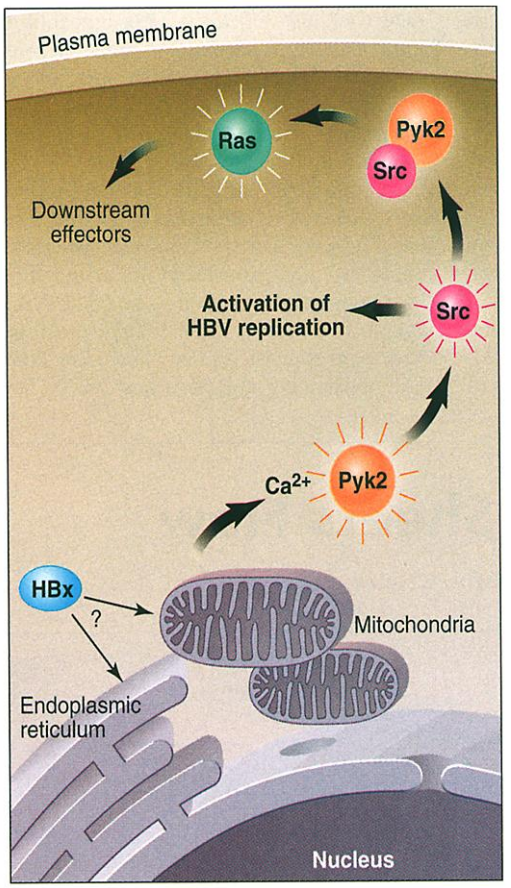
PERSPECTIVES: VIROLOGY

The X Files—One Step Closer to Closure

Don Ganem

One might have thought that a viral pathogen like hepatitis B virus (HBV), whose DNA genome is only 3.2 kb in length, would have been able to harbor few secrets from the prying eyes of virologists. And indeed, over the past two decades, many remarkable insights have been gleaned into the inner workings of this pathogen. We now know, for example, that viral DNA replication proceeds by reverse transcription of an RNA intermediate, and that inhibition of the responsible viral polymerase results in clinically effective therapy for established HBV infection. Additionally, cloning and expression of HBV envelope proteins have yielded an enormously successful vaccine to prevent new infections. But despite all of this progress, the function of one viral gene, appropriately named X, has proven elusive. On page 2376 of this issue, Schneider and colleagues (*1*) provide major new insights into the action of the product of this enigmatic viral gene.

HBV produces acute and chronic infections of the liver. Although HBV replication does not kill cultured liver cells, infection in vivo often is associated with liver injury, which results from attack of virus-infected cells by the host immune system. Chronic liver injury, in turn, triggers chronic liver regeneration and confers a striking risk of developing primary hepatic cancer (*2*). HBV engenders all of this with only four open reading frames (ORFs; DNA sequences that encode proteins). Three of these ORFs encode the capsid, envelope, and polymerase proteins of the virion. The fourth is ORF X, so named because its predicted amino acid sequence suggested no clues to its potential function. The X gene is clearly essential in vivo: Null mutations in this gene ablate the ability of the virus to replicate and spread in the liver in animal models of HBV (*3*). In at least some liver cell lines, X gene mutations lead to a substantial block in viral DNA synthesis, which would be consistent with the phenotype observed in animals (*4*). Expression of the X gene in cultured cells often produces effects on cell growth and susceptibility to apoptosis, but a number of these effects have been inconsistently observed, and it is not clear whether they are primary or secondary consequences of X protein action (*2*).



What HBx does in the cytoplasm. HBx acts on either mitochondria or the endoplasmic reticulum (or both) to trigger release of Ca^{2+} into the cytosol. This transient flux of Ca^{2+} triggers activation of the Pyk2 kinase, which then binds and activates Src-family kinases. Activated Src kinases, in their turn, stimulate Ras by promoting its engagement with Shc and Grb2. Activated Ras then turns on Raf–mitogen-activated protein kinase pathways, leading to nuclear transcription factor activation and other downstream effects. Activated Src kinases also up-regulate HBV reverse transcription, an effect that does not appear to involve Ras (*4*).

The product of ORF X (referred to as HBx protein) is poorly immunogenic and displays a short half-life, two properties that have greatly impeded its study. The first biological activity reproducibly linked to HBx was the activation of reporter genes driven by a variety of promoters. Activation was relatively weak—3- to 10-fold in most studies—but quite broad-spectrum. Most HBx-responsive promoters harbor binding sites for particular transcription factors, such as AP-1, AP2, CREB, NF-κB, c/EBP, or NFAT. Reporter genes that are dependent upon these factors individually can be activated by coexpression of HBx. This led to early speculation that HBx might be a nuclear transcription factor, and indeed, a small fraction of the intracellular pool of HBx can be found in the nucleus (*5*). In fact, in vitro evidence suggests that HBx binds to the bZIP domain of CREB and promotes its oligomerization and subsequent binding to DNA, thus providing one potential task for the nuclear isoform of this viral protein (*6*, *7*). However, there is good reason to believe that only some of HBx's effects are attributable to its nuclear form. For example, deliberate targeting of all HBx to the nucleus by fusing it to a heterologous nuclear localization sequence results in loss of its ability to activate AP-1–dependent gene expression (*5*).

So what does cytoplasmic HBx do? Attempts to clarify this by yeast two-hybrid screening and other methods that detect protein-protein interactions have generated a morass of claims and counterclaims (*2*). A more fruitful approach has been to demonstrate that for many of the transcription factors activated by HBx, the process begins in the cytosol with the induction of distinct signaling cascades. For example, dominant-negative mutations in the Ras or Raf signaling proteins (which block their activity) blunt the activation of AP-1 by HBx (*8*). Also, HBx expression leads to a modest but reproducible activation of Ras, chiefly by enhancing the production of the intermediate Ras-GTP (*9*). This activation of Ras may be indirect, because HBx does not seem to associate with either Ras, Ras-GAP, or upstream activators of these signaling molecules such as Sos, Grb2, or Shc, even though HBx expression leads to enhanced formation of Grb2-Shc-Sos activation complexes (*9*).

How then does Hbx expression trigger Ras activation? In earlier work from the Schneider group, nonspecific inhibition of tyrosine kinases with genistein was found to block HBx-induced Ras activation, suggesting that these kinases may be involved. Because nonreceptor tyrosine kinases of the Src family can activate Ras in other contexts (for example, through phosphorylation of Shc, resulting in Grb2 association), these kinases

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were clearly potential targets of HBx. Sure enough, HBx expression was soon found to be associated with activation of both c-*src* and *fyn* genes, which encode the Src and fyn nonreceptor tyrosine kinases, respectively (*9*).

As they could detect no direct interaction between HBx and either of these Src kinases, or the carboxyl-terminal Src kinase (Csk), Schneider and co-workers turned their attention to other upstream regulators of the Src family. This led them to the kinase Pyk2, a cytosolic kinase that is activated by transient calcium ion (Ca^{2+}) fluxes and is known to activate Src kinases. In the new work, these investigators make a compelling case for involvement of this Ca^{2+}-dependent Src kinase signaling pathway in at least some of HBx's activities (*1*). First, HBx production leads to activation of Pyk2 through addition of phosphate groups (phosphorylation). Dominant-negative mutations in Pyk2 block HBx induction of AP-1 activity, and also produce a substantial decline in fyn kinase activity. In addition, expression of dominant-negative mutant Pyk2 in cells replicating wild-type HBV has exactly the same effect as seen in X-null mutant cells: reduced viral DNA synthesis in the presence of normal levels of viral RNA. These findings are consistent with the notion that HBx acts upstream of Pyk2, perhaps on a pathway that leads to Ca^{2+} release. Consistent with this notion are two further lines of evidence. First, molecules that chelate intracellular Ca^{2+} also block the HBx induction of Pyk2 activation. Second, the impaired replication of HBx-deficient HBV mutants can be partially overcome by agents (Ca^{2+} ionophores and thapsigargin) that increase the concentration of cytosolic Ca^{2+}.

A signaling pathway that depends on Ca^{2+} fluxes is an attractive biochemical locus for HBx action because it affects so many cellular processes, including transcription, translation, cell cycle control, and apoptosis. Many of these cellular processes have been suggested to be affected by HBx. The outstanding questions now are (i) how does HBx influence the release of Ca^{2+} and from which organelles (see the figure), and (ii) are the effects of Ca^{2+} release limited to those caused by Pyk2 activation, or does HBx activate other Ca^{2+}-dependent signaling events? If the latter is true, as seems likely, it is conceivable that many of the other phenotypic effects of ORF X expression that have been described may be attributable to this activity. Time will tell.

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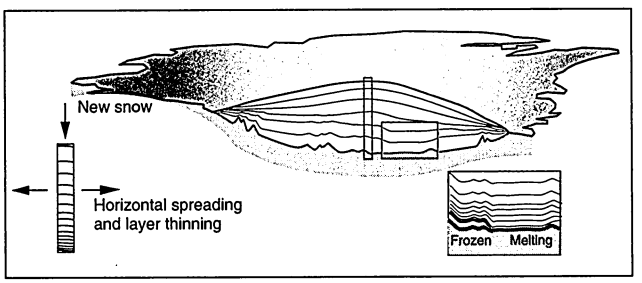
PERSPECTIVES: GLACIOLOGY

How Ice Sheets Flow

Christina L. Hulbe

Visitors to alpine glaciers routinely marvel at the torrents of melt water rushing from under the ice. As early as the late 18th century, alpine geologists had surmised the importance of this liquid water to glacier flow. In his epic *Voyages in the Alps* (*1*), Horace-Bénédict de Saussure observed "Almost all glaciers [...] of any appreciable size, have beneath them, even in winter, streams of water that flow between the ice and the bed that supports it. One therefore understands that these ice masses, driven by the loss of contact with the bed on which they rest, freed by the loss of contact with the bed on which they rest, freed by water from contact that ice could make with that same bed, sometimes even lifted by this water, must little-by-little, slide and descend following the valley slope or ridge they cover."

It is perhaps less obvious that basal melt water is also important to the much larger, and considerably colder, polar ice sheets. Yet the effects of water beneath ice sheets can be profound, as exemplified by the unexpectedly fast-flowing networks of ice streams embedded within the West Antarctic Ice Sheet (*2*) and by the flotillas of icebergs cast into the North Atlantic by surges of the Laurentide Ice Sheet, which covered much of North America during the Pleistocene glacial cycles (spanning the last 2 million years) (*3*).



Peering into an ice sheet. Internal layers thin with depth (and age) because of ice flow. Where basal melting occurs, ice is lost at the bottom of the ice sheet and layers experience relatively less thinning than they would over a frozen bed.

On page 2338 of this issue, Fahnestock *et al.* (*4*) report a striking example of the power of basal melting in an unexpected place: the northeast sector of the Greenland Ice Sheet. They use an innovative combination of remote observation and theory. The technical aim of their work is narrow: to deduce surface snow accumulation and basal melting rates from the geometry of ice layers within the ice sheet, measured remotely by airborne radar. But the mark they hit is much broader.

Snow falling on the ice sheet surface forms layers that move downward and densify to become glacier ice as new snow falls above them. The downward-moving layers thin as a result of horizontal spreading of the flowing ice (see the figure). The process is easy to imagine for a simple scenario in which the ice sheet is frozen to its bed and is close to a steady-state mass balance. In that case, the rate of new snow accumulation is matched by the spreading rate and the ice layers thin with depth (and thus age) in a predictable way. But when Fahnestock *et al.* (*4*) applied a commonly used model of that process to a set of well-dated layers in the Greenland Ice Sheet, the model failed in some interesting locations.

The model failed because the downward-moving layers do not thin as much as they ought to thin. The implication, which the authors demonstrate quite elegantly, is that the bottom of the ice sheet is melting, in some places at rates of up to 20 cm/year. The largest inferred melting rates exceed what can be explained by typical heat flow in old (and hence relatively cold) cratonic crust. The basal melting anomaly is large enough in both magnitude and spatial distribution to suggest that a feature similar to North America's Yellowstone caldera lies beneath the ice sheet. Such calderas are caused by the collapse of massive magma chambers, indicating substantial geophysical activity. The area where large basal melting begins lies upstream of a heretofore unexplained

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