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Features

Open Question

Does muscle activation occur by direct mechanical coupling of transverse tubules to sarcoplasmic reticulum?

Anthony H. Caswell and Neil R. Brandt

Our knowledge of the physiological and biochemical constituents of skeletal muscle excitation has increased greatly during the last few years but this has not led to a consensus of the physiological mode of muscle activation. Three hypotheses of transmission, involving either transmitter–receptor interaction or direct mechanical coupling, are still under active consideration. The hypothesis of direct mechanical coupling currently being evaluated proposes that the dihydropyridine receptor in the transverse tubules serves as a voltage sensor that communicates directly with the junctional foot protein/Ca^{2+} channel of sarcoplasmic reticulum to initiate opening of the channel.

During the last 20 years three basic hypotheses of the mechanism of excitation–contraction coupling in skeletal muscle have been proposed. These have found favor and disfavor in a cyclical fashion much as the waves of the sea. Our understanding of the physiological and biochemical processes of muscle activation has increased vastly during this period but, we cannot as yet delineate unequivocally between the theories.

The three basic hypotheses of muscle excitation expressed in general terms are:

(1) Direct or capacitive electrical communication between the transverse tubule (T-tubule) and the terminal cisternae causes the passage of a wave of potential change that is responsible for the opening of the Ca^{2+} channel in the sarcoplasmic reticulum (SR).
(2) Depolarization of the T-tubule causes the release of a transmitter into the junctional space which binds to a receptor and causes Ca^{2+} channel opening in the SR.
(3) Direct mechanical communication occurs between the T-tubule and the terminal cisternae of the SR in which depolarization of the T-tubule causes a conformational alteration of the spanning structure, thus opening the Ca^{2+} channel of SR.

A. H. Caswell and N. R. Brandt are at the Department of Pharmacology, University of Miami School of Medicine, Miami, FL 33101, USA.

At present, the first of these hypotheses has little experimental support. It is hard to define within the context of the known ionic gradients across the SR membrane and the transverse tubular membrane[1]. The other two hypotheses have been involved in a long running dogfight for supremacy. This review questions whether direct mechanical communication can account for muscle activation. The answer should be viewed in the context of alternative modes of excitation–contraction coupling.

The transmitter–receptor hypothesis may be expressed more specifically in terms of two potential transmitters for muscle activation: (1) Ca^{2+}; and (2) inositol 1,4,5-trisphosphate (IP_3). These two hypotheses reduced to their minimal constituents are illustrated in Fig. 1. Variations of each hypothesis are possible but have as yet little experimental support. For example, Ca^{2+} may be released from intracellular storage sites on muscle depolarization rather than being transported across the membrane.

Trigger Ca^{2+}-induced Ca^{2+} release

A remarkable feature of skeletal muscle is the high content of dihydropyridine receptor in the transverse tubules. In other cellular systems, this receptor has been associated with Ca^{2+} channel activity. Experimental data in skeletal muscle support the view that drugs which bind to and inhibit this receptor also inhibit the slow Ca^{2+} channel. Estimates of dihydropyridine receptor content in T-tubules vary from 20 to 200 pmol per mg protein[2]. Assuming each receptor is a channel, this content would probably not be quite adequate to supply the Ca^{2+} necessary for contraction. This number of channels, however, could supply a source of trigger Ca^{2+} which, in its turn, stimulates opening of the Ca^{2+} channel of SR. In a similar context, experiments with skinned muscle fibers (in which the plasma membrane has been mechanically removed or chemically rendered leaky), as well as experiments in isolated SR demonstrate that Ca^{2+} release from SR may be activated in the presence of micromolar concentrations of Ca^{2+} and inhibited in very low Ca^{2+} concentrations[3–5]. The rate of Ca^{2+} release from the SR initiated by this process although under the non-physiological conditions of low Mg^{2+}, is adequate to account for the rapidity of muscle contraction[5].

Why, then, is this hypothesis not generally accepted as the mode of muscle activation? The initial evidence against this hypothesis was presented by Armstrong *et al.*[6] who showed that muscle could continue to contract when immersed in a solution containing the Ca^{2+} chelator EGTA. Subsequent experiments have supported this observation but more sophisticated experimental approaches have demonstrated a reduction in Ca^{2+} release associated with EGTA treatment[7]. In addition, Baylor and Hollingworth[8] have shown that muscle contraction is not significantly affected by the inclusion in the cytoplasm of high concentrations of the Ca^{2+}-chelating agent, FURA-2. Thus, in skeletal muscle the curious situation

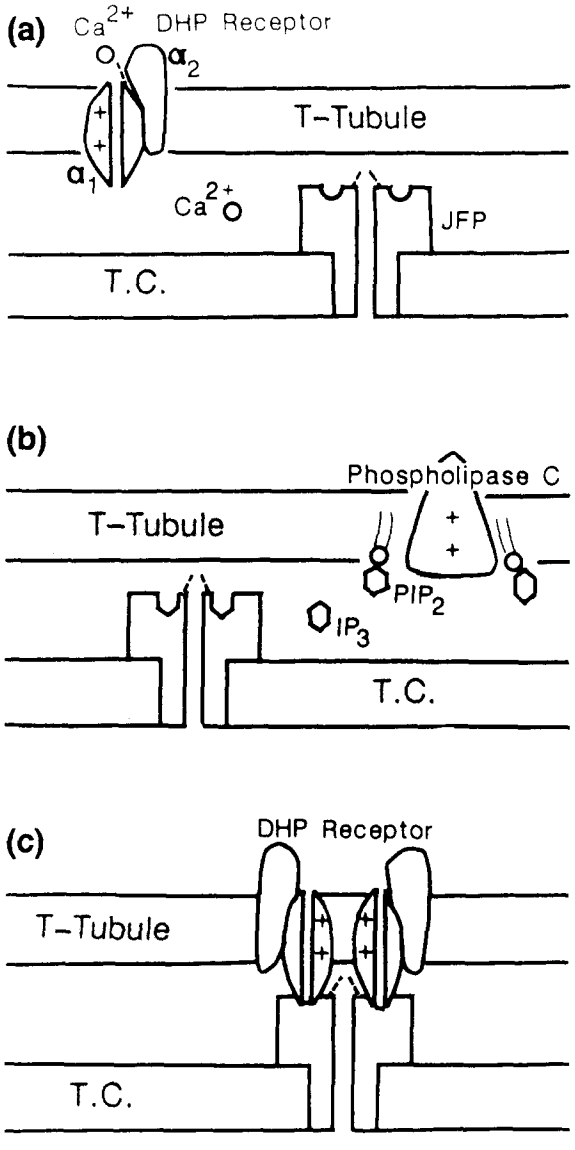

Fig. 1. Basic schemes for excitation–contraction coupling (**a**) *Ca^{2+}-triggered Ca^{2+} release. Ca^{2+} passes from the lumen of the T-tubule through the voltage-sensitive dihydropyridine receptor (DHP) and binds to the junctional foot protein (JFP) thereby activating channel opening in the terminal cisternae (T.C.), of the sarcoplasmic reticulum.* (**b**) *A voltage-sensitive phospholipase C in T-tubules activates the conversion of PIP_2 to IP_3. The latter binds to the junctional foot protein and activates Ca^{2+} channel opening.* (**c**) *The dihydropyridine receptor binds directly to the JFP. On depolarization of the transverse tubule the dihydropyridine receptor alters its conformation and thereby alters the conformation of the JFP so as to gate the channel opening.*

exists that the whole apparatus for Ca^{2+}-induced Ca^{2+} release appears to exist but, nevertheless, does not appear to operate.

Inositol trisphosphate induced Ca^{2+} release

The more recently postulated alternative, IP_3, is a superficially attractive candidate for initiating Ca^{2+} release since this is a known activator of Ca^{2+} release from SR or the equivalent organelle in non-muscle tissue[9,10]. It should, however, be recognized that the Ca^{2+} release initiated in these other cells is hormonally activated and takes place on a relatively slow time scale. In contrast, Ca^{2+} release from skeletal muscle is activated by depolarization. It begins and is terminated within a few milliseconds of depolarization[11]. The evidence in favor of IP_3-induced Ca^{2+} release is that injection of this compound into skinned muscle fibers induces contraction of the muscle[12,13]. However, these observations are still fraught with experimental inconsistencies among different investigators. Critical issues such as the concentration of IP_3 required to initiate release have not been unequivocally answered. The most efficacious response reported indicates that a concentration of ~0.5 μM is adequate to activate Ca^{2+} release[14]. The kinetics have not been studied directly using IP_3 since diffusion time limits the response to the treatment. Walker *et al.*[15] have administered an inactive precursor (caged IP_3) to skeletal muscle and converted it by a photoreaction into IP_3. They observed a very slow onset of contraction. Similarly, in cardiac muscle Fabiato[16] has shown that the rate of IP_3 induced contraction is quite slow. It is interesting that, as with Ca^{2+}-induced Ca^{2+} release, the T-tubules contain the apparatus for synthesis of IP_3 even though there is no evidence of the existence of hormone receptors which might use the cascade as occurs in other cellular systems. Phosphatidylinositol (PI) kinase and phosphatidylinositol 4-phosphate (PIP) kinase are localized in the T-tubule, although PI kinase appears also to be present in sarcoplasmic reticulum[17,18]. A phospholipase C acting on PIP_2 is associated with the T-tubule. The quantitative requirements for IP_3 to be a transmitter are particularly stringent in view of the rapidity of Ca^{2+} release in normal contraction, and the necessity for regeneration of IP_3 to permit repetitive firing of the muscle in a short period of time. Current evidence indicates PI kinase and PIP kinase are present in concentrations similar to those of other cells but these concentrations are not adequate to account for the required rapidity of regeneration of the precursor PIP_2. In addition, phospholipase C activity has been described as Ca^{2+} dependent in the physiological range[18]. Therefore, its action may be the result of stimulation rather than the cause. It is doubtful that the concentration of PIP_2 provides an adequate store to generate IP_3 at the concentration needed in the cytoplasm, unless the diffusion of IP_3 is restricted to the triadic region. Current evidence, therefore, does not favor the view that IP_3 is the activator of contraction, although it may play a role in modulating or modifying contraction.

Direct mechanical coupling hypothesis

An alternative scheme of contraction by direct coupling between T-tubules and SR has been proposed to bypass the limitations of transmitter–receptor interaction. Among the attractions of this hypothesis, originally proposed by Schneider and Chandler[19], is the favorable morphology of the junction. It has been known for a considerable period that the T-tubule is held in immediate juxtaposition to the SR by electron-dense protrusions called junctional feet. It has also become apparent largely through the work of Meissner *et al.*[20], that Ca^{2+} release is associated predominantly with the terminal cisternae portion of the SR. It is this domain which is held in apposition to the transverse tubule. Thus, direct mechanical coupling between the T-tubule and the SR could occur through the junctional structures which, in their turn, may interact with or be a part of the Ca^{2+} channel of the SR.

The original hypothesis of direct mechanical communication arose through the observation that a non-linear capacitive current was observable when the membrane potential of the muscle was altered. The size of this charge movement was half-maximal at a level of depolarization which gave half-maximal activation of muscle contraction. The charge movement was originally modeled as a plunger which contained a charge constituent joining the T-tubule to the terminal cisternae. The movement of the charge constituent caused the opening of the Ca^{2+} channel of the terminal cisternae by unblocking the pore[21]. In many ways

this crude but simple model has withstood the test of time. Most particularly, charge movement is very closely connected to muscle activation not only as a function of membrane potential but also in the response to pharmacological manipulation. The limitation of the model was that it did not lead to the defining of a specific moiety through which the message of transmission occurred. The details of this model, as well as the alternative protocols, has been fleshed out through a growing knowledge of the molecular constituents of the triad junction.

The Ca^{2+}-release site

Our ability to obtain molecular insight into the triad junction has arisen through fractionation procedures at the level of the vesicular components for the junction and, subsequently, at the level of the protein constituents of these vesicles. Sarcoplasmic reticulum has been physically separated into longitudinal reticulum and terminal cisternae. The terminal cisternae fraction has been shown to contain T-tubules associated in the form of a triadic junction, and the triadic junction has itself been separated into its component organelles. The T-tubules have been shown to be an extremely rich source of dihydropyridine receptor, the putative Ca^{2+} channel. The identification of the junctional constituent has been achieved through two complementary approaches. Cadwell and Caswell[22] identified a high molecular weight protein (confined to the terminal cisternae) that exhibited expected properties of the junctional feet. This protein was subsequently extracted and isolated[23]. At the same time, several investigators have followed the pharmacological approach of identifying a drug which binds and reacts specifically with the Ca^{2+} release channel of sarcoplasmic reticulum. One drug (ryanodine) has now been radiolabeled as a means to the isolation of the channel. The recent isolation of the channel has shown that it is identical to the protein previously identified as the junctional feet[24–26]. This now allows us to see the model of direct mechanical coupling in a more specific context: The feet processes themselves contain the Ca^{2+} channel and this channel is in immediate juxtaposition to the T-tubule. Therefore, the logic of direct mechanical coupling becomes more compeling, but the caveat remains that the understanding of muscle contraction has been bedeviled by compeling logic which has not translated into direct demonstration.

The voltage sensor

A further insight into the mechanism of muscle contraction has arisen through the development of our understanding of the role of the dihydropyridine receptor, the putative Ca^{2+} channel of T-tubules. The existence of this channel was originally invoked to support the concept of Ca^{2+}-induced Ca^{2+} release, but it soon became apparent that the mode of action of this protein is more complicated. The early experiments on drugs which inhibited this channel failed to elicit a significant inhibition of muscle contraction. In addition, the Ca^{2+} current associated with the action potential was switched on by muscle depolarization significantly after the contractile event had begun. Furthermore a long-standing discrepancy existed between the biochemical data in which dihydropyridine drugs, such as nitrendipine, gave a K_d for the receptor in the nanomolar range while inhibition of the slow Ca^{2+} current requires much higher concentration.

Hui *et al.*[27] demonstrated that the Ca^{2+}-blocking drug, D600, was indeed able to inhibit contraction although this inhibition occurred only after the muscle had been subjected to K^+ contracture and cooled. Rios and Brum[28] subsequently reported that inhibition of contraction could be elicited with nitrendipine. These authors added an interesting corollary, postulating that the dihydropyridine receptor might be acting not in the capacity of a Ca^{2+} channel but in the role of a voltage sensor. The earlier electrophysiological experiments demonstrated that the opening of the Ca^{2+} channel occurred on depolarization of the fiber. Rios and Brum[28] have argued that the dihydropyridine receptor can have a dual role. In skeletal muscle the main role of this channel is to sense the electrical potential of the T-tubule and to transmit, via a conformational change, the information to the SR without necessarily initiating Ca^{2+} influx.

The physiological evidence germaine to this hypothesis rests largely on a requirement for the following correlations:

(1) activation or inhibition of the dihydropyridine receptor is associated with concomitant activation or inhibition of charge movement ($Q\beta$) and the two are temporally linked;
(2) activation or inhibition of charge movement is associated with concomitant activation or inhibition of Ca^{2+} release from SR and contraction;
(3) activation or inhibition of Ca^{2+} movement into the cell is not associated with concomitant activation or inhibition of charge movement and contraction.

Most of the data currently available support these conclusions although with some reservation. Two classes of agents which are known to bind to and inhibit the dihydropyridine receptor's action as a Ca^{2+} channel are also known to inhibit charge movement and contraction. The conditions in which D600 blocks contraction require that the muscle first be depolarized and cooled and the inhibition can be reversed by hyperpolarization. A possible explanation for these requirements is that D600 only binds the receptor when it is in an inactive state after depolarization. The blockage of charge movement and of contraction are both complete. In the presence of dihydropyridine drugs blockage of charge movement and Ca^{2+} release from the SR appears to be partial and critically dependent on the condition of the fiber. When the holding potential of the muscle fiber is hyperpolarized (-100 mV) the blockage by the dihydropyridine is quite limited, but when the holding potential is at -70 mV a blockage by about 70% is observed. Rios and Brum[28] have shown that this is associated with a concomitant inhibition of the rate and extent of Ca^{2+} release from the SR. The concentrations required to produce even a partial blockade are considerably in excess of the K_d estimated from isolated T-tubules. Lamb[29] has found that increasing dihydropyridine to extremely high concentration does not create more than partial blockage. In neonatal muscle there is evidence that the half-maximal blocking concentration effected by the dihydropyridine, PN200-110, is considerably decreased by depolarization of the fiber. If blockade by PN200-110 of charge movement is similarly voltage dependent this may explain the discrepancy with the biochemical K_d. The physiological half-maximal concentration for the dihydropyridine effect on charge movement may be high because the muscle is polarized, while in biochemical experiments the drug binds with higher affinity to its receptor because it is in its inactive (depolarized) state. What is not yet clear is whether that portion of

the charge movement that is refractory to dihydropyridines represents a separate component of charge movement or whether it simply represents the fact that under the experimental conditions employed, only a portion of the dihydropyridine receptor has been placed in an inactive state capable of binding the drug. In addition, Lamb[29] has found, in rabbit muscle fibers, that although charge movement is consistently blocked (to approximately 50%) by nifedipine, the effect on contraction is quite variable, some fibers being completely refractory to the effects of the drug while others are completely paralysed. This, in its turn, raises the issue as to whether the charge movement invariably reflects the activation of the muscle.

Evidence of a temporal link between activation or inactivation of the dihydropyridine receptor and muscle activation is, as yet, unavailable. The dihydropyridine-sensitive Ca^{2+} channel is switched on with a slow time course. It could be argued, however, that this represents a multistage process in which a rapid conformational response to a voltage change may precede the slow gating of the channel. When the receptor is operating as a voltage sensor for muscle activation, only the conformational change may be required. It is also unclear whether blockage of the receptor by drugs gives a time-correlated blockade of activation. It is possible that the inhibition of muscle activity may be consequent not to direct inhibition of the receptor but to the modified Ca^{2+} homeostasis following channel inhibition. T-tubules contain an active Ca^{2+}-extrusion pump which may deplete cytoplasmic stores directly and SR stores indirectly if Ca^{2+} entry is blocked.

The third requirement for the hypothesis that the dihydropyridine receptor directly mediates the message of excitation to the SR is that there should be a dissociation between Ca^{2+} influx and activation. There is no doubt that under normal conditions a considerable influx of Ca^{2+} is elicited by an action potential but this Ca^{2+} current is slow in onset. A second component of much more rapid activity has also been discerned in neonatal muscle[30]. This fast Ca^{2+} current is uninfluenced by Ca^{2+}-blocking agents and, therefore, cannot play a role in dihydropyridine blockage of contraction. The slow Ca^{2+} current is too slow to account for the rapidity of Ca^{2+} release from the SR. In addition, Baylor and Hollingworth[8] have observed that Ca^{2+} release from the SR may take place in a stimulated muscle even when the cytoplasmic environment is bathed with a rapidly binding Ca^{2+}-chelating agent, further suggesting that any Ca^{2+} influx which may occur is not directly responsible for Ca^{2+} release.

A biochemical model?

If this hypothesis of direct mechanical coupling expressed in its present form is to fulfill the role of excitation–contraction coupling then the physiological data must be supported by biochemical evidence that direct mechanical communication does occur. In many ways the hypothesis is rendered more attractive by the observation that Ca^{2+} release is elicited by the junctional foot protein. For this now requires of the mechanical coupling hypothesis only that there be communication between this protein and the voltage sensor in the T-tubules as illustrated in Fig. 1c. This could be fulfilled if the junctional foot protein is physically attached to the dihydropyridine receptor. One would further expect that the attachment should be specific in the sense that all junctional foot particles would have an associated dihydropyridine receptor or receptor cluster.

Block *et al.*[31] have observed tetrads of large intercalated particles in freeze-fracture replicas of skeletal muscle T-tubules. The large size of the intercalated particle is consistent with the view that this particle may be the dihydropyridine receptor whose total molecular weight must be in the range of 400 000. An unexpected feature of the observation of these authors is that there is one tetrad per two feet indicating that only half the feet are associated with these particles.

The biochemical evidence to support direct connection between the dihydropyridine receptor and the junctional foot protein is weak. We have employed three techniques to determine which proteins in the T-tubule bind to the foot protein. These are:

(1) affinity chromatography, employing the isolated protein and dissolved T-tubular vesicles;
(2) gel overlay of T-tubular proteins that have been blotted onto nitrocellulose membranes, employing radiolabeled isolated junctional foot protein;
(3) crosslinking using hetero bifunctional crosslinking agents.

In each case we have clear evidence for an association between the glycolytic enzymes, glyceraldehyde phosphate dehydrogenase and aldolase, with the junctional foot protein. We have not been able to observe any direct association between the junctional foot protein and the dihydropyridine receptor or, for that matter, any intrinsic T-tubular protein. Chadwick *et al.*[32], using a hetero bifunctional agent, have described an association between the junctional foot protein and a protein of molecular weight 72 000 in the T-tubule. This is not a subunit of the dihydropyridine receptor and is, in any event, an extrinsic protein. Using gel overlay procedures, we have observed an association between glyceraldehyde phosphate dehydrogenase and the α_1-subunit of the dihydropyridine receptor; this, however, is not specific in the sense that the glycolytic enzyme binds to a number of other T-tubular constituents. Thus, there remains the possibility that the junctional foot protein binds indirectly through the glycolytic enzymes to the DHP receptor but it is doubtful that this could serve for direct mechanical coupling.

It needs to be emphasized that the hypothesis that the dihydropyridine receptor is the voltage sensor for muscle excitation does not, in itself, require that the receptor binds directly to the junctional feet. Communication could take place through indirect means; we are, therefore, currently in the ambiguous situation that our knowledge on the processes of muscle excitation has increased vastly but we still are limited by our inability to produce a theory that is fully consistent with the existing data, although much of our current knowledge is in accord with the hypothesis of direct mechanical coupling. The molecular mechanism of direct mechanical coupling has not been explained in skeletal muscle but could be analogous to the sliding of subunits which has been described in gap junctions.

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Talking Point

How 'hidden' reading frames are expressed

Roberto Cattaneo

Secondary reading frames, 'hidden' under other reading frames, are used for coordinated expression of proteins in several eukaryotic viruses. In some genes, ribosomal frameshifting and initiation or reinitiation of protein synthesis on internal AUG codons are translational mechanisms allowing access to such 'hidden' reading frames. In others, secondary reading frames are translated from alternatively spliced or edited mRNAs.

Eukaryotic mRNAs are generally monocistronic, but for several viral transcripts[1–8] as well as for one cellular transcript[9], it has been shown that proteins encoded in different reading frames are expressed. Since eukaryotic ribosomes typically start protein synthesis at the first AUG codon only[4], it is not immediately evident how secondary reading frames overlapping with or following the first reading frame can be expressed.

Ribosomal frameshifting

A situation present in many viral eukaryotic transcripts is illustrated in Fig. 1: the first open reading frame (top, stippled box) overlaps with a second one (top, black box). Thus, if translation begins at the first AUG codon, only one protein will be expressed (bottom left, stippled box). Ribosomal frameshifting, subsequent to initiation on the first AUG codon, may enable expression of the overlapping reading frame, creating a fusion protein (bottom right, fused stippled and black boxes). Since only a fraction of the ribosomes change frame at a frameshift signal, fusion proteins are produced in addition to, rather than instead of, the 'normal' protein.

Frameshifting in the −1 frame is used by most retroviruses to access the reverse transcriptase reading frame[5,6], hidden in the gag mRNA, and by coronaviruses to express their RNA replicase[10], encoded by two different reading frames. Until now, no clear-cut case of ribosomal frameshifting has been observed in cellular genes of higher eukaryotes, and it seems that cellular eukaryotic genes avoid sequences on which frameshifting could occur[6]. In contrast, the yeast retroviral-like element Ty shifts to the +1 frame to express its reverse transcriptase[7], and the ribosomes of *E. coli* can slip, shift, step backward and hop forward (−2, −1, +1, +2, +5 and +6 frameshifts)[11].

Internal initiation

The use of an internal AUG codon for initiation of translation is an alternative to ribosomal frameshifting, and does not result in production of fusion proteins (Fig. 1, bottom center, black box). Several conditions may allow the use of an internal AUG codon. (1) An internal AUG is sometimes used in cases where the first AUG occurs in an unfavourable context for translation initiation[4]. (2) Termination of protein synthesis at a stop codon may lead to reinitiation at a nearby AUG in another frame[9,12]. (3) A 'ribosome landing pad' may direct the ribosome to an internal position in the mRNA, as described for the uncapped genomic RNA of picornaviruses[13]. (4) In a capped mRNA of a paramyxovirus, some ribosomes pass from the cap directly to an initiation codon far downstream[14].

It is interesting to note that the cousins of retroviruses and the yeast Ty elements, the hepatitis B-like viruses (HBVs), also have the reverse transcriptase reading frame hidden under an overlapping reading frame initiating upstream. However, in contrast to the retroviruses, HBVs avoid ribosomal frameshifting and instead use an internal AUG codon to express reverse

R. Cattaneo is at the Institut für Molekularbiologie I, Universität Zürich, Hönggerberg, 8093 Zürich, Switzerland.