

Research article

## Functional analysis of a frame-shift mutant of the dihydropyridine receptor pore subunit ( $\alpha_{1S}$ ) expressing two complementary protein fragments

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### Abstract

**Background:** The L-type  $\text{Ca}^{2+}$  channel formed by the dihydropyridine receptor (DHPR) of skeletal muscle senses the membrane voltage and opens the ryanodine receptor (RyR1). This channel-to-channel coupling is essential for  $\text{Ca}^{2+}$  signaling but poorly understood. We characterized a single-base frame-shift mutant of  $\alpha_{1S}$ , the pore subunit of the DHPR, that has the unusual ability to function voltage sensor for excitation-contraction (EC) coupling by virtue of expressing two complementary hemi- $\text{Ca}^{2+}$  channel fragments.

**Results:** Functional analysis of cDNA transfected dysgenic myotubes lacking  $\alpha_{1S}$  were carried out using voltage-clamp, confocal  $\text{Ca}^{2+}$  indicator fluorescence, epitope immunofluorescence and immunoblots of expressed proteins. The frame-shift mutant (fs- $\alpha_{1S}$ ) expressed the N-terminal half of  $\alpha_{1S}$  (M1 to L670) and the C-terminal half starting at M701 separately. The C-terminal fragment was generated by an unexpected restart of translation of the fs- $\alpha_{1S}$  message at M701 and was eliminated by a M701I mutation. Protein-protein complementation between the two fragments produced recovery of skeletal-type EC coupling but not L-type  $\text{Ca}^{2+}$  current.

**Discussion:** A premature stop codon in the II-III loop may not necessarily cause a loss of DHPR function due to a restart of translation within the II-III loop, presumably by a mechanism involving leaky ribosomal scanning. In these cases, function is recovered by expression of complementary protein fragments from the same cDNA. DHPR-RyR1 interactions can be achieved via protein-protein complementation between hemi- $\text{Ca}^{2+}$  channel proteins, hence an intact II-III loop is not essential for coupling the DHPR voltage sensor to the opening of RyR1 channel.

### Background

The dihydropyridine receptor (DHPR) of skeletal muscle consists of  $\alpha_{1S}$ ,  $\alpha_2$ ,  $\beta_{1a}$  and  $\gamma_1$  subunits [1]. The  $\alpha_1$  subunit is a large four-repeat transmembrane protein of ~220 kDa that contains the basic functional elements of the L-type  $\text{Ca}^{2+}$  channel, including the  $\text{Ca}^{2+}$  selective pore and S4

"voltage-sensing" transmembrane segments in each of the four internal repeats [2].  $\beta$  subunits are ~65 kDa cytosolic proteins essential for membrane trafficking, modulation of channel kinetics, and for excitation-contraction (EC) coupling [3,4]. The  $\alpha_2$  subunit is a highly glycosylated ~175 kDa protein formed by two disulfide-linked pep-

tides [5], whereas the  $\gamma_1$  subunit is a ~32 kDa skeletal muscle-specific protein of four presumptive transmembrane domains with almost unknown function [6,7].

Skeletal muscle cells utilize the voltage sensors formed by the S4 segments to trigger a rapid elevation of cytosolic  $\text{Ca}^{2+}$ , thus coupling membrane excitation to muscle cell contraction. Subsequent to charge movements in the voltage sensors, a conformational change in the DHPR is transmitted to the ryanodine receptor (RyR1), presumably, via protein-protein interactions [8]. Ultimately, there is a brief opening of the RyR1 channel resulting in the release of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum (SR). Numerous observations have lent support to this view, and especially significant are the functional expression studies in dysgenic myotubes lacking  $\alpha_{1S}$ . The dysgenic myotube is devoid of L-type  $\text{Ca}^{2+}$  current, charge movements and EC coupling. All three are restored in the dysgenic myotube by expression of  $\alpha_{1S}$ [9–11]. These results corroborated the essential role of  $\alpha_{1S}$  in the mechanism of EC coupling of skeletal muscle cells.

The mechanism by which the DHPR signals the RyR1 is poorly understood [12,13]. Domains in the cytoplasmic linker between repeats II and III have been clearly implicated [14–19], and some regions such as Thr671-Lue690 were suggested to trigger RyR1 opening by binding to RyR1 [15]. However, extensive deletions within the II-III linker that eliminate the RyR1 binding region, and other suggested signaling regions in the II-III loop [16], do not entirely eliminate EC coupling [20,21]. Hence additional domains of  $\alpha_{1S}$  and/or other DHPR subunits appear to be engaged by the voltage sensor and contribute to an EC coupling signal. In this respect, the contribution of the  $\beta_1$  subunit of the DHPR to EC coupling in skeletal muscle cells has been extensively documented [4,22–24].

In the present report, we characterized a frame-shift mutant of  $\alpha_{1S}$  that expresses two complementary fragments of  $\alpha_{1S}$ . Complementation between the two  $\alpha_{1S}$  fragments produced recovery of EC coupling in dysgenic muscle cells lacking  $\alpha_{1S}$ . The results suggest the EC coupling voltage sensor of skeletal muscle is modular in function and can be assembled from separate hemi- $\text{Ca}^{2+}$  channel fragments.

## Results and Discussion

### Expression of a frame-shift mutation of $\alpha_{1S}$ in dysgenic myotubes

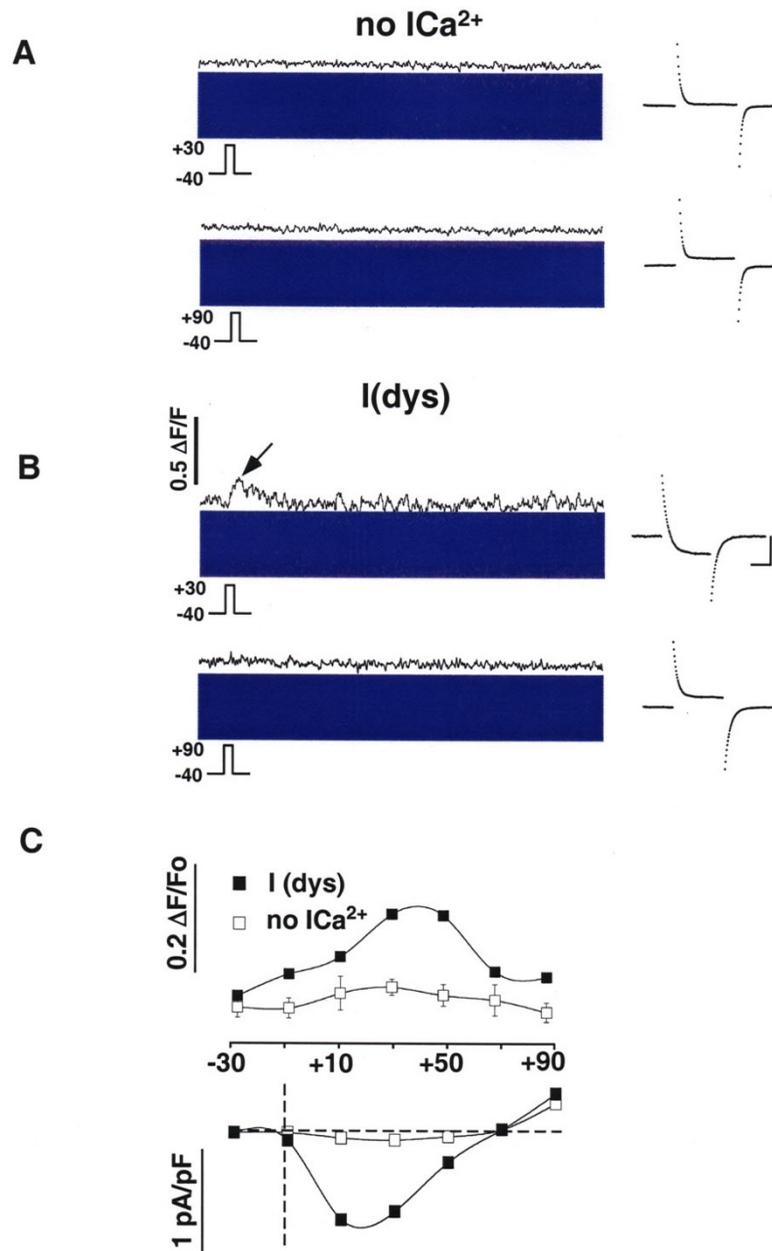
Primers for the frame-shift mutant, fs- $\alpha_{1S}$ , were originally designed to delete the 20-mer Thr671-Leu690 in the cytosolic loop between repeats II and III of  $\alpha_{1S}$  and to generate a full-length  $\alpha_{1S}$  carrying this internal deletion. A proofreading error during a PCR reaction resulted in an amplified DNA with the desired deletion but also contain-

ing an additional thymidine following the TTC codon for Leu670 (Fig. 1A). The one-base shift in reading frame introduced a serine at position 671 followed immediately by a stop codon (Fig. 1B). This frame-shift mutation was re-ligated into an otherwise full-length  $\alpha_{1S}$ , subcloned into the mammalian expression vector pSG5, and transfected into dysgenic ( $\alpha_{1S}$  null) myotubes. Fs- $\alpha_{1S}$  was abundantly expressed in primary dysgenic myotubes in culture (Fig. 1C) and produced the expected truncated  $\alpha_{1S}$  protein (Fig. 1D). Western blots using N-terminus T7-tagged fs- $\alpha_{1S}$  and T7-tagged full-length  $\alpha_{1S}$  showed that the expressed full-length  $\alpha_{1S}$  protein migrated with an apparent molecular weight of approximately 185 KDa under reducing conditions. This result is consistent with the mobility of the native purified skeletal muscle  $\alpha_{1S}$  subunit [25]. The fs- $\alpha_{1S}$  migrated with a molecular weight of approximately 90 KDa which is entirely consistent with the theoretical molecular weight of the expressed fragment which was 85.6 KDa. Furthermore, 5-fold overloading of the SDS-PAGE gel failed to detect any fragment of a size comparable to full-length  $\alpha_{1S}$  (not shown).

### Recovery of EC coupling by the frame-shift $\alpha_{1S}$ cDNA

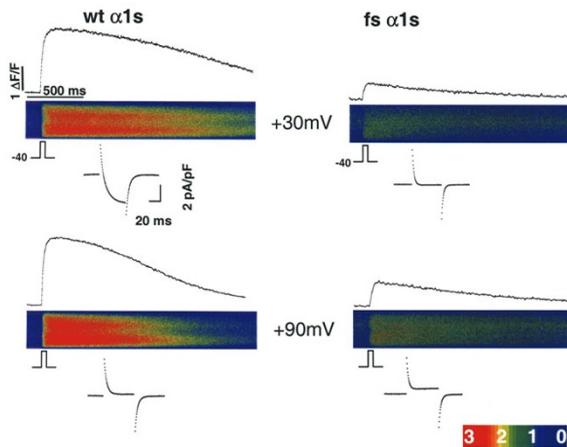
EC coupling was investigated in voltage-clamped myotubes with simultaneous monitoring of intracellular  $\text{Ca}^{2+}$  using confocal fluorescence of fluo-4 [26]. Controls shown in Fig. 2A indicated that the overwhelming majority of non-transfected dysgenic myotubes (13 of 15 cells) did not produce detectable  $\text{Ca}^{2+}$  transients ( $<0.1 \Delta\text{F}/\text{Fo}$ ) or  $\text{Ca}^{2+}$  currents ( $<20 \text{ pA}/\text{cell}$ ) in response to depolarization under voltage-clamp. This is shown in the line-scan images of fluo-4 fluorescence in Fig. 2A and the corresponding traces of  $\text{ICa}^{2+}$  during a 50-ms depolarization to +30 mV and +90 mV delivered at the start of the line scan in the same cell. However, in two cells (2 of 15 cells) we observed Idys, the low-density endogenous  $\text{Ca}^{2+}$  current previously described in dysgenic myotubes [27,28]. The reason for the low abundance of this current in these cultured myotubes is unknown.  $\text{Ca}^{2+}$  currents and stimulated fluorescence for one of the cells expressing Idys is shown in Fig. 2B. We observed a peak  $\text{ICa}^{2+}$  density of approximately 0.8 pA/pF and a barely detectable fluorescence signal which in Fig. 2B is indicated by the arrow in the trace of integrated fluorescence at +30 mV. This small fluorescence signal disappeared entirely at +90 mV, suggesting it might be contributed directly by Idys or might be due to SR  $\text{Ca}^{2+}$  release induced by Idys. The voltage dependence of the fluorescence signal and  $\text{ICa}^{2+}$  are compared in Fig. 2C for the two cells expressing Idys and for the vast majority of cells which altogether did not express intracellular  $\text{Ca}^{2+}$  transients or  $\text{ICa}^{2+}$ . The maximum fluorescence signal contributed by Idys, when Idys was present, was  $<0.2 \Delta\text{F}/\text{Fo}$  units. Furthermore, the shape of the fluorescence vs. voltage relationship was bell-shaped and a mirror image of the  $\text{ICa}^{2+}$  vs. voltage curve. These





**Figure 2**

Absence of EC coupling in non-transfected dysgenic myotubes. The confocal line-scan images in color show fluo-4 fluorescence across myotubes in response to a 50-ms depolarization from a holding potential of -40 mV. Line scan images have a constant temporal dimension of 2.05 s (horizontal) and a variable spatial dimension (vertical) depending on the cell size. Traces immediately above each line scan show the time course of the fluorescence change in resting units ( $\Delta F/F_0$ ). The amplitude and the timing of the depolarization are indicated under each line-scan. Arrow indicates a small  $Ca^{2+}$  transient elicited in 1 of 2 cells found to express  $I_{dys}$ . Traces next to lines-cans show  $ICa^{2+}$  during the 50 ms depolarization used to stimulate the  $Ca^{2+}$  transient. Current calibration bars are 10 ms and 1 pA/pF. A) Absence of  $Ca^{2+}$  transients and  $ICa^{2+}$  in a typical dysgenic cell. B) Minor  $Ca^{2+}$  transient and  $ICa^{2+}$  in a cell expressing  $I_{dys}$ . Note that fluorescence calibration bar is 0.5  $\Delta F/F_0$ . A 16-color calibration bar in  $\Delta F/F_0$  units is included in Fig. 3 for visual reference. C) Voltage dependence of the mean ( $\pm$  SEM)  $ICa^{2+}$  and mean peak  $Ca^{2+}$  transient ( $\pm$  SEM) in 13 cells not expressing  $ICa^{2+}$  and 2 cells expressing  $I_{dys}$  (mean only).



**Figure 3**

$\text{Ca}^{2+}$  transients in dysgenic myotubes transfected with  $\text{fs-}\alpha_{1S}$ . The confocal line-scan images in color show fluo-4 fluorescence across myotubes in response to a 50-ms depolarization from a holding potential of  $-40$  mV to  $+30$  mV (top) and  $+90$  mV (bottom). Line scan images have a constant temporal dimension of 2.05 s (horizontal) and a variable spatial dimension (vertical) depending on the cell size. Traces immediately above each line scan show the time course of the fluorescence change in resting units ( $\Delta F/F_0$ ). Traces under lines cans show  $\text{ICa}^{2+}$  during the 50 ms depolarization used to stimulate the  $\text{Ca}^{2+}$  transient. The amplitude and the timing of the depolarization are indicated under each line scan. Note that fluorescence calibration bar is  $1 \Delta F/F_0$ . A 16-color calibration bar in  $\Delta F/F_0$  units is included for visual reference.

controls indicated that non-transfected dysgenic myotubes are low-background cells that do not express voltage-activated  $\text{Ca}^{2+}$  signals of major consequence for the present studies.

Fig. 3 shows that  $\text{fs-}\alpha_{1S}$  recovered a significant fraction of the voltage-activated  $\text{Ca}^{2+}$  transient compared to that expressed by full-length  $\text{wt-}\alpha_{1S}$ . The magnitude of the fluorescence signal expressed by  $\text{fs-}\alpha_{1S}$  was approximately 5-fold larger than the largest  $\text{Ca}^{2+}$  transient detected in non-transfected myotubes expressing  $\text{Idys}$ , >20-fold larger than the average  $\text{Ca}^{2+}$  transient detectable in non-transfected cells, and about 1/3 of the maximum SR  $\text{Ca}^{2+}$  release expressed by the control  $\text{wt-}\alpha_{1S}$  construct. Thus, we are confident that the voltage-evoked  $\text{Ca}^{2+}$  transient in cells transfected by  $\text{fs-}\alpha_{1S}$  cells was a direct consequence of the expressed protein. Also shown in Fig. 3 is  $\text{ICa}^{2+}$  activated by the 50-ms depolarization used to activate the  $\text{Ca}^{2+}$  transient.  $\text{fs-}\alpha_{1S}$  did not express L-type  $\text{Ca}^{2+}$  current even though it was consistently able to activate the  $\text{Ca}^{2+}$  transient in 15 of 15 cells. Absence of  $\text{ICa}^{2+}$  was further verified using longer 500-ms depolarizing pulses (not

shown). The skeletal nature of the EC coupling expressed by  $\text{fs-}\alpha_{1S}$  is shown in Fig. 4A. The peak  $\text{Ca}^{2+}$  vs. voltage relationship expressed by  $\text{fs-}\alpha_{1S}$ , like that of  $\text{wt-}\alpha_{1S}$ , was sigmoidal in shape reaching a maximum at large positive potentials ( $>50$  mV), a range in which  $\text{ICa}^{2+}$  is progressively small. The line scans of Fig. 4B further confirmed that a  $\text{Ca}^{2+}$  transient of similar shape and magnitude was observed in a  $\text{fs-}\alpha_{1S}$  transfected myotube in the absence of external  $\text{Ca}^{2+}$ . Hence the signaling mechanism, like that reported in normal myotubes and dysgenic myotubes expressing  $\text{wt-}\alpha_{1S}$ , was  $\text{Ca}^{2+}$  entry independent [29]. We also expressed  $\text{fs-}\alpha_{1S}$  in cultured myotubes from two available gene knock-out (KO) mice, lacking the endogenous  $\beta 1a$  isoform of the skeletal muscle DHPR [30] and lacking RyR1 [31]. As shown in Fig. 4B, we failed to detect EC coupling in these KO cells transfected with  $\text{fs-}\alpha_{1S}$ . In summary, the EC coupling expressed by  $\text{fs-}\alpha_{1S}$  is strictly skeletal-type and requires RyR1 and DHPR  $\beta 1a$ .

#### ***fs-α<sub>1S</sub> expresses two complementary protein fragments***

The EC coupling recovered by  $\text{fs-}\alpha_{1S}$  could be due either to the activity of the N-terminal half of  $\alpha_{1S}$  alone or to protein-protein complementation between the N-terminal half and a fragment expressing the C-terminal half of  $\alpha_{1S}$ . The C-terminal half of  $\alpha_{1S}$  could have been translated by the  $\text{fs-}\alpha_{1S}$  expression vector if the ATG codon (Met701), which is downstream from the TGA termination codon and is in-frame with the wild-type message (Fig 1B) served as open reading frame for translation of the second half of the wt message. Although this would be unusual, the fact that the codon for Met701 is only 25 bases downstream from the termination codon could have substantially increased the possibility of a re-start of the translation of the second half of the message at Met701. This phenomenon has been described in eukaryotic cells and in viral-infected mammalian cells and is known as translation by leaky ribosomal scanning [32,33]. To test this explanation, the presumptive restart codon, Met701, was mutated to Ile701 in the  $\text{fs-}\alpha_{1S}$  template. If  $\text{fs-}\alpha_{1S}$  recovered EC coupling by virtue of expressing a single protein fragment, then  $\text{fs-}\alpha_{1S}\text{M701I}$  should also recover EC coupling since the mutation was introduced downstream from the stop codon. Fig. 5 shows that this was not the case.  $\text{Fs-}\alpha_{1S}\text{M701I}$  did not recover  $\text{Ca}^{2+}$  transients in 9 of 9 tested cells, consistent with leaky ribosomal scanning. As a positive control, we coexpressed  $\text{fs-}\alpha_{1S}\text{M701I}$  and the C-terminus half of  $\alpha_{1S}$ , namely  $\alpha_{1S}\Delta 1-700$ , cloned into a separate pSG5 vector. The results in Fig. 5 indicated that  $\alpha_{1S}\Delta 1-700$  alone was inactive. However, when myotubes were cotransfected with  $\text{fs-}\alpha_{1S}\text{M701I}$  and  $\alpha_{1S}\Delta 1-700$ , each in a separate pSG5 vector, there was a robust recovery of  $\text{Ca}^{2+}$  transients in 5 of 5 cells. Fig. 6A shows fluorescence vs. voltage relationships for the  $\text{fs-}\alpha_{1S}\text{M701I}$  mutant and for this mutant coexpressed with  $\alpha_{1S}\Delta 1-700$ . The combined expression of the two complementary frag-

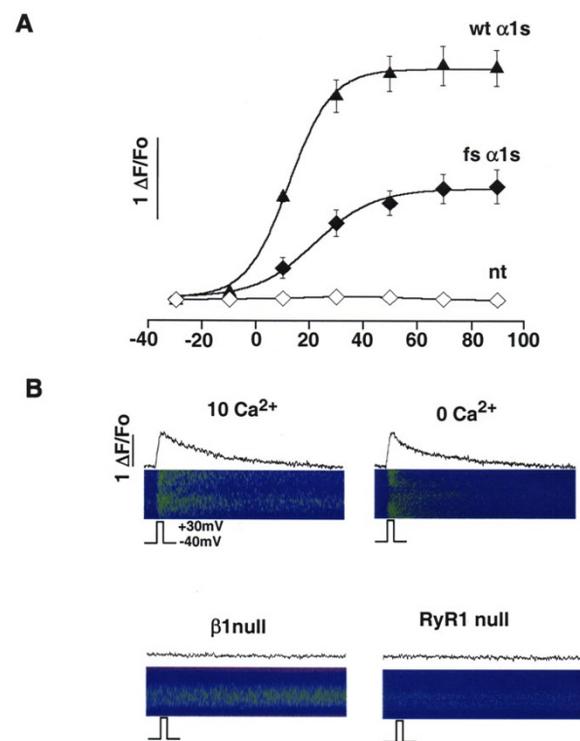
ments of  $\alpha_{1S}$  resulted in a robust recovery of EC coupling with sigmoidal  $\text{Ca}^{2+}$  release vs. voltage characteristics. A summary of the maximum fluorescence during the  $\text{Ca}^{2+}$  transient in response to a depolarization to +90 mV is shown in Fig. 6B. The magnitude of the  $\text{Ca}^{2+}$  transient expressed by fs- $\alpha_{1S}$ M701I +  $\alpha_{1S}\Delta 1-700$  was indistinguishable from that of wt- $\alpha_{1S}$  (t-test significance  $p = 0.671$ , see figure legend). To confirm expression of the C-terminus half of  $\alpha_{1S}$  in cells transfected with fs- $\alpha_{1S}$ , we used the II-III loop polyclonal antibody SKI [34] directed against epitope Ala739-Ile752 which is downstream from Met701. Fig. 6C shows that the II-III loop antibody recognized the C-terminus half when cells were transfected with fs- $\alpha_{1S}$  but not when myotubes were transfected with fs- $\alpha_{1S}$ M701I. The C-terminal protein migrated with a molecular weight of approximately 126 KDa which is consistent with the theoretical molecular weight of 132 KDa. Finally, Fig. 6D shows that fs- $\alpha_{1S}$ M701I was abundantly expressed in myotubes in the absence or presence of the C-terminal fragment. This indicated that the absence of EC coupling observed in myotubes expressing fs- $\alpha_{1S}$ M701I was not due the production of a labile protein. In summary, the recovery of EC coupling by coexpression of two functionally inactive proteins (Fig. 5) taken together with the immunoblots (Fig. 6C) favor the explanation that 1) fs- $\alpha_{1S}$  recovers DHPR function by virtue of expressing two complementary fragments of  $\alpha_{1S}$  and 2) the expression of the C-terminal half of  $\alpha_{1S}$  by fs- $\alpha_{1S}$  is likely to occur by leaky ribosomal scanning.

#### Implications for EC coupling in skeletal myotubes

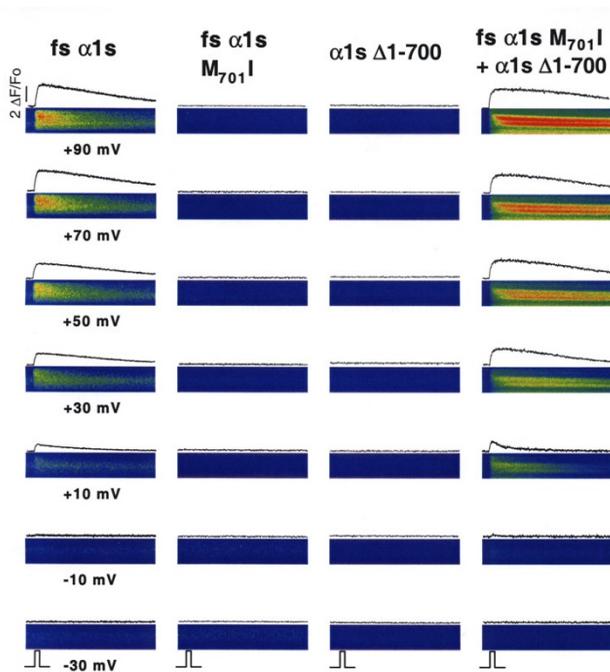
Except for the magnitude, the SR  $\text{Ca}^{2+}$  release signal expressed by fs- $\alpha_{1S}$  was entirely typical of skeletal myotubes with sigmoidal voltage-dependence, preceding in the absence of external  $\text{Ca}^{2+}$  and requiring RyR1. A comparison of the maximum fluorescence ( $\Delta F/\text{Fo}$  max) at +90 mV (Fig. 6B) shows that the signal generated by fs- $\alpha_{1S}$  was significantly smaller than that generated by the control construct (wt- $\alpha_{1S}$  vs. fs- $\alpha_{1S}$  t-test significance  $p = 0.014$ ) and smaller than that generated by the two coexpressed fragments (fs- $\alpha_{1S}$  vs. fs- $\alpha_{1S}$ M701I +  $\alpha_{1S}\Delta 1-700$  t-test significance  $p = 0.013$ ). These observation suggests that the magnitude of the  $\text{Ca}^{2+}$  release appears to be limited by the low yield of expression of the C-terminal fragment achieved by leaky ribosomal scanning of the second half of the fs- $\alpha_{1S}$  message. To test this explanation further, we coexpressed fs- $\alpha_{1S}$  and the C-terminal half of  $\alpha_{1S}$  each in a separate pSG5 vector. We found that fs- $\alpha_{1S}$  and  $\alpha_{1S}\Delta 1-700$  together expressed  $\text{Ca}^{2+}$  transients with a  $\Delta F/\text{Fo}$  max similar to wt- $\alpha_{1S}$  control (not shown). Thus we are certain that the EC coupling expressed by fs- $\alpha_{1S}$  is mechanistically similar to control skeletal-type EC coupling but limited in magnitude by a comparatively lower density of functional DHPRs that are assembled in cells expressing fs- $\alpha_{1S}$ . It is conceivable that the functional integrity of the

fragmented  $\alpha_{1S}$  protein is maintained in part by the  $\beta$  subunit of the DHPR which spans both halves of the  $\alpha 1$  pore subunit by binding to the I-II loop and the C-terminus [35,36]. Consistent with this explanation, we failed to detect EC coupling recovery when fs- $\alpha_{1S}$  was expressed in  $\beta 1$ -null myotubes.

Earlier studies in the voltage-gated  $\text{Na}^+$  channel had shown that pore function was not compromised when the II-III linker or III-IV linker was cut and the two fragments were coexpressed each in a separate vector [37]. We would thus conclude that in the case of the  $\text{Ca}^{2+}$  channel, an in-



**Figure 4** Skeletal-type EC coupling expressed by fs- $\alpha_{1S}$ . A) Voltage-dependence of peak  $\text{Ca}^{2+}$  for 5 control myotubes expressing wt- $\alpha_{1S}$  and 5 myotubes expressing fs- $\alpha_{1S}$ .  $\text{Ca}^{2+}$  transients for 15 non-transfected myotubes (NT) are included for reference. The sigmoidal lines are a Boltzmann fit with parameters  $\Delta F/\text{Fo}$  max = 2.9, 1.4  $\Delta F/\text{Fo}$ ;  $V_{1/2} = 11.7, 20$  mV;  $k = 8.7, 13.2$  mV, for wt- $\alpha_{1S}$  and fs- $\alpha_{1S}$  respectively. B) Line-scans (horizontal dimension is 2.05 seconds) and traces of integrated fluorescence in  $\Delta F/\text{Fo}$  units for depolarizations to +30 mV. Top line-scans are for the same fs- $\alpha_{1S}$  transfected myotube in standard external solution (10 mM  $\text{CaCl}_2$ ) and the same solution without added  $\text{CaCl}_2$  (0  $\text{Ca}^{2+}$ ). Bottom line-scans show fs- $\alpha_{1S}$  transfected KO myotubes lacking DHPR  $\beta 1a$  or lacking RyR1 in standard external solution.



**Figure 5**  
EC coupling generated by two complementary fragments of  $\alpha_{1S}$ . Line scans (horizontal dimension is 2.05 seconds) of fluo-4 fluorescence show  $\text{Ca}^{2+}$  transients in response to the indicated 50-ms depolarization from a holding potential of -40 mV. Trace of integrated fluorescence in  $\Delta F/F_0$  units is shown for each line scan. Each set of depolarizations is from a separate dysgenic myotube expressing the  $\alpha_{1S}$  construct(s) indicated at the top of each column. A 16-color calibration bar in  $\Delta F/F_0$  units is included in Fig. 3 for visual reference.

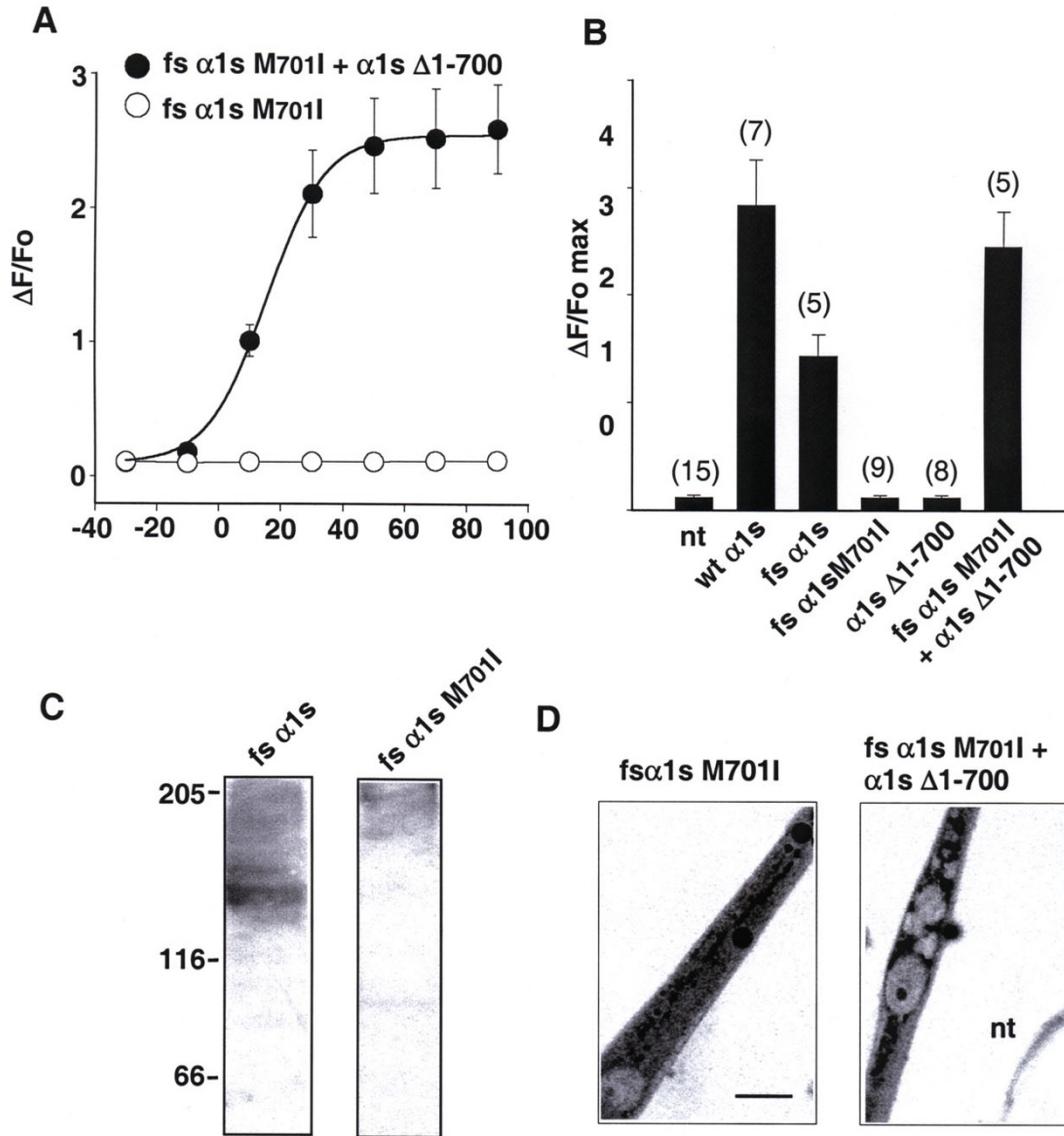
tact II-III loop is essential for this function since neither fs- $\alpha_{1S}$  nor the combined expression of the two truncated fragments (not shown) was able to rescue  $\text{Ca}^{2+}$  current. This result is entirely consistent with the identification of the II-III loop as critical for enhancement of L-type  $\text{Ca}^{2+}$  current expression by the RyR1 [38]. However EC coupling, per se, can clearly proceed with a cut in the II-III loop. This was shown here by the behavior of the fs- $\alpha_{1S}$  construct and elsewhere by expressing the N-terminal half ( $\alpha_{1S}1-670$ ) and the C-terminal half ( $\alpha_{1S}701-1873$ ), each with entirely wt sequence and each in a separate expression vector [20]. The fact that Thr671-Leu690 region known as Peptide A [15] is missing in fs- $\alpha_{1S}$  suggests this 20-mer domain is not critical for the conformation change transmitted from the DHPR to the RyR1. To test this further, we generated an in-frame deletion of this region, as originally intended, that showed normal function [20]. Another domain critical for EC coupling is Csk35 downstream from Peptide A (Leu720-Gln765). This region was identified using chimeras of  $\alpha_{1S}$  and  $\alpha_{1C}$  [16]. Since Csk53 is present in  $\alpha_{1S}\Delta 1-700$  and was detected by

the II-III loop antibody which is directed against the center portion of Csk53 [34], the participation of this domain in the EC cannot be ruled-out.

In eukaryotic cells, translation starts at the AUG codon nearest to the 5' end of the mRNA, and this initiator site is found by sequential ribosomal scanning in the 5' to 3' direction [32]. However, translation initiation at internal AUGs due to leaky ribosomal scanning has been documented, especially for mRNAs consisting of a short leader ORF upstream from the main ORF. In some cases, re-assembly of a new ribosomal initiation complex after the terminator codon of the leader ORF serves to re-initiate translation at the initiator AUG codon for the main ORF, and thus two proteins are generated [39]. In other cases, leaky scanning by the ribosomal initiation complex bypasses the 5'-located leader ORF entirely, and only the "internal" ORF is translated [32]. In the case of fs- $\alpha_{1S}$ , we are not entirely certain which mechanism best applies. A bypass of the ORF at the 5' end of the  $\alpha_{1S}$  mRNA in favor of a presumed ORF at Met701 is unlikely because a C-terminal fragment of the size expressed by  $\alpha_{1S}\Delta 1-700$  has not been detected in skeletal muscle with the SKI antibody [34] or other antibodies [25]. Thus Met701 is not an internal initiation site under normal circumstances. Entry of a new ribosomal complex at Met701 seems a more likely explanation. However, re-initiation after a stop has only been described for cases in which the leader ORF is no longer than 30 codons because initiation factors fall off shortly after recognition of the initiator AUG [32]. In the case of fs- $\alpha_{1S}$ , what can be considered the "leader" ORF encodes for a protein of 671 residues. Hence we are not certain if re-initiation of translation after a stop signal, as currently described in the literature, would apply here. At the same time, it is important to point out that in the present studies, fs- $\alpha_{1S}$  expression is under the control of a viral promoter and that in this hybrid viral-mammalian expression system, the rules pertaining to leaky ribosomal scanning may be different. The mechanism of translation of the fs- $\alpha_{1S}$  clearly deserves closer scrutiny in the future.

## Conclusions

The present studies show EC coupling recovery by a frame-shift mutant of  $\alpha_{1S}$  due to protein-protein complementation of the N-terminal and C-terminal halves of  $\alpha_{1S}$ . The N-terminal half houses repeats I and II with the adjoining cytosolic loop and the C-terminal half houses most of the II-III loop, along with repeats III and IV with the adjoining loop. Protein-protein complementation between the N-terminal and C-terminal fragments produced a DHPR capable of functioning as EC coupling voltage sensor, thus suggesting the presence of at least two functional modules within  $\alpha_{1S}$ . Recent evidence suggests that the four internal repeats of the voltage-gated  $\text{Na}^+$  channel, which is closely related to the L-type  $\text{Ca}^{2+}$  channel encod-



**Figure 6**

Expression of C-terminal fragment of  $\alpha_{1S}$  is essential for EC coupling. A) Voltage dependence of peak  $Ca^{2+}$  during the  $Ca^{2+}$  transient for dysgenic myotubes transfected with the indicated constructs. The sigmoidal curve is a Boltzmann fit with parameters  $\Delta F/F_o$  max = 2.45;  $V_{1/2}$  = 15.4 mV;  $k$  = 9.3 mV for 5 cells coexpressing fs- $\alpha_{1S}$ M701I +  $\alpha_{1S}$  $\Delta$ 1-700. Absence of response is shown for 9 cells expressing fs- $\alpha_{1S}$ M701I alone. B)  $\Delta F/F_o$  max (mean  $\pm$  SEM) obtained from a depolarization to +90 mV is shown for the indicated number of cells. NT denotes non-transfected dysgenic myotubes. Compared to wt- $\alpha_{1S}$  (control), the statistical significance in unpaired t-Student test was  $p = 1.6 \times 10^{-6}$  (non-transfected, NT); 0.014 (fs- $\alpha_{1S}$ ); 0.0002 (fs- $\alpha_{1S}$ M701I); 0.0008 ( $\alpha_{1S}$  $\Delta$ 1-700); 0.671 (fs- $\alpha_{1S}$ M701I +  $\alpha_{1S}$  $\Delta$ 1-700). Compared to fs- $\alpha_{1S}$ , the statistical significance was  $p = 1 \times 10^{-6}$  (non-transfected, NT); 0.014 (wt- $\alpha_{1S}$ ); 0.00019 (fs- $\alpha_{1S}$ M701I); 0.0008 ( $\alpha_{1S}$  $\Delta$ 1-700); 0.013 (fs- $\alpha_{1S}$ M701I +  $\alpha_{1S}$  $\Delta$ 1-700). C) Immunoblots using a polyclonal antibody directed to the II-III loop epitope Ala739-Ile752 [34] in cultures of dysgenic myotubes expressing fs- $\alpha_{1S}$  and fs- $\alpha_{1S}$ M701I. Indicated are 3 of 7 molecular weight markers run in the same gel. D) Confocal images of cells transfected with the CD8 cDNA plus T7-tagged fs- $\alpha_{1S}$ M701I or T7-tagged fs- $\alpha_{1S}$ M701I + untagged  $\alpha_{1S}$  $\Delta$ 1-700. Cells were incubated with CD8 antibody beads, fixed, and stained with T7 primary/ fluorescein-conjugated secondary antibodies. Pixel intensity was converted to a 16-level inverted gray scale with high-intensity pixels in black color. NT indicates a non-transfected myotube. CD8 antibody beads have a diameter of 4.5 microns. Calibration bar is 10 microns.

ed by the DHPR, have non-equivalent functional roles because the S4 segments of repeats I and II move much faster than those of repeats III and IV [40]. By analogy, the "fast-moving" module of the DHPR would be represented by the N-terminal fragment and the "slower-moving" module by the C-terminal fragment. Interactions between these two modules are likely to be critical for intramembrane charge movements in the assembled four-repeat channel and for coupling the movement of the S4 gating charges to the opening of the RyR1 channel. Future studies of gating currents in each hemi-Ca<sup>2+</sup> channel fragment should provide valuable information on how the "fast" and "slow" gating modules interact during EC coupling in skeletal muscle.

The C-terminal fragment was generated by an unusual restart of translation of the fs- $\alpha_{1S}$  message at M701, presumably by leaky ribosomal scanning, and was eliminated by a M701I mutation. Hence, a premature stop codon in the II-III loop upstream of M701 may not necessarily cause a loss of DHPR function because in these cases, function would be recovered by complementation between protein fragments expressed by the same cDNA. From a methodological perspective, leaky scanning could be further used as a means to control protein expression to desired levels, since restart of translation after a premature stop codon is sensitive to the number of nucleotides separating the stop and restart codons [39]. By changing the position of the restart methionine relative to the premature stop codon, it might be possible to significantly change the level of expression of the distal protein fragment and hence functional protein as a whole. Thus, leaky scanning remains as an attractive possibility for boosting or depressing protein levels in a transfected cell.

## Materials and Methods

### Primary cultures of mouse myotubes

Primary cultures were prepared from hind limbs of day 18 embryos (E18) as described previously [23]. cDNAs of interest and a separate expression vector encoding the T-cell membrane antigen CD8 were subcloned into the mammalian expression vector pSG5 (Stratagene, CA) and were mixed and cotransfected with the polyamine LT-1 (Panvera, WI). Whole-cell recordings and immunostaining were done 3–5 days after transfection. Cotransfected cells were recognized by incubation with CD8 antibody beads (Dynal, Norway). The coincidence of expression of CD8 and a cDNA of interest was >85%.

### $\alpha_{1S}$ cDNA constructs

All cDNA constructs were sequenced twice or more using BigDye technology (Perkin Elmer, CA) at a campus facility. For epitope tagging and expression in mammalian cells, the unmodified full-length rabbit  $\alpha_{1S}$  cDNA encoding residues 1–1873 (Genebank #M23919 nucleotide co-

ordinates nt 226 to nt 5847) was fused in frame to the first 11 amino acids of the phage T7 gene 10 protein in pSG5 using *AgeI* and *NotI* cloning sites. All constructs were made using the T7 tagged  $\alpha_{1S}$  as template in PCR-based strategies, some previously described [20,21]. All primers were HPLC-purified (Operon, CA) and a phosphate was tagged to the 5'-end of the sense primer. Genebank #M23919 nucleotide coordinates are used below to describe primers.

### pSG5 wt- $\alpha_{1S}$

A unique silent *HindIII* site was introduced by PCR at nt 2228 in the full-length  $\alpha_{1S}$  template and cloned into the T7- $\alpha_{1S}$  pSG5 vector using *AgeI* and *XhoI* sites. The *HindIII-XhoI* fragment (nt 2228 to nt 2878) encompassing the II-III loop was subcloned into pCR 2.1 TOPO TA (Invitrogen, CA) and this plasmid was further used for PCR reactions.

### pSG5 fs- $\alpha_{1S}$

PCR reactions for deletion of residues 671–690, consisted of 10 nanograms pCR 2.1 TOPO/*HindIII-XhoI* insert, 15 pmoles of each primer, 0.5 mM dNTPs, 1X cloned *Pfu* buffer (Stratagene) and 2.5 U cloned *Pfu* DNA polymerase (Stratagene). The antisense primer was complementary to nt 2202 to nt 2235 and the sense primer was nt 2296 to nt 2326. Amplification was carried out for 30 cycles at 95°C for 45 seconds, 60°C for 2 minutes and 72°C for 2 minutes/kb of plasmid. The PCR reaction was treated with 10 U of *DpnI* (Stratagene) and recircularized with T4 DNA ligase (Stratagene). Once amplified by PCR, the *HindIII-XhoI* digest was ligated into the T7- $\alpha_{1S}$  pSG5 vector using the same restriction sites.

### pSG5 fs- $\alpha_{1S}$ M701I

The construct was produced by a two-step PCR reaction using fs  $\alpha_{1S}$  as template. Using conditions as above, the sense primer (nt 1932 to nt 1959) was paired with antisense primer 5'TCCAGCTTCTTGCGATCACAGACTTCTCC3' carrying the point mutation. In a separate reaction, sense primer 5'GGAGAAGTCTGTGATCGCCAAGAAGCTGGA3' was paired with antisense primer (nt 3100 to nt 3081). The two PCR products were diluted 1:500 in ddH<sub>2</sub>O and then hybridized to each other for 2 minutes at 95°C, 1 minute at 43°C and 1 minute at 72°C for 4 cycles. 15 pmol of nt 1932 to nt 1959 primer and 15 pmol of nt 3100 to 3081 primer were added and further cycled for 4 minutes at 95°C, then 30X of 2 minutes at 95°C, 1 minute at 60°C, 1 minute at 72°C and finally 10 minutes at 72°C. The PCR product was then cloned into the fs  $\alpha_{1S}$  construct using *HindIII* and *XhoI* sites.

### pSG5 $\alpha_{1S}\Delta 1-700$

The construct was produced by cutting pSG5 fs  $\alpha_{1S}$  with *AgeI* and *HindIII* enzymes and filling-in the overhangs

with klenow fragments. The plasmid was religated using DNA T4 ligase.

### Whole-cell voltage-clamp

Whole-cell recordings were performed as described previously [22] using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). All experiments were performed at room temperature. Patch pipettes had a resistance of 1–2 M $\Omega$ . The external solution was (in mM) 130 TEA-Methanesulfonate, 10 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES-TEA(OH), pH 7.4. The pipette solution was (in mM) 140 Cs-aspartate, 5 MgCl<sub>2</sub>, 0.1 EGTA (for Ca<sup>2+</sup> transients) or 5 EGTA (for Ca<sup>2+</sup> current), 10 MOPS-CsOH, pH 7.2. The voltage dependence of peak intracellular Ca<sup>2+</sup> ( $\Delta F/F_0$ ) was fitted according to a Boltzmann distribution (Eqn. 1)  $A = A_{\max}/(1 + \exp(-(V - V_{1/2})/k))$ .  $A_{\max}$  is  $\Delta F/F_{0\max}$ ;  $V_{1/2}$  is the potential at which  $A = A_{\max}/2$ ; and  $k$  is the slope factor.  $\Delta F/F_0 = (F - F_0)/F_0$  where  $F$  is the fluorescence during a Ca<sup>2+</sup> transient and  $F_0$  is the resting fluorescence of the cell immediately before the stimulation.

### Confocal fluorescence microscopy

Line-scans were performed as described [26] in cells loaded with 4 mM fluo-3 AM (fluo-3 acetoxymethyl ester, Molecular Probes, OR) for ~30 minutes at room temperature. Cells were viewed with an inverted Olympus microscope with a 20X objective (N.A. 0.4) and a Fluoview confocal attachment (Olympus, NY). Excitation light was provided by a 5 mW Argon laser attenuated to 20% with neutral density filters. For immunofluorescence, confocal images had a dimension of 1024 by 1024 pixels (0.35 microns/pixel) and were obtained with a 40X oil-immersion objective (N.A. 1.3).

### Immunostaining

Cells were fixed and processed for immunofluorescence as described [4,20]. The N-terminal fragment expressed by fs- $\alpha_{1S}$  or wt- $\alpha_{1S}$  was identified with a mouse monoclonal antibody against a T7 epitope fused to the N-terminus of  $\alpha_{1S}$ . The anti-T7 antibody (Novagen, WI) was used at a dilution of 1:1000. Secondary antibodies were a fluorescein-conjugated goat anti mouse IgG (Boehringer Mannheim, IN) used at a dilution of 1:1000 and a fluorescein-conjugated donkey anti-rabbit IgG (Chemicon, CA) used at a dilution of 1:1000.

### Western blots

The C-terminal fragment was identified with SKI, a rabbit polyclonal antibody against the II-III loop of  $\alpha_{1S}$  (Ala739-Ile752) previously characterized [34]. Cells were scrapped from tissue cultures dishes with cold PBS plus protease inhibitors and spun in a cold table-top centrifuge. Cells were homogenized in a glass-teflon homogenizer in a minimal volume of PBS and diluted 1:1 (vol:vol) with SDS-gel loading buffer composed 100 mM Tris-Cl (pH 6.8), 200

mM dithiothreitol, 4% SDS, 0.2% bromophenol blue and 20% glycerol. Samples were incubated at 100°C for 20 minutes. Approximately 10 mg of total protein was applied to a 5–15% SDS polyacrylamide gel and electrophoresed for 2 hours at 40 mA. Proteins were transferred to PVDF membranes and analyzed with either anti-T7 or SKI antibodies and the appropriate secondary antibodies. The subunits were visualized using SuperSignal ECL reagent (Pierce, Rockford, IL). The images were captured on a Chemi-Imager (Alpha Innotech, San Leandro, CA) set to a level just below saturation.

### Abbreviations

DMSO (dimethyl sulfoxide); EGTA (ethylene glycol bis-minoethylether tetraacetic acid); HEPES (2-hydroxyethyl piperazine 2-ethane sulfonic acid); MOPS (3N-Morpholino-propane sulfonic acid); PVDF (polyvinylidene difluoride); TTX (tetrodotoxin); TEA (tetraethylammonium); ORF (origin of replication).

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