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The depolarization response element in acetylcholine receptor genes is a dual-function E box

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Abstract **All acetylcholine receptor subunit genes contain E boxes and are blocked by membrane depolarization. We have used transfected C2C12 myogenic cells to investigate the response, to electrical stimulation and KCl, of wildtype and mutant regulatory regions of the chick acetylcholine receptor α, γ and δ subunit, and the mouse MLC genes. Point mutations revealed that E boxes function as activating elements targeted by the depolarization signal. These experiments suggest, and insertion of a depolarization response element into an unrelated promoter confirms, that plasma membrane depolarization switches the depolarization response element from an activating to a repressive mode.**

Key words: Depolarization; E box; Excitation-transcription coupling; Muscle; Repression

1. Introduction

The electrical activity of the plasma membrane affects many processes of excitable cells, including gene activity; consequently, signaling pathways must be present that couple membrane depolarization to transcription. This link between plasma membrane and genome has remained largely unexplored, with the notable exception of the depolarization-triggered induction of the expression of immediate early genes, especially c-*fos*, in neurons [1,2], and the action potential-dependent inhibition of genes coding for the γ isoform of the acetylcholine receptor (AChR) in skeletal muscle [3,4].

It has been known since the early 1970s that the depolarization accompanying intermittent electromechanical activity of skeletal muscle blocks the expression of extrajunctional AChR [5–7]. Electrostimulation of muscle of high extrasynaptic receptor density leads to down-regulation of the receptor by a mechanism that involves rapid shutdown of receptor subunit genes [8]. Considerable research effort has gone into the identification of *cis* elements involved in this depolarization response. A plausible candidate is the CANNTG motif or E box of which at least one copy is present in all known regulatory sequences that impart depolarization sensitivity: promoter fragments of the genes coding for the chick AChR α subunit [9–12]; mouse AChR δ subunit [13,14]; rat AChR δ subunit [15]; mouse AChR ε subunit [11,16]; and mouse [12,17] and chick [18] myogenin.

Recently Bessereau et al. [19] and Tang et al. [20] reported that E box mutation abolishes the denervation response of reporter constructs driven by the chick α and the mouse δ promoter, respectively. Since it is believed that the activation of AChR genes upon nerve section arises from the cessation of electrical activity, E boxes are likely to function as activity (and inactivity) response elements. However, E boxes are also present in many muscle-specific genes that are not stimulated by the lack of electrical activity. Examples are the myosin light chain (MLC) gene, used by Merlie and colleagues as a negative control in studies of the effects of muscle denervation [9,12], and the muscle creatine kinase (MCK) gene whose expression is affected neither by denervation [14,21] nor by electrical stimulation [15]. These observations imply that if E boxes in the 5′ flanking region of muscle-specific genes can mediate effects of plasma membrane activity and act as depolarization response elements (DRE) they must belong to a subset with distinct regulatory properties.

Using mutational analysis in an experimental paradigm in which short-term effects of electrical stimulation of cultured muscle cells are investigated we confirm the role of E boxes in AChR subunit enhancers. We also show that the depolarization response element can function in an activating as well as an inhibitory mode.

2. Materials and methods

2.1. Cell culture and transfections

Mouse muscle C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) GIBCO BRL-Life Technologies, Gaithersburg, MD) containing 5% fetal calf serum and 10% supplemented calf serum (HyClone, Logan UT) at 37°C under a 95% air, 5% CO_2, water-saturated atmosphere. For transgene activity analysis, 60-mm dishes were transfected, at about 80% confluency, with 5 μg of individual reporter gene constructs and 5 μg control gene construct (pSV2CATXbaI) using the calcium phosphate precipitation technique. The day after transfection, medium was replaced with differentiation medium (2% horse serum in DMEM); thereafter cells were maintained in differentiation medium. At 48 h after transfection, cells were electrically stimulated with the A-M SYSTEMS isolated pulse stimulator model 2100 (A-M SYSTEMS Inc., Everett WA); stimulation was for 30 min in 100-Hz trains, 2 s in duration and applied once every minute.

2.2. Isolation of nuclei and transcript elongation analysis

At the desired time after onset of electrical stimulation, cells were processed, and nuclei isolated as described [22]. Transcript elongation was carried out with freshly prepared or liquid nitrogen-stored nuclei, in a previously described solution hybridization/ribonuclease protection version of the commonly used run-on assay [18,22–24].

2.3. Plasmid construction

The α2kbCAT reporter contains 2 kb of a α-subunit upstream region fused to CAT; the shortest fully active 5′ deletion derived from it and extending downstream of −116 was cloned into the CAT vector to generate α116CAT [23]. The enhancer sequence containing the two E boxes is GGCCCT*CAGCTG*TCATGCCTGGAA*CAGGTG*GTG. To prepare the template for α-subunit promoter E box mutagenesis, the 116-bp *Hin*dIII/*Sma*I fragment was cloned into the *Hin*dIII/*Sma*I site of bacteriophage M13mp18 (New England Biolabs, Beverly, MA) generating m13-α116. αML is the construct carrying a mutated (substitu-

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tions underlined) upstream or left E box (GGCCCTCCTAGGGAATGCCTGGAACAGGTGGTG); αMR has the downstream or right E box corrupted (GGCCCTCAGCTGTCATGCCTGGAAGCTAGGGTG); and αMLR lacks both E boxes (GGCCCTCCTAGGGAATGCCTGGAAGCTAGGGTG). γ926CAT was the construct reported previously [25], containing a 926-bp fragment of the upstream region of the chick AChR γ-subunit, including the enhancer sequence AGAA*CAGCTG*ATGGGGTCAGGGA*CAGCTG*TCCC. The E box mutants are those described earlier [25]: AGAACAGAGTATGGGGTCAGGGACAGCTGTCCC (γML), AGAACAGCTGATGGGGTCAGGGAACTCTGTCCC (γMR), and AGAACAGAGTATGGGGTCAGGGAACTCTGTCCC (γMD). The δ690CAT reporter was constructed by inserting a segment of chick AChR δ-subunit genomic DNA (extending from −690 to +60 relative to the start site and containing *Bgl*II sites at both ends) into the CAT vector [26]. A 650-bp *Hin*dIII/*Bgl*II fragment of δ690CAT was excised and subcloned into the *Hin*dIII/*Bam*HI site of bacteriophage M13mp18 (*Bam*HI site blunt-ended by Klenow fill-in) to produce m13-δ650. Site-directed mutagenesis yielded the following mutants: δMS, in which all 4 E boxes upstream of the enhancer are converted to a CTCGAG sequence; δM1, in which the single E box within the enhancer sequence [26] is changed from CAGCTG to CAGTTA; and δM2 in which the E box upstream of the transcription start site is mutated from CACCTG to AATCTG. The mutant δ-subunit promoter E boxes were obtained by digestion with *Hin*dIII/*Sma*I and inserted back into the *Hin*dIII/*Bgl*II site of the CAT vector (with *Bgl*II site blunt-ended by Klenow fill-in).

MCKCAT (pCK4800) comprises 4,800 bp of 5′ flanking region from the mouse MCK fused to CAT [27]. MLCCAT contains a 180-bp fragment of mouse myosin light chain (MLC) enhancer; in the MLCMABCCAT plasmid derived from it the E boxes A, B, and C are mutated [28]. RSVCAT was derived from pOPI3CAT (STRATAGENE, La Jolla CA) by deleting the *Hin*dIII/*Hin*dIII fragment from the vector containing the Rous sarcoma virus LTR. The 50-bp fragment of the chick AChR α-subunit promoter (extending from −116 to −81 relative to the start site and containing *Sma*I sites at both ends), after Klenow fill-in, was inserted into the *Bst*XI site of RSVCAT to generate α50-RSVCAT, or the *Bgl*II site to generate RSV-α50CAT. α4CAT consists of α4, four tandemly arranged α50 units spliced to SV40 minimal promoter, and the CAT coding region; 4RTkCAT harbors 4RTk, a multimerized MCK high-affinity E box immediately upstream of the thymidine kinase gene (*tk*) basal promoter [29]; and [E5-E2]$_4$CAT contains [E5-E2]$_4$, four copies of the immunoglobulin gene enhancer μE2/μE5 site cloned 5′ of an alkaline phosphatase TATA box, linked to CAT [30]. The pSV2CATXbaI plasmid was obtained from pSV2CAT by deleting the 3′ end of the CAT gene with *Xba*I; the resulting plasmid contains 190 bp of the 5′ end of the gene [23]. The riboprobe template for CAT was prepared as described; the probe protects a region of 250 bp [23].

3. Results

3.1. Membrane depolarization inactivates AChR subunit genes via E box motifs

To investigate the role of the E box motif in membrane potential-dependent gene regulation we examined appropriate chicken AChR promoter CAT constructs in C2C12 cells. A 116-bp segment of the chick α-subunit promoter, including the 36-nt enhancer with its two E box motifs, was silenced by electrical stimulation as efficiently as the endogenous α-subunit gene [23]. Mutation of a single E box caused loss of ~70% of transcriptional activity, but did not affect response to membrane depolarization, whereas a double mutant loses not only most of its activity (~90%) but also its ability to respond to membrane excitation (Fig. 1; Table 1). The γ-subunit promoter resembles the α-subunit promoter in that it contains two tandemly arranged E boxes in its enhancer [25]; these E boxes were mutated and tested for involved in depolarization-transcription coupling. As can be seen from Table 1, either single E box mutant retains >70% of the activity of the wild type promoter, and their response to depolarization is little affected. The double E box mutant, however, not only loses >60% of its activity, but its responsiveness to depolarization as well. Similar experiments with the δ-subunit promoter focused on a 690-bp 5′ flanking sequence harboring six CANNTG motifs including the single E box within the previously described enhancer [26]. Mutation of the enhancer E box resulted in inactivation and loss of the depolarization response (Table 1), whereas mutations at other enhancer sites including a CAGGGG element impaired activation but not the ability to respond to electrical

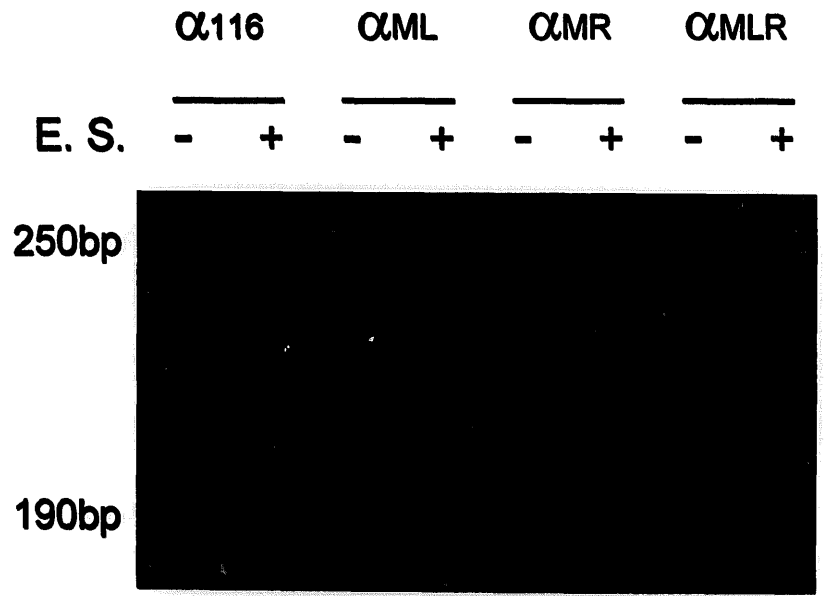

Fig. 1. Analysis of membrane depolarization effects by run-on/ribonuclease protection assay. The α116CAT construct and the indicated mutants (ML, construct carrying a mutated upstream or left E box; MR, construct with mutated downstream or right E box; MLR, both E boxes mutated) were transfected into C2C12 cells; 48 h after transfection, cultured cells were treated with electrical stimulation for 30 min. Nuclei were then isolated, and gene activity determined. Autoradiographs of ribonuclease protection analysis are shown; the top protected band (250 bp) reflects the strength of reporter gene, and the bottom one (190 bp) reveals activity of the SV40 promoter (pSV2CATXbaI, internal control).

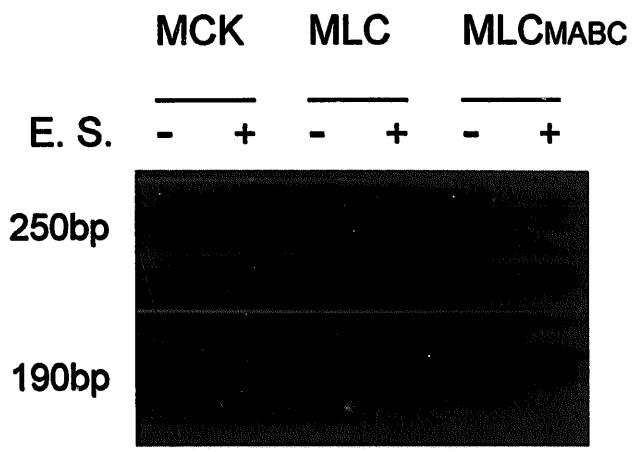

Fig. 2. Depolarization does not shut down MCK and MLC genes. The MCK enhancer-CAT construct, MLC enhancer-CAT construct, and the CAT construct containing the MLC triple E box mutant were transfected into C2C12 cells and 48 h later analyzed as described for α116CAT in the legend to Fig. 1.

activity (data not shown). Likewise, neither an E box downstream of the enhancer nor four E boxes upstream in the previously described silencer region [26] function as depolarization response elements (Table 1). These results indicate that only a subset of E box motifs participate in AChR subunit gene inactivation by membrane activity. Upon deletion or corruption of these elements, promoters exhibit higher activities after membrane depolarization than their wildtype counterparts, suggesting that such E boxes not only function as positive elements, but under certain conditions may suppress the activity of the promoters in which they are embedded. In all cases, treatment with 50 mM KCl mimicked the effects of electrical stimulation (data not shown).

3.2. *E box motifs present in denervation-insensitive genes do not respond to membrane depolarization*

From the findings described so far it could be argued that all E boxes that mediate gene activation may also be involved in the depolarization response. However, both muscle creatine kinase (MCK) and myosin light chain (MLC) enhancers, which contain E boxes important for tissue-specific gene expression, are largely unaffected by membrane activity (Fig. 2). Elimination of three E boxes (A, B, C [28]) from the MLC enhancer reduces activity to about half of wildtype, regardless of whether the cells are stimulated or not. Taken together with the analysis of the δ-subunit upstream region, these results confirm that only a subset of functionally active E boxes in muscle genes are involved in this type of regulation.

3.3. *The α-subunit enhancer inserted into a control gene imparts depolarization sensitivity*

If the DRE functions as an inhibitory element in an AChR subunit gene, it should likewise function in an unrelated gene that is not normally turned off in response to electrical activity. To test this proposition, α50, the enhancer element from the AChR α-subunit promoter, which harbors two depolarization-sensitive E boxes, was implanted into RSVCAT, a construct that is active in all cell types regardless of membrane potential. Two such chimeras were generated, having the α-subunit insert either at the 5′ end of the RSV promoter or embedded between promoter and reporter gene. In either case, depolarization of the host muscle cell membrane resulted in prompt silencing of the gene (Fig. 3). The presence of the implant did not in any significant way affect the activity of the construct in non-muscle (3T3, HeLa) cells, either in the presence or absence of KCl or phorbol ester (data not shown), thus establishing the insensitivity of the viral promoter to the structural modifications. To rule out the possibility that the MCK promoter is unresponsive to the depolarization signal because of compensatory activities of other *cis* elements within its 4.8-kb extent, a multimerized MCK enhancer in combination with a minimal promoter was also tested; similar constructs involving the α-subunit and Ig enhancers were analysed for comparison (Table 1, bottom).

4. Discussion

E boxes are plausible candidates for mediators of the depolarization response: they are known to be responsible for the stage- and tissue-specificity of AChR promoters; they are the only discernable elements common to depolarization-responsive promoters; and they are targets of M proteins whose participation in the depolarization response has been suspected based on (a) denervation- and activity-induced changes in M protein expression, and on (b) the likely participation, in the activity response, of an autocatalytic factor, such as MyoD and myogenin are believed to be.

Table 1
The effect of depolarization on promoter activity[a]

Promoter	Untreated	E.S.[b]
α116	100.0%	5.6% ± 1.3%
αML	27.6% ± 3.3%	4.8% ± 2.3%
αMR	32.7% ± 4.5%	5.7% ± 1.5%
αMLR	10.9% ± 1.2%	11.2% ± 2.8%
γ926	100.0%	5.4% ± 1.5%
γML	78.4% ± 8.1%	7.9% ± 2.7%
γMR	76.4% ± 9.2%	8.2% ± 1.6%
γMD	30.5% ± 3.7%	31.6% ± 4.2%
δ690	100.0%	8.3% ± 1.1%
δMS	67.0% ± 8.8%	1.8% ± 1.2%
δM1	25.3% ± 5.8%	31.4% ± 3.2%
δM2	97.7% ± 8.2%	9.7% ± 2.5%
MCK	100.0%	88.5% ± 18.5%
MLC	100.0%	87.9% ± 22.7%
MLC_{MABC}	36.4% ± 9.2%	40.2% ± 7.6%
RSV	100.0%	78.4% ± 24.2%
RSV-α50	62.4% ± 29.2%	7.1% ± 1.6%
α50-RSV	80.9% ± 30.7%	6.5% ± 4.2%
α4	100.0%	5.3%
4R-tk	100.0%	107%
$[E5\text{-}E2]_4$	100.0%	103%

[a]Gene activities were determined as described in Fig. 1. Each value, corrected for expression of pSV2CATXbaI and normalized to the activity of the wild type promoter CAT construct in untreated cells, represents the mean and S.E.M. of 3 to 6 independent experiments (except for α4-CAT, 4R-tk-CAT and E4-CAT).
[b]E.S., electrical stimulation.

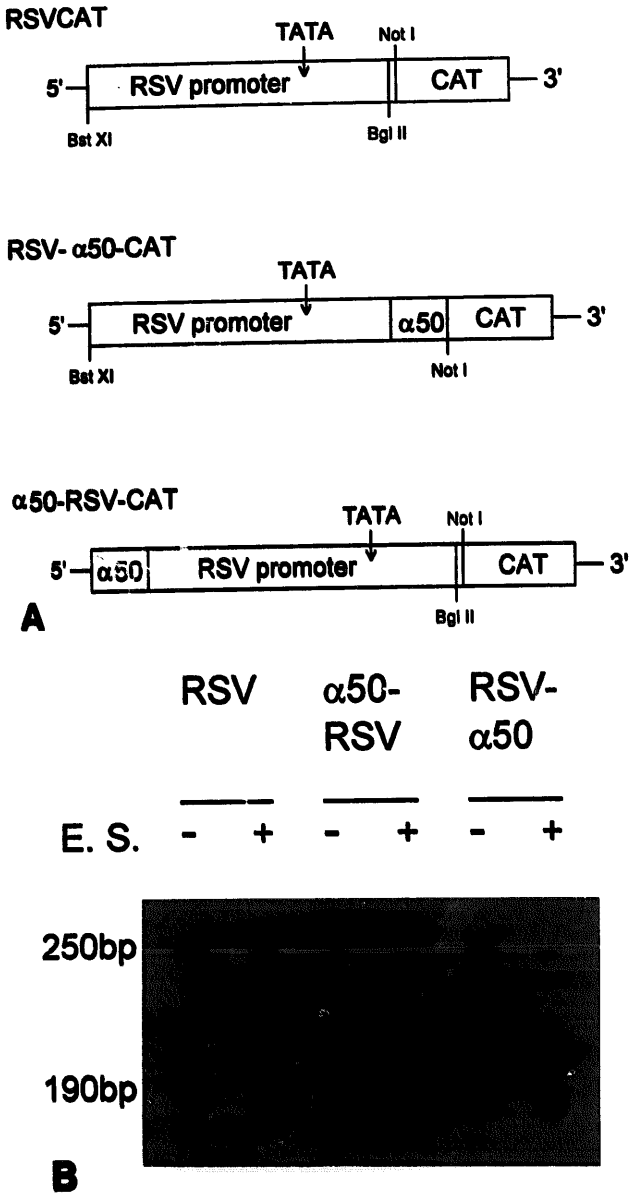

Fig. 3. Insertion of the α-subunit enhancer imparts depolarization sensitivity to an unrelated promoter. α50, an α-subunit promoter segment containing both E boxes with flanking sequences was cloned into the *Bgl*II and *Bst*XI site of RSVCAT. The constructs were then transfected into C2C12 cells and analyzed as described in the legend to Fig. 1. (A) Preparation of RSVCAT derivatives. (B) Effect of electrical stimulation.

Previous work has suggested a role for E boxes in depolarization transcription coupling. Our observations on the chick AChR α-subunit enhancer confirm and extend the findings of Bessereau et al. [19] who recently showed that, in transgenic animals, constructs carrying *lacZ* under the control of an 850-bp α-subunit promoter are stimulated by denervation only when the 3′ E box in the α-subunit enhancer is intact. Tang et al. [20] were able to eliminate the response to denervation in a transgene composed of the human growth hormone coding region and a mouse AChR δ-subunit regulatory region in which the single E box had been mutated. Chahine et al. [15] observed that a rat δ-subunit promoter construct truncated to 102 bp and containing a single CANNTG motif remains sensitive to direct electrical stimulation of the primary muscle cells harboring the transgene. Our studies on the chicken δ promoter extend these observations by showing that five additional E boxes in the vicinity of the enhancer (four of them in the silencer region, one close to the transcription start site) *do not* participate in the depolarization response; one of them (near the cap site) constributes to the tissue specificity of the promoter by lowering activity in non-muscle cells.

The identification of E boxes as mediators of the depolarization response is plausible as well as perplexing: plausible, because E boxes serve as targets for the MyoD family of transcription regulators, and perplexing, because clearly muscle genes that are little affected by membrane activity as well as those exceedingly sensitive to it contain E boxes as functional elements in their promoters. Examples of insensitive E boxes are those present in the enhancers of the genes for MLC and MCK and presumably a large number of muscle proteins which either do not respond to changes in impulse frequency or do so at a rate completely different from the genes coding for AChR subunits.

The solution to the E box dilemma must lie in the multiplicity of E boxes and of transactivator complexes associating with them. Since there are four M proteins (MyoD, myogenin, myf5, and herculin/MRF4 – for review of myogenic factors see [31,32], it is conceivable that one of them, possibly in conjunction with a cofactor, e.g. a specific E protein as dimerization partner, serves as the transactivating factor responsive to, i.e. inhibited by, membrane depolarization. This presupposes that individual E boxes are preferentially recognized by a specific transactivator. That individual M proteins have subtle predilections for flanking and central nucleotides not specified in the basic CANNTG motif has been shown using selection and amplification of binding sites ('SAAB' [33]) or cycles of amplification and selection ('CASTing' [34,35]) of appropriate target sequences. It remains to be seen, what specific features render an E box depolarization-sensitive; based on our findings, the discriminating elements may well lie within one to two dozen nucleotides flanking the E box.

Upon depolarization, AChR promoters are silenced beyond the effect that the deletion of a *cis* element would have, i.e. to an extent that cannot be explained only by the removal or neutralization of a transactivator. The simplest explanation is that the DRE binding protein is a transcriptional regulator that *activates* the dependent gene in the resting cell, but *inactivates* it upon receiving a signal from the depolarized membrane (Fig. 4). In fact, the inhibitory nature of the α enhancer was directly demonstrated through transplantation into the RSV promoter. That the same *cis* element should have both stimulatory and suppressive function is not entirely novel. The E box in the mouse δ-subunit promoter for example has been shown to activate the gene in muscle cells and to inhibit it in myoblasts and non-muscle cells [36]. The transcription factor responsible for these effects differs from the DRE binding protein in that it does not seem to depend on membrane excitation for its silencing activity and that its opposite modes of function are expressed in different cell backgrounds. There are precedents for one and the same gene encoding transcriptional activators and repressors targeted at the same site; however the functional

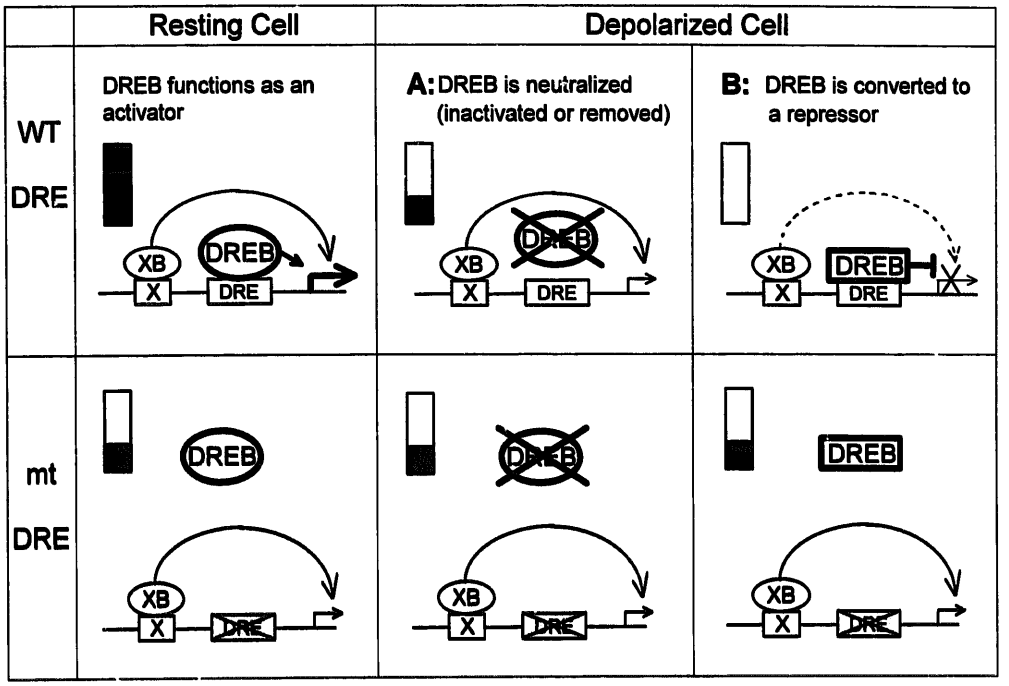

Fig. 4. A model of the depolarization response element (DRE) and its function. In the left panel, activity of a DRE-binding protein (DREB) is shown in an unstimulated cell. If an intact DRE is present in the promoter, DREB, along with other transcriptional activators, here donated as XB (for *X* element-*B*inding protein), stimulate gene activity. In the center and on the right, two hypothetical responses to membrane depolarization are shown. In (A) DREB is neutralized by the depolarization signal, and activity drops to the level seen with a mutated DRE. In (B) DREB is converted to a repressor (indicated by the rectangular shape) and eliminates not only the DRE-dependent, but also the DRE-independent, *X*-dependent activity. The inset bars represent reporter gene activities; arrows denote activation or transcript initiation. Mechanism B is compatible with the observed results.

switch in these cases requires de novo synthesis of the *trans*-acting protein, through alternative splicing or the utilization of alternative (internal) initiation codons, leading to either the expression or the excision of an activation domain [37]. The case of DRE binding protein differs in that the switch can be actuated on the time scale of intracellular signaling and probably involves posttranslational modification, e.g. phosphorylation, of a transcription factor such as myogenin.

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