Cloning of a human *ether-a-go-go* potassium channel expressed in myoblasts at the onset of fusion

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Abstract An early sign of human myoblast commitment to fusion is the expression of a non-inactivating delayed rectifier K^+ current, $I_{K(NI)}$, and an associated membrane potential hyperpolarization. We have isolated the full-length coding region of a human *ether-a-go-go* K^+ channel (h-*eag*) from myoblasts undergoing differentiation. The h-*eag* gene was localized to chromosome 1q32–41, and is expressed as a ~9 kb transcript in myogenic cells and in adult brain tissue. Forced expression of h-*eag* in undifferentiated myoblasts generates a current with remarkable similarity to $I_{K(NI)}$ indicating that h-*eag* constitutes the channel responsible for this current in vivo.

Key words: Human EAG; Potassium channel; Myoblast fusion

1. Introduction

Myoblast fusion is a complex pattern of coordinated and temporally separable events which include withdrawal from the cell cycle, cell-cell interactions, adhesion, alignment, and final membrane fusion to form the multinucleated skeletal muscle fiber.

Studies on myogenic cells from various species have shown that their membrane resting potential undergoes a hyperpolarization from approximately −10 mV in undifferentiated proliferating myoblasts to values ranging between −55 mV and −70 mV in multinucleated myotubes [1–4]. When human myoblasts are induced to differentiate, we observe that their resting potential hyperpolarizes through the sequential expression of two voltage-gated potassium currents [5]. The first is a non-inactivating delayed rectifier current ($I_{K(NI)}$) that hyperpolarizes the cell to an intermediate resting potential of approximately −32 mV [6]. Then, slightly before fusion, an inward rectifier current is expressed ($I_{K(IR)}$) and drives the potential further down to approximately −65 mV, i.e. similar to that measured for multinucleated myotubes. A pharmacological blockade of $I_{K(IR)}$ depolarizes myoblasts to a resting potential of about −30 mV and prevents their fusion [5]. The role of $I_{K(NI)}$ in myoblast fusion is more difficult to assess, as there are, as yet, no pharmacological agents available to specifically block this current.

The present work was undertaken to elucidate the molecular nature of the $K_{(NI)}$ channel that is activated at the onset of human myoblast differentiation. As $K_{(NI)}$ channels produce a current whose electrophysiological characteristics closely resemble those of the current evoked by the rat *ether-a-go-go* (r-*eag*) potassium channel [7–10], we searched for an *eag* homolog in human myogenic cells. We report here the cloning of a human cDNA encoding a member of the *ether-a-go-go* potassium channel family and show that it constitutes the $K_{(NI)}$ channel expressed in vivo.

2. Materials and methods

2.1. Cell cultures

Biopsies of human skeletal muscle were obtained during corrective orthopedic surgery of young patients (9 months to 17 years old) without any known neuromuscular disease, in accordance with the guidelines of the ethical committee of the University Hospital of Geneva, Switzerland (written informed consent was obtained from the patients or their legal guardians). Myoblast clonal cultures were prepared from satellite cells, proliferated in a growth medium and induced to fuse by transfer to a differentiation medium (DM) as described previously [11]. Fusion-competent myoblasts were obtained by plating myoblasts at a very low density in DM. Under these conditions, the cells are induced to differentiate but prevented from fusing [12].

2.2. h-eag cDNA cloning and sequencing

Template cDNA was prepared from human differentiating myoblast RNA, isolated as described [13]. RT-PCR was performed using oligonucleotide primers EAGF1 (5′-AACGACACTAATTTTGTGTTGGG-3′) and EAGR1 (5′-CTTCTTGTACATCAGAATTTCG-3′) corresponding to nucleotides 285–307 and 494–515 of rat *eag*, respectively. Touchdown PCR conditions (60°C to 50°C over 30 cycles) produced a 230 bp fragment which was cycle-sequenced, and used to generate a radiolabeled probe to screen a custom-made ZAP express cDNA library (Stratagene) prepared from RNA isolated from human differentiating myoblasts (myoblasts cultured in DM for 24–48 h; clonal cultures were from several biopsies). Plaque lifts were performed on nylon membranes, and hybridization carried out at 65°C according to the manufacturer's protocol (DuPont NEN). Further library screening was performed using a PCR probe derived from the 3′-most region of the clone isolated in the initial screening (generated with primers EAGF6: 5′-GGAGATGTGTTCTGGAAGGAA-3′; and EAGR8: 5′-ACGTCGCTGATCTTCCGGAACACA-3′). Sequences were determined on both strands.

2.3. Northern blot analysis

Human myogenic cell RNA was probed with a radiolabeled 765 bp PCR product generated from S5 transmembrane segment and C-terminal sequences of h-*eag*. Hybridization was performed as described [14]. Hybridizations of a multiple tissue Northern blot (Clontech) were performed as per manufacturer's protocol. A 182 bp PCR-radiolabeled probe was generated from the 5′ untranslated and 5′ coding sequence of h-*eag*. To probe for h-*eag*2, a 264 bp PCR-radiolabeled probe to the 3′ end of the gene and encompassing the stop codon (with 224 bp of 3′ untranslated region) was used.

2.4. Chromosome mapping of h-eag

The chromosomal location of h-*eag* was determined by PCR using two different somatic cell hybrid panels: the monochromosomal hy-

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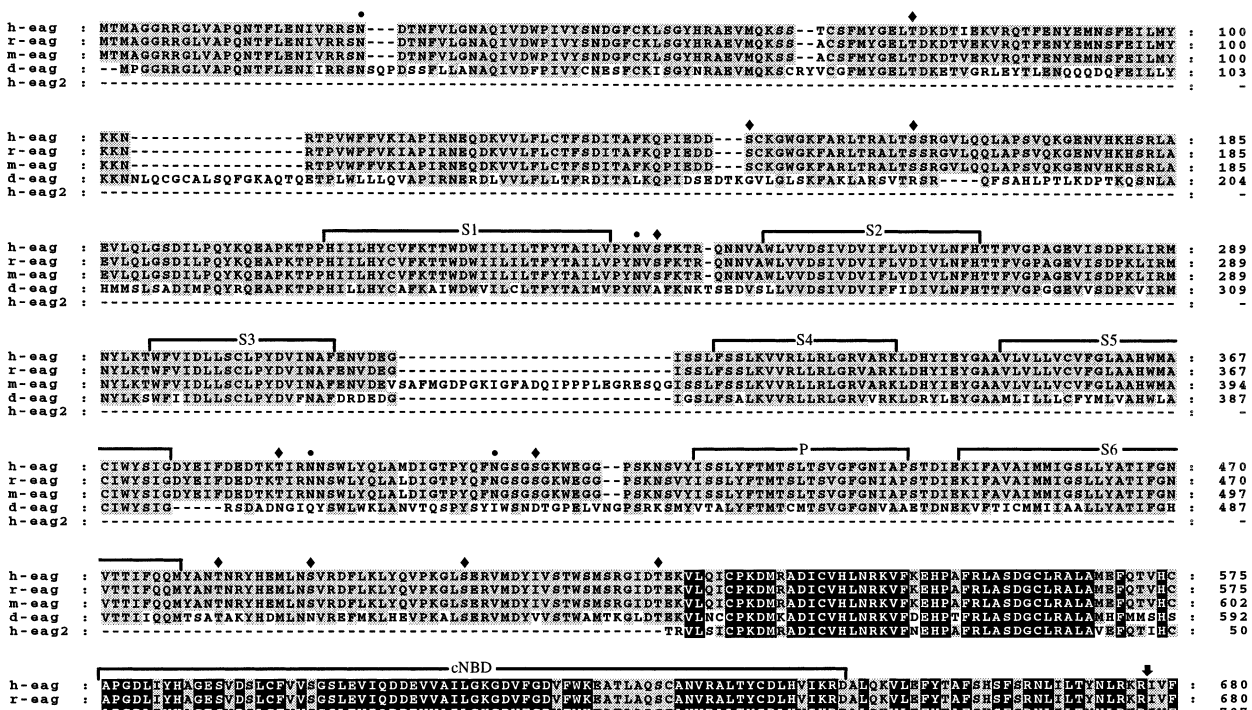

Fig. 1. Alignment of the deduced amino acid sequence of human *eag* (h-*eag*) with rodent homologs (r-*eag* and m-*eag*), the prototype *Drosophila eag* (d-*eag*) and the partial sequence of a presumptive second human *eag* (h-*eag*2). Sequence alignments were created using ClustalW [38] and shaded using the Genedoc program (at http://www.cris.com/~ketchup/gddl.htm). Black shading indicates identity in all sequences when alignment includes h-*eag*2. Shading is in gray when residues are identical in three or four of the sequences. Amino acid residues are numbered on the right. Gaps in the alignment are indicated by dashes. The double slash (//) indicates interruption in the d-*eag* sequence (only the first 1001 residues were aligned). The six putative transmembrane segments (S1–S6), the pore-forming region (P) and the putative cyclic nucleotide binding domain (cNBD) are overlined. Putative post-translational modification sites for h-*eag* are indicated: (●) for *N*-glycosylation, (♦) for protein kinase C, and (*) for cAMP- and cGMP-dependent protein kinases. Only those sites that are presumably accessible are indicated. The arrow indicates the position of an intron found unspliced in one h-*eag* cDNA clone. The h-*eag* cDNA sequence has been submitted to the EMBL data bank (accession number AJ001366).

brid cell panel of human-rodent hybrid cell lines (NIGMS [15]) and the Genebridge 4 Radiation Hybrid DNA panel from the HGMP Resource Centre, UK [16]. The PCR oligonucleotides used were EAGF6, derived from the h-*eag* coding sequence, and EAGR13 (5′-ATGATCCAGCTAGGGCATGAGG-3′) derived from a downstream intron. Cell lines giving the expected 300 bp PCR product were identified by Southern analysis using a labeled exonic 205 bp PCR product generated using primers EAGF6 and EAGR8. Results were analyzed at http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl. The position of h-*eag* relative to the Genethon chromosome 1 linkage map (July, 1997) was deduced using Mapview 2.0 from the Genome database (http://www.gdb.org).

2.5. Bicistronic h-eag-GFP expression vector construction

A full-length cDNA clone was reconstructed in pBluescript II (Stratagene) to contain the full open reading frame (2886 bp), 116 bp of 5′ untranslated sequence and 43 bp of 3′ untranslated sequence. The h-*eag*-GFP bicistronic vector was generated by inserting a 3 kb fragment of h-*eag* into the expression vector pcDNA3/IRES-GFP. The latter vector was constructed by inserting an IRES-GFP cassette in pcDNA3 (Invitrogen). The IRES element of EMCV was excised from a vector kindly provided by E. Wimmer (State University of New York, Stony Brook, NY), and the GFP DNA was excised from phGFP-S65T (Clontech).

2.6. h-eag expression in human myoblasts

Human proliferating myoblasts (seeded in 35 mm Costar dishes) were transfected at 50–70% cell confluence with a 1:2 ratio of DNA (3–5 μg plasmid) to DAC-30 transfection agent (Eurogentec), in 1 ml of medium (in the presence of FCS), for 9–14 h at 37°C, after which the medium was completely replaced by fresh growth medium. Recordings were done 36–48 h after transfection. Control cells were mock-transfected with a pcDNA3/IRES-GFP vector without a first cistron.

2.7. Electrophysiology

Whole-cell configuration of the patch-clamp technique [17] was used to measure ionic currents as described in [6]. Recordings were

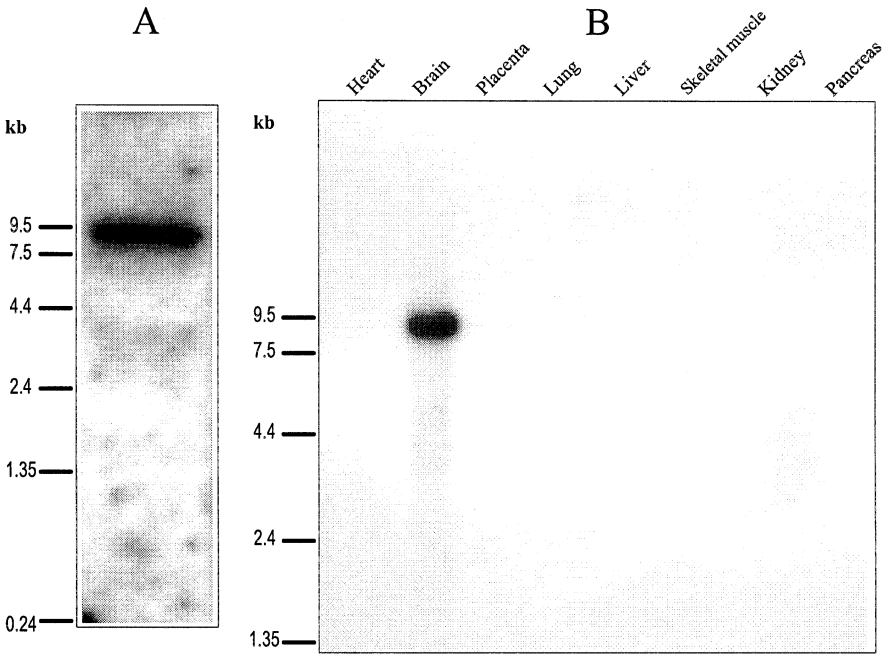

Fig. 2. Northern blot analysis of h-*eag* expression in cultured myogenic cells and various adult tissues. A: Total RNA (20 μg) extracted from freshly formed myotubes were loaded and a ^{32}P-PCR-labeled 765 bp product was used as probe. B: Multiple human adult tissue Northern blot (Clontech, 2 μg of poly(A)$^+$ RNA per lane) probed with a ^{32}P-labeled 182 bp probe. Tissue origin as stated at the top of each lane. Migration of an RNA ladder is indicated on the left of each blot.

performed in absence of extracellular Ca^{2+} and in presence of intracellular BAPTA (20 mM) to prevent activation of Ca^{2+}-activated potassium channels present in human myoblasts [4]. The extracellular solution was composed of (in mM): NMG-Cl 100, KCl 5, $MgCl_2$ 3, HEPES 5, NaOH 50, acetic acid 50, and glucose 8. The pH was adjusted to 7.4 with NMG. The intracellular (pipette) solution was (in mM): KCl 110, NaCl 5, $MgCl_2$ 1, HEPES 5, BAPTA 20, and glucose 5. The pH was adjusted to 7.4 with KOH. Statistics: all data are expressed as the means ± S.E.M.

3. Results

3.1. Isolation of h-eag from human myoblasts undergoing differentiation

A DNA alignment was carried out between the *eag* superfamily potassium channel members rat *eag* [7], mouse *eag* and human *erg* [18]. Oligonucleotides for PCR amplification were designed to a conserved area in the 5′ regions of the sequences such that primer sequences were identical between rat and mouse *eag* but different in human *erg*. A PCR product of the expected size (230 bp) was obtained from human myogenic cell cDNA. Its sequence had 87% and 89% nucleotide identity to rat and mouse *eag* and thus it was assumed to be the human *eag* (h-*eag*). This PCR product was used as a probe to screen a cDNA library made from human differentiating myoblasts (900 000 phages). The single positive h-*eag* clone isolated had an insert of 2033 bp which lacked the initiating methionine and stop codon. Further screening of 1.2 million phages, using the same probe as above, as well as a 205 bp probe derived from the 3′-most end of the original clone, resulted in the isolation of a further 12 positive clones, among which two separate clones contained an initiating ATG codon, consistent with the rat and mouse *eag* sequences, and a termination codon.

The combined sequence of these overlapping clones revealed an open reading frame of 2886 bp, encoding a deduced h-*eag* protein of 962 amino acids with a predicted molecular mass of 108 kDa. The protein shows 97% and 94% overall amino acid identity with the rat and mouse *eag* proteins respectively, confirming its identity as human *eag* (Fig. 1). A polymorphism at nucleotide 2225 [C or T], which does not change the coding amino acid (Asp-685), was observed in different cDNA clones. One cDNA clone, derived from an incompletely spliced *eag* RNA, showed the presence of an intron of at least 1.3 kb at nucleotide 2201 of the cDNA sequence (arrow at Arg-667 in Fig. 1). A recent study on gene structure of another member of the *eag* superfamily, mouse *erg* [19], showed the presence of an intron at the equivalent position (at Thr-865 of mouse *erg*).

Alignment of the h-*eag* protein sequence with other *eag* members shows that it shares the structural features of the *eag* superfamily of potassium channels (Fig. 1): a central hydrophobic core containing six putative transmembrane segments (S1–S6) flanked by long presumptive cytoplasmic N- and C-termini, the putative pore region (P) between transmembrane segments S5 and S6, and a highly conserved putative cyclic nucleotide binding domain (cNBD) downstream from S6 [20]. Further analysis using the PROSITE program [21] shows six consensus sites for *N*-glycosylation, as well as three consensus phosphorylation sites for cAMP- and cGMP-dependent protein kinases, 18 sites for casein kinase II, and 16 consensus sites for protein kinase C.

3.2. h-eag mRNA is expressed in myogenic cells and adult brain

Northern blot analysis revealed a h-*eag* transcript of approximately 9 kb in cultured myogenic cells (Fig. 2A) and in adult brain (eight adult tissues tested, Fig. 2B). After a longer exposure, a faint band of the same size appeared in placental mRNA but no transcript could be detected in adult

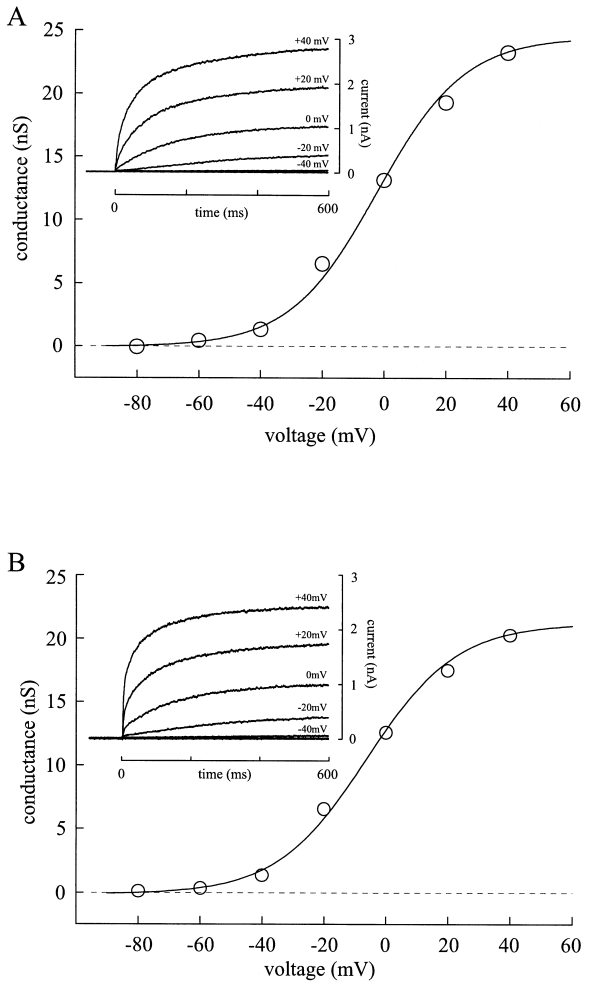

Fig. 3. Properties of h-*eag* current. Recordings were performed in green fluorescent undifferentiated myoblasts (A) or COS-7 cells (B), 48 h after transfection with a bicistronic h-*eag*-GFP vector. Whole-cell outward currents were measured at the end of 600 ms steps and leak current subtracted. The leak current was determined in presence of 90 mM TEA and 5 mM Ba^{2+}. Steady-state conductances were plotted against step potentials and were well described by a Boltzmann equation. Insets: Voltage-dependent activation of h-*eag* channels at different voltage steps. Cell capacitance was 24 pF for A, and 35 pF for B.

skeletal muscle mRNA suggesting that h-*eag* transcripts are expressed at low levels in the adult tissue, or that h-*eag* channels are transiently expressed during muscle development (see Section 4).

3.3. *h-eag maps to chromosome 1q32–41*

The chromosomal localization of the h-*eag* gene was carried out by PCR analysis of two different human-rodent somatic cell hybrid panels (see Materials and Methods). A human specific PCR product was obtained using a primer in h-*eag* coding sequence and a primer in intronic sequence, found in the incompletely spliced cDNA clone (data not shown). The gene was found to map to human chromosome 1 and was further fine mapped between the genetic markers D1S217 and D1S204, corresponding to region 1q32–41.

3.4. *A presumptive second eag gene product was not detected in human differentiating myoblasts*

While screening for human *eag* in our system, BLAST homology searches revealed the existence of a human EST (F05455) in the expressed sequence tag database generated from sequencing studies in the human genome project. This EST, isolated from an adult brain cDNA library, presented significant homology to the C-terminal region of r-*eag*, and its corresponding I.M.A.G.E. Consortium (LLNL) Clone (ID 21313 [22]) was mapped to human chromosome 14 (between D14S997 and D14S63 [23]). We obtained and fully sequenced this clone which contains an insert of 1704 bp, including a stop codon at base 1400, a polyadenylation signal and a poly A tail. This clone was recently sequenced independently by other researchers and submitted to GenBank (accession number U69185).

An alignment of overlapping areas (see Fig. 1) shows that h-*eag* and the *eag*-like I.M.A.G.E. clone share 58% amino acid identity over a total overlap of 467 amino acids. To determine whether this putative second *eag* member might also be expressed in myogenic cells, we performed RT-PCR using several PCR conditions, but failed to amplify this product in myogenic cells. In addition, the 3′-most h-*eag* probe used to screen our cDNA library was highly homologous to the I.M.A.G.E. clone sequence, and, in the conditions used, is likely to have hybridized to any *eag*-like clones derived from this second transcript. Instead, it only detected h-*eag* clones, suggesting that the presumptive h-*eag*2 is not expressed in myoblasts undergoing differentiation. Furthermore, analyses by Northern blotting could not show presence of h-*eag*2 transcripts in myogenic cells, but detected a transcript of >11 kb in adult brain tissue only (using a multiple adult human tissue blot; data not shown).

3.5. *Expression of h-eag current recorded in h-eag-transfected undifferentiated human myoblasts*

To test whether h-*eag* mRNA could encode functional $K_{(NI)}$ channels, we overexpressed h-*eag* in undifferentiated myoblasts. At that stage, the cells are proliferating and do not express active $K_{(NI)}$ channels, or only at a very low density [6], nor do they express any delayed rectifier current ($I_{K(DR)}$ [4]). For these expression studies, a bicistronic h-*eag*-GFP vector was constructed by inserting the h-*eag* sequence upstream of an internal ribosomal entry site [24] followed by the sequence encoding green fluorescent protein [25].

Fig. 3A illustrates whole-cell characteristics of the outward current (I_{h-eag}) recorded in transfected undifferentiated myoblasts. A whole-cell outward current of the amplitude shown in Fig. 3A (inset) was never observed in untransfected myoblasts, nor in mock-transfected cells. For comparison, the mean conductance at +40 mV in h-*eag*-transfected myoblasts is approximately 100 times larger than that occasionally observed in some undifferentiated myoblasts expressing a very low density of $I_{K(NI)}$.

Like $I_{K(NI)}$, I_{h-eag} activates above -50 mV and does not inactivate during sustained depolarization. The steady-state conductances were computed by dividing the current recorded during various voltage steps (see inset) by the driving force on potassium ions ($E_K = -78$ mV), and the conductance-voltage relationship was fitted with a Boltzmann equation (Fig. 3). In 10 h-*eag*-transfected myoblasts, the gating charge of I_{h-eag} was 1.55 ± 0.03 times the elementary charge, V_o was -4.1 ± 2.1 mV, and $G_{\infty,max}$ was 1401 ± 365 pS/pF. Except for $G_{\infty,max}$, which is about 10 times larger than for $I_{K(NI)}$ in fusion-competent cells, reflecting a high level of h-*eag* expres-

sion in the transfected cells, these results are similar to those obtained for $I_{K(NI)}$ recorded in fusion-competent myoblasts [6]. COS-7 cells, which do not express any endogenous $I_{K(NI)}$ or $I_{K(DR)}$, were transfected in parallel and, as shown in Fig. 3B, the currents elicited by h-*eag* in these cells ($n = 5$) were identical to those obtained with transfected myoblasts.

4. Discussion

The possibility to examine the evolution of membrane electrical properties during human myoblast fusion using primary cultures at defined stages of differentiation has allowed us to show that an initial hyperpolarization of the resting potential is associated with the expression of a non-inactivating delayed rectifier potassium current, $I_{K(NI)}$ [6]. Undifferentiated myoblasts that are actively proliferating do not express $I_{K(NI)}$ or only at a very low density. When the cells are induced to differentiate, the current density increases rapidly and markedly, and then steeply declines upon formation of myotubes, indicating that $I_{K(NI)}$ expression peaks before the cells fuse [5]. Similarities between $I_{K(NI)}$ and that elicited by r-*eag* channels [7–10] prompted us to search for an *eag* homolog in myoblasts undergoing differentiation.

The human *eag* sequence isolated produces a functional non-inactivating delayed rectifier potassium channel, whose properties at the whole-cell and single-channel levels are indistinguishable from those of the endogenous $I_{K(NI)}$ expressed in fusion-competent myoblasts [10], indicating that h-*eag* is the molecular entity constituting the $K_{(NI)}$ channel expressed at onset of human myoblast fusion. The presence of h-*eag* transcripts in cultured myogenic cells and the lack of detectable transcripts in adult skeletal muscle mRNA (see Fig. 2) correlate with the changes of $I_{K(NI)}$ density [5], reinforcing our belief that h-*eag* forms the $K_{(NI)}$ channel and that it may be transiently expressed during myoblast differentiation. Alternatively, h-*eag* transcripts may be too scarce in adult tissues to be detectable by Northern blotting, and, although $I_{K(NI)}$ density is downregulated upon myotube formation, it does not completely disappear in freshly formed myotubes.

A presumptive second *eag* gene product could not be detected in myogenic cells. Cloning of the full-length h-*eag*2 cDNA is required to ascertain if it indeed encodes a protein having the structural features of *eag* potassium channels. The detection of transcripts of two *eag* genes in adult brain tissue raises the hypothesis that, if found colocalized in the nervous system, the products of two *eag* genes could coassemble to form heteromultimeric channels. Coassembly of two isoforms of another member of the *eag* superfamily has been suggested to occur in the mouse cardiac muscle [19].

Comprehensive mapping of the h-*eag* gene was performed to determine whether its chromosomal location maps close to any identified genetic syndrome. Several human genetic diseases, such as certain forms of long-QT syndrome [26,27] and of inherited epilepsy [28–30], have recently been demonstrated to be due to mutations in potassium channel genes. The h-*eag* gene was found to map to chromosome 1q32–41, an area to which one form of a rare condition called rippling muscle disease (RMD [31]) has been linked in one family. However, it appears unlikely that mutations in the *eag* gene would be a direct cause of this pathology as the symptoms presented (muscle contraction waves unaccompanied by electrical signals) are not those that would be expected for an ion channel-linked deficiency at the level of the plasma membrane. A search for mutations in the h-*eag* gene in RMD patients will confirm whether there is any involvement in this disorder.

Myoblast fusion is intimately coupled with irreversible cell-cycle arrest. The timing of cell-cycle withdrawal and the extent of myoblast fusion clearly depend on environmental signals and information provided by surrounding cells [32–34]. When proliferating human myoblasts are transferred to differentiation-promoting medium, we observe that their membrane resting potential becomes rapidly hyperpolarized due to the expression of $I_{K(NI)}$, and the activation of this current appears to coincide with cell-cycle arrest. How the cell cycle can exert an influence on plasma membrane electrical properties and conversely, how channel activity and membrane resting potential exert an influence on the cell cycle are questions that are receiving increasing attention [35–37]. The identification of h-*eag* as the molecular entity of the potassium current that contributes to the initial hyperpolarization of myoblasts will allow examination of how h-*eag* expression and channel activity are regulated during myoblast differentiation and coordinated with permanent cell-cycle withdrawal. These future studies shall provide insight on the precise role of membrane potential hyperpolarization in the sequence of events that leads to myoblast fusion.

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