Aberrant gating, but a normal expression pattern, underlies the recessive phenotype of the deafness mutant Connexin26M34T

I. M. Skerrett,* W.-L. Di,† E. M. Kasperek,* D. P. Kelsell,† and B. J. Nicholson‡

*Department of Biological Sciences, University at Buffalo, State University of New York, Buffalo, NY, 14260; †Centre for Cutaneous Research, Barts and the London School of Medicine and Dentistry, Queen Mary, 2 Newark St., Whitechapel, London E1 2AT, UK; ‡Department of Biochemistry, University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX

Corresponding author: Department of Biochemistry, University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX 78229. E-mail: nicholsonb@uthscsa.edu

ABSTRACT

Mutations in the gene GJB2, encoding the gap junction protein Connexin26 (Cx26), are the most prevalent cause of inherited hearing loss, and Cx26M34T was one of the first mutations linked to deafness (Kelsell et al., 1997; Nature 387, 80–83). We report the first characterization of the gating properties of M34T, which had previously been reported to be nonfunctional. Although homotypic mutant channels did not produce detectable currents, heterotypic pairings with wtCx26 confirmed that M34T formed intercellular channels, although the gating properties were altered. Cx26M34T displayed an inverted response to transjunctional voltage ($V_j$), mediating currents that activate in a time- and $V_j$-dependent manner. These characteristics suggest that the channel population is only partially open at rest, consistent with previous reports that dye transfer in M34T-expressing cells is reduced or abolished (e.g., Thonnissen et al., Human Genet. 111, 190–197). To investigate the controversial recessive/dominant behavior of this mutant, we coexpressed M34T with wtCx26 RNA at equimolar levels, mimicking the situation in heterozygotic individuals. Under these conditions, M34T did not significantly reduce Cx26/Cx26 coupling, or alter the electrophysiological properties of the wt channels, consistent with the recessive nature of the allele. Overexpression of the mutant did have some inhibitory effects on conductance, possibly explaining some of the previous reports in exogenous expression systems and some patients. Consistent with its electrophysiological behavior, we also show that M34T localizes to cell junctions in both transfected HeLa cells and patient-derived tissue.

Key words: Connexin26 • gap junction • channel gating

In humans, mutations in the gene encoding connexin26 (Cx26) primarily cause autosomal recessive nonsyndromic deafness (1) (Connexin-deafness home page: http://www.crg.es/deafness). Cx26 is a member of the large connexin family of proteins, the major structural component of gap junction channels that connect the intracellular environments of neighboring cells, allowing the passage of ions and cellular metabolites. In the inner ear, Cx26 is expressed in cells within and surrounding the cochlea, initially suggesting a role for Cx26 in endolymph potassium recycling (2). However, results from both conditional knockout-and transgenic mice have shown that a
lack of functional Cx26 in the inner ear or overexpression of the dominant R75W mutation, respectively, leads to degeneration of the sensory hair cells (3, 4).

Cx26M34T was one of the first connexin mutations to be associated with deafness (1) and its properties have been investigated previously. Although the genetic nature of Cx26M34T in humans led to an initial proposal that it could be a dominant mutation (1), M34T heterozygotes were subsequently identified with normal hearing (5). Further genetic observations identified individuals with mild to moderate prelingual hearing impairment who were homozygous for M34T, suggesting it is a recessive GJB2 allele (6, 7). Individuals who inherited M34T in trans with either of the recessive GJB2 mutations 167delT or 35delG, had hearing in the normal range (8, 9), suggesting that either the resulting phenotype is dependent on genetic background or that it is a benign polymorphism. Exogenous expression studies initially argued against the latter, as the mutant appeared incapable of forming functional gap junction channels (10). In addition, it was reported that M34T did not traffic to the cell surface in transfected cells (11) and that it exerted a dominant negative influence on wild-type channels when coexpressed in oocytes (10), consistent with the dominant role initially postulated for M34T in humans. However, Thonnissen et al. (12) observed low levels of dye transfer between HeLa cells coupled by M34T, providing the first evidence that this mutant could traffic to the cell membrane and form intercellular channels, although with reduced efficiency. More recently, Oshima et al. (13) have reported that assembly of M34T in HeLa and Sf9 cells resembles that of wtCx26 and that dye transfer in these cells is near normal.

In the current study, we present electrophysiological evidence that Cx26M34T is capable of forming intercellular channels. M34T, when expressed in Xenopus oocytes, was capable of forming functional heterotypic channels with Cx32, but with abnormal gating properties, suggesting that the channels are not fully open at rest. In addition, coinjection of M34T and wtCx26 in oocytes supported a recessive role for the M34T allele, as a 1:1 ratio of mutant:wt-type RNA (as found in heterozygotic individuals) did not significantly reduce wild-type coupling. Although this result is contradictory to a previous study of M34T in oocytes, we do find that overexpression of the mutant did cause a reduction in conductance, potentially explaining the disparity. Because of previous inconsistency in the literature with regard to M34T localization and function, we further confirmed the localization of M34T to cell junctions in HeLa cells and present the first evidence for intercellular localization of M34T in biopsied patient material.

METHODS

Expression and recording from oocytes

In brief, oocytes were removed from ovulating, Xenopus laevis females and the follicular layer partially removed with collagenase. The remaining follicular cells were removed using fine forceps before oocytes were coinjected with 4–20 ng of connexin RNA mixed with 4 ng of antisense oligonucleotide directed against nucleotides 327–353 of the endogenous Xenopus Cx38 (14). For preparation of cRNA, Human Cx26 and Cx26M34T were cloned into the Hind III site of pcDNA3 (Invitrogen, San Diego, CA). RNA was prepared using the Amplicap Kit™ of Epicentre Technologies (Madison, WI) or the mRNA Machine protocol of Ambion Inc. (Austin, TX).
Approximately 24 h after injection, oocytes were stripped of their vitelline membrane and paired overnight in agar wells. Oocytes were continuously bathed in half-strength L15 media, which included 89 mM KCl and 0.2 mM CaCl₂ (Sigma, St. Louis, MO). Junctional currents were assessed using the dual oocyte voltage clamp technique as described previously (15). Oocytes were clamped at approximately −30 mV using two Geneclamp Amplifiers (Axon Instruments, La Jolla, CA), one cell was then pulsed in 10 or 20 mV increments to establish \( V_j \)s up to ±100 mV. Data were acquired and analyzed using Pclamp8 software (Axon Instruments, La Jolla, CA). For calculation of junctional conductance (\( G_j \)), currents were measured within the first 100 ms of the voltage pulse (instantaneous current) and at the end of a 4–10 s pulse (steady state current).

**Dominant negative studies in oocytes**

For studies in oocytes, cRNA concentration was determined spectrophotometrically and from ethidium bromide-stained agarose gels. To test potential interactions between wtCx26 and M34T, RNA was mixed at ratios of 1:1 or 1:2, while injection volume was maintained constant at 40 nl/oocyte. Hence, M34T was present at an equal concentration to that of wtCx26 (1:1 ratio), and at a concentration double that of wtCx26 (1:2 ratio). Because the amount of Cx26 RNA varied for different ratios, each ratio was compared with the conductance of oocytes injected with the same amount of Cx26RNA (Cx26 dil). For example, if the 1:1 ratio resulted in injection of 2 ng of Cx26 RNA and 2 ng of M34T RNA per oocyte, the corresponding control oocytes were injected with 2 ng of Cx26 RNA only. Cells were first preinjected with antisense oligonucleotide directed against XeCx38 (1 ng/oocyte), and all oocytes were paired with an oocyte injected with the undiluted Cx26 standard.

To control for the possibility that decreases in conductance at the 1:2 ratio resulted from a saturation of the translational machinery of the oocyte, cRNA of an invertebrate gap junction protein (C. elegans INX-3) was coinjected with wtCx26 at a 1:2 ratio. In oocytes, INX-3 forms functional channels with itself, but not with Cx26. As for the M34T/Cx26 experiments, the coinjected oocytes were paired with those injected with a high concentration of Cx26 (Cx26/Cx26+INX-3) eliminating the possibility of INX-3 channel formation.

**cDNA analysis in keratinocytes heterozygous for M34T**

Previously, we have reported the identification of M34T heterozygosity in one primary keratinocyte culture and in four cell lines (7). Total RNA from the primary keratinocytes was extracted using Trizol reagent (Life Technologies, UK). 1 µg RNA was reverse transcribed to first-strand cDNA by moloney murine leukemia virus reverse transcriptase and random hexamer (Perkin Elmer, UK) in 20 µL of reaction mixture, and 5 µl of RT product was applied to 35 cycles of PCR using Taq DNA polymerase (Perkin Elmer, UK) with forward primer CAGCGCAGAGACCCCAACGC and reverse primer GACACGAAGATCAGCTGCAG (5′-3′). The RT-PCR product was sequenced using BigDye Sequencing chemistry (Perkin-Elmer, UK), and the relative transcribed levels of M34T and wild-type allele were compared by eye.

**Visualization of Cx26 and M34T in HeLa cells**

HeLa Ohio cells (ECACC, Cancer Research, UK) were cultured in DMEM, supplemented with 10% FCS and 100U/100 µg/mL penicillin/streptomycin. After reaching confluence, these cells
were plated on 13 mm glass coverslips for transfection. HeLa cells were transfected with the pcDNA2 encoding either wild-type Cx26 or M34T using the Transfast liposome system (Promega, USA). Cells (0.5–1×10^5/13 mm coverslip) were transfected with 0.5 µg of plasmid DNA in 1:2 Transfast reagent. Twenty-four hours post-transfection, the cells were fixed and processed for immunocytochemical staining using the Cx26 antibody as described previously (16). Images were recorded using a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss, Germany) and processed using Adobe Photoshop 6.

**Immunofluorescence staining and microscopy of human sweat glands**

Skin biopsy material was obtained from a consenting patient homozygous for the Cx26M34T mutation and from a healthy individual, with ethical approval. Immunofluorescence staining was performed as described previously (16). Briefly, cells were fixed with 4% paraformaldehyde for 15 min, then rinsed with phosphate-buffered saline (PBS) and incubated in PBS containing 0.1% Triton X-100 (Sigma Poole, UK) for 10 min at room temperature. After rinsing, slides were incubated in a humidified chamber at 37°C for 2 h with monoclonal Cx26 antibody (Zymed, USA) diluted 1:100. Following several rinses with PBS, slides were incubated in the dark for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Molecular Probes, USA) diluted 1:100. Propidium iodide (Molecular Probes, USA) diluted 1:1000 in PBS was added after washing off the secondary antibody in order to highlight the nuclei. Following two minutes incubation, three 5 min washes with PBS were performed. The slides were then mounted in mowiol reagent containing 10% mowiol D-488 (Calbiochem, UK), 25% glycerol and 2.5% 1.4-diazabicyclo [2.2.2] octane (Sigma, Poole, UK) in 50 mM Tris/HCl, pH 8.5. Images were recorded using a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss, Germany) and processed using Adobe Photoshop 6.

**RESULTS**

**Expression of hCx26 and hCx26M34T in oocytes**

**Properties of wild-type and M34T channels**

Oocytes injected with humanCx26 cRNA established junctional conductances in the 1–50 µS range after overnight pairing, depending on the amount of RNA injected. Fig. 1A shows a typical recording of Cx26 mediated currents induced by 10 mV transjunctional voltage steps ranging from –100 mV to +100 mV. The slow decay of current in response to \( V_j \) > 80 mV of either polarity is similar to that reported previously for ratCx26 (14, 17).

In Fig. 1D, conductance is plotted as a function of transjunctional voltage (\( V_j \), relative to the pulsed oocyte), for Cx26 pairs. For \( V_j \)s at ±100 mV and for short-pulse durations (<20 s), Cx26 displays very little voltage sensitivity, consistent with previous reports that half-maximal voltage-dependent inactivation occurs at \( V_j = ±90 \) mV (17). A slight asymmetry in the steady state conductance–voltage relationship is consistent with previous analysis of Cx26 and has been reported to result from a slight sensitivity of Cx26 to transmembrane voltage (Vm, or Vt-o, e.g., 14).

Homotopic (mutant/mutant) pairings of M34T failed to form functional channels, as also reported by White, et al. (10) after expression of M34T in *Xenopus* oocytes. Currents could not
be induced between oocytes even when the amount of injected cRNA was increased to a level 4 times greater than that required for high coupling levels with wild-type protein. However, analysis of heterotypic pairings of mutant with Cx26 (Cx26/Cx26M34T) revealed that the mutant was capable of forming gap junction channels but with modified gating properties (Fig. 1B). The channel population was partially open at rest ($V_j = 0$), as illustrated by small steps in current in response to application of voltage of either polarity. However, while junctional currents showed the characteristic decay in response to $V_j$ when the wild-type-expressing oocyte was relatively positive, when the M34T-expressing oocyte was relatively positive, junctional currents activated in a time- and voltage-dependent manner. Conductance is plotted as a function of voltage for these heterotypic Cx26/M34T channels in Fig. 1E. As for Cx26, conductance was normalized to the instantaneous current at $–20 \text{ mV}$. At positive transjunctional voltages with respect to M34T, $G/j/G_j\text{max}$ increased by >twofold, while a reduction to ~0.5 was seen at negative potentials.

**Interactions between M34T and wtCx26 in oocytes**

To determine the physiological significance of the altered M34T gating properties in heterozygotic individuals and to test for possible interactions between wild-type Cx26 and the Cx26M34T mutant, oocytes were coinjected with cRNA’s encoding M34T and hCx26. Given the possibility of a mutant gating phenotype, where homotypic currents cannot be recorded, we paired the coinjected cells heterotypically with wtCx26. However, surprisingly, the coexpression of M34T in oocytes did not significantly alter the gating profile of Cx26 channels (Fig. 1C, and 1F). The wt/mutant coinjection ratios were based on cDNA ratios in heterozygotic individuals. Analysis of genomic and cDNA sequences in cultured keratinocytes derived from heterozygotic M34T individuals, revealed that wild-type and mutant alleles were transcribed with equal efficiency. This was based on the ratio of T to C in reversed transcribed cDNA (switching codon 34 in the sequence from encoding M to T) at the position marked “N” in Fig. 2B ($n=3$ with one example presented in Fig. 2). Hence, a 1:1 ratio of wild-type:mutant RNA was considered to most closely mimic the situation in vivo.

In addition to the lack of any changes in the gating properties of the junction when Cx26M34T was added to wtCx26 (cf. Fig. 1A and 1C; Fig. 1D and 1F), coinjected oocytes (Cx26/Cx26+Cx26M34T) showed no decrease in intercellular coupling compared with wt pairings in three different oocyte batches (Fig. 3). The apparent lack of contribution by Cx26M34T to the junctional properties could be linked to several different properties of the mutant. For instance, the translational or trafficking efficiency of M34T could be significantly lower than that of Cx26, or the properties of Cx26 gating could dominate the channel behavior at the connexin ratio established by equimolar levels of RNA. Previous evidence indicates that M34T induces a complete knockout of Cx26 coupling when the expression system is designed to produce equal protein levels. Hence, we tested the effect of a slightly higher ratio of M34T for coexpression. When the oocytes received twice as much M34T RNA as wild-type (wild-type:mutant ratio = 1:2), a reduction in conductance occurred to approximately one-sixth that of wild-type pairs (Fig. 3). However, even at this ratio, the residual coupling was not significantly different from wtCx26, and there was no evidence of the activating junctional currents characteristic of M34T (see the conductance–voltage relationship in Fig. 1E). This suggests that prejunctional interactions between M34T and Cx26 might prevent the efficiency with which Cx26 forms channels. We were able to rule out the possibility that the two RNAs were competing for translational machinery in the oocyte, because coinjection of an invertebrate gap
junction protein (*C. elegans* innexin 3) with wtCx26 at the same ratio had no effect (data not shown).

**Localization of Cx26M34T in cells**

Because of previous inconsistency in the literature with regard to localization of M34T, we assessed the localization of Cx26 and Cx26M34T in HeLa cells and, for the first time, in patient-derived tissue. HeLa cells were transfected with Cx26 and M34T and the transfected protein localized by immunocytochemistry. HeLa cells transfected with wtCx26 showed punctate staining and aggregation of protein at the plasma membrane, especially in regions of cell–cell contact (Fig. 4A). However, cytoplasmic staining, particularly associated with the golgi, was also evident, possibly as a product of overexpression. For cells transfected with Cx26M34T, the pattern of staining was similar (Fig. 4B). Although it was not possible to quantitatively compare relative cytoplasmic or membrane distributions of the proteins, particularly as this is affected by expression levels, and hence transfection efficiency, it was clear that in transfected cells, M34T could localize to regions of cell–cell contact.

The localization of Cx26 and M34T was also examined in sweat glands derived from healthy individuals and patients homozygous for M34T (Fig. 5). Glands stained with a FITC-conjugated Cx26 antibody clearly demonstrate the expression of wtCx26 (Fig. 5A) and M34T (Fig. 5B). Although a quantitative comparison is not possible between different tissue samples (as factors such as body site, age, and sample processing can effect expression or staining), M34T appears to be expressed at levels comparable to that of wtCx26. In addition, staining at cell interfaces is clearly apparent in the gland derived from an M34T patient, indicating that M34T is capable of forming gap junction plaques in vivo.

**DISCUSSION**

**Gating characteristics of M34T**

Wild-type gap junction channels have a high open probability in the absence of transjunctional voltage and typically gate to a “residual” low conductance state in response to $V_j$. On a macroscopic scale, the M34T substitution in Cx26 induces an apparent reversal of this response, where currents activate, rather than inactivate in response to $V_j$. Because the macroscopic recordings obtained from *Xenopus* oocytes represent the behavior of many thousands of channels, it is not possible to ascertain whether the time-dependent increases in $G_j$ occur as a result of increased channel open probability (Po) or increased channel conductance ($\gamma$). However, the phenotype is shared by Cx32M34T, which is also associated with disease in humans. Single-channel analysis of Cx32M34T revealed that these channels reside predominantly in a low conductance state, similar to the residual conductance state associated with voltage gating (20). In addition, the M34T substitution in Cx32 has been shown to induce a constriction of the pore (19), further supporting the likelihood that these channels are only partially open at rest. However, single-channel analysis is ultimately required to determine whether $V_j$ induces an increase in Po or $\gamma$ for Cx26M34T.

Regarding the complete absence of coupling when M34T channels were assessed homotypically, several possibilities exist. The channels may not dock properly or open after docking. However, the properties of M34T heterotypic junctions (Cx26/M34T) may be consistent with the finding
that (M34T/M34T) homotypic gap junctions are nonfunctional. The gating characteristics of the heterotypic channels suggest that, if formed, M34T homotypics would remain partially closed, even when \( V_j \) is applied. The activating current typical of the heterotypic channels would not be observed because the M34T hemichannels on one side of the junction would remain in a low residual conductance state even if channels on the other side were induced to open. In addition, one would predict that the instantaneous conductance observed in the M34T/Cx26 pairs would be reduced by at least half, by combining two M34T channels in series. Combined with the likelihood that mutant protein levels from similar levels of cRNA are lower than wt protein, the absence of coupling in M34T homotypics is not surprising.

In heterotypic pairings with wild-type Cx26, M34T formed channels that activated in a time- and voltage-dependent manner in response to relatively positive \( V_j \). This phenotype commonly results from point mutations within the transmembrane domains of connexins (18) and may reflect a decrease in stability of the channel open state, and/or an increase in stability of the closed state. Because several other mutations linked to human disease display similar \( V_j \)-activated currents (18, 20), understanding the structure and consequences of these channels is important. The gating behavior of the channels suggests that they remain predominantly closed at rest but can be opened with applied voltage. On the basis of our analysis of Cx32, mutations in M1, M2, M3, and M4 are all capable of inducing \( V_j \)-activated currents (unpublished observation). Several of the corresponding sites in Cx26 are linked to deafness (e.g., S138 and R142 in the third transmembrane domain), raising the possibility that gating alterations may underlie the symptoms associated with other mutations in Cx26 where channel properties have not yet been tested.

Recently, a region of pore constriction was mapped in Cx32M34T, which also mediates \( V_j \)-activated currents (19). The fact that the channel gate can be localized in the absence of applied \( V_j \) in these channels suggests that these mutant channels reside predominantly in a closed state at rest. The consequence of a mutation that closes, or partially closes the channel, is a change in channel permeability. Because of the diverse nature of gap junction channels and their many permeants, including ions and metabolites, physiological aspects of altered permeability are difficult to ascertain. Dye permeability and exclusion studies may reveal changes in the pore diameter, but changes in pore-lining configuration are likely to alter permeability in other ways, by affecting natural interactions between permeants and the channel. Further analysis of M34T and other mutations that cause disease without abolishing gap junction formation will ultimately provide interesting information about the role of gap junctions in hearing, although such analyses may first require advances in techniques for assessing gap junction permeability.

**Interactions between Cx26 and M34T**

Initial studies suggested that M34T displayed dominant properties and that heterozygous individuals suffered from the disease (1). Subsequent analysis revealed that the M34T carriers with profound hearing loss in the small family described in this initial study were in fact compound heterozygotes for an additional GJB2 mutation, D66H (21). Furthermore, genetic analysis of larger populations indicated homozygotic, but not heterozygous individuals, suffer from severe hearing loss (6, 7). Contrary to these more recent genetic findings, original in vitro analyses of the mutation were more consistent with a dominant mode of action. The mutant inhibited wild-type coupling when coexpressed in *Xenopus* oocytes (10) and in cells failed to form identifiable gap junctions at the cell surface (11).
To further examine the consequences of interactions between M34T and wtCx26, we designed a coinjection protocol that would closely mimic the situation in heterozygous individuals, where cDNA analysis showed that wild-type and mutant alleles are transcribed with equal efficiency. Under these circumstances, M34T expression did not influence the function of wtCx26, consistent with the recessive nature of the allele. At higher levels, M34T did influence Cx26 in a negative manner (such as the 2:1 ratio of M34T:Cx26 cRNAs), suggesting that M34T and wild-type protein interact, as demonstrated by Oshima et al. (13). This interaction can result in a negative influence on Cx26 coupling, but only when mutant RNA expression significantly exceeds wild-type. This could explain the apparent discrepancy between the results presented here and the dominant negative effects of Cx26 M34T reported by White et al. (10). In the latter study, mutant and wild-type protein were expressed in both cells (rather than only one as done here), enhancing any inhibitory effect that could have been caused by overexpression of the mutant. It is possible that the lack of dominant-negative interactions between M34T and wtCx26 reported here when the RNA’s are equimolar, may be related to lower M34T protein levels than wild-type, either due to degradation of partially misfolded mutant proteins in oocytes, or lower translation levels of the mutant cRNA. With respect to the latter, it should be noted that in the previous study by White et al. (10), both mutant and wild-type coding regions were fused downstream of *Xenopus* β-globin 5′untranslated sequences to ensure equal and efficient translation, while in the current study, the endogenous 5′untranslated regions were used to mimic as closely as possible the in situ condition. Unfortunately, direct comparisons of the levels of mutant and wt proteins cannot be made in coinjected cells. Protein levels have previously been inferred from singly injected cells (10). However, qualitative comparisons of Figs. 4 and 5, suggest that mutant and wild-type protein are made at similar levels endogenously.

Our finding that M34T inhibits wild-type coupling when present at higher quantities, but not at the equal RNA levels which occur in vivo, suggests that some systems may be inappropriate for studying the functional consequences of protein interactions. The range of results reported for exogenous expression of M34T is likely due to a lack of consistency in controlling for relative amounts of cDNA or RNA. Because it is difficult or impossible to control these levels in transfected cells, they may not be the most appropriate system for assessing the functional consequences of protein interactions in vivo.

**Localization of M34T**

The reexamination of Cx26M34T expression presented here suggests that Cx26M34T localizes to cell junctions in exogenous expression systems (Figs. 1, 3, and 4), and in vivo (Fig. 5). The observation that significant amounts of M34T protein localize to cell junctions in cells derived from an individual homozygous for M34T demonstrates that in humans, the link between M34T and deafness is unlikely to be related to low protein levels, or trafficking defects. The consequences of the mutation are more likely related to altered, or nonfunctional, channels. This is consistent with recent analyses of M34T in exogenous expression systems which all report good localization of M34T to regions of cell–cell contact (12, 13), although the function of the channels has been variable.

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Figure 1. Intercellular currents recorded from Xenopus oocytes expressing wtCx26 (A) heterotypic pairings of wt Cx26 with Cx26M34T (B), or a mixture of wild-type and mutant proteins within one oocyte (C). Corresponding conductance–voltage relationships are shown (D–F). For recording of intercellular currents, both oocytes were clamped at –30 mV. \(I_j\) was then recorded from one of the cells, which was continuously clamped at –30 mV, while its partner was pulsed in 10-mV increments to +70 mV and –130 mV. Wild-type Cx26 channels (A and D) are minimally voltage-sensitive within this range, with half-maximal inactivation occurring at \(V_j = \pm 90\) mV (14). The currents from the Cx26/M34T pair (B and C) were recorded relative to the mutant-expressing oocyte, and the M34T channels display time-dependent activation in response to relatively positive transjunctional potentials. Recordings from the coinjected pairs (C and F) were also made relative to the mutant-expressing oocyte and showed no significant differences from wild-type currents. In the conductance–voltage plots in (D–F), each data point represents three low conductance cell pairs (\(G_j < 5\) \(\mu\)S), sampled at 20 mV increments between transjunctional voltages of –100 mV and +100 mV. Solid squares represent instantaneous conductance, measured within 100 ms of the start of a voltage pulse, and solid triangles represent steady-state conductance, which was measured at the end of an 8–10-s pulse.
Figure 2. A typical sequence electropherogram used to compare the frequency of wild-type and mutant alleles for genomic DNA (A) and cDNA (B) from keratinocytes derived from an individual heterozygous for M34T in Cx26. The T-C nucleotide substitution at the site indicated by the bold “N” in the sequence at the top of each panel alters the amino acid sequence from a methionine to a threonine residue. (A) Genomic DNA sequence. (B) cDNA sequence. Note the almost equal contribution of both alleles to the sequence trace in (A and B), the latter profile indicating that both alleles are transcribed at similar levels.
Figure 3. Coupling levels for oocytes expressing Cx26 and M34T. All were preinjected with antisense oligonucleotide directed against XeCx38, and in order to discern decreases in channel-forming ability from nonfunctional channels, all pairings included an oocyte injected with a high concentration (~10 ng) of wtCx26 (except M34T/M34T). Homotypic pairings of wtCx26-induced high levels of oocyte coupling after overnight pairing, while homotypic M34T failed to induce oocyte coupling. Heterotypic pairings (M34T/Cx26) induced levels of coupling that were ≤40% that of wild-type proteins. Coinjection of M34T with wt did not reduce coupling levels for wt:mutant cRNA ratios of 2:1 or 1:1. However, coupling levels were reduced by a factor of ~6 when the wt and mutant cRNAs were injected at a ratio of 1:2. The number of oocyte pairs that is shown above each bar is derived from three independent batches of oocytes.
Figure 4. Localization of transfected wtCx26 (A) and M34T (B) in Hela Cells. Cells were transfected with Cx26 or Cx26M34T, and 24 hours post-transfection, the cells were fixed and processed for immunocytochemical staining using a Cx26 antibody as described previously (16). Localization to regions of cell contact was clearly observed in both wild-type and M34T transfectants.
Figure 5. The expression pattern of Cx26 protein in sweat glands from normal patients (A) and patients homozygous for M34T (B). Cells were fixed and immunostained with a monoclonal, FITC-conjugated Cx26 antibody. Nuclei were counterstained in red with propidium iodide. Again, both tissues clearly express considerable quantities of connexin protein, including protein localized to cell junctions.