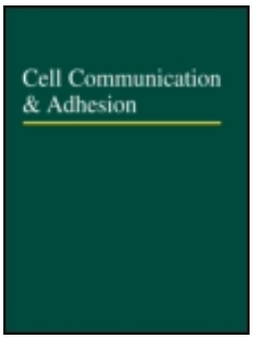

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Cloning and Functional Expression of a Novel Human Connexin-25 Gene

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Gap junctions are intercellular, water-filled channels composed of transmembrane proteins called connexins, six of which are arranged radially and dock with six homologous proteins in an adjacent cell to form an approximate 16 A pore. Through this pore cell-to-cell transfer of small water-soluble molecules up to about 1000 daltons occurs along concentration gradients. Connexins comprise a multigene family that share consensus sequences in the trans-membrane domains and the first and second extracellular loops. Comparison of the protein sequences of known human connexins with the draft nucleotide sequence of the human genome revealed two clones from chromosome 6 which showed strong similarity to highly conserved connexin sequences. Detailed analysis revealed the presence of a 672 nt open reading frame in these clones, encoding a 223 amino acid polypeptide with a predicted molecular weight of about 25 kD. This is smaller than other known human connexins. The ORF of the potential connexin25 was amplified by semi-nested PCR using human genomic DNA as a template. To confirm that this new gene encodes a connexin, Cx25 was transfected into a gap junction deficient subclone of the human HeLa cell line. After selection of transformants, cells were microinjected with the fluorescent dye Lucifer yellow. Transfectants but not controls successfully transferred dye, demonstrating that this new gene encodes a functional connexin.

Keywords Cloning, connexin-25 gene, protein

INTRODUCTION

The phenomenon of gap junctional communication was discovered serendipitously with the observation that cultured cells were electrically coupled when in contact. It was later discovered that many tumor cells lacked this property (Loewenstein and Kanno 1966). This led to the still-developing hypothesis of growth control through junctional communication (Loewenstein 1979). The cloning and functional expression of a widely expressed connexin gene, connexin43, allowed analysis of gene structure and gene function (Beyer et al. 1987). It also led to the discovery of a family of connexin genes with tissue and development specific expression (Beyer et al. 1990). To date, a family of about

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20 members have been reported in the literature (Simon and Goodenough 1998). The functions of individual connexins have been probed using knockout mice in which the gene has been deleted, studies which have been complicated by extensive redundancy among connexin family members (Willecke et al. 1999; Houghton et al. 1999). Additional information regarding function has been aided by the discovery of mutations in connexin genes that are linked to numerous human diseases. Multiple mutations in connexin32, which is expressed in the Schwann cells which surround and insulate the axon, have been linked to the human peripheral neuropathy X-linked Charcot-Marie-Tooth disease (Krutovskikh and Yamasaki 2000). Mutations in the connexin50 gene, expressed in the lens, have been shown to be a risk factor for congenital cataract development (Shiels et al. 1998). Several different mutations in connexin26, a gene expressed in the cochlea, have been identified as the molecular bases for nonsyndromic deafness (Rabionet et al. 2000). There is growing evidence that cells of the immune system also communicate via gap junctions, although the physiological functions of such communication are not well established. In lymph nodes, expression of connexins 43 and 40 has been reported. Extensive communication between follicular dendritic cells and germinal lymphocytes has been demonstrated. Connexin43 is also expressed in circulating T and B lymphocytes, and functioning junctions were detected when these two cell types were co-cultured. Interestingly, antigenic stimulation with phytohaemagglutinin and lipopolysaccharide, respectively, increased expression of connexin43, while blockade of junctional communication resulted in decreased expression of IgM. These results suggest that junctional communication acts to coordinate the immune response (Oviedo-Orta et al. 2000).

In the bone marrow there is normally strong expression of connexin43 which is increased during bone marrow regeneration. The functional significance of this expression is indicated in connexin43 knockout mice which exhibit impairment of terminal stages of primary T and B lymphopoiesis. Even in heterozygous mice, the regeneration of lymphoid and myeloid cells was found to be impaired after cytotoxic damage to the marrow (Montecino-rodriquez et al. 2000). Gap junctions thus appear to play a major role in modulating immune responsiveness and ensuring adequate generation of T and B lymphocytes.

METHODS

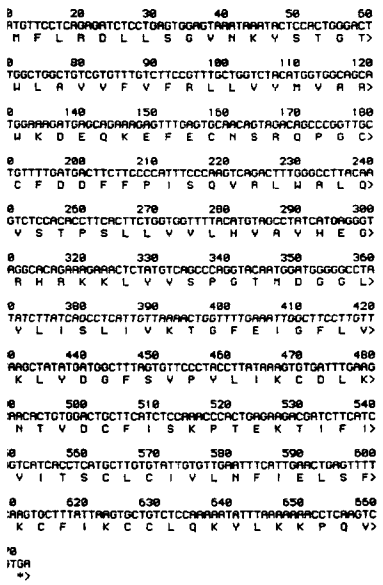
Primary PCR was conducted in 100 ul of 1x Taq buffer containing 10 mM $MgCl_2$, 100 pmol of each dNTP, 5 U Taq enzyme (Promega), 500 ng of human genomic DNA, and 100 pmol each of 25-EF (5′-ACATATCTGAGGCTGTGGCAC-3′) and 25-ER (5′-ACAGCACTTAATAAAGCACTTC-3′) primers. PCR reaction conditions were as follows: 94°C for 1 min, followed by 35 cycles (94°C for 30 sec, 54°C for 30 sec, 72° for 1 min) and final elongation at 72°C for 7 min. 1 ul of the primary PCR reaction served as template for a nested secondary PCR reaction with primers 25-OF (5′-ATGAGTTGGATGTTCCTC-3′) and 25-OR (5′-TCACACACTGAGGACTTG-3′), conducted as described above. The PCR product was gel purified and cloned into the mammalian expression vector pTarget (Promega). Positive clones containing the Cx25 ORF in the plus orientation with respect to the vector's CMV promoter were identified by PCR using T7 and 25-OR primers, conducted as described above. Positive results were confirmed by restriction digestion of plasmid DNA and DNA sequencing. Plasmid DNA for transfection of the communication-deficient subclone of the human HeLa cell line was purified using a DNA Midi Prep kit (Qiagen). Cells were plated at a density of 2.5×10^6 cells in 100 mm dishes and transfected with 50 ul GenePORTR transfection reagent (Gene Therapy Systems Inc.) plus 10 mg plasmid DNA. Cells were subcloned and selected with G418 for 2 weeks. The functionality of the putative Cx25 gene was assayed by microinjecting the fluorescent dye Lucifer yellow CH (dilithium salt) as previously

(King et al. 2000). Dye transfer to adjacent recorded digitally.

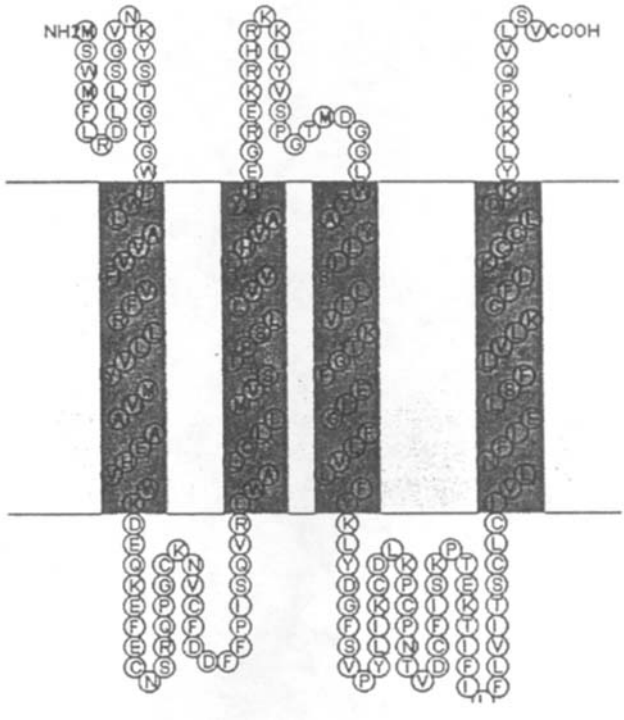
'S AND DISCUSSION

rison of protein sequences of known hu- ıexins with the unfinished nucleotide se- ɔf the human genome (tblastn version of ٧CBI) demonstrated strong similarity with 7180.5 and AL160258.3 clones from chro- 6 ("htgs" NCBI Genbank database). De- lysis showed the presence of a 672 nt open ame (ORF) in these clones, encoding a pre- 3 amino acid polypeptide with homology to nnexins (Figure 1A). The molecular weight dicted protein was about 25.8 kD, smaller known human connexin. Analysis of the protein topography, using the "SOSUI" program (Tokyo University of Agriculture and Technology) at <http://sosui.proteome.bio.tuat.ac.jp/cgi-bin/sosui.cgi?/sosui_submit.html>, revealed structures also common for other connexin family members (Figure 1B).

To clone the ORF of the potential connexin25.8 (Cx25), nested PCR was performed. Based on the nucleotide sequences of AL137180.5 and AL160258.3 clones, two sets of primary and nested primers were designed. A single band of the expected size was cloned and expressed under control of the CMV promoter in a gap junction deficient subclone of the HeLa cell line (King et al. 2000). After selection of G418 resistant clones, cells were grown to monolayers and the ability of this novel gene to function as a connexin was determined by microinjection of single cells with the gap junction permeable fluorescent dye Lucifer yellow. As shown in Figure 2, extensive transfer of Lucifer yellow



B



.. Alignment of nucleotide and amino acid sequences of human connexin25. (Numbers indicate nucleotide positions. Symbol *> ɔp codon.) B. Predicted secondary structure of connexin25 protein. Predicted transmembrane regions in green. Upper, predicted : sequences; lower, predicted extracellular. (See Color Plate I).

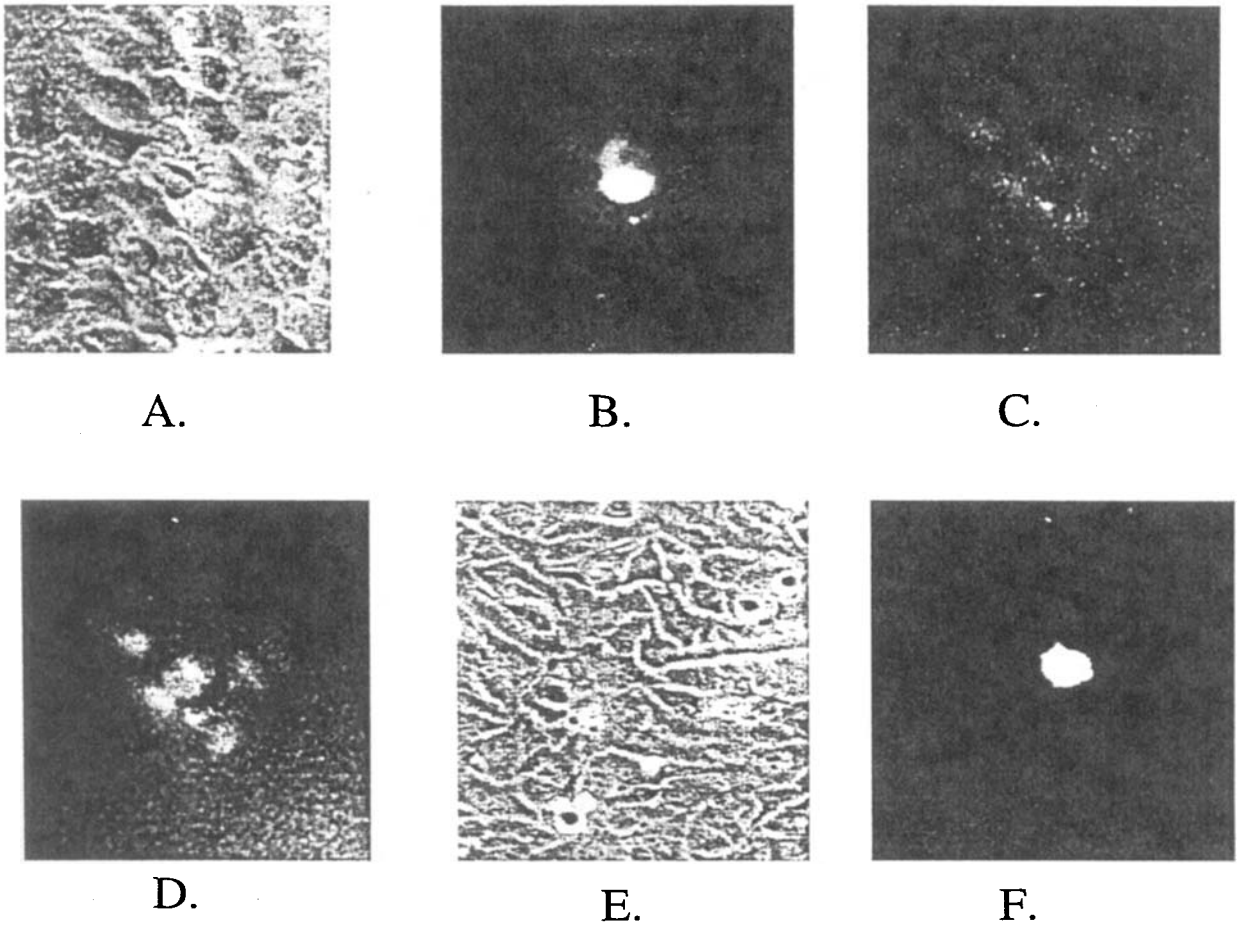


FIG. 2. Connexin25 mediated cell-to-cell Lucifer yellow dye spreading. A. Photomicrograph of connexin25 expressing Hela cells in visible light. B.–D. Fluorescent image of the same field 0, 1 and 5 min after microinjection of Lucifer yellow. E. Image of original gap-junction deficient HeLa cells in visible light. F. Fluorescent image of the same cells 5 min after microinjection of Lucifer yellow. (See Color Plate II).

was detected from the injected cell to surrounding neighboring cells. Non-transfected cells were, as expected, negative for dye transfer. This observation confirmed that Cx25, when expressed in a junctionally-non-competent HeLa cell line, was able to re-establish junctional communication and is an additional member of the connexin family. That this gene is transcriptionally active in humans is indicated by the detection of an expressed sequence tag (EST AA7492214, NCBI Genbank database) with complete sequence identity to Cx25 that originated from a B-cell germinal center of human tonsil. The transcription and lack of inactivating mutations indicates that this gene does not represent a pseudogene. We predict that the corresponding full length mRNA is translated into functional protein. Its predicted expression in lymphoid tissue opens the question of the participation of this gene in immune function and how this function differs from those of connexins 40 and 43 which, as discussed above, are also expressed in immune tissues (Oviedo-Orta et al. 2000).

Analysis of both the gene structure and predicted protein topography revealed many similarities between Cx25 and other family members. For example, analysis of the ORF of Cx25 indicates that the entire ORF is encoded by a single exon. This structure

is unusual but is typical for connexin family members (Henneman et al. 1992). There are four putative hydrophobic transmembrane domains, the third of which is amphipathic and, in analogy with other connexins, is believed to line the water-filled pore. Assuming a cytoplamic location of both the N- and C-termini of this protein as demonstrated for other connexins (Rahman et al. 1993), the two extracellular domains are the same size as the majority of connexins. These domains each have three conserved cysteines which are believed to dock with corresponding cysteines in the extracellular loops presented by connexins in adjacent cells (Foote et al. 1998). Interestingly, the central cytoplasmic loop of Cx25 is the shortest of all known connexins. As a consequence, despite Cx25 being the smallest of all connexins so far discovered, its C-terminal cytoplasmic domain is longer than the next largest connexin (connexin26) and contains potential sites for phosphorylation at tyrosine-214 and serine-222 (Lampe and Lau 2000). The presence of three cysteines in this domain raises the question of potential internal disulfide bonds.

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