

Transcriptional Downregulation of Gap-junction Proteins Blocks Junctional Communication in Human Mammary Tumor Cell Lines

Sam W. Lee,*[‡] Catherine Tomasetto,*[‡] David Paul,[§] Khandan Keyomarsi,*^{||} and Ruth Sager*[‡]

*Dana-Farber Cancer Institute, Boston, Massachusetts 02115; and [‡]Departments of Microbiology and Molecular Genetics, [§]Anatomy and Cellular Biology, and ^{||}Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Abstract. Subtractive hybridization, selecting for mRNAs expressed in normal human mammary epithelial cells (NMECs) but not in mammary tumor cell lines (TMECs), led to the cloning of the human gap junction gene connexin 26 (Cx26), identified by its sequence similarity to the rat gene. Two Cx26 transcripts derived from a single gene are expressed in NMECs but neither is expressed in a series of TMECs. Northern analysis using rat Cx probes showed that Cx43 mRNA is also expressed in the normal cells, but not in the tumor lines examined. Connexin genes Cx31.1, Cx32, Cx33, Cx37, and Cx40 are not expressed in either normal cells or the tumor lines examined. In cell-cell communication studies, the normal cells transferred Lucifer yellow, while tumor cells failed to show dye transfer. Both Cx26 and Cx43 proteins were immunolocalized to membrane sites in normal cells but

were not found in tumor cells.

Further analysis demonstrated that Cx26 is a cell-cycle regulated gene expressed at a moderate level during G1 and S, and strongly up-regulated in late S and G2, as shown with lovastatin-synchronized NMECs. Cx43, on the contrary is constitutively expressed at a uniform low level throughout the cell cycle. Treatment of normal and tumor cells with a series of drugs: 5dB-cAMP, retinoic acid, okadaic acid, estradiol, or TGF β , had no connexin-inducing effect in tumor cells. However, PMA induced re-expression of the two Cx26 transcripts but not of Cx43 in several TMECs. Thus Cx26 and Cx43 are both downregulated in tumor cells but respond differentially to some signals. Modulation of gap-junctional activity by drug therapy may have useful clinical applications in cancer.

CANCER develops in multiple genetic steps, at many of which cells lose opportunities for tumor suppression (16, 24, 30). One such step can be the loss of gap-junctional communication. A role for cell-cell communication in tumor suppression was first proposed in the 1960's by Stoker (27) and by Loewenstein (15). Stoker showed that polyoma virus-transformed BHK fibroblasts could be growth arrested by cocultivation with untransformed BHK cells; and Loewenstein demonstrated electrical coupling between adjacent normal rat liver cells that was lost in four rodent hepatomas.

Subsequent studies by many investigators have established a correlation between gap-junctional intercellular communication (GJIC)¹ and growth control or suppression of the transformed phenotype using rodent fibroblasts in culture (for reviews see 18, 19, 29, 32). In human cancer, examples of tumor cells with and without communicating ability have

been described, but reports of the loss of this property are much more frequent than its retention. In early studies of breast cancer, cell lines were examined for their communicating ability using ³H-uridine transfer (9, 27). Most tumor cells showed no homologous transfer between cells of the same cell line. On the contrary, normal breast epithelial cells showed strong homologous transfer. These findings obtained before the connexin genes and proteins had been identified foreshadowed the results reported here.

In this laboratory a selection system was established to recover tumor suppressor genes, using subtractive hybridization to isolate mRNAs expressed in normal but not in closely related human mammary carcinoma cells (13, 14, 24). An important assumption underlying this approach is that normal tumor suppressor genes are preferentially or uniquely expressed in normal cells, and lost in tumor cells, as exemplified by retinoblastoma, normal p53, and Wilms' tumor genes (16, 24, 30).

Using subtractive hybridization (6, 13, 14), we have isolated the gene Cx26 encoding a gap-junctional structural protein, connexin 26, identified by its sequence similarity to rat Cx26 (33). It is shown here that Cx26 is expressed in normal human mammary epithelial cells but not in a series of

S. W. Lee's present address is Department of Radiation Oncology, Division of Cancer Biology, The University of Michigan Medical School, Ann Arbor, Michigan 48109.

1. *Abbreviations used in this paper:* dd, double distilled; NMEC, normal mammary epithelial cell; TMEC, tumor-derived mammary epithelial cell.

breast cancer cell lines, including the 21MT-2 line used in the subtraction (13). Consistent with the Northern hybridization data, the tumor cells showed no junctional communicating ability as assayed by Lucifer yellow dye transfer whereas the normal cells were active in GJIC.

The evidence from this study that connexins are transcriptionally downregulated in tumor cells compared with their normal mammary epithelial counterparts focuses interest on the regulatory mechanisms involved. To understand the regulation of connexin gene expression is central to investigations of gap-junctional functions in development and in disease. Preliminary studies presented here of messenger stability, cell cycle regulation, and the differential responses of Cx26 and Cx43 to drug treatments open new avenues for further research.

Materials and Methods

Cells and Cell Cultures

Normal human mammary epithelial cells strains (70N, 76N, 81N) were derived from reduction mammoplasty tissues (2). Human breast tumor cell lines were obtained from ATCC BT549 (HTB123), MDA-MB-231 (HTB26), SKBR3 (HTB30), T47D (HTB133), ZR75-1 (CRL1500). The ATCC numbers are indicated parenthetically. The MCF7 line was obtained from the Michigan Cancer Foundation (Detroit, MD). 21T series (21PT, 21NT, 21MT2, and 21MT1) were established in this laboratory (4). All mammary epithelial cells were maintained in DFCI-1 complete medium (4) or in α -MEM supplemented with insulin, hydrocortisone, EGF, and 10% FCS (Hyclone Laboratories Inc., Logan, UT) (3). All cells were cultured at 37°C in a humidified atmosphere with 6.5% CO₂/93.5% air.

Exponentially growing cells were treated in separate experiments with the following drugs: dBc-cAMP, 1 mM in double distilled (dd) H₂O; PMA, 100 ng/ml in DMSO; all-trans retinoic acid, 1 μ M in absolute ethanol (experiment performed in the dark); Actinomycin D, 5 μ g/ml in dd H₂O; cycloheximide, 10 μ g/ml in dd H₂O; β -estradiol, 2 nM in absolute ethanol (all the above from Sigma Chemical Co., St. Louis, MO); TGF β , 1 ng/ml in dd H₂O (from Collaborative Research Inc., Bedford, MA); and okadaic acid (Tanaka Biochemical Co., Japan), 5 ng/ml in dimethyl formamide. Controls were treated with solvent alone for the times indicated in the text. Total RNA was extracted as indicated.

DNA and RNA Analysis

High molecular weight genomic DNA was isolated from mammary epithelial cells as previously described (14). DNA (10 μ g) was digested with restriction enzymes (BamHI or BglII), and then subjected to Southern analysis (14). Total RNA was extracted from exponentially growing cells. For Northern analysis, total RNA (20 μ g/lane) was denatured, electrophoresed through a 1.0–1.2% agarose-formaldehyde gels, transferred to nylon-membrane (Zeta-probe, BioRad, Richmond, CA), and hybridized as previously described (14). All probes were labeled to a specific activity of 10⁹ cpm/ μ g DNA. Poly(A)⁺ RNA was purified by oligo(dT) cellulose chromatography. cDNA was synthesized with the Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions (14). The probe 36B4 was used as a loading control (11).

Isolation of Human Cx26 (Cx26) cDNA

Human Cx26 cDNA was isolated by subtractive hybridization, using cDNA from normal mammary epithelial cells (NMEC), strain 76N, and total mRNA from the tumor-derived mammary epithelial cell line, 21MT2 (TMEC) (13, 14). To isolate sequences which are only expressed in the normal cells, a subtracted cDNA probe from 76N cells recovered after two rounds of subtraction was labeled by random priming, and used to screen a 76N cDNA library made in λ ZapII (Stratagene Inc., La Jolla, CA). ~60,000 recombinant phage (2–4 \times 10³ per 150-mm dish) were transferred in duplicate to nitrocellulose filters (Schleicher and Schuell Inc., Keene, NH), and the DNA on filters was immobilized by baking at 80°C for 1 h. The filters were prehybridized for 12 h or more at 42°C in 5 \times SSC,

5 \times Denhart's solution, 1% SDS, 10 μ g/ml of poly(A)⁺, and 100 μ g/ml salmon sperm DNA. One set of filters was hybridized with the ³²P random labeled subtracted probe and the other with a TMEC specific cDNA probe. After 24 h at 42°C, the filters were washed sequentially at low and high stringency and after radiography plaques that showed positive hybridization only with subtracted probe, they were purified and subjected to secondary and tertiary screening. Duplicate filters were hybridized with a 76N cDNA probe and with a TMEC cDNA probe. 50 clones were recovered, and further analyzed to confirm the differential expression pattern on Northern blots. Inserts from positive recombinants were amplified by polymerase chain reaction (PCR) directly from phage lysates using T3 and T7 sequences as primers. Differentially hybridizing clones were transferred into plasmids using the phagemid excision protocol (Stratagene Inc.). The full-length coding sequence of human Cx26 was isolated by rescreening the 76N library using a partial cDNA insert.

Sequence Analysis

Progressive unidirectional deletions of the human Cx26 full-length cDNA clone were generated using exonuclease III (Erase-A-Base System; Promega Corp., Madison, WI). The original cDNA clone and the deleted derivatives were sequenced by the dideoxy termination sequencing method (25) with T7 DNA polymerase (Pharmacia Fine Chemicals, Piscataway, NJ) or Sequenase (version 2.0; US Biochemical Co., Cleveland, OH). The sequence was identified as closely related to rat Cx26 in GenBank.

Immunofluorescence

Confluent cells grown on coverslips were treated as described (18). Rabbit polyclonal anti-sera against rat Cx26 (33) and rat Cx43 (20) were used for immunostaining.

Cell Communication Assays

Intercellular dye transfer was studied by iontophoresing the fluorescent dye Lucifer yellow CH (Sigma Chemical Co.) into cells and following the dye movement into surrounding cells with epiillumination fluorescence optics. The specimen stage was warmed to 37°C and a stream of 95% O₂/5% CO₂ was directed over the specimen throughout the experiment. High resistance microelectrodes (80–120 Mohm) were filled from tip to shoulder with 4% Lucifer yellow in water, then back filled with 3 M LiCl. After cell penetration, dye was iontophoresed with 0.5 s, 5 na pulses at 1 Hz. 15 separate cells of each type were injected. Injections were performed for 6 min and then immediately photographed.

Scrape loading was performed for rough measurement of cell communication according to a procedure development by El-Fouly et al. (8). The cells were grown to confluence in 35-mm plates, washed three times with serum-free medium, incubated for 1 min with 40 μ l of 1% Lucifer yellow-CH and 1% Rhodamine dextran (Molecular Probes, Inc., Eugene, OR). The cells were scratched at 2–3-mm intervals, using a fine needle, washed three times with serum free medium, observed, and photographed on a Zeiss-Axioscope using the appropriate filters (Carl Zeiss Inc., Thornwood, NY).

Cell Synchronization of NMECs

Cells were plated in 24-well dishes and in 150-mm plates at 6.7 \times 10³ cells/cm². For all conditions the volume of media used was 1.5 ml/well of Zywel dishes and 20 ml/150 mm plates. 36–48 h after plating, cells were washed and incubated with fresh medium containing lovastatin (15 μ M) for 24 h. Under these conditions, cells are arrested in G₁ as determined by [³H] thymidine incorporation and positive staining of cells with Ki-67 antibody (11). At time zero cells were re-fed with fresh medium containing mevalonic acid (2.0 mM) and harvested at the indicated times (0–33 h); DNA synthesis and cell density were measured.

Results

Isolation of a cDNA Clone Encoding the Human Gap Junction Protein Cx26 by Subtractive Hybridization

Subtractive hybridization was carried out to select for genes expressed in NMECs (76N) but not in mammary carcinoma cells (21MT-2). Briefly, single stranded cDNA from the nor-

GATTTAATCCTATGACAACTAAGTT -175

GGTTCTGCTTTCACCTGTTTTGGTGGAGTTGTGTAAGAGTTGGTGTGCTCAGGAAGAGATTTAAGCATGCTTGCTTACCCAGACT -88

CAGAGAAGTCTCCCTGTTCTGCTAGCTAGTGTATTCCTGTGTTGTGTCATTGCTCTTTCCAGAGCAACCCGCCAGACTAGAAG -1

ATG GAT TGG GGC ACG CTG CAG ACG ATC CTG GGG GGT GTG AAC AAA CAC TCC ACC AGC ATT GGA AAG 66

1 M D W G T L Q T I L G G V N K H S T S I G K

S

ATC TGG CTC ACC GTC CTC TTC ATT TTT CGC ATT ATG ATC CTC GTT GTG GCT GCA AAG GAG GTG TGG 132

23 I W L T V L F I F R I M I L V V A A K E V W

GGA GAT GAG CAG GCC GAC TTT GTC TGC AAC ACC CTG CAG CCA GGC TGC AAG AAC GTG TGC TAC GAT 198

45 G D E Q A D F V C N T L Q P G C K N V C Y D

CAC TAC TTC CCC ATC TCC CAC ATC CGG CTA TGG GCC CTG CAG CTG ATC TTC GTG TCC AGC CCA GCG 264

67 H Y F P I S H I R L W A L Q L I F V S S P A

M T

CTC CTA GTG GCC ATG CAC GTG GCC TAC CGG AGA CAT GAG AAG AAG AGG AAG TTC ATC AAG GGG GAG 330

89 L L V A M H V A Y R R H E K K R K F I K G E

M

ATA AAG AGT GAA TTT AAG GAC ATC GAG GAG ATC AAA ACC CAG AAG GTC CGC ATC GAA GGC TCC CTG 396

111 I K S E F K D I E E I K T Q K V R I E G S L

N

TGG TGG ACC TAC ACA AGC AGC ATC TTC TTC CGG GTC ATC TTC GAA GCC GCC TTC ATG TAC GTC TTC 462

133 W W T Y T S S I F F R V I F E A A F M Y V F

T V

TAT GTC ATG TAC GAC GGC TTC TCC ATG CAG CGG CTG GTG AAG TGC AAC GCC TGG CCT TGT CCC AAC 528

155 Y V M Y D G F S M Q R L V K C N A G T W P C P N

I N F

ACT GTG GAC TGC TTT GTG TCC CGG CCC GAG AAG ACT GTC ACA ATG TTC ATG ATT GCA GTG 594

177 T V D C F V S R P T E K T V F T V F M I A V

I S

TCT GGA ATT TGC ATC CTG AAT GTC ACT GAA TTG TGT TAT TTG CTA ATT AGA TAT TGT TCT GGG 660

199 S G I C I L L N V T E L C Y L L I R Y C S G

I F

AAG TCA AAA AAG CCA GTT TAA 681

221 K S K K P V -

R

CGCATGGCCAGTTGTTAGATTAAGAAATAGACAGCATGAGAGGGATGAGGCAACCCGTCCTCAGCTGTCAAGGCTCAGTCGCCAGC 768

ATTGCCAACACAAGATTCTGACCTTAAATGCAACCAATTTGAAACCCCTGAGGCCCTCAGGTGAACTCCAGATGCCACAATGAGC 855

TCTGCTCCCTAAAGCCCTCAAACAAGGCCAATTTCTATGCGCTGCTTAAATTTTCTTCACTTAAAGTTAGTTCCACTGAGACCCCA 942

GGCTGTAGGGGTTATGGTGAAGTACTTTTCATATTTTAAACAGAGGATATCGGCATTTGTTCTTCTCTGAGGACAAGAGAAA 1029

AAAGCCAGGTTCCACAGAGACACAGAGAAGGTTGGGTGCTCCTCGGGGTTCTTTTGGCAACTTCCCCACGTTAAAGGTGAAC 1116

ATTGGTTCTTTCATTTGCTTTGGAAGTTTAAATCTCTAACAGTGGACAAAGTTACCAGTGCCTTAAACTCTGTTACACTTTTGGAA 1203

GTGAAACTTTGATGATGATAGGTTATTTGATGTAAGAGATGTTCTGGATACCATTATATGTTCCCTGTTTCAGAGGCTCAGAT 1290

TGTAATATGTAATGGTATGTCATTGCTACTATGATTTAATTTGAAATATGGTCTTTTGGTTATGAATACTTTCAGCACAGCTGA 1377

GAGAGCTGCTGTTGTTATTCATTGTTGGTCATAGCACCTAACCAACATTTAGTGCCTCAATCGAGTGAGACAGACTAGAAGTCTCTAGT 1464

TGGCTTATGATAGCAAAATGGCCTCATGTCAAATATAGATGTAATTTTGGTAAGAAATACAGACTGGATGTACCACCACTACTAC 1551

CTGTAATGACAGGCTGTCCAACACATCTCCCTTTTCCATGCTGGTAGCCAGCATCGGAAAGAACGGTGATTTAAGAGGTGAGC 1638

TTGGAAATTTTATGACACAGTACCTTTAATGTTGGGAGACAAAATGTTGGGCCCAGGGGAGGAGAAGTTCTGTGCTTAAACAGAG 1725

TTTGGAAAGACTGGACTCTAAATCTGTTGATTTAAGATGAGCTTTGTCTACCTTCAAAGTTTGTGTTGCTTACCCCTCAGCCCT 1812

CAATTTTTTAAAGTAAATAAATAAATGTAAGAAAGTAAAGTAAAGTAAAGTAAAGTAAAGTAAAGTAAAGTAAAGTAAAGTAAAG 1899

ATTGAACCTGAATATGCCATTTATGCTTGACATGGTTTCCAAAAATGGTACTCCACATACTTCAGTGAGGTAAGTATTTTCTGCT 1986

TGTCAGAATAGCATTGTAAGCAATTTTGTAAATATAAAGAAATAGCTTTAATGATATGCTTGAACATAAAATAATTTGTAATGTA 2073

TCAATATACATTTAACAATTAATAATAATCTCTATAAT 2112

Figure 1. Nucleotide and deduced amino acid sequence for human Cx26 cDNA. Numbers to the left and right of the sequence show the positions of amino acids and nucleotides, respectively. A consensus splice acceptor signal is underlined at 23 nucleotides upstream from the ATP translation start site. In the 3' non-coding region polyadenylation signal sequences AATAAA and ATTAATA are underlined and the putative instability sequence ATTTA is also underlined. The amino acid differences in the rat sequence are shown below the human sequence. These sequence data are available from EMBL/GenBank/ DDBJ under accession number M86849.

mal cells (76N) was hybridized with a large excess of tumor mRNA and the nonhybridizing pool of 76N cDNAs was recovered as the subtracted probe. Further screening of this pool by differential hybridization yielded 15 clones that hybridized uniquely with the subtracted 76N library. These clones were partially sequenced, and one of them (called clone 1-3) was found to have high homology with the rat Cx26 sequence reported in GENBANK (29). Using this clone as probe, ~60,000 plaques of the total 76N library were screened, and among the clones recovered, one contained the full-length Cx26 cDNA.

Sequence Analysis of Human Connexin 26

The full-length clone was sequenced on both strands. Comparison of its peptide sequence to the GENBANK data base revealed close homology to rat gap junction protein connexin 26 (33). The nucleotide and deduced amino acid sequence of the coding region is shown in Fig. 1. This cDNA clone has a single long open reading frame that extends to a stop codon at base 881, and encodes a putative protein of 226

amino acid residues with a predicted molecular mass of ~26 kD.

Preceding the initiator ATG, 23 nucleotides upstream from ATG, is a consensus splice acceptor signal (TTTCCAG). This signal sequence is not present in the 5' region of the rat Cx26 sequence (33) which does not produce two transcripts. The 3' untranslated region contains a possible polyadenylation signal sequence AATAAA positioned 87 nucleotide upstream from the poly(A)⁺ tail. A putative instability sequence, ATTTA, which may be involved in mRNA instability, is present at nucleotide positions 1326, 1623, 1664, and 2082 (26).

Sequence Comparison of Human Cx26 to Rat Cx26

The overall nucleotide homology between human and rat Cx26 is 86.2% within the open reading frame. The amino acid sequence deduced from human is 92.5% identical to rat Cx26. Of the 226 amino acids, there are only 15-amino acid differences between the two species. These substitutions are indicated in Fig. 1. Structural characteristics are conserved.

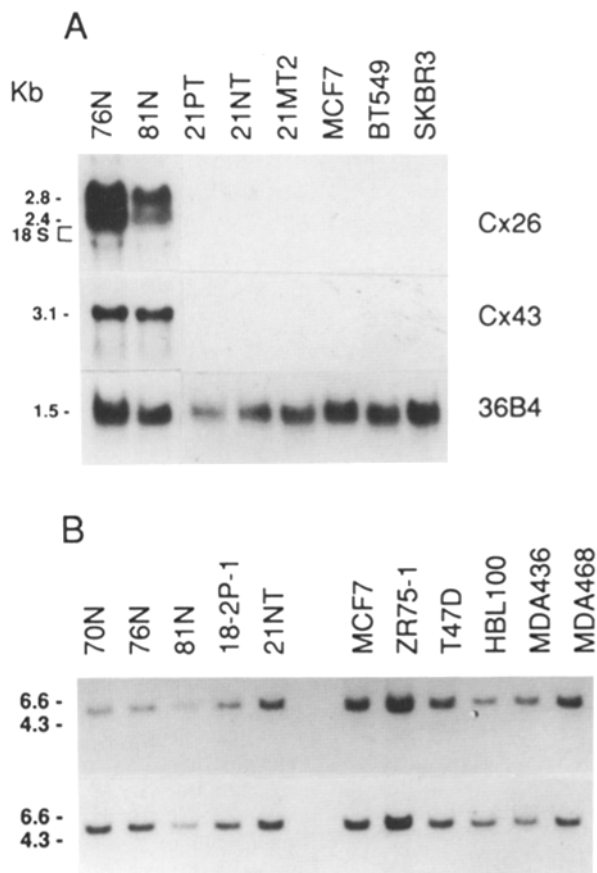


Figure 2. Characterization of human Cx26 in NMECs and TMECs. (A) Northern blot analysis. P^{32} -labeled cDNAs from human Cx26, rat Cx43, and 36B4 (loading control) were hybridized with total RNAs extracted from exponentially growing cells: NMEC, 76N and 81N; TMEC, 21PT, 21NT, 21MT2, MCF7, BT549, and SKBR3. Transcript sizes and position of 18S RNA are indicated. (B). Southern analysis of human Cx26 genomic DNA. Genomic DNA was isolated, digested with restriction enzymes (BamHI in upper line and BglI in lower line), separated by 0.8% agarose gel electrophoresis, and transferred to a nylon membrane and hybridized with human Cx26 cDNA. 70N, 76N, and 81N, NMEC; 18-2P-1, HPV immortalized mammary epithelial cells; 21NT, MCF7, ZR75-1, T47D, HBL100, MDA-MB-436, and MDA-MB-468, TMEC. Position and size of λ HindIII fragments are shown on the left.

In addition, the potential consensus sites for kinases are also conserved in human Cx26 peptides (Tyr⁹⁷ and Tyr²¹⁷ for tyrosine kinase, Ser²¹⁹ for Ca²⁺-dependent kinase) (33). However, the 5' and 3' untranslated regions show no significant similarity between human and rat.

Expression of Cx26 and Cx43 in Human Normal and Tumor Mammary Epithelial Cells

Cx26 cDNA was examined for mRNA expression in a series of NMEC and TMEC. Northern blot analysis revealed the expression of two major mRNA transcripts, 2.4 and 2.8 kb, in the NMEC but not in the TMEC series (Fig. 2 A). Transcript sizes were determined by reference to ribosomal RNAs as seen by ethidium bromide staining. The weak lowest band is a distortion of the gel by the 18S ribosomal RNA. A weak upper band may represent incompletely processed mRNA. It is not seen consistently in other gels.

The two major bands were abundantly expressed in normal cells 76N and 81N (as well as 70N and 94N not shown), and very weakly in primary TMECs 21NT and 21PT. In contrast, no expression was seen in any of the tested TMECs shown in Fig. 2 A (i.e., 21 MT-2, MCF7, BT549, SKBR3 or in 21MT-1, BT-20, MDA-MB-157, -231, -435, -436, -468, T47D, or ZR-75-1 [not shown]).

The possibility that a different gap junction protein might be expressed in human breast cells was examined by Northern blot analysis using the rat cDNA probes for Cx26 (33), Cx32 (22), and Cx43 (5, 22) (Fig. 2 A). Cx43 was expressed only in the normal cells in parallel with Cx26. Cx32 was not expressed by any of these normal or tumor cells (data not shown). After the cloning of new connexins (10, 23, 31), we examined the expression of Cx31.1, Cx33, Cx37, Cx40, and Cx46 in several normal and tumor lines but no expression was seen in a long exposure at moderate stringency of DNA-RNA hybridization.

To examine the possibility of a deletion or gross rearrangement in the human Cx26 gene of the tumor cells, genomic DNA from normal and tumor cells was digested with restriction enzymes (BamHI, BglI) and subjected to Southern blot analysis using a full-length human Cx26 cDNA probe (Fig. 2 B). After hybridization, a single band (\sim 5.7 kb from BamHI, \sim 4.8 kb from BglI) was detected in all digests, indicating that the Cx26 gene is present in both normal and tumor cells without detectable gross rearrangement. Thus, loss of connexin mRNA in tumor cells seems to result primarily from transcriptional regulation or mRNA instability. The presence of a single band in Southern blots suggests that the two mRNA transcripts are derived from the same gene, and may result from alternate splicing, leading to insertion or deletion of additional sequences or to differences in polyadenylation. Studies of mRNA stability in normal cells with actinomycin D and cycloheximide, presented below, indicate that the upper transcript is quite stable, whereas the lower transcript decreases within 30 min.

Immunolocalization of Gap Junction Proteins Cx26 and Cx43

The intracellular localization of the gap junction proteins was examined by indirect immunofluorescence using anti-peptide antibodies specific for either Cx26 (33) or Cx43 (12). The NMEC line 76N, displayed regions of punctate fluorescence as well as diffuse intracellular staining for both Cx26 and Cx43 (Fig. 3, D and H, respectively). The labeling patterns for Cx26 and Cx43 were similar. Comparison of phase-contrast and fluorescence images suggested that the punctate labeling often corresponded to regions of cell-cell contact (Cx26, Fig. 3, C and D; Cx43, Fig. 3, G and H). Pre-immune controls did not display punctate labeling but indicated that some of the diffuse intracellular labeling may be nonspecific (Fig. 3, B and F). No specific labeling for either connexin was observed in 21MT2 cells (data not shown), consistent with the lack of connexin mRNA expression in this tumor line (Fig. 2 A).

Junctional Communication of NMECs and TMECs

Dye transfer was used to detect the presence of functional gap junctions. A series of micro-iontophoretic injections of Lucifer yellow were performed in the normal cell line 81N

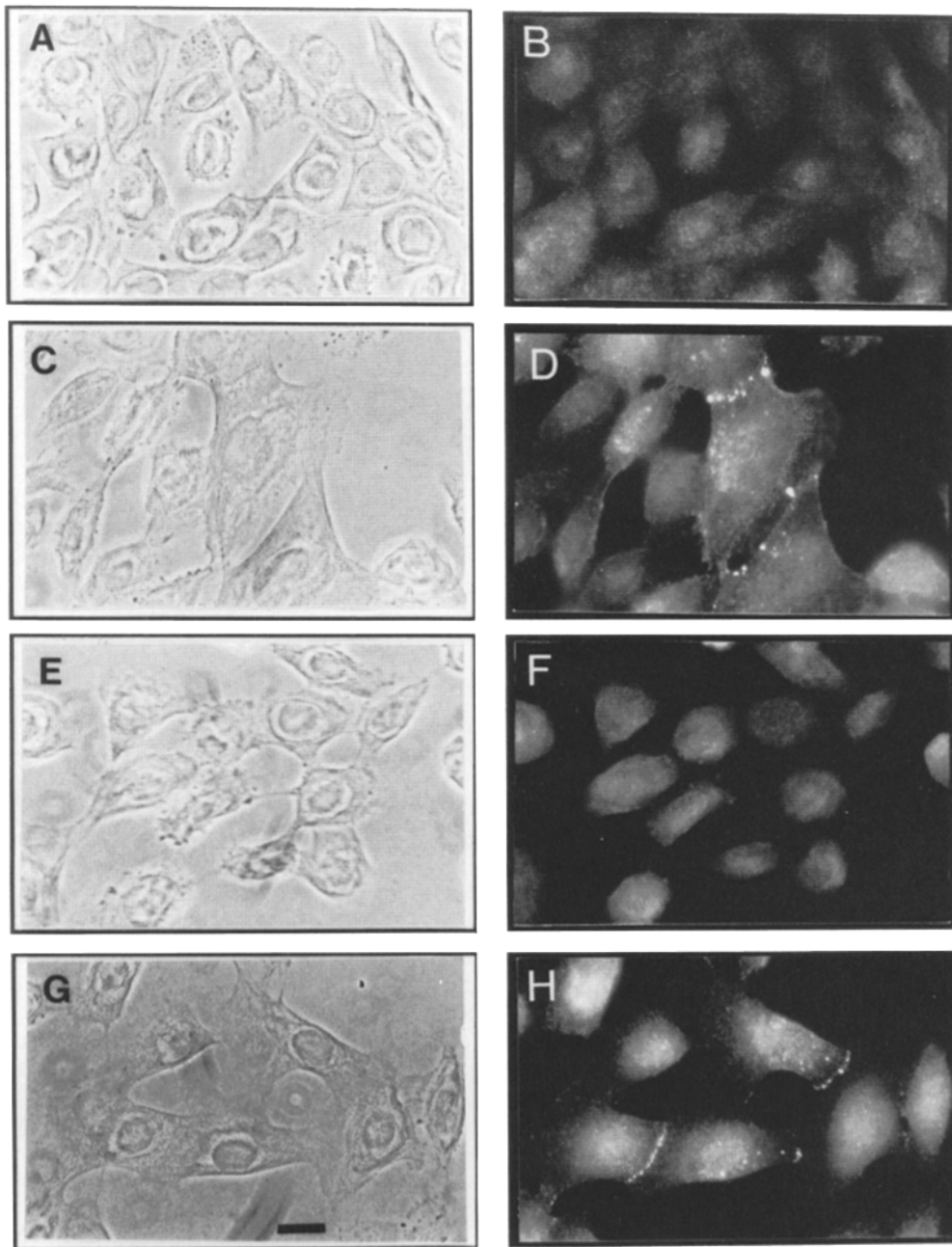


Figure 3. Immunolocalization of gap-junction proteins (Cx26, Cx43) in human mammary epithelial cells (76N) by indirect immunofluorescence. Cells were fixed in paraformaldehyde and reacted with anti-Cx26 or Cx43. (A and B) 76N cells incubated with pre-immune serum; (C and D) 76N cells incubated with anti-Cx26 antibody; (E and F) 76N cells incubated with pre-immune serum; (G and H) anti-Cx43 antibody staining. A, C, E, and G are phase-contrast micrographs of 76N cells, and B, D, F, and H are indirect immunofluorescence images. Cx26 and Cx43 specific staining was found at cell-cell interface junction areas in normal human mammary epithelial cells. Bar, 10 μ m.

(Fig. 4, A and B) and the metastatic tumor line 21 MT-2 (Fig. 4, C and D). Injected cells are marked with an asterisk. In the normal cells, dye could always be detected in at least third order neighboring cells. The tumor line was never observed to pass dye. Thus, the presence of Cx26 and 43 mRNAs correlated well with the presence of gap junctional communication.

Stability of Cx26 and Cx43 mRNAs after Actinomycin D and Cycloheximide Treatment

To look for differences in mRNA stability of the two Cx26 transcripts and the Cx43 transcript, 76N cells were exposed to actinomycin D to block further transcription. Samples of total RNA, prepared at various times (0–12 h) after addition of the drug, were analyzed on a Northern blot (Fig. 5). Differential regulation of mRNA levels was observed for the

two Cx26 transcripts. Although the 2.8-kb upper transcript was slightly decreased upon actinomycin D treatment, the level of lower transcript (2.4 kb) was rapidly reduced with a half-life of <30 min. Thus, the upper band showed persistence of preexisting message. Rehybridizing of the blots with Cx43 cDNA showed that Cx43 mRNA remained stable over the 12-h period (data not shown).

Treatment of 76N cells with cycloheximide for 1–24 h also revealed differences in regulation of the two Cx26 transcripts. The upper band was stable for 12 h and then decreased, whereas the lower band decreased during the 12-h period and was gone at 24 h. No detectable effect of cycloheximide could be seen on Cx43 mRNA over the 12-h period. These results demonstrate relative stability of the upper Cx26 and Cx43 transcripts in contrast to the lower Cx26 transcript in NMECs. In TMECs, however, neither ac-

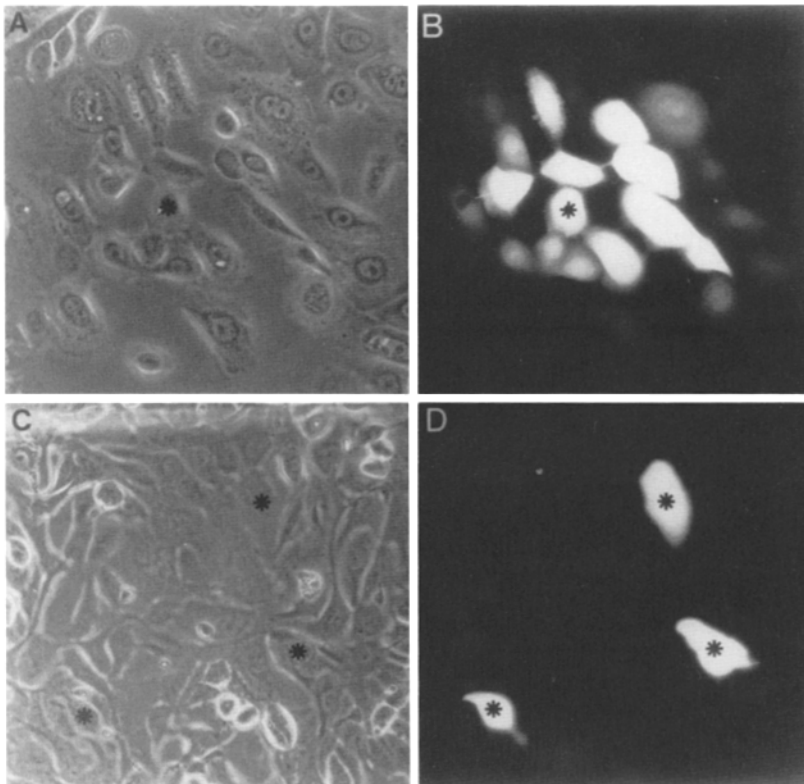


Figure 4. Dye transfer. Normal mammary epithelia but not mammary tumor lines produce functional gap junctions that allow dye transfer. (A and B) Phase-contrast and corresponding fluorescence micrographs of 81N normal mammary epithelial cells. The injected cell is marked with an asterisk. Rapid and extensive transfer of dye is observed. (C and D) Phase-contrast and corresponding fluorescence micrograph of 21 MT-2 tumor cells. Three cells were separately injected. No transfer of dye is detected under these conditions.

tinomycin D nor cycloheximide induced expression of either Cx26 or Cx43 mRNAs (data not shown).

Cell Cycle Expression of Cx26 and Cx43 in Synchronized Cells

To assess the relative periodicity of connexin gene expression during the cell cycle, NMECs were synchronized in G₁ by lovastatin (15 μ M/24 h), released from lovastatin-induced arrest by the addition of 2 mM mevalonate (11). Cells were sampled at 3-h intervals over the next 33 h and Cx26 and Cx43 transcript levels were analyzed by Northern blot analysis. (Fig. 6 A). Progression of the cells through the cell cycle was monitored by [³H]thymidine incorporation and by the level of histone H4 mRNA in Northern blot analysis. The appearance of histone H4 message coincided with the 18-h

peak of DNA synthesis as measured by [³H]thymidine incorporation (Fig. 6 B).

In these cells the cycle time was 27 h and the G₁/S boundary was \sim 12 h after addition of mevalonate, as shown by [³H]thymidine incorporation. Both Cx26 transcripts increased about twofold at 6 h, and remained relatively constant until 21 h when another 2.5-fold increase in the upper transcript and 3.5-fold increase in the lower transcript occurred. What is most significant is that both transcripts showed a sharp rise in late S phase. In contrast to Cx26 mRNA, Cx43 expression during the cell cycle was relatively invariant (Fig. 6 A). Thus Cx26 and Cx43 mRNAs are differentially regulated in the normal mammary epithelial cell cycle.

Phorbol Ester (PMA) Stimulation of Cx26 mRNA Expression in Human Mammary Tumor Cells

The possibility was examined that expression of Cx26 or Cx43 might be induced in tumor cells by drugs that affect signal transduction pathways. Accordingly, normal and tumor cells were treated with PMA, retinoic acid, dB-cAMP, forskolin, okadaic acid, or TGF β . Of these drugs, only PMA was effective in inducing Cx26 mRNA expression in tumor cells. As shown in Fig. 7 A, there was induction of both Cx26 transcripts in the treated cells. The level of both transcripts increased at 3 h and reached its peak at 6 h. In contrast, neither PMA nor any of the other treatments induced Cx43 mRNA expression in these tumor cells. We also examined the effect of PMA in several TMEC lines over a treatment period of 3–12 h. Exposure of tumor cells (21PT, ZR75-1, and MDA-MB-231) to PMA induced the expression of Cx26 mRNA (Fig. 7 B), but not the expression of Cx43 (data not shown).

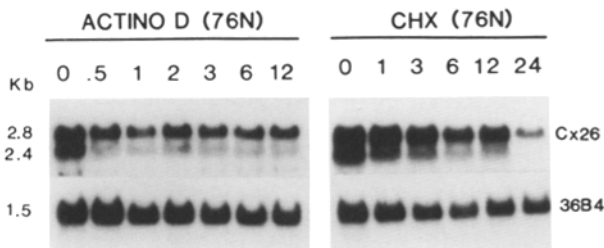


Figure 5. Effect of actinomycin D and cycloheximide on transcription of Cx26 in 76N. Cells were treated in the presence and absence of actinomycin D (5 μ g/ml) for 12 h or cycloheximide (10 μ g/ml) for 24 h. Total RNA was prepared from cells sampled at times indicated, and 20 μ g samples were analyzed by Northern blot hybridization with P³²-labeled human Cx26 and 36B4 (loading control) probes.

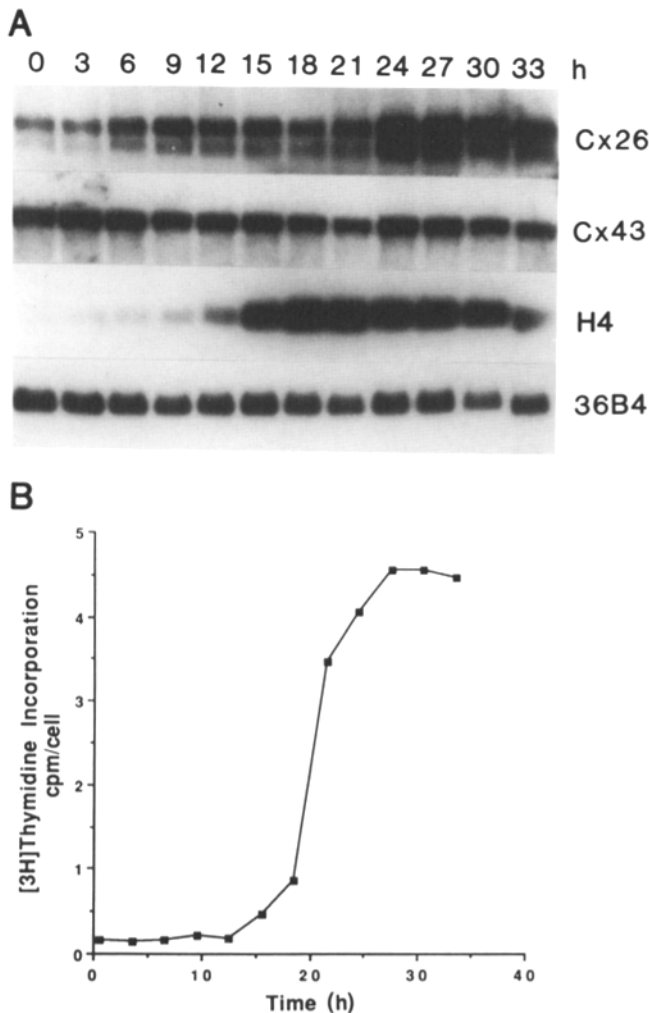


Figure 6. Cx26 and Cx43 mRNAs during cell cycle progression. (A) Analysis of Cx26 and Cx43 transcripts in normal mammary epithelial cells (70N) undergoing synchronous cell cycle progression after release in early G₁ from lovastatin-induced arrest. Total RNA was extracted at indicated times (0–33 h), and 20 µg/lane was separated on 1% formaldehyde-agarose gels and analyzed by Northern blots using Cx26, Cx43, histone H4, and 36B4 (loading control) as probes. (B) Rate of DNA synthesis measured by [³H]thymidine incorporation. Relative rates were estimated by measuring the incorporation of [³H]thymidine during cell progression of a synchronous population.

The effect of PMA on connexin gene expression was also examined in NMECs (81N). Unlike tumor cells, the expression of Cx26 mRNA was decreased by PMA at 6 h whereas longer exposures resulted in subsequent upregulation to pretreatment levels by 24 h. However, the expression of Cx43 mRNA in the same experiment was gradually diminished to barely detectable levels by 24 h (data not shown).

To see whether Cx26 mRNA stimulation in PMA-treated tumor cells leads to protein synthesis, immunofluorescence staining with anti-Cx26 antibody and scrape-loading dye transfer experiments were performed at various times after PMA treatment, using several TMEC lines. Cx26 proteins were not detected at cell–cell junctional areas nor was junctional communication detected between cells (data not shown). Neither method was sensitive enough to detect a very weak signal.

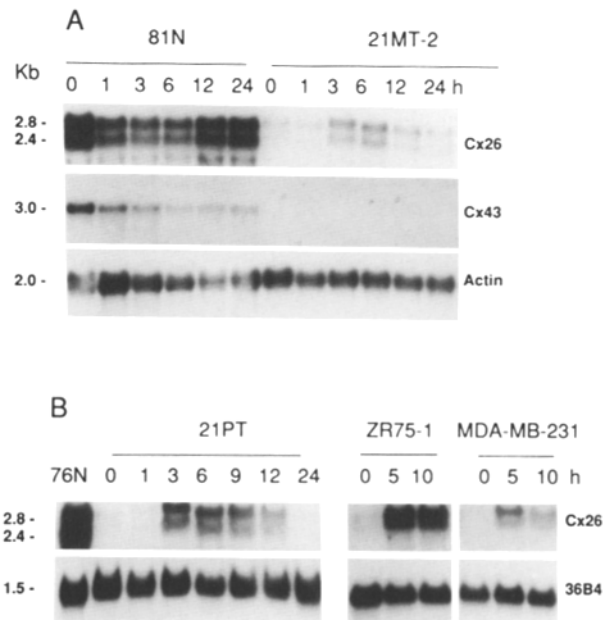


Figure 7. Effect of PMA on expression of Cx26 and Cx43 mRNAs in normal and tumor mammary epithelial cells. (A) 81N and 21MT2 cells were treated with 100 ng/ml of PMA for the indicated times, and total RNA was isolated and analyzed by Northern hybridization (20 µg/lane total RNA). The same blot was hybridized successively with ³²P-labeled Cx26, Cx43, and β-actin probes. (B) Expression of Cx26 mRNA in PMA-treated (100 ng/ml) tumor cells. At each indicated time, RNA was extracted, and analyzed by Northern blot hybridization (20 µg total RNA/lane). The same blot was hybridized with 36B4 as a loading control.

Discussion

This paper has shown that normal human mammary epithelial cells express two connexin genes, Cx26 and Cx43. Neither transcript is found in mammary tumor cell lines, although a trace is present in two lines of recent origin from a primary tumor. The normal cells express both genes at the mRNA and protein levels, and show abundant gap junctional communication as judged by dye transfer using Lucifer yellow. The two genes are differentially regulated at the RNA level in the cell cycle and in response to actinomycin D, cycloheximide, and PMA.

Of particular importance, we have shown that the loss of expression in tumor cells does not involve physical loss of the genes, but is primarily at the level of transcription or mRNA stabilization. The evidence for transcriptional regulation is: (a) the Cx26 and Cx43 genes appear unaltered in restriction fragment analysis of DNA from tumor cells; (b) connexin mRNAs are faintly expressed in primary tumor cells but not in metastatic lines; (c) Cx26 (upper band) and Cx43 mRNAs are stable in the presence of actinomycin D, indicating that these transcripts do not undergo rapid degradation in normal cells; and (d) Cx26 mRNA expression can be induced in tumor cells by treatment with PMA.

The human Cx26 cDNA described here for the first time, is closely related to rat Cx26 at both the DNA and protein levels. The six cysteine residues found in all rat connexins so far described (10) are also conserved in human Cx26. In all, there are 15 amino acid substitutions between human and rat Cx26 coding regions; none are significant for structure as currently understood.

Are Connexin Genes a Class of Tumor-Suppressor Genes?

The hypothesis that gap-junctional communication contributes to growth control and the corollary that loss of junctions in tumor cells contributes to their malignant potential, has been the subject of experimentation and speculation since it was formulated in the 1960's (1, 7, 9, 15, 17-19, 27, 29, 32, 34). The experimental evidence, based largely on growth inhibition studies with rodent fibroblasts, has been supportive of the hypothesis, but very indirect. Recently, transfection of C6 glioma cells with Cx43 (34) and of the human hepatoma cell line SKHepl with Cx32 (7) have provided preliminary evidence of decreased growth of transfectants in culture (34) or in nude mice (7). In other experiments, transfection of weakly communicating mouse fibroblasts with Cx43 on a retroviral vector led to strong overexpression of Cx43 mRNA, and to enhancement of cell-cell communication and decreased growth (19). All of these studies support the hypothesis that gap-junctional communication can play a role in growth control, and therefore that connexins may have tumor suppressor functions. The importance of this mode of growth control in human cancer remains to be shown. At this time genes encoding connexins remain potential tumor-suppressor genes.

We have previously divided tumor suppressor genes into two classes: Class I, in which the gene in itself is altered at the DNA level by loss or mutation; and Class II, in which the gene is not lost, but rather downregulated (13). On this scheme, the connexin genes described here would be of Class II (13), as is the CaN19 gene encoding a calcium-binding protein which we have recently described (14).

Regulation of Connexin Expression

Cx26 and Cx43 are differentially regulated as shown by their cell cycle expression, and by their responses to actinomycin D, cycloheximide, and PMA. To examine cell cycle regulation, normal cells (70N) were synchronized by lovastatin (11). Cx43 mRNA remained at a constant steady-state level throughout the cell cycle, whereas the Cx26 transcripts increased in G₁ and again near the end of S phase, suggesting their participation in events of G₂ and M. These results contrast with the increase in Cx32 mRNA and protein that occurs after proliferation of hepatocytes induced by partial hepatectomy (20).

Gap junctions may play an important role in preparation for mitosis, and for junctional activity in the new daughter cells. The fact that the two Cx26 transcripts show different stabilities, suggests that their functions may be different. Further studies are required to clarify the differences in sequence and regulation of the two transcripts. In addition, the constitutive expression of Cx43 in the cell cycle needs to be understood. We have shown that either Cx26 or Cx43 cDNAs will restore cell-cell communication when transfected into tumor cells (Tomasetto, C., and R. Sager, unpublished observations), showing that although both are present in normal epithelial cells, both are not required in the cell culture environment. It seems likely, therefore, that the two connexins have separate functions within their *in vivo* settings. Further support for this view is based on sequence differences between the Cx26 and Cx43 proteins. Cx43 channels are regulated posttranscriptionally by phosphorylation of a site not present in Cx26 protein (20, 21, 28, 33).

The transcriptional effects of actinomycin D and cycloheximide were examined to determine mRNA stability in NMECs, and look for induction of connexin expression in TMECs. Cycloheximide did not superinduce either Cx26 or Cx43, indicating that protein synthesis was required for their transcription. The relative stability of the upper Cx26 transcript was seen in the presence of cycloheximide and during actinomycin D treatment. In contrast the lower Cx26 band was inhibited by both drugs. The Cx43 transcript was stable in the presence of either actinomycin D or cycloheximide, consistent with its stability in the cell cycle studies. Neither drug induced connexin expression in tumor cells.

Of particular interest, we found that the tumor promoter PMA induced Cx26 expression in tumor cells. PMA is a well known inducer of protein kinase C, elevating PKC mRNA transiently, but leading to its downregulation within 24 h. Here we have found that in normal cells (81N) the Cx26 mRNA was strongly decreased within an hour, and began to recover at 12-24 h, thus showing an inverse response to that reported for PKC expression. In the tumor cells, however, the Cx26 mRNA increased in the period of 3-12 h and then decreased.

Thus the effects of PMA on Cx26 mRNA show a similar time course as PKC expression, transiently depressed in normal cells and elevated in tumor cells. These results suggest that Cx26 mRNA expression is regulated by the PKC signal-transduction pathway and that contrary to its inhibitory effect on Cx26 and Cx43 expression in normal cells, it may be readily inducible in tumor cells by agents that act downstream of PKC.

Concluding Remarks

The results presented in this paper contribute to a research theme introduced some 25 years ago (15, 27): a search for evidence that direct cell-cell communication is a homeostatic mechanism operating in normal cells and lost in tumor cells. Recent studies have put the connexin genes and proteins on a firm molecular basis, but have not yet established their functional contribution to the regulation of tumor growth. The fact that Cx26 expression is cell-cycle regulated may be important in contributing to growth control.

This paper describes the differential transcriptional regulation of connexin expression in normal and tumor-derived human mammary epithelial cells. We show that the absence of gap junctions in tumor cells is the result of reversible downregulation of Cx26 mRNA and that its re-expression can be induced by treatment of cells with PMA. Although the stimulation is transient, it suggests that a more permanent effect may be achieved with a different agent. The differences we have found in regulation and stability of Cx26 and Cx43 transcripts provide important leads towards understanding the regulation of gap junctional expression. Thus, the fact that the connexin genes are downregulated in tumor cells rather than irreversibly mutated, opens new opportunities for research in the context of remedial chemotherapy.

Analysis of the mechanisms of transcriptional and post-transcriptional regulation should contribute to developing ways to regulate expression exogenously, and towards understanding the detailed functions of gap junctions in proliferating and in quiescent conditions.

We thank Mark Neveu for editorial advice and Susan Harper for preparing the manuscript.

C. Tomasetto is a recipient of French Institut National de la Sante et de la Recherche Medicale fellowship. This work was supported by National Institutes of Health Grant CA39814 to R. Sager.

Received for publication 24 February 1992 and in revised form 1 June 1992.

References

1. Azarnia, R., S. Reddy, T. E. Kmiecik, D. Shalloway, and W. R. Loewenstein. 1988. The cellular *src* gene product regulates junctional cell-to-cell communication. *Science (Wash. DC)*. 239:398-401.
2. Band, V., and R. Sager. 1989. Distinctive traits of normal and tumor-derived human mammary epithelial cells expressed in a medium that supports long-term growth of both cell types. *Proc. Natl. Acad. Sci. USA*. 86:1249-1253.
3. Band, V., D. Zajchowski, G. Stenman, C. C. Morton, V. Kulesa, J. Connolly, and R. Sager. 1989. A newly established metastatic breast tumor cell line with integrated amplified copies of *erbB2* and double minute chromosomes. *Genes Chromosomes & Cancer*. 1:48-58.
4. Band, V., D. Zajchowski, K. Swisshelm, D. Trask, V. Kulesa, C. Cohen, J. Connolly, and R. Sager. 1990. Tumor progression in four mammary epithelial cell lines derived from the same patient. *Cancer Res*. 50:7351-7357.
5. Beyer, E. C., J. Kistler, D. L. Paul, D. A. Goodenough. 1988. Antipeptide antisera directed against Connexin 43 react with a 43-kD protein localized to gap junctions in myocardium and other tissues. *J. Cell Biol*. 108:595-605.
6. Davis, M. M., D. I. Cohen, E. A. Nielsen, M. Steinmetz, W. Paul, and L. Hood. 1984. Cell-type-specific cDNA probes and the murine I region. *Proc. Natl. Acad. Sci. USA*. 81:2194-2198.
7. Eghbali, B., J. A. Kessler, L. M. Reid, C. Roy, and D. C. Spray. 1991. Involvement of gap junctions in tumorigenesis: transfection of tumor cells with connexin 32 cDNA retards growth in vivo. *Proc. Natl. Acad. Sci. USA*. 88:10701-10705.
8. El-Fouly, M. H., J. E. Trosko, and C. C. Chang. 1987. Scrape-loading and dye transfer: a rapid and simple technique to study gap junctional intercellular communication. *Exp. Cell Res*. 168:422-430.
9. Fentiman, I. S., J. Hurst, R. L. Ceriani, and J. Taylor-Papadimitriou. 1979. Junctional intercellular communication pattern of cultured human breast cancer cells. *Cancer Res*. 39:4739-4743.
10. Haefliger, J.-A., R. Bruzzone, N. A. Jenkins, D. J. Gilbert, N. G. Copeland, and D. L. Paul. 1992. Four novel members of the connexin family of gap junction proteins: molecular cloning, expression and chromosome mapping. *J. Biol. Chem.* 267:2057-2064.
11. Keyomarsi, K., L. Sandoval, V. Band, and A. B. Pardee. 1991. Synchronization of tumor and normal cells and G1 to multiple cell cycles by lovastatin. *Cancer Res*. 51:3602-3609.
12. Kumar, N. M., and N. B. Gilula. 1986. Cloning and characterization of human liver cDNAs coding for a gap junction protein. *J. Cell Biol*. 103:767-776.
13. Lee, S. W., C. Tomasetto, and R. Sager. 1991. Positive selection of candidate tumor suppressor genes by subtractive hybridization. *Proc. Natl. Acad. Sci. USA*. 88:2825-2829.
14. Lee, S. W., C. Tomasetto, K. Swisshelm, K. Keyomarsi, and R. Sager. 1992. Down-regulation of a new member of the S100 gene family in mammary carcinoma cells and reexpression by azadeoxycytidine treatment. *Proc. Natl. Acad. Sci. USA*. 89:2504-2508.
15. Loewenstein, W. R. 1979. Junctional intercellular communication and the control of growth. *Biochem. Biophys. Acta*. 560:1-65.
16. Marshall, C. J. 1991. Tumor suppressor genes. *Cell*. 64:313-326.
17. Mehta, P. P., and W. R. Loewenstein. 1991. Differential regulation of communication by retinoic acid in homologous and heterologous junctions between normal and transformed cells. *J. Cell Biol*. 113:371-379.
18. Mehta, P. P., J. S. Bertram, and W. R. Loewenstein. 1986. Growth inhibition of transformed cells correlates with their junctional communication with normal cells. *Cell*. 44:187-196.
19. Mehta, P. P., A. Hotz-Wagenblatt, B. Rose, D. Shalloway, W. R. Loewenstein. 1991. Incorporation of the gene for a cell-cell channel protein into transformed cells leads to normalization of growth. *J. Membr. Biol*. 124:207-225.
20. Musil, L. S., and D. A. Goodenough. 1990. Gap junctional intercellular communication and the regulation of connexin expression and function. *Cur. Opin. Cell Biol*. 2:875-880.
21. Musil, L. S., and D. A. Goodenough. 1991. Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap-junctional plaques. *J. Cell Biol*. 115:1357-1374.
22. Paul, D. L. 1986. Molecular cloning of cDNA for rat liver gap junction protein. *J. Cell Biol*. 103:123-134.
23. Paul, D. L., L. Ebihara, L. J. Takemoto, K. I. Swenson, and D. A. Goodenough. 1991. Connexin46, a novel lens gap junction protein, induces voltage-gated currents in non-junctional plasma membrane of *Xenopus* oocytes. *J. Cell Biol*. 115:1077-1089.
24. Sager, Ruth. 1989. Tumor suppressor genes: the puzzle and the promise. *Science (Wash. DC)*. 246:1406-1412.
25. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*. 74:5463-5467.
26. Shaw, G., and R. Kamen. 1986. A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell*. 46:659-667.
27. Stoker, M. G. P. 1967. Transfer of growth inhibition between normal and virus-transformed cells: autoradiographic studies using marked cells. *J. Cell Sci*. 2:293-304.
28. Swenson, K. I., H. Piwnica-Worms, H. McNamee, and D. L. Paul. 1990. Tyrosine phosphorylation of the gap junction protein connexin43 is required for the pp60^{src}-induced inhibition of communication. *Cell Regul*. 1:989-1002.
29. Trosko, J. E., C. C. Chang, and B. V. Madhukar. 1990a. Symposium: cell communication in normal and uncontrolled growth. *Radiat. Res*. 123:241-251.
30. Weinberg, R. A. 1991. Tumor suppressor genes. *Science (Wash. DC)*. 254:1138-1146.
31. Willecke, K., R. Heynkes, E. Dahl, R. Stutenkemper, H. Hennemann, S. Jungbluth, T. Suchyna, and B. J. Nicholson. 1991. Mouse connexin37: cloning and function expression of a gap junction gene highly expressed in lung. *J. Cell Biol*. 114:1049-1057.
32. Yamasaki, H. 1990. Gap junctional intercellular communication and carcinogenesis. *Carcinogenesis (Eynsham)*. 11:1051-1058.
33. Zhang, J.-T., and B. J. Nicholson. 1989. Sequence and tissue distribution of a second protein of hepatic gap junctions, CX26, as deduced from its cDNA. *J. Cell Biol*. 109:3391-3401.
34. Zhu, D., S. Caveney, G. M. Kidder, and C. C. G. Naus. 1991. Transfection of C6 glioma cells with connexin 43 cDNA: analysis of expression, intercellular coupling, and cell proliferation. *Proc. Natl. Acad. Sci. USA*. 88:1883-1887.