Transcription of the Gene for the Gap Junctional Protein Connexin43 and Expression of Functional Cell-to-Cell Channels are Regulated by cAMP

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Submitted December 20, 1991; Accepted June 9, 1992

We investigated the mechanism by which cyclic AMP (cAMP) induces gap junctional communication via cell-to-cell channels in a communication-deficient rat Morris hepatoma cell line. We found that under basal conditions, the cells transcribe *cx43* at a low level but do not transcribe *cx26* or *cx32*. Elevation of intracellular cAMP, which induced communication, increased *cx43* mRNA 15- to 40-fold and the rate of *cx43* transcription 6-fold. Cx43 protein was detected by immunostaining in junctions of only those cells in which communication had been induced. We found the regulation by cAMP also in other cell lines; namely, in those with a low basal level of *cx43* mRNA.

INTRODUCTION

The cells in most tissues are interconnected by channels in their membrane junctions (*gap junctions*) that provide a direct intercellular pathway for molecules up to 1500–2000 kDa (Flagg-Newton *et al.*, 1979; Schwartzmann *et al.*, 1981). Evidence is mounting that these cell–cell channels transmit growth controlling signals and thus are instrumental in the maintenance of growth control in normal cells (see Loewenstein and Rose, 1992). Many transformed cells exhibit deficiencies in this form of intercellular communication (for a review, see Loewenstein, 1979). Such deficiencies may have various causes; for instance, the cell–cell channels may be missing because the channel protein is not expressed or cannot be properly assembled into (functional) channels, or the channels may be present but are kept closed by a condition prevailing in the transformed cells. The cloning in recent years of several genes coding for cell–cell channel proteins (connexins; see Beyer *et al.*, 1990) has made it possible to begin to investigate, at the molecular level, the causes underlying the communication deficiency in transformed cells. With the available connexin (*cx*) cDNAs, one can ask whether *cx* gene transcription is altered in the transformed cells, and with

antibodies against connexin proteins, one can assess whether the connexin protein is synthesized and is inserted into the junctional membrane.

Here we study the Morris hepatoma H5123 cells, a transformed rat cell line that ordinarily has no intercellular communication (Borek *et al.*, 1969). We show that communication can be induced in these cells by elevation of intracellular cyclic AMP (cAMP). cAMP has been shown to increase intercellular communication in normal cultured cell lines as well as in a communication-deficient transformed cell line, and this communication increase was associated with an increase in gap junctional area (Azarnia *et al.*, 1981; Flagg-Newton *et al.*, 1981). The mechanism of this action of cAMP has not been clear, although the time course of cAMP action on communication level and the enlarged gap junction area pointed to de novo formation of channels, perhaps de novo synthesis of connexin protein(s). The Morris hepatoma H5123 cells, which in basal condition lack communication altogether, provided an ideal system to investigate the basis for their communication incompetence, as well as the mechanism by which cAMP induces communication in these cells. Specifically, we investigate whether connexins are expressed at all in basal condition and whether cAMP induces functional cell–cell channels by stimulating transcription of one or more *cx* genes. We examine the action of cAMP also in a number of other transformed and normal cells.

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METHODS

Materials

All culture media were from GIBCO BRL (Life Technologies, Gaithersburg, MD); calf and fetal bovine serum were from Hyclone Lab (Logan, UT); tissue culture plasticware was from Nunc (Roskilde, Denmark); Seakem GTG agarose was from FMC Bio Products (Rockland, ME); guanidinium isothiocyanate, phenol (molecular biology grade), yeast tRNA, Poly(A), poly(C), and salmon sperm DNA were from Boehringer Mannheim Biochemicals (Indianapolis, IN); ultrapure formamide was from Clontech Laboratories (Palo Alto, CA); and Lucifer Yellow CH was from Molecular Probes (Eugene, OR). Rabbit serum containing polyclonal antibody against amino acid 252–271 of rat heart connexin43 was a gift from Dr. Joan Brugge (Ariad Pharmaceuticals) and phosphodiesterase inhibitor Ro-20-1724 was a gift from Dr. P. Sorter, Hoffman-LaRoche (Nutley, NJ). Rhodamine- or fluorescein isothiocyanate-labeled goat antirabbit IgG, forskolin, and dideoxy forskolin were from Boehringer Mannheim; all other reagents (Molecular Biology grade or highest purity) were from Sigma (St. Louis, MO). GeneScreen Plus nylon membranes, ^{32}P UTP, and [α - ^{32}P]dCTP were from New England Nuclear DuPont (Wilmington, DE); restriction enzymes were from Boehringer Mannheim or from New England Biolabs (Beverly, MA).

Cells

We used the following cell lines: Morris hepatoma H-5123 (Borek *et al.*, 1969); C3H10T1/2 clone 8; methylcholanthrene transformed 10T1/2, MCA-4D (Reznikoff *et al.*, 1973; Mehta *et al.*, 1986); simian virus 40-transformed SV40C98-10T1/2 cells (Sompayrac and Danna, 1983; Mehta *et al.*, 1986); 3T3 A31 (CCL 163, American Type Culture Collection, Rockville, MD); *v-mos* transformed 3T3 MA31 (Croy and Pardee, 1983); *v-src* transformed SRD 3T3 (Rose *et al.*, 1986); rat C-6 glioma (CCL 107, American Type Culture Collection); two rat liver-derived cell lines, WBF 344 (Tsao *et al.*, 1984) and RL clone 9 (CRL 1439, American Type Culture Collection).

Cell Culture

All cells except SRD 3T3 and WBF344 were grown at 37°C in basal minimal essential medium supplemented with 5% serum—a mixture of 2.5% calf and 2.5% fetal bovine serum—and 50 $\mu\text{g}/\text{ml}$ penicillin, streptomycin, and kanamycin in an atmosphere of 5% $\text{CO}_2/95\%$ air. SRD 3T3 were grown in Dulbecco's modified Eagle's medium and WBF344 in Richter's medium with 10% fetal bovine serum.

Stock cultures were maintained in 10 ml medium in 10-cm dishes and passaged weekly at 10^5 cells/dish, with a medium change at 3- or 4-day intervals.

Treatments

Cells were treated when near-confluent by replacing their medium with fresh medium containing the relevant drug at the desired concentration. Forskolin, dideoxyforskolin, dbcAMP, 8-Br-cAMP, and Ro-20-1724 were added from stock solutions in dimethyl sulfoxide (DMSO), with final DMSO not exceeding 0.4%, a concentration that did not affect any of the parameters we measured. For controls, cultures received fresh medium with 0.2–0.4% DMSO added.

DNA Probe Synthesis

For synthesis of radiolabeled DNA probes, the relevant DNA fragments were excised from the plasmids and gel purified and electroeluted twice. One hundred to 200 ng of the fragment were then nick-translated with [α - ^{32}P]dCTP to a specific activity of 10^8 – 10^9 cpm/ μg DNA. The ^{32}P -labeled probes were precipitated twice with 2.5 M ammonium acetate and ethanol to eliminate free nucleotides and used at 5–10 ng/ml hybridization buffer.

Northern Blot Analysis

Total RNA of cultured cells was extracted from one 15-cm or two 10-cm dishes of freshly confluent cells by the acid-phenol guanidinium isothiocyanate procedure (Chomezynski and Sacchi, 1987) (absorbance ratio at 260/280 nm was 1.8–2.0), and RNA integrity was checked on minigels. From rat liver and heart, total RNA was isolated using the guanidinium isothiocyanate procedure either by repeated precipitations with ethanol or by spinning at 35 000 rpm at 20°C for 16 h (Chirgwin *et al.*, 1979). For Northern blots, total RNA was electrophoresed in 1% agarose/formaldehyde gels (Lehrach *et al.*, 1977) in 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 10 mM sodium acetate, 1 mM EDTA, pH 7.0, at 2 V/cm for 12–16 h at room temperature and transferred by capillary action onto GeneScreen Plus Nylon membranes in $12.5\times$ SSC ($1\times$ SSC: 0.015 M NaCl, 0.015 M trisodiumcitrate, pH 7.4) for 24–48 h. Blots were prehybridized for ≥ 1 h (hybridization buffer: $5\times$ SSC, 0.01 M sodium phosphate, pH 6.8, 0.2% sodium dodecyl sulfate (SDS), $1\times$ Denhardt's reagent, 50 $\mu\text{g}/\text{ml}$ yeast tRNA, 2.5 $\mu\text{g}/\text{ml}$ each of poly A and poly C, 100 $\mu\text{g}/\text{ml}$ sonicated salmon sperm DNA, 5–10% polyethylene glycol mol wt 8000) and hybridized with nick-translated ^{32}P -labeled DNA probes for either *cx43*, *cx32*, *cx26*, or GAPDH for 16–48 h at 44°C and washed with $0.1\times$ SSC for 1–2 h or until background radioactivity was negligible. Blots were exposed to AR or RP film (X-Omat, Eastman Kodak, Rochester, NY) with intensifying screen (Cronex Lightening Plus, DuPont) for 2 h to 2 d.

Reprobing of the blots was done by boiling them for 30 min either in distilled water containing 10 mM EDTA or in $0.1\times$ SSC containing 1 mM EDTA and 0.1% SDS to remove the probe, and then were probed as described above.

RNA Dot Blot Analysis

A known amount of total RNA (usually 20 μg), suspended in dot blot buffer (50% formamide, 2.2 M formaldehyde, 0.02% bromophenol blue, 0.02% xylene cyanol FF), was vortexed briefly, denatured for 30 min at 70°C, and applied to the Nylon membrane in the dot blot apparatus (Bio-Rad, Richmond, CA) according to manufacturer's instructions. After drying, the RNA dots were marked individually with pencil, and the blots were prehybridized and hybridized as described above and exposed to x-ray film. After exposure, the Nylon membrane was cut into equal-sized squares, each containing an RNA dot, and the radioactivity of each membrane square was counted in scintillation fluid (Ultimafold, Packard Instr., Chicago, IL).

Measurement of Transcription Rate by Nuclear Runoff

Nuclear runoff experiments were done essentially as described by Marzuluff and Huang (1984), with some modifications. Cells were treated with 30 μM forskolin in such a manner as to permit simultaneous harvesting of cells for all treatment time points (six 15-cm dishes per time point). Cells were scraped in cold phosphate-buffered saline (PBS) and pelleted at $500\times g$ for 5 min at 4°C. Pellets were vortexed gently for ~ 10 s and resuspended in 5 ml cold buffer I (10 mM tris (hydroxymethyl) aminomethane [Tris]HCl, pH 7.4, 10 mM NaCl, 3 mM CaCl_2 , 2 mM magnesium acetate, 0.1 mM EDTA, 1 mM dithiothreitol [DTT], 0.5% NP40) by gentle vortexing. Cells were lysed on ice for 5 min and then spun at $500\times g$ at 4°C to pellet the nuclei. The supernatant was saved for RNA isolation. Nuclei were resuspended in cold buffer II (25% glycerol, 5 mM magnesium acetate, 0.1 mM EDTA, 5 mM DTT, 50 mM TrisHCl, pH 7.6). The number of nuclei (flow cytometer counts) was adjusted to 2×10^8 nuclei/ml and immediately processed for in vitro transcription.

Transcription was initiated by addition of 300 μl nuclei-suspension in buffer II (6×10^7 nuclei) to 300 μl $2\times$ nuclear transcription buffer ($1\times$: 2 mM each of ATP, GTP, CTP; 0.15 M KCl, 10 mM magnesium acetate, 0.1 mM S-adenosylmethionine) and 15 μl α - ^{32}P -UTP (760 mCi/mmol; final radioactivity, 10 mCi/ml) in 30-ml tubes. Transcription was allowed to proceed for 45 min at room temperature after

which time it was terminated by addition of 4 ml 5 M guanidinium isothiocyanate (brought to pH 4.0 with 0.4 ml 2 M Na acetate). The tubes were vortexed to lyse the nuclei, and the labeled RNA was extracted as for Northern blots. Precipitated RNA was dissolved in 100 μ l sterile H₂O containing 0.1% SDS and 1 mM EDTA. The trichloroacetic acid (TCA) precipitable counts ranged 2.1×10^7 to 3×10^7 counts per minute (CPM) per sample (i.e., per 6×10^7 nuclei).

For hybridization with the labeled RNA, 10 μ g of the appropriate linearized and denatured plasmid DNA, in $2.5\times$ SSC containing 0.02% bromophenol blue and 0.02% xylene cyanol FF, was spotted onto GeneScreen Plus membrane in Bio-Rad's dot blot apparatus, and the spots were marked after drying. Each dot was hybridized with an equal count (2×10^7 cpm) of TCA-precipitable ³²P-labeled nuclear RNA at 44°C for 48 h in buffer consisting of 0.25 M sodium phosphate, pH 7.2, 0.25 M NaCl, 7% SDS, 1 mM EDTA, 50% formamide, 10% polyethylene glycol (8000 mol wt). The membranes were washed, exposed to x-ray film, and processed for radioactivity counts of each dot as described above for dot blot analysis.

Immunostaining

For immunostaining, cells were seeded on coverslips; at barely confluent state they were fixed at -20°C for 20 min in 50:50 acetone:methanol and 5% glacial acetic acid, washed in PBS (pH 7.2), incubated in blocking solution (PBS containing 10% Carnation nonfat dry milk or 1% goat serum) at 4°C for 1 h, incubated in PBS/1% goat serum containing polyclonal (rabbit) antibody raised against a peptide representing amino acids 252–271 of rat heart connexin 43 (affinity-purified with the peptide bound to MAC 25 membrane filters, Amicon, Danvers, MA) either for 6–16 h at 4°C or 1–2 h at room temperature, washed three times with PBS, incubated with rhodamine-labeled goat anti-rabbit IgG (1:100 dilution) for 1 h, and washed three times with PBS. Alternatively, cells were immunostained with or without Triton-X 100 extraction, following the protocol of Musil and Goodenough (1991). To ascertain specificity of the antibody for *cx43* protein, we also immunostained RL cells, which have abundant *cx43*-containing junctions (see Fig. 8), with staining solution containing 25 μ g/ml of the peptide in addition to the primary antibody. No immunostaining was seen in those cases. Immunostained cells were immersed in 50% glycerin/50% PBS and viewed on a fluorescence microscope (Diaphot, Nikon, Garden City, NY) with a 100 $\times$ oil immersion objective. Images were captured on an optical disk recorder (Panasonic model TQ-2026F, Panasonic, Garden City, NJ) with an SIT66 (Dage MTL, Michigan City, IN) videocamera and reproduced on a videoprinter (Hitachi, Woodbury, NY).

Electrical Measurements

For measurement of electrical coupling between cells, two contiguous cells were impaled with microelectrodes (≥ 100 M Ω ; 0.5 M K₂SO₄) connected to WPI bridge amplifiers with 100 M Ω resistance compensation. Current pulses of 2–4 nA, 100-ms duration, were applied alternately to the two cells at 200-ms intervals. The voltage and current traces from both cells were displayed on a storage oscilloscope (≤ 1 mV or 0.1 nA resolution). Cells were considered electrically coupled when the voltage trace in one cell showed a deflection of ≥ 2 mV in response to current pulsed into the other cell. Membrane potentials ranged -5 to -50 mV.

Junctional Transfer of Lucifer Yellow

Cells with four to seven direct neighbors were injected with Lucifer Yellow (lithium salt, Molecular Probes; 5% aqueous solution) by sharp (≤ 1 s) pneumatic pressure pulses. Cells were viewed through a Nikon Diaphot fluorescence microscope (at 430 nm excitation and 520 nm emission) with the aid of a low light level TV camera (Dage 66 SIT) at fixed gain, displayed on a TV monitor together with a continuously running digital clock, and recorded on a videocassette recorder or optical memory disk recorder. The number of cells fluorescent (excluding the injected one) was noted at 60 s and again at 6 min after

injection. Cells with at least one fluorescent neighbor at 6 min were considered transfer or communication competent, and we tabulated or plotted the percent injected cells that showed junctional transfer after 6 min. Trials where the fluorescence of the injected cell had faded considerably without junctional transfer to other cells having occurred were considered as having damaged the injected cell and were not counted. So were trials in which fluorescence spread immediately and to equilibration to only one cell neighbor—such injections were considered to have been done into a recently mitotic cell still connected to its daughter cell by a cytoplasmic bridge. The incidence of such injections was $\leq 1\%$ in untreated cultures that had the highest number of mitotic figures.

RESULTS

Forskolin Induces Cell–Cell Communication

Morris hepatoma cells grown under normal culture conditions showed no cell–cell communication as assayed by measurement of electrical coupling or by cell-to-cell transfer of microinjected Lucifer Yellow, confirming earlier reports of lack of communication between these cells (Borek *et al.*, 1969; Azarnia and Loewenstein, 1971). After addition to the medium of forskolin, an agent that raises intracellular cAMP by activating adenylate cyclase, electrically coupled cells could be detected in a small fraction of the cells within 3.5 h. By 8 h, this fraction had risen to 50% (Table 1). Concomitant with the appearance of electrical coupling, some of the cells also began to transfer the 443 Da fluorescent dye Lucifer Yellow (Figure 1). Junctional transfer, as indexed by the fraction of injected cells which transferred Lucifer Yellow, rose steadily, reaching half-maximum at ~ 6 h and a peak of 60–80% at ~ 12 –15 h of continuous forskolin treatment. The transfer declined eventually but was still detectable at 48 h of forskolin treatment (Figure 2). That decline ensued regardless of the presence of forskolin in the medium; it could not be prevented by reapplication of forskolin.

Table 1. Appearance of electrical coupling in Morris hepatoma cells after treatment with forskolin^a

Treatment time (h)	Percent electrically coupled cells	n
0	0	20
3	0	12
3.5	21	24
4	15	13
5	7	14
6	31	16
7	45	20
8	50	30

^a Cells were considered electrically coupled when a 2–5 nA, 250 ms current pulse administered to one cell caused a voltage change of ≥ 2 mV in the neighbor cell. Voltage resolution was 1 mV or better. Resting potentials ranged -5 to -50 mV. Subconfluent cultures; forskolin, 40 μ M.

Figure 1. Induction of junctional transfer by forskolin. Brightfield (left) and fluorescence images (right) of Morris hepatoma cells. (a) Control (treated with 0.02% DMSO); (b) treated 14 h with 40 μ M forskolin. The fluorescence images were taken 6 min after Lucifer Yellow injection into cells marked by asterisks

Junctional transfer was assessed here 6 min after injection of Lucifer Yellow. We found that with this interval, the Lucifer Yellow injection technique was about as sensitive in detecting communication-competent cells as was the measurement of electrical coupling; electrically coupled and Lucifer Yellow transferring cells appeared at about the same time of forskolin treatment, and their respective fractions rose with a similar time course (Figure 2).

The preceding effects of forskolin are attributable to the well-known action of forskolin on stimulating adenylate cyclase. When we used the analogue dideoxyforskolin that does not stimulate the cyclase, no induction of communication was detected (Table 2, D).

Dibutyryl cAMP (db-cAMP) also induced communication but was much less effective than forskolin, even in the presence of a phosphodiesterase inhibitor, Ro-20-1724 (Table 2).

Elevation of cx43-Transcript Level by Forskolin, db-cAMP, and 8-Br-cAMP

The enhancement of junctional communication developed over several hours, hinting that cAMP here might induce functional channels by *de novo* synthesis of connexin(s), possibly by activating the transcription of a *connexin* gene. We analyzed total RNA from forskolin-treated and from untreated confluent cultures of Morris hepatoma cells by Northern blot for the presence of mRNA for three rat connexins: *cx26*, *cx32*, and *cx43*. The *cx43* probe hybridized with a band at ~ 3 kilobase, the size expected for *cx43* (Beyer *et al.*, 1987). This band was usually barely detectable in RNA blots of untreated cells (Figure 3A, lanes a and b). After cell treatment with forskolin, the band's intensity increased manyfold. The increase was detectable already at 0.5 h, the shortest

treatment time tested (Figure 3A, lane c) and peaked at ~ 4 –6 h. With longer treatments, the *cx43* transcript level declined progressively (lanes i–k), approaching control level at 16 h.

The dose response to forskolin is shown in the Northern blot of Figure 3B for which RNA was extracted from cells after 1 h and after 3 h of treatment with forskolin, in concentrations ranging from 1 to 100 μ M. Maximum response was seen at 5–10 μ M (lanes d and k and l).

Also shown in the blot of Figure 3B is the *cx43* transcript level of a normal rat liver cell line, RL Clone 9 (lane g). These cells have a very high level of intercellular communication (junctional transfer is 100% even when scored as early as 10 s after Lucifer Yellow injection, with all first-order and additional second-order neighbors of the injected cell fluorescent at that time) (see also Yada *et al.*, 1985). The level of *cx43* transcript in these cells far exceeds that of even the maximally forskolin-stimulated Morris hepatoma cells (compare lane g with lanes k–n). The *cx43* transcript levels in these two cell lines seem to reflect the cells' relative junctional permeabilities; the more *cx43* mRNA, the higher the junctional permeability and, as will be seen later on,

Figure 2. Time course of induction of junctional transfer by forskolin. Plotted are the mean percentages of Lucifer Yellow-injected cells that showed transfer of Lucifer Yellow to at least one cell neighbor 6 min after injection (Junctional Transfer, %; ●). Each data point represents the mean from an individual culture dish, where 10–17 cells were injected during a 30-min period. (Fifteen experiments, where each experiment comprised several treatment durations.) The data point at time zero represents the results from >10 dishes; in no case was transfer observed. Forskolin, 40 μ M, was applied at time zero. For a time course comparison, the percent electrically coupled cells (data from Table 1) are also plotted (Δ).

Table 2. Effect of dibutyryl cAMP, forskolin, and dideoxyforskolin on junctional transfer of Lucifer Yellow^a

Experiment	Junctional transfer				
	Duration (h)	db-cAMP, 1 mM	db-cAMP + Ro ² , 1 mM + 50 μ M	Forskolin, 40 μ M	Dideoxyforskolin, 40 μ M
A	5	11 (9)	—	25 (12)	—
	15	—	42 (12)	—	—
	18	0 (9)	17 (12)	60 (10)	—
B	7	0 (21)	0 (19)	23 (26)	—
C	26	0 (9)	20 (10)	58 (12)	—
D	6	—	—	20 (10)	0 (8)
	17	—	—	52 (29)	0 (27)
	19	—	—	61 (27)	0 (32)

^a Percent injected cells that had ≥ 1 fluorescent neighbor(s) 6 min after Lucifer Yellow injection. Number of injection trials in parentheses. Where $n \geq 12$, data were pooled from two dishes. Data within experiments A–D were from dishes treated in parallel.

the more anti-cx43 immunostaining at the cell junctions (see Fig. 8).

Other agents that act to elevate intracellular cAMP, namely phosphodiesterase inhibitor Ro-20-1724, db-cAMP, and 8-Br-cAMP, also elevated cx43 mRNA (compare lanes with equivalent GAPD signals, namely g and h, j and k, and m–p in Figure 4 for forskolin, Ro-20-1724, and db-cAMP, respectively; see Figure 5 for 8-Br-cAMP).

In probeds for transcripts of the other two connexins, no bands were detected either in untreated or in treated cultures (Figure 4). And this was so also when poly(A) RNA instead of total RNA was examined in Northern blots. The cells thus express cx43 at a relatively low level but do not express either cx26 or cx32; and only the cx43 transcript level is elevated by cAMP.

Quantitative dot blot analysis (Figure 5) of cx43 mRNA of cAMP-stimulated and control cells revealed that cx43 mRNA was increased ~ 25 -fold after 4–6 h forskolin treatment. db-cAMP and 8-Br-cAMP were even more effective, increasing cx43 mRNA 30- to 40-fold. Phosphodiesterase inhibitor heightened the response considerably when applied in conjunction with the other agents; this increase was more than additive, indicating a synergistic action. The synergistic action was more pronounced at the 6-h than at 4-h treatments. No increase in cx43 transcript was seen with dideoxy-forskolin treatment.

Forskolin Increases Rate of cx43 Transcription

Higher mRNA levels could be the result of increased rate of transcription or of increased stability of the message or both. We assayed for rate of transcription by nuclear runoff experiments (see METHODS). Within 1 h of forskolin treatment, the transcription rate for cx43 had increased sixfold. This high rate was maintained for ≥ 3 h and then declined within 12 h to near the

control rate (Figure 6), roughly reflecting the observed time course of mRNA increase. The transcription rate of GAPD, a constitutively expressed protein known not to be under the control of cAMP and serving here as control, did not change significantly. We also probed for cx32 mRNA in this experiment; the counts remained essentially at background level.

Protein Synthesis is not Required for the Forskolin-Induced cx43 mRNA Elevation

One mechanism by which cAMP elevation might induce cx43 transcription could be via induction of the expression of transcription-regulatory protein(s). In that event, protein synthesis would be required for the cAMP effect on cx43 transcription. If, however, cAMP induces cx43 transcription via an already existing regulatory protein, say, by phosphorylation of such a protein, it would do so in the absence of protein synthesis. This is exactly what we found. The cx43 transcript level of cells exposed simultaneously to forskolin and to the protein synthesis inhibitor cycloheximide was not reduced compared with that of cells exposed to forskolin alone (Figure 7A; compare lanes i, m, and q with lanes h, l, and p). In fact, cycloheximide enhanced the transcript level, both in combination with forskolin and by itself (compare lanes g, k, and o with lanes b, f, and j). Such an effect of mRNA elevation by cycloheximide has been reported before and is thought to result from an increase in mRNA stability (Cochran *et al.*, 1983). A 2-h pretreatment with cycloheximide also did not reduce the forskolin effect. At the concentration of cycloheximide used (60 μ g/ml), protein synthesis was reduced to 5% as determined by ³⁵S-methionine labeling (3 h) in the absence or presence of cycloheximide, of TCA-precipitable counts.

Effect of Forskolin is Long-Lasting

The cx43 transcript stayed elevated several hours after washout of forskolin. This was seen in experiments of

Figure 3. Forskolin increases *cx43* mRNA. Northern blots of total RNA extracted from Morris hepatoma cells that had been treated for various durations with 30 μ M forskolin (A) or with various concentrations of forskolin (B). Blots were first probed with 32 P-labeled *cx43* cDNA and then reprobbed for GAPD. Controls in A were extracted from cells at time zero and at 20 h after treatment with 0.02% DMSO (lanes a and b in A). For a comparison, lane g in B shows RNA from rat liver clone 9 (RL CL9), a cell line that is highly communicating. Lane h contained RNA from rat liver (RL), as a control for *cx32* and *cx26* mRNA expression during reprobings of the blots for these two transcripts (not shown). Total RNA loaded in A and in lanes a–n in B was 20 μ g. Lanes o–r were loaded with sequential dilutions of the same RNA as in lane m. All Northern blots were routinely probed for GAPD, a constitutively expressed gene, whose transcript thus can serve as an indicator of RNA content of the individual lanes. Although all RNA concentrations were equalized for gel loading, pipetting variations may arise.

pulse treatments in which forskolin was applied for various periods and RNA was extracted after a further 3 or 6 h (Figure 7B). The long-lasting effect on *cx43* mRNA level most likely is not due to continued elevation of cAMP, because elevation of cAMP by forskolin is reversible within minutes after forskolin washout (Seamon and Daly, 1981). It may be due to continued protein kinase A activity and/or diminished phosphatase action (Ingebritsen and Cohen, 1983).

Immunohistochemical Localization of Connexin43 at Cell Junctions of Forskolin-Treated Cells

We immunostained Morris hepatoma cells and RL CL9 cells with polyclonal antibody against a peptide corre-

sponding to amino acids 252–271 of rat heart connexin43. RL CL9 cultures exhibited abundant staining at cell junctions (Figure 8e). This staining was absent when 25 μ g/ml of the antigen peptide was included in the antibody staining solution before addition to the cells. In untreated Morris hepatoma cultures (Figure 8a) or in Morris hepatoma controls treated with dideoxyforskolin, no immunostaining was seen at cell junctions. However, in Morris hepatoma cultures treated with forskolin, punctate immunostaining was seen at cell contact regions (Figure 8b). Some cells in untreated Morris hepatoma cultures showed diffuse cytoplasmic staining (Figure 8a, asterisk), but most cells had little or none. In contrast, most treated cells showed diffuse staining, and some had very intensely stained cytoplasmic regions (Figure 8b, asterisk). When Morris hepatoma cells were permeabilized with Triton-X 100 before fixation (Musil and Goodenough, 1991) in a procedure that is expected to remove all connexin43 not assembled into gap junctions, the punctuate staining at cell junctions of treated cells remained (Figure 8d), whereas the cytoplasmic staining was reduced. The junctional immunostaining was not seen at each cell contact region in the treated cultures. This is not surprising considering that not all cells responded to forskolin (see Figure 2).

The finding that connexin43 could be detected at Morris hepatoma cell junctions only after forskolin treatment strongly suggests that cell–cell communication

Figure 4. Phosphodiesterase inhibitor and dibutyryl cAMP also increase *cx43* mRNA. Northern blots of total RNA from Morris hepatoma cells treated 2, 4, or 8 h with phosphodiesterase inhibitor Ro-20-1724 (Ro; 10 μ M), forskolin (Fk; 30 μ M), dibutyryl cAMP (Db; 1 mM), forskolin (30 μ M) + Ro-20-1724 (10 μ M), or dibutyryl cAMP (1 mM) + Ro-20-1724 (10 μ M). Also shown are the probings of this blot for *cx32* and *cx26*. Bands are seen only in the last two lanes, which contain RNA from (whole) liver. Total RNA loaded nominally was 20 μ g in lanes a–p, 2.5 μ g in lane q, and 5 μ g in lane r. However, from the GAPD probings, it is evident that the loadings varied here considerably. In particular, lanes b and i contain essentially no RNA. For comparison of relative *cx43* mRNA levels, consider only lanes with equal GAPD signals, e.g., a and c; d–f, g and h, j–l, and m–p.

Figure 5. Quantitative dot blot analysis of the *cx43* mRNA increase. Total RNA (20 μ g) from Morris hepatoma cells treated 6 h with Ro-20-1724 (10 μ M), dideoxyforskolin (DDF; 30 μ M), forskolin (30 μ M), dibutyryl cAMP (1 mM), 8-Br-cAMP (1 mM), or Ro-20-1724 (10 μ M) in combination with the other agents was spotted in triplicate on GeneScreen Plus nylon membrane, hybridized with 32 P-labeled *cx43* probe, and exposed to x-ray film. After exposure, dots were cut from the nylon membrane and counted individually. Plotted are the means of the background-corrected counts per min per dot (3 dots/treatment; mean background, 99 ± 7 ; standard deviations were 10% or less in all cases).

by elevation of intracellular cAMP is brought about by de novo formation of cell-cell channels.

Forskolin Induces *cx43* Transcription and Communication in Cell Lines With Low Basal Level of *cx43* mRNA

Connexin43 transcription and junctional communication were found to be regulated by cAMP in a number of other cell lines (Figure 9A and Table 3), namely normal 10T1/2 cells and the transformed 10T1/2 lines MCA-4D (methylcholanthrene transformed) and C-98 (SV40 transformed); C-6 glioma, and 3T3 A31. All these responsive cell lines have a low basal level of *cx43* mRNA. Cell lines with relatively high levels of *cx43* transcript in the untreated (basal) state did not respond with an increase in *cx43* message (Figure 9B). Three of these cell lines have high junctional permeability in the basal condition and, at least in the *v-mos* transformed 3T3 MA31, cAMP does not increase it further (Table 3; other 2 cell lines not tested here). In Rous Sarcoma Virus-transformed SRD 3T3, junctional permeability is low and remains low even when treated with forskolin. This presumably is a consequence of tyrosine phosphory-

lation of the gap junctional *cx43* protein by pp60^{v-src} (Filson *et al.*, 1990; Swenson *et al.*, 1990). None of the cell lines, treated or untreated, had detectable levels of Cx26 or Cx32 mRNA.

DISCUSSION

Of the three murine connexin genes *cx43*, *cx32*, and *cx26*, only *cx43* was found to be transcribed in the transformed communication-deficient rat Morris hepatoma cells and at a level very much lower than in a normal communication-competent rat liver cell line, RL Cl9. The *cx43* protein itself was not detectable immunohistochemically in the junctions of these hepatoma cells in basal conditions, nor was communication detectable electrically or by dye transfer. Thus, whatever amount of connexin43 may be expressed, it seems not to be assembled into detectable channels.

cAMP had a powerful inducing effect on *cx43* mRNA and on communication and also resulted in immunohistochemically detectable *cx43* protein at cell junctions. The good correlation between communication competence and detectability (by immunostaining) of *cx43* protein at the Morris hepatoma cell junctions and the further correlation of abundance of such immunostaining with high degree of communication, such as in RL Cl9 cells, make it highly likely that in these cells communication is mediated by channels composed of *cx43* protein. The lack of communication in Morris hepatoma cells, therefore, seems to be due to lack of channels rather than to a condition prevailing in the cells that keeps channels closed. A similar paucity of *cx43* mRNA

Figure 6. Forskolin increases rate of transcription of *cx43* gene. Cells were treated with 30 μ M forskolin, and at various times of treatment, cell nuclei were isolated. RNA was synthesized in vitro by the isolated nuclei in the presence of α - 32 P UTP. Equal counts of labeled RNA were hybridized with the linearized plasmid DNAs for *cx43*, *cx32*, or GAPD that had been spotted onto Genescreen Plus nylon membranes. After exposure to x-ray film, dots were excised from the membrane and their radioactivity was counted and plotted against cell treatment duration. The stippled lines indicate the range of background counts.

Figure 7. Protein synthesis and continued presence of forskolin are not required for the increase in *cx43* mRNA. (A) Cycloheximide treatment. Northern blots of total RNA (20 μ g/lane) of Morris hepatoma cells treated for the durations indicated with 0.3% DMSO (DM); 60 μ g/ml cycloheximide (CHX); 30 μ M forskolin (FK); or with both 30 μ M forskolin and 60 μ g/ml cycloheximide (FK + C). Note that cycloheximide by itself raises *cx43* mRNA (compare lanes g, k, and o with lanes f and j) and strengthens the effect of forskolin (compare lanes e, i, m, and q with d, h, l, and p). Lane r was loaded with 10 μ g total RNA from rat heart (R-HRT). (B) Pulse treatment with forskolin. Morris hepatoma cells were treated with 30 μ M forskolin for 0, 20, 40, 60, or 180 min as indicated, washed thrice, and total RNA was harvested after a further 3 or 6 h incubation in normal medium. Forty μ g RNA/lane.

and lack of immunostaining has been observed in transformed communication-deficient mouse L929 L-cells (Musil *et al.*, 1990).

The onset of cAMP-induced communication in Morris hepatoma cells was preceded by a sixfold increase in the rate of transcription of *cx43* and by a 15- to 40-fold higher *cx43* mRNA level. This elevation of *cx43* transcript occurred also in the presence of cycloheximide and hence does not involve synthesis of an intermediary protein, suggesting that the *cx43* gene is one of the primary response genes induced by cAMP.

Our results clearly showed that the rate of *cx43* transcription is elevated by cAMP, but it is possible that cAMP stabilizes *cx43* mRNA in addition. In rat hepatocytes that express *cx32*, a cAMP-induced increase in *cx32* mRNA level was observed that was not associated with an increase in transcription rate. That increase may have been due to such stabilization (Saez *et al.*, 1989).

We could not detect any *cx32* transcript with or without cAMP elevation in Morris hepatoma cells, indicating that in these cells *cx32* expression is not regulated by cAMP.

The functional expression of cell-cell channels after *cx43* mRNA elevation took considerable time; maximum communication was reached at ~ 12 h, i.e., ~ 6 h after the maximum *cx43* mRNA level. This delay probably reflects the various stages involved in getting the transcript functionally expressed, i.e., translation, shuttling of the protein subunits to the membrane, and their final assembly into open channels.

Communication lingered on for ≥ 28 h after the *cx43* mRNA had returned to the low control level. The half-life of connexin43 has been shown to be 1–2 h, and that seems to apply also to the phosphorylated, i.e., membrane-associated (but not necessarily to the junctional plaque-associated) form of *cx43* (Musil *et al.*, 1990;

Figure 8. Immunohistochemical localization of connexin 43 at cell junctions. (a and c) Morris hepatoma cells, untreated; (b and d) Morris hepatoma cells treated 14 h with 40 μ M forskolin. (a and b) Normal fixation; (c and d) fixation after Triton-X 100 extraction. Brightly fluorescent punctate staining was seen only in forskolin-treated cultures; it occurred mainly at cell contact regions and presumably represents gap junctional plaques. (e) RL C19 cells, normal fixation. All cultures were treated and processed for immunostaining in parallel.

Figure 9. Effect of forskolin or 8-Br-cAMP on cx43 mRNA level in other cell lines. (A) Increase in cx43 transcript in cell lines with a relatively low basal level of cx43. The various cell lines were treated with forskolin or 8-Br-cAMP for the durations indicated, at which time RNA was extracted. Twenty μ g RNA/lane. (B) No effect on cx43 RNA in four cell lines with high basal cx43 RNA level. For a comparison, RNA from RL C19 and from rat heart was loaded onto the same gel as RNA from 3T3 MA31 cells. Twenty μ g RNA/lane for all cell lines.

Laird *et al.*, 1991; Musil and Goodenough, 1991). The prolonged maintenance of communication would thus indicate that the half-life of the channel or of the channels assembled into gap junctional plaques is considerably longer, at least in the presence of cAMP. cAMP may indeed retard degradation of cx43 once it is assembled into channels. Green *et al.* (1991), for instance, have found that forskolin approximately doubled the half-life of acetylcholine receptor channel subunits under conditions where (heteromeric) subunit aggregation was possible, but it did not prolong the half-life when such aggregation could not occur. It is thus possible that cAMP increases the half-life also of the cx43 protein either by permitting, after phosphorylation, assembly of cx43 into connexons, into complete channels, or into

gap junctional plaques or by stabilizing such assembly with phosphorylation.

Formation of functional channels involves more than synthesis of the channel protein. For instance, Mege *et al.* (1988) found that expression of cell adhesion molecules induced gap junctions and communication, and Musil *et al.* (1990) found that this induction correlated with the cells' ability to convert the connexin43 protein expressed by these cells into a serine-phosphorylated form. More recently, Musil and Goodenough (1991) showed that cx43 in the triton-insoluble fraction, which represents cx43 in the gap junctional plaques, exists mainly in the phosphorylated P₂ form and that the phosphorylation takes place after cx43 incorporation into the (nonjunctional) membrane. This implies that

Table 3. Effect of forskolin on communication in a variety of cell types^a

Cell type	Fluorescent neighbors		Fold change
	Control	Treated	
3T3 A31	4.7 ± 3.1 (24)	12.4 ± 5.9 (22)	+2.6*
C6 Glioma	0.8 ± 1.4 (20)	11.8 ± 5.6 (17)	+14.8*
10T1/2	5.2 ± 3.4 (25)	13 ± 5.9 (26)	+2.6*
MCA-4D	3.3 ± 1.9 (22)	8.9 ± 4.8 (22)	+2.7*
3T3 MA31	12.9 ± 4.5 (16)	14.9 ± 5.8 (17)	+1.2
3T3 SRD	1.0 ± 1.1 (21)	0.2 ± 0.4 (25)	-4.2

^a Number of fluorescent cell neighbors 1 min after injection of Lucifer Yellow; mean ± SD. In parentheses, the number of injection trials. Cells were seeded at 5×10^5 cells/6 cm-dish in 4 ml medium. Freshly confluent cells were treated with 30 μ M forskolin (with medium change) and communication was probed at 12–16 h of treatment. Asterisks indicate cell lines in which the cAMP-induced change in communication was statistically significant ($P < 0.02$).

serine protein kinase action is required posttranslationally for the assembly of cx43 into functional channels. If cAMP-dependent protein kinase A is the enzyme required for such phosphorylation, then cAMP has a dual role in the regulation of (cx43-mediated) junctional communication: activating transcription of the cx43 gene and mediating phosphorylation of the cx43 protein, converting it into the channel-assembling form. Stimulation of cx43 transcription in Morris hepatoma cells thus would not, by itself, be sufficient for induction of functional channels. Indeed, Morris hepatoma cells that were infected with cx43-harboring retrovirus and that highly transcribed cx43 in untreated condition were communication incompetent unless treated with forskolin or 8-Br-cAMP (Mehta and Rose, unpublished observations).

In addition to mediating cx43 phosphorylation, cAMP could induce or activate another protein that facilitates channel formation or stabilizes functional channels. Specific glycoprotein adhesion molecules may play such a role; they have been implicated in the formation of functional cell–cell channels (Loewenstein, 1967; Keane *et al.*, 1988; Mege *et al.*, 1988; Musil *et al.*, 1990; Jongen *et al.*, 1991).

Although forskolin, 8-Br-cAMP, and db-cAMP were nearly equally effective in raising the cx43 transcript level, db-cAMP had little effect on communication, even in the presence of a phosphodiesterase inhibitor. This differential effect on transcription and on posttranscriptional events may indicate that the cx43 transcription and the formation of open channels are regulated by different cAMP-dependent protein kinases. The regulatory subunits of protein kinase A isoenzymes PKA I and PKA II are known to have different affinities for various cAMP analogues, such as 8-Br-cAMP and db-

cAMP (Cho-Chung, 1990). Alternatively, the difference may be due to some action of a by-product of db-cAMP metabolism.

Our finding of enhanced cx43 transcription by cAMP in a number of cell lines, normal and transformed, suggests that this upregulation may be a more general physiological phenomenon, although some cell lines, namely those with a high basal level of cx43 mRNA, were not responsive to either forskolin or 8-Br-cAMP. It is possible that these cells have a high level of cAMP in basal condition. In any event, this lack of response does not mean that the cx43 gene is not regulatable otherwise in these cells. In the responsive mouse 10T1/2 fibroblasts, for instance, junctional communication and cx43 mRNA rise also after treatment with retinoids (Mehta *et al.*, 1989; Rogers *et al.*, 1990), a class of agents that exerts its effects by binding to specific nuclear receptor proteins (Giguere *et al.*, 1987) with DNA-binding domains similar to those of steroid and thyroid hormone receptors (Evans, 1988).

Several peptide hormones and neurotransmitters that use a cAMP-dependent signal transduction pathway exert their transcriptional effects by modulating the activity of multiple *trans*-acting factors (Bolander, 1989; Goodman, 1990). During the last several years, numerous cAMP-responsive genes have been analyzed, and a *cis*-acting DNA sequence referred to as “cAMP response element” has been characterized in many of these genes (Roesler *et al.*, 1988). Our findings that the cx43 gene is transcriptionally upregulated by cAMP suggest that the cx43 gene may harbor such a *cis*-acting DNA sequence. Consensus sequences for two such cAMP response elements have been found in the rat cx32 gene (Miller *et al.*, 1988) (genomic rat cx43 DNA sequences have not been worked out). But because cx32-related mRNA was not induced by forskolin or 8-Br-cAMP in any of the cell lines we have investigated, we assume that additional tissue-specific transcriptional factors (Maniatis *et al.*, 1987), which may be required for transcriptional regulation of cx32 by cAMP, are missing in these cell lines.

Because the cells in many tissues—heart, uterus, kidney, smooth muscle, and brain, to name a few (see Bennett *et al.*, 1991)—express cx43, the regulation by cAMP of the formation of functional cell–cell channels, and consequently of intercellular communication, has broad physiological implications. The extent of intercellular transfer of ionic currents and of cell–cell channel-permeable cytoplasmic messenger molecules will be influenced if not determined by the cells’ previous and existing cAMP concentration. Even a relatively short-lived (10–30 min) surge in intracellular cAMP, such as might occur in tissues that respond to hormones that use cAMP as second messengers, could have a long-lasting (several hours) effect on communication, and thus modulate the tissues’ response to subsequent electrical or chemical

signals by determining the signals' spatial and temporal distribution pattern in the tissue.

ACKNOWLEDGMENTS

We thank Eric Beyer, Joan Brugge, Bruce Nicholson, David Paul, and Sven Pahlman for gifts of plasmids and antibodies; Tomas Lopez for excellent technical support; and Werner R. Loewenstein for critical discussions. This work was supported by NCI CA14464.

REFERENCES

- Azarnia, R., Dahl, G., and Loewenstein, W.R. (1981). Cell junction and cyclic AMP. III. Promotion of junctional membrane permeability and junctional membrane particles in a junction-deficient cell type. *J. Membr. Biol.* 63, 133–146.
- Azarnia, R., and Loewenstein, W.R. (1971). Intercellular communication and tissue growth. V. A cancer cell strain that fails to make permeable membrane junctions with normal cells. *J. Membr. Biol.* 6, 368–385.
- Bennett, M.V.L., Barrio, L.C., Bargiello, T.A., Spray, D.C., Hertzberg, E., and Saez, J.C. (1991). Gap junctions: new tools, new answers, new questions. *Neuron* 6, 305–320.
- Beyer, E.C., Paul, D.I., and Goodenough, D.A. (1990). Connexin family of gap junction proteins. *J. Membr. Biol.* 116, 187–194.
- Bolander, F.F. (1989). *Molecular Endocrinology*. New York: Academic Press.
- Borek, C., Higashino, S., and Loewenstein, W.R. (1969). Intercellular communication and tissue growth. IV. Conductance of membrane junctions of normal and cancerous cells in culture. *J. Membr. Biol.* 1, 274–293.
- Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979). Isolation of biological active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18, 5294–5299.
- Cho-Chung, Y.S. (1990). Role of cyclic AMP receptor proteins in growth, differentiation, and suppression of malignancy: new approaches to therapy. *Cancer Res.* 50, 7093–7100.
- Chomezynski, P., and Sacchi, N. (1987). Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156–159.
- Cochran, B.H., Reffel, A.C., and Stiles, C.D. (1983). Molecular cloning of gene sequences regulated by platelet-derived growth factor. *Cell* 33, 939–947.
- Croy, R.G., and Pardee, A.B. (1983). Enhanced synthesis and stabilization of M-68,000 protein in transformed Balb 3T3 cell: candidate for restriction point control of cell growth. *Proc. Natl. Acad. Sci. USA* 80, 4699–4703.
- Evans, R. (1988). The steroid and thyroid receptor superfamily. *Science* 240, 889–895.
- Filson, A.J., Azarnia, R., Beyer, E.C., Loewenstein, W.R., and Brugge, J.S. (1990). Tyrosine phosphorylation correlates with inhibition of cell-to-cell communication. *Cell Growth & Differ.* 1, 661–668.
- Flagg-Newton, J.L., Dahl, G., and Loewenstein, W.R. (1981). Cell junction and cyclic AMP. I. Upregulation of junctional membrane permeability and junctional membrane particles by administration of cyclic nucleotide or phosphodiesterase inhibitor. *J. Membr. Biol.* 63, 105–121.
- Flagg-Newton, J.L., Simpson, I., and Loewenstein, W.R. (1979). Permeability of the cell-to-cell membrane channels in mammalian cell junction. *Science* 205, 404–407.
- Giguere, V., Ong, E.S., Segui, P., and Evans, R.M. (1987). Identification of a receptor for the morphogen retinoic acid. *Nature* 330, 624–629.
- Goodman, R.H. (1990). Regulation of neuropeptide gene expression. *Annu. Rev. Neurosci.* 13, 111–127.
- Green, W.N., Ross, A.F., and Claudio, T. (1991). cAMP stimulation of acetylcholine receptor expression is mediated through posttranslational mechanisms. *Proc. Natl. Acad. Sci. USA* 88, 854–858.
- Ingebritsen, T.S., and Cohen, P. (1983). Protein phosphatases: properties and role in cellular regulation. *Science* 221, 331–338.
- Jongen, W.M.F., Fitzgerald, D.J., Asamoto, M., Piccoli, C., Slaga, T.J., Gros, D., Takeichi, M., and Yamasaki, H. (1991). Regulation of connexin 43-mediated gap junctional intercellular communication by Ca^{2+} in mouse epidermal cells is controlled by E-cadherin. *J. Cell Biol.* 114, 545–555.
- Keane, R.W., Mehta, P.P., Rose, B., Honig, L.S., Loewenstein, W.R., and Rutishauser, U. (1988). Neural differentiation, NCAM-mediated adhesion and gap junctional communication in neuroectoderm. A study in vitro. *J. Cell Biol.* 106, 1307–1319.
- Laird, D.W., Puranam, K.L., and Revel, J.-P. (1991). Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes. *Biochem. J.* 273, 67–72.
- Lehrach, H.D., Diamond, D., Wozney, J.M., and Boedtker, H. (1977). RNA molecular weight determination by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry* 16, 4743–4751.
- Loewenstein, W.R. (1967). On the genesis of cellular communication. *Dev. Biol.* 15, 503–520.
- Loewenstein, W.R. (1979). Junctional intercellular communication and the control of growth. *Biochim. Biophys. Acta* 560, 1–65.
- Loewenstein, W.R., and Rose, B. (1992). The cell-cell channel in the control of growth. *Semin. Cell Biol.* 3, 59–79.
- Maniatis, T., Goodbourn, S., and Fischer, J.A. (1987). Regulation of inducible and tissue-specific gene expression. *Science* 236, 1237–1244.
- Marzuluff, W.F., and Huang, C.C.R. (1984). Transcription of RNA in isolated nuclei. In: *Transcription and Translation: A Practical Approach*, eds. B.D. Hames and S.J. Higgins, Oxford: IRL Press, 89–129.
- Mege, R.M., Matsuzaki, F., Gallin, W.J., Goldberg, I., Cunningham, B.A., and Edelman, G.M. (1988). Construction of epitheloid sheets by transfection of mouse sarcoma cell cells with cDNAs for chicken cell adhesion molecules. *Proc. Natl. Acad. Sci. USA* 85, 7274–7278.
- Mehta, P.P., Bertram, J.S., and Loewenstein, W.R. (1986). Growth inhibition of transformed cells correlates with their junctional communication with normal cells. *Cell* 44, 187–196.
- Mehta, P.P., Bertram, J.S., and Loewenstein, W.R. (1989). The actions of retinoids on cellular growth correlate with their actions on gap junctional communication. *J. Cell Biol.* 108, 1053–1065.
- Miller, T., Dahl, G., and Werner, R. (1988). Structure of a gap junction gene: rat connexin-32. *Biosci. Rep.* 8, 455–464.
- Musil, L.S., Cunningham, B.A., Edelman, G.M., and Goodenough, D.A. (1990). Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. *J. Cell Biol.* 111, 2077–2088.
- Musil, L.S., and Goodenough, D.A. (1991). Biochemical analysis of connexin43 intracellular transport, phosphorylation and assembly into gap junction plaques. *J. Cell Biol.* 115, 1357–1374.

- Reznikoff, C.A., Bertram, J.S., Brankow, D.W., and Heidelberger, C. (1973). Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. *Cancer Res.* 33, 3229–3249.
- Roesler, W.J., Vandenbark, G.R., and Hanson, R.W. (1988). Cyclic AMP and the induction of eukaryotic gene transcription. *J. Biol. Chem.* 263, 9063–9066.
- Rogers, M., Berestecky, J.M., Hossain, M.Z., Guo, H., Kadle, R., Nicholson, B., and Bertram, J.S. (1990). Retinoid-enhanced gap-junctional communication is achieved by increased levels of connexin43 mRNA and protein. *Mol. Carcinog.* 3, 335–343.
- Rose, B., Yada, T., and Loewenstein, W.R. (1986). Downregulation of cell-to-cell communication by the viral *src* gene is blocked by TMB-8 and recovery of communication is blocked by vanadate. *J. Membr. Biol.* 94, 129–142.
- Saez, J.Z., Gregory, W.A., Watanabe, T., Dermietzel, R., Hertzberg, E.L., Reid, I., Bennett, M.V.L., and Spray, D.C. (1989). cAMP delays disappearance of gap junctions between pairs of rat hepatocytes in primary culture. *Am. J. Physiol.* 257, C1–C11.
- Schwartzmann, G.O.H., Wiegandt, H., Rose, B., Zimmerman, A., Ben-Haim, D., and Loewenstein, W.R. (1981). The diameter of the cell-to-cell junctional membrane channels, as probed with neutral molecules. *Science* 213, 551–553.
- Seamon, K.B., and Daly, J.W. (1981). Forskolin: a unique diterpene activator of cAMP-generating systems. *J. Cyclic Nucleotide Res.* 7, 201–224.
- Sompayrac, L., and Danna, K.J. (1983). A simian virus 40 d1884/tsA58 double mutant is temperature sensitive for abortive transformation. *J. Virol.* 46, 620–625.
- Swenson, K.I., Piwnica-Worms, H., McNamee, H., and Paul, D.L. (1990). Tyrosine phosphorylation of the gap junction protein connexin43 accounts for the pp60^{v-src}-induced inhibition of communication in *Xenopus* oocyte pairs. *Cell Regul.* 1, 989–1002.
- Tsao, M.-S., Smith, J.D., Nelson, K.G., and Grisham, J.W. (1984). A diploid epithelial cell line from normal rat liver with pphenotypic properties of "oval" cells. *Exp. Cell Res.* 154, 38–52.
- Yada, T., Rose, B., and Loewenstein, W.R. (1985). Diacylglycerol downregulates junctional membrane permeability. TMB-8 blocks this effect. *J. Membr. Biol.* 88, 217–232.