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Unexpected Induction of the Human Connexin 43 Promoter by the Ras Signaling Pathway Is Mediated by a Novel Putative Promoter Sequence

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ABSTRACT

Connexin 43 (Cx43) is essential for survival and is tightly regulated at the transcriptional and post-transcriptional levels. A number of previous studies have demonstrated altered expression in malignant tissues, and in the presence of carcinogenic factors. We examined the effect of protooncogenes of Cx43 expression, and found no effect on Cx43 promoter activity in cells transformed with Src or erbB2. On the other hand, we identified and characterized a novel sequence that mediates Cx43 promoter regulation in cell lines engineered to overexpress H-Ras. Compared with wild-type NIH3T3 cells, both Cx43 mRNA and protein levels are increased in NIH3T3-Ras cells. The H-Ras+ cells also have enhanced Cx43 promoter activation, which is inhibited by the MEK1 inhibitor 2′-amino-3′-methoxyflavone (PD98059), suggesting that Ras-mediated Cx43 overexpression is via the mitogen activated protein kinase kinase/extracellular signal-regulated pathway. Deletion analysis of the Cx43 promoter revealed a 200-bp region downstream of the Cx43 transcription start site as the minimal sequence essential for the Ras-mediated Cx43 up-regulation. Using this 200-base pair fragment in electrophoretic mobility shift assays, we identified one main protein complex that binds efficiently and is more abundant in nuclear extracts from NIH3T3-Ras and MCF7-Ras cells compared with their matched controls. This complex selectively recognizes a consensus sequence, AGTTCAATCA, located at positions +149 to +158 of the Cx43 promoter. Supershift assays identified the 90-kDa heat shock protein (HSP90) and c-Myc as constituents of this DNA-binding complex. Treatment of cells with the HSP90 inhibitor geldanamycin resulted in repression of the Cx43 promoter activity, and inhibits binding of the complex to the Cx43 promoter. Coimmunoprecipitation studies confirmed the interaction between endogenous HSP90 and c-Myc. This study provides evidence that the transcriptional up-regulation of Cx43 by Ras-Raf-MAPK is mediated via the interaction of a novel Cx43 promoter element with a protein complex that contains both HSP90 and c-Myc.

The gap junction (GJ) is an important cell-cell communication structure that has a broad physiological function including the regulation of cell growth, cell differentiation, and the maintenance of tissue homeostasis (Bruzzone et al., 1996; Zhu et al., 1991, 1992). Several second messengers and small molecules are transported through gap junctions, including cAMP, cGMP, inositol trisphosphate, glutathione, and Ca^{2+} ions (Charles et al., 1992; Kam et al., 1998). GJ is composed of hemichannels formed by two connexons from adjacent cells coming together at the point of cell contact. Each connexon is a hexamer of connexins (Cx), the building blocks of the GJ (Laird et al., 1995; Goodenough et al., 1996). At least 14 members of the connexin gene family have been characterized in mammalian cells, including Cx26, Cx30, Cx30.3, Cx31, Cx31.1, Cx32, Cx33, Cx37, Cx40, Cx43, Cx45, Cx46, and Cx50. A series of post-translational phosphorylations, and a complex intracellular trafficking scenario, are critical to the development of functional connexins. Connexon hexamers can belong to the same or distinct connexin genes. The genes that are most characterized are connexin 43 (Cx43), connexin 26, and connexin 32.

The impairment of gap junctional intercellular communication (GJIC) is a common marker of transformed and cancer cell lines (Yamasaki, 1990; Yamasaki et al., 1995; Laird et al., 1999). We and others have shown that Cx43 is undetectable in early stage human breast cancer tissue compared

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ABBREVIATIONS: GJ, gap junction; Cx, connexin; GJIC,gap junctional intercellular communication; MEK, mitogen activated protein kinase kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PD98059, 2′-amino-3′-methoxyflavone; wt, wild type; DMSO, dimethyl sulfoxide; HSP90, 90-kDa heat shock protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AhR, aryl hydrocarbon receptor; bp, base pair(s); EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; SSC, standard saline citrate; RRCxE, Ras-responsive Cx43 element.

with adjacent normal tissue (Nicolson et al., 1988; Lee et al., 1992; Holden et al., 1997; Laird et al., 1999). Similar results are observed in other cancer tissues, such as ovarian cancer, lung cancer, and neuroblastomas (Albright et al., 1990; Tsai et al., 1996; Huang et al., 1999; Umhauer et al., 2000). This loss in Cx43 is believed to be among the earliest events by which transformed cells acquire independence from stimuli from neighboring cells. Cx can regulate apoptotic mechanisms (Trosko and Goodman, 1994; Trosko and Ruch, 1998), and enhance metabolic cooperation (Freeman et al., 1993; Mesnil et al., 1996; Carystinoset al., 1999). Restoration of Cx43 and GJIC in cancer cells has been shown to reverse phenotypes of tumorigenicity, including inhibition of cell proliferation and induction of cell differentiation (Mehta et al., 1991; Rose et al., 1993; Proulx et al., 1997).

Some protooncogenes have been shown to alter regulation of GJIC and Cx43 (Brissette et al., 1991; Hofer et al., 1996; Hossain et al., 1998). Activated c-Src leads to an increase in Cx43 phosphorylation and to a reduction in GJIC and Cx43 levels (Postma et al., 1998; Loo et al., 1999; Toyofuku et al., 1999; Zhou et al., 1999). Human keratinocytes engineered to express human papillomavirus showed Cx43 gene expression is inhibited by HPV16E5 expression (Tomakidi et al., 2000). The data for the effect of Ras are less clear, because its signaling pathway is shared by a number of receptor kinases that have different effects on Cx43 expression. Ras isoforms can transform cells and are often found to be mutated and constitutively activated in human tumors (Lundberg et al., 2002). The Ras signaling pathway includes several effectors, such as the Raf family of proteins, phosphatidyl inositol 3-kinase, and members of the Ral family of proteins (reviewed in Campbell et al., 1998; Vojtek and Der, 1998). Raf activation stimulates the MEK-ERK kinase cascade, which plays a very important role in cell-cycle control, as well as cell transformation (Burgering and Bos, 1995; Marshall, 1996). Among its downstream targets are the transcription factors jun, fos, Elk-1, nuclear factor κB, serum response factor, ATF-2, Cdc42, and myc (Campbell et al., 1998; Kerkhoff et al., 1998; Vojtek and Der, 1998).

A role for MAPK in the regulation of Cx43 is supported by earlier studies showing that EGF induces a transient Cx43 phosphorylation via activation of MEK1 (Warn-Cramer et al., 1996; Warn-Cramer et al., 1998). Also, PDGF induces Cx43 phosphorylation and reduced GJIC by activating the MAPK pathway (Hossain et al., 1998, 1999a), although MEK1 stimulation alone is not sufficient for Cx43 phosphorylation and degradation (Hossain et al., 1999b). In one study, the MEK1 inhibitor PD98059 was found to decrease Cx43 expression (Bao et al., 2000). Other reports demonstrate that although Ras-transformation leads to a decrease in overall GJIC, it can also increase Cx43 protein (Huang et al., 1999). Thus the effect of Ras on Cx43 seems to be complex and not entirely understood.

The human Cx43 promoter contains several important regulatory sequences, including a TATA box and an activator protein-1 site, yet its mode of regulation is still not fully characterized (Geimonen et al., 1996). Putative responsive elements include Sp1 regulation of basal Cx43 expression in NRK cells (Fernandez-Cobo et al., 2001), T cell factor/lymphoid enhancer binding factor, E-box, ERE half-sites, AP-2, cAMP-responsive element binding protein, and Ets-1 sites, which can serve as transcription factor targets. In rat Cx43, there is a recently identified putative regulatory element (rCx-480) that binds thyroid hormone receptor/retinoid X receptor α and mediates induction of the gene by 3,3′,5-triiodo-L-thyronine (Stock and Sies, 2000). There is as well a recent description both of sequences situated just upstream of the transcription start site and in the 3′-untranslated region that are responsive to parathyroid hormone in rodent models (Mitchell et al., 2001). Neither of these have been described in the human gene. In this study, we examined the mechanisms by which the Ras signaling pathway regulates Cx43 gene transcription.

Materials and Methods

Cell Lines and Reagents. The mouse fibroblast stable cells NIH3T3-wt (wild type), NIH3T3-Ras (stably expressing the constitutively active oncogene H-Ras-V12), NIH3T3-Src (stably expressing the Src oncogene), and NIH3T3-ErbB2 (stably expressing the ErbB2 oncogene) were obtained from Dr. Stephane Richard (Lady Davis Institute, Montreal, PQ, Canada) and were grown in Dulbecco's modified Eagle's medium with 10% calf serum and 1% penicillin/streptomycin at 36.6°C in a 5% (v/v) CO_2 atmosphere. The human mammary epithelial cancer lines MCF-7-neo (control), MCF-7-A4 and MCF-7-A6 (stably expressing the constitutively active oncogene H-Ras-V12) were obtained from Dr. Lee (Georgetown University Medical Center, Washington, DC), and were grown in RPMI with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin and 400 μg/ml G418 (Invitrogen, Burlington, ON, Canada) in 36.6°C with 5% (v/v) CO_2. The MEK1 inhibitor PD98059 (Calbiochem, CA) was dissolved in DMSO at a concentration of 10 mM and was used at a final concentration of 50 μM (Janssen et al., 1998; Miele et al., 2000). Treatment with PD98059 always occurred after cells were serum-starved overnight. The stock solution of the HSP90 inhibitor geldanamycin (Calbiochem, San Diego, CA) was made in DMSO at a concentration of 1 mM and was used at a final concentration of 2 μM (Schulte et al., 1997). The vectors pDCR (empty vector), RasN17 (expressing the dominant negative H-Ras-N17), and RasV12 (expressing the constitutively active H-Ras-V12) have been described previously (Tabin et al., 1982). The human Cx43 cDNA and promoter were a generous gift from Drs. G. I. Fishman (Mount Sinai School of Medicine, New York, NY) and J. Anderson (School of Medicine, State University of New York, Stony Brook, NY) (Geimonen et al., 1996). The luciferase vectors pGL3-basic (promoterless vector containing firefly luciferase) and pRL-0 (promoterless vector containing *Renilla reniformis* luciferase) were purchased from Promega (Madison, WI). The Cx43 antibody was a kind gift from Dr. D. W. Laird (University of Western Ontario, London, ON, Canada). The rabbit anti Cx43 antibody, recognizing nonphosphorylated and phosphorylated forms, was purchased from Zymed Laboratories (South San Francisco, CA). The antibodies to GAPDH, AhR, and Sp1, and the oligonucleotides of known *cis*-elements (Ets-1/PEA3, Ets, Myc-Max, Sp1, AP2) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The c-Myc antibody was purchased from Calbiochem. Finally, rat and mouse antibodies to HSP90 were obtained by StressGen Biotechnologies (Victoria, BC, Canada) and from Dr. D. Toft (Department of Biochemistry, Mayo Graduate School, Rochester, MN), respectively.

DNA Constructs. The DNA constructs are summarized in Table 1. A 2400 bp fragment of the human Cx43 promoter was excised from the vector pCx2400CAT (Geimonen et al., 1996) using the *Bam*HI [subsequently filled with DNA polymerase I large (Klenow) fragment (Promega)] and *Xho*I restriction enzymes and subcloned into the vector pGL3-basic at the *Sma*I and *Xho*I sites, resulting in the vector pCx2400luc. Also, pCx2400CAT was excised with a *Hin*dIII partial digestion (and subsequent Klenow reaction) and an *Xho*I full digestion. Of the resulting fragments, the 600-bp fragment was gel-extracted and inserted in the pGL3-basic at the sites *Sma*I and *Xho*I, resulting in the vector pCx600luc. The smaller fragments of approx-

imate sizes 350 and 200 bp (called Cx350 and Cx200, respectively) were designed by polymerase chain reaction, using pCx2400luc as the template, the downstream primer 5′-TACCGGAATGCCAAGCTTAC-3′ (which binds downstream of the pGL3-basic polycloning site) and the upstream primers 5′-ATATACGCGTACTGCTGCTCTTTGCCTCTT-3′ (containing the site *Mlu*I) and 5′-ATATACGCGTAAGCTTTTACGAGGTATC-3′ (containing the site *Mlu*I), respectively. The resulting polymerase chain reaction products were digested with *Mlu*I and *Xho*I and subcloned to pGL3-basic at the *Mlu*I and *Xho*I sites, resulting in the vectors pCx350luc and pCx200luc, respectively. The pCx600M fragment was designed by restriction of the pCx600luc vector with *Hin*dIII and *Xho*I, followed by Klenow treatment and religation.

Annealing of DNA Oligonucleotides. Annealing of single-stranded oligonucleotides was performed to produce double stranded DNA molecules of a desired sequence to be used in EMSA studies (Table 2). Single stranded DNA oligonucleotides were designed (Invitrogen) according to our sequence requirements and dissolved to a final concentration of 5 μg/μl. The two complementary strands of the oligonucleotides were mixed in a microfuge tube at a ratio of 1:1 and a final volume of 20 μl. The mixture was incubated at 85°C for 10 min in a hot block, and the temperature was allowed to slowly return to room temperature overnight. The resulting annealed oligonucleotides were run in a 10% nondenaturing polyacrylamide gel and were gel-purified by incubating the gel slices (containing the DNA of interest) with Tris/EDTA overnight in a 37°C shaking incubator. The DNA was subsequently precipitated out of the Tris/EDTA buffer by sodium acetate and ethanol, dissolved in distilled water, and quantified.

Promoter Assays. Cells were seeded in 24-well plates at a density of 40,000 cells/well and were incubated overnight. The following day, 1 μg of DNA (either 1 μg of a Cx43 promoter construct or 0.5 μg of a promoter construct plus 0.5 μg of a Ras construct) was cotransfected with 0.2 μg pRL-0 and 4 μg of LipofectAMINE (Invitrogen) in 200 μl of serum-free media per well. After 5 h, the transfection mix was removed and cells were overlaid with 400 μl of complete medium per well. Cells were allowed to recover overnight. Depending on the treatment, cells were lysed at various times after transfection. Lysis was performed as described in the dual-luciferase reporter assay system manual (Promega) by using 100 μl of 1× passive lysis buffer per well (Promega). The dual luciferase assay was performed as described in the dual-luciferase reporter assay manual using the Lumat LB-9507 luminometer (PerkinElmer Instruments, Rodgau-Juegesheim, Germany). The transfection conditions were different for PD98059 treatment. In this case, cells were overlaid with serum-complete media for 3 h after transfection to recover. Subsequently, cells were overlaid with serum-free media and serum-starved overnight. The following day, PD98059 was added and allowed to be taken up by the cells for 1.5 h; serum was then added (to a final concentration of 10%) to all wells. Cells were lysed the following day. Luciferase activity was calculated as the ratio of firefly luciferase activity (of the promoter luciferase construct) to *R. reniformis* luciferase activity (of the vector pRL-0). The transfection efficiency control vector pRL-0 was used based on previous reports with Ras (Behre et al., 1999). All assays were done in triplicate, and all transfections and luciferase assays were repeated in at least three independent experiments. For each figure of a promoter assay, all treatments and conditions shown were assayed concurrently to control for variability introduced by the instability of the luciferase assay reagents.

Western Blotting for Cx43. A rabbit polyclonal antibody against connexin 43 was used at a dilution of 1:500. Treated cells were washed with PBS, collected by trypsinization, and centrifuged for 30 s at 12,000*g*. Each pellet was resuspended in 0.4 ml of 1× SDS buffer containing 50 mM Tris-Cl buffer, pH 6.8, 100 mM dithiothreitol, 2% SDS, supplemented with 0.5 mM phenylmethylsulfonyl fluoride, 1 μM sodium-orthovanadate, 0.01 μg/ml leupeptin, 0.01 μg/ml pepstatin, and 0.01 μg/ml aprotinin. The sample was then incubated on ice for 15 min, and centrifuged for 10 min at 12,000*g*. The soluble fraction was collected and assayed for protein content using the Bradford assay (Bio-Rad, Hercules, CA). Equal protein amounts were size-fractionated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The immunoblots were processed as described previously (Brissette et al., 1991), and immune complexes were detected by horseradish peroxidase conjugates. A mouse antibody against GAPDH was used as a control for protein loading.

Northern Blotting. To measure the level of Cx43 RNA, total RNA was isolated from exponentially growing cells using the high pure RNA isolation kit (Roche Molecular Biochemicals, Indianapolis, IN). RNA was size-separated through a 1% formaldehyde-agarose gel and transferred to a nitrocellulose membrane by capillary action for 18 h in 20× SSC. Filters were prehybridized for 2 h at 42°C in prehybridization buffer [50% (v/v) formamide, 5× SSC, 5× Denhardt's buffer, 250 mg/ml sonicated calf thymus DNA, and 0.5% SDS]. Probe was labeled to a high specific activity with [^{32}P]dCTP using an oligonucleotide labeling kit (Amersham Biosciences, Montreal, ON, Canada) and added to the blots at a concentration of 10^6 cpm/ml in hybridization buffer. Hybridization was carried out for 20 h at 42°C in hybridization buffer [dextran sulfate/prehybridization buffer, 1:4 (v/v)]. Membranes were washed three times for 10 min at room temperature in 1× SSC containing 0.1% SDS, 3 times at 60°C for 10 min in 0.1× SSC containing 0.1% SDS, and subjected to autoradiography. The human Cx43 probe was the insert from the Cx43 cDNA vector provided by Dr. Fishman, and the H-Ras probe was the insert from the RasV12 vector. To control for RNA loading, ribosomal-RNA bands were visualized by ethidium bromide staining.

Electrophoretic Mobility Shift Assays. Nuclear extracts were prepared as described previously (Osborn et al., 1989), quantified, and stored at −80°C. EMSAs were performed as described previously. Connexin43 promoter constructs were excised from the luciferase vectors, end-labeled with [α-^{32}P]ATP and polynucleotide kinase, and purified in a G-50 Sephadex column (Amersham Biosciences). Nuclear extracts were incubated with 0.2 ng of labeled DNA in a buffer containing 1 μg poly(dI-dC), 20 mM HEPES, pH 7.9, 5% glycerol, 0.1 M KCl, 0.2 mM EDTA, pH 8.0, 0.2 mM EGTA, pH 8.0, and 2 μM dithiothreitol. The incubation was performed at room temperature for 20 min. Samples were run in a 4% nondenaturing polyacrylamide gel (60:1) for 2 h. The gel was subsequently vacuum-dried, and labeled DNA was visualized by autoradiography. The protocol was modified for competition and supershift assays. To perform competition assays, protein extracts were incubated with 100- to 200-fold excess oligonucleotide for 10 min before addition of labeled DNA and further incubation for 20 min. To carry out supershift assays, protein extracts were incubated with 2 to 5 μg of

TABLE 1

DNA constructs of the human Cx43 promoter

Human Cx43 promoter deletions were inserted upstream of the firefly luciferase of the pGL3-basic vector (Promega). The distance of the 5′ and 3′ ends of the inserts relative to the human Cx43 transcription start site (+1) is indicated. The name of the inserts, where indicated, will be used when describing EMSAs, because those fragments were excised from their respective constructs and used as linear DNA fragments. All 3′ inserts were introduced at the *Xho*I site of the pGL3-basic vector.

Construct	5′ End	3′ End	Name of Insert
pCx2400luc	−2200	+209	
pCx600luc	−405	+209	
pCx350luc	−158	+209	Cx350
pCx200luc	+7	+209	Cx200
pCx600M	−405	+11	

antibody for 20 min at room temperature, followed by the addition of labeled DNA and 20-min incubation.

Coimmunoprecipitation Assays. Protein extracts from NIH3T3-wt and NIH3T3–Ras cells (500 μg per sample) were incubated with 3 to 5 μg of the appropriate antibody in an immunoprecipitation buffer containing 50 mM Tris-Cl, pH 8.0, 5% glycerol, 0.2 mM EDTA, 0.01% Nonidet P-40, 50 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μM sodium orthovanadate, 0.01 μg/ml leupeptin, 0.01 μg/ml pepstatin, and 0.01 μg/ml aprotinin. The incubation was performed in microcentrifuge tubes on a rotating plate at 4°C overnight. The following day, 50 μl of protein G Sepharose (1:1) was added to the mixture, which was further incubated for 3 h at 4°C. Subsequently, the protein-antibody-Sepharose mix was washed five times with the immunoprecipitation buffer at 4°C. Finally, the protein-antibody-Sepharose complex was resuspended in SDS-PAGE loading buffer, boiled at 95°C for 15 min, vortexed, and centrifuged at 12,000*g* for 5 s. Samples were size-separated by SDS-PAGE and transferred to a nitrocellulose filter. Western blotting was performed using the appropriate antibodies. The antibodies used for the immunoprecipitation are mouse c-Myc and mouse HSP90. The resulting protein blots were then incubated with mouse HSP90 and mouse c-Myc antibodies, respectively, and Western blotting was performed as described previously. Purified HSP90β protein was run next to the c-Myc-immunoprecipitated proteins, to better localize the HSP90 protein during Western blotting.

Results

H-Ras Induces Cx43 Expression. To examine the effect of H-Ras overexpression on Cx43, we compared Cx43 expression in NIH3T3-wt and NIH3T3-Ras cells by Western and Northern blotting analysis. Figure 1 shows that NIH3T3-Ras cells had increased Cx43 RNA (Fig. 1A) and protein (Fig. 1B) levels, in comparison with NIH3T3-wt cells. Exposure of cells to the MEK1 inhibitor PD98059 at a concentration of 50 μM led to a decrease in Cx43 protein in both NIH3T3-wt and NIH3T3-Ras cells (Fig. 1C). The concentration of PD98059 used to block MEK1 activity of NIH3T3 cells (50 μM) did not affect cell survival (data not shown) and was based on previous studies (Janssen et al., 1998; Miele et al., 2000).

H-Ras Regulates Cx43 at the Promoter Level. To examine whether the H-Ras-mediated induction of Cx43 protein and RNA levels originates at the transcriptional level, promoter assays were performed. Figure 2A illustrates the Cx43 promoter activities of NIH3T3-wt, NIH3T3-ErbB2, NIH3T3-Ras, and NIH3T3-Src cells transiently transfected with pCx2400luc and pRL-0. Ras-overexpression induced Cx43 promoter activity, whereas overexpression of Src did not affect Cx43 promoter activity. erbB2 overexpression reduced Cx43 promoter activity slightly, as previously suggested by studies in rat liver (Jou et al., 1995). Subsequent studies focused on the Ras induction observation. NIH3T3-wt and NIH3T3–Ras cells were transiently cotransfected with Cx43 promoter constructs (Table 1), pRL-0, and either the dominant-negative mutant RasN17 or the control vector pDCR. Figure 2B indicates that the activities of all the Cx43 promoter constructs were greater in NIH3T3-Ras cells compared with NIH3T3-wt cells. Cotransfection of Cx43 promoter constructs with RasN17 reduced promoter activity in both cell lines, compared with cotransfection with the control vector pDCR. The smallest promoter fragment tested was pCx200luc, which contained the Cx43 promoter area between positions +7 and +209 of the transcription start site (Table 1), and also contained the site responsible for the H-Ras mediated transactivation. Transient assays confirmed that Cx43 promoter activity was greatly reduced after 24 h of PD98059 treatment in both NIH3T3-wt and NIH3T3-Ras cells, indicating that MEK1 is important in Cx43 promoter activity (Fig. 2C). Transient promoter assays were also performed in MCF-7 cells (Fig. 3) that were either devoid of mutated H-Ras (MCF7-neo) or stably expressed H-RasV12 (MCF7-A4 and MCF7-A6) to look for this effect in a different experimental model. Similarly to NIH3T3 cells, the activities of all Cx43 promoter constructs (Table 1) were greater in MCF7-A4 and MCF7-A6 cells compared with MCF7-neo cells (Fig. 3A). Also, cotransfection with RasN17 reduced Cx43 promoter activity in MCF7-A4 and MCF7–A6 cells, whereas cotransfection with RasV12 led to an increase in Cx43 promoter activity in MCF7-neo cells, providing further evidence that the Cx43 promoter activities are affected by H-Ras status. Treatment of these cells with the MEK1-inhibitor PD98059 also led to a reduction in Cx43 promoter activity (Fig. 3B). We did not examine Cx43 protein in the MCF-7 cells, because we have previously shown that despite production of a normal transcript, Cx43 protein cannot be identified in these cells using these antibodies (Laird et al., 1999). Our data here showed that even in the presence of this post-translational defect, the Ras regulation of Cx43 was still observed in MCF-7 cells.

Detection of a Specific Cx43 Promoter-Binding Complex. EMSAs were performed to test various nuclear extracts for the presence of a protein complex that may interact with the Cx43 promoter. Figure 4A indicates that Cx200 was recognized by a protein complex, existing in nuclear extracts from the cells tested. The binding of the protein complex to Cx200 was greater in cells overexpressing the H-Ras oncogene (NIH3T3-Ras, MCF7-A4, and MCF7-A6), relative to

TABLE 2

DNA oligonucleotides used to compete Cx200 for transcription complex binding.

The listed DNA oligonucleotides were designed by annealing as described under *Materials and Methods* and used to compete Cx200 in EMSAs. With the exception of FR3-B, they are homologous to the human Cx43 promoter, between positions +140 and +182, and contain the underlined base substitutions. FR3-B is homologous to the human Cx43 promoter, between positions +181 and +209. The promoter element binding to the transcription complex is shown in bold, and is referred to as RRCxE.

Cx43 promoter	5′-TCATCCTCCAAGG**AGTTCAATCA**CTTGGCGTGACTTCACTACTTTTAAGCAA-3′
FR3	CCTCCAAGGAGTTCAATCACTTGGCGTGACTTCACTACTTTTA
FR3-M1	CCTCCAAGGAGTTCAATCACTGAGCGTGACTTCACTACTTTTA
FR3-M2	CCTCCAAGGAGTTCAATCACTTGGGAAGACTTCACTACTTTTA
FR3-M3	CCTCCAAGGAGTTCAATCGGGTGGCGTGACTTCACTACTTTTA
FR3-M4	CCTCCAAGGAGTTCAGGGACTTGGCGTGACTTCACTACTTTTA
FR3-M5	CCTCCAAGGAGTTCAATCACTTGGCGTCGGTTCACTACTTTTA
FR3-M6	CCTCCAAGGAGTGGGATCACTTGGCGTGACTTCACTACTTTTA
FR3-M7	CCTCCAGCCAGTTCAATCACTTGGCGTGACTTCACTACTTTTA
FR3-B	TAAGCAAAAGAGTAAGTTTTTAAAAAATG

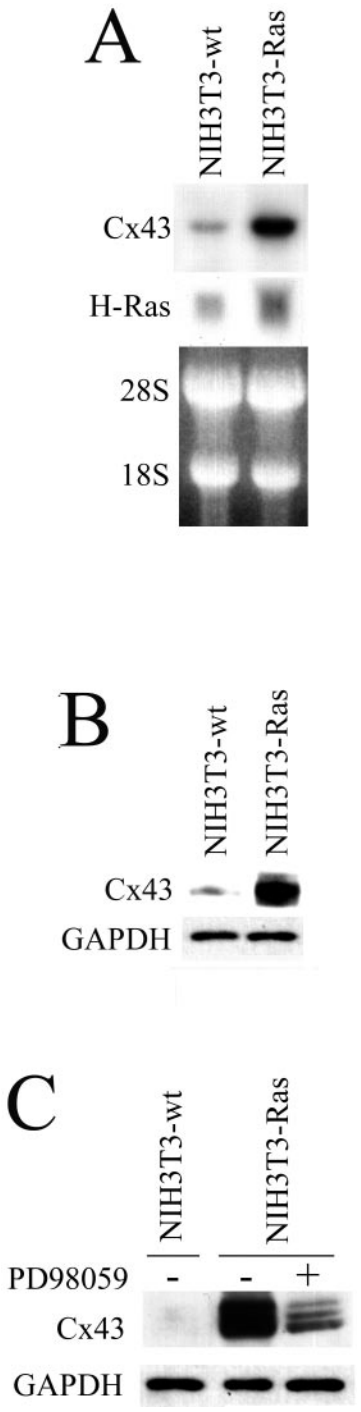


Fig. 1. Connexin 43 RNA (A) and protein (B and C) levels of NIH3T3-wt and NIH3T3-Ras cells. A, total RNA (20 μg per lane) was size-separated and transferred to nitrocellulose membranes. Northern blotting was performed on the membranes using a ^{32}P-labeled human Cx43 cDNA or a ^{32}P-labeled human H-Ras DNA. The 28S and 18S rRNA bands were visualized with the use of ethidium bromide staining and were used to control for RNA loading and degradation. B, whole-cell protein extracts (10 μg per lane) from NIH3T3 cells were size-separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blots were performed using a mouse Cx43 antibody or a mouse GAPDH antibody (loading control). C, NIH3T3-wt and NIH3T3-Ras cells were serum-starved for 24 h before addition of 50 μM PD98059 and serum (10% v/v) and lysed for 24 h after treatment. Cell extracts (20 μg per lane) from NIH3T3 cells treated with and without 50 μM PD98059 were size-separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blots were performed using a rabbit Cx43 antibody or a mouse GAPDH antibody.

control cells. Binding of this protein complex was diminished upon treatment of NIH3T3 cells with PD98059 for 24 h (Fig. 4B), suggesting that its binding was dependent on the MEK-ERK pathway. Additional gel-shift assays were performed using the Cx43 promoter fragments Cx350 and Cx200 (Table 1). Figure 5A indicates that there is one main protein complex from NIH3T3-Ras cells that interacts with fragment Cx350. The binding to Cx350 was competed by excess unlabeled Cx200. In addition, promoter assays were performed to

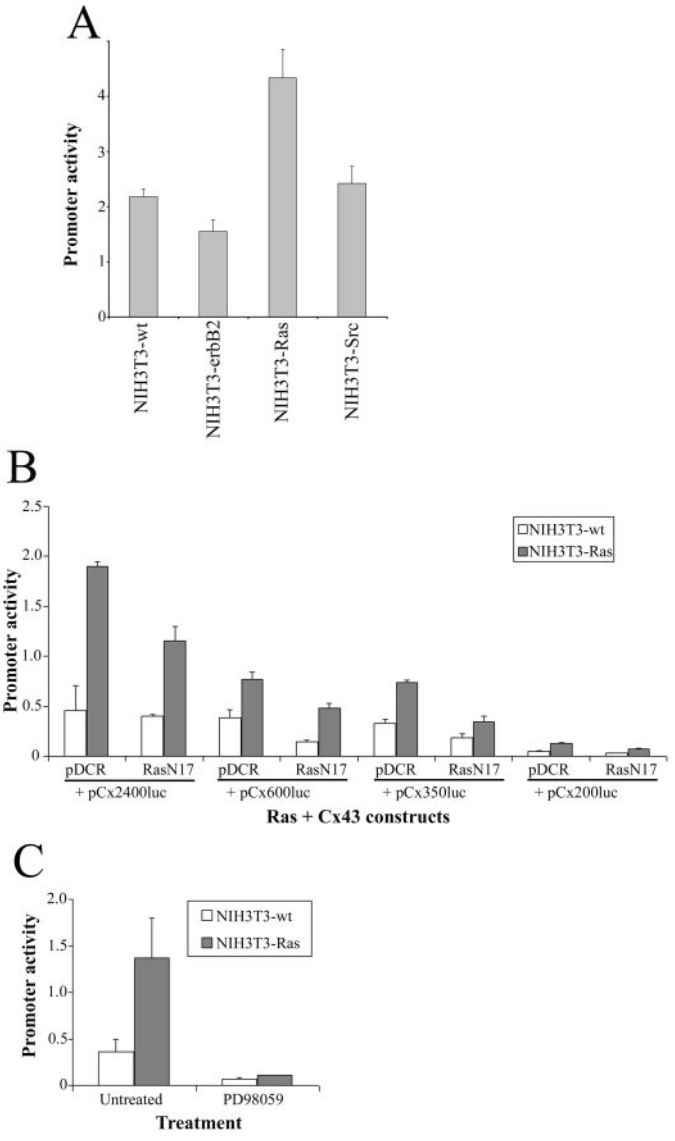


Fig. 2. Transient promoter assays on NIH3T3 cells. A, NIH3T3-wt, NIH3T3-erbB2, NIH3T3-Ras, and NIH3T3-Src cells were cotransfected with 1 μg of pCx2400luc and 0.2 μg of pRL-0 (transfection efficiency control) and lysed 2 days after transfection to perform luciferase assays. B, NIH3T3-wt and NIH3T3-Ras cells were cotransfected with 0.5 μg of Cx43 promoter constructs (Table 1), 0.5 μg H-Ras constructs (as described under *Materials and Methods*), and 0.2 μg of pRL-0 and lysed 2 days after transfection to perform luciferase assays. C, NIH3T3-wt and NIH3T3-Ras cells were cotransfected with 1 μg of pCx2400luc and 0.2 μg of pRL-0 and serum-starved overnight. On the next day, PD98059 (50 μM) was added where appropriate, and serum was added in all cells [final concentration, 10% (v/v)]. Cells were lysed 24 h after treatment with PD98059, and luciferase assays were performed. Promoter activity is a measure of firefly luciferase (Cx43 promoter luciferase constructs, Table 1) divided by *R. reniformis* luciferase (pRL-0) activities. Error bars represent S.D.

compare the activity of pCx600luc, pCx600M (pCx600luc after removal of Cx200), and pGL3-basic (Table 1) in NIH3T3-wt and NIH3T3-Ras cells. As shown in Fig. 5B, pCx600luc activity was greater in NIH3T3-Ras cells compared with NIH3T3-wt cells. On the other hand, pCx600M and pGL3-basic activities were equal in both NIH3T3-wt and NIH3T3-Ras cells. The Cx200 fragment is therefore important for the Ras-mediated induction of the Cx43 promoter and contains the recognition sequence to the protein complex that is more abundant in H-Ras-overexpressing cells.

Identification of the Cx43 Promoter Element Binding to the Protein Complex. Various double stranded oligonucleotides were designed and tested by competition-EMSAs to identify the sequence within Cx200 that is responsive to Ras. Preliminary competition studies (data not shown) revealed that the promoter sequence responsible for binding resides between positions +140 and +182 of the Cx43 promoter, where the oligonucleotide FR3 (Table 2A) competes

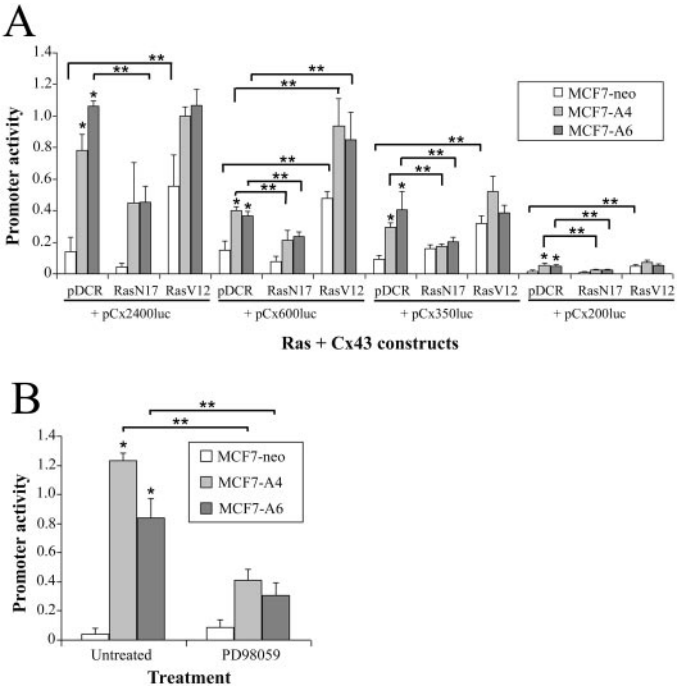


Fig. 3. Transient promoter assays on MCF7 cells. A, cells were cotransfected with 0.5 μg of Cx43 promoter constructs, 0.5 μg H-Ras constructs, and 0.2 μg of pRL-0 and lysed 2 days after transfection to perform luciferase assays. B, cells were cotransfected with 1 μg pCx2400luc and 0.2 μg pRL-0, and serum-starved overnight. On the next day, PD98059 (50 μM) was added where appropriate, and serum [final concentration, 10% (v/v)] was added in all cells. Cells were lysed 24 h after treatment with PD98059, and luciferase assays were performed. Promoter activity is a measure of firefly luciferase divided by *R. reniformis* luciferase (pRL-0) activities. Error bars represent S.D.

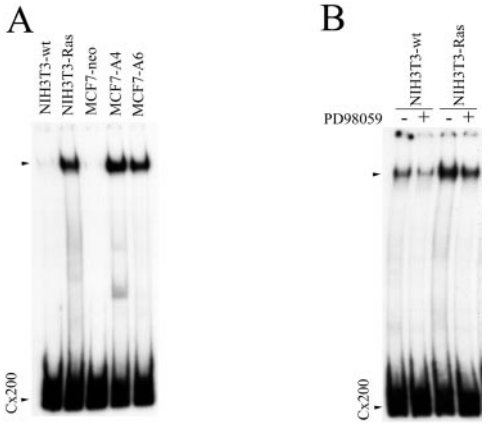


Fig. 4. Binding of nuclear components to Cx200. EMSAs were performed using the Cx43 promoter fragment Cx200 and nuclear extracts from various cell lines and treatments. A, nuclear extracts (5 μg per lane) from NIH3T3-wt, NIH3T3-Ras, MCF7-neo, MCF7-A4, and MCF7-A6 were incubated with Cx200. B, nuclear extracts (10 μg per lane) from NIH3T3-wt and NIH3T3-Ras. Cells were serum-starved overnight and subsequently treated with or without 50 μM PD98059 plus serum [final concentration, 10% (v/v)] for 24 h. Top arrows indicate shift, and bottom arrows indicate free radiolabeled Cx200.

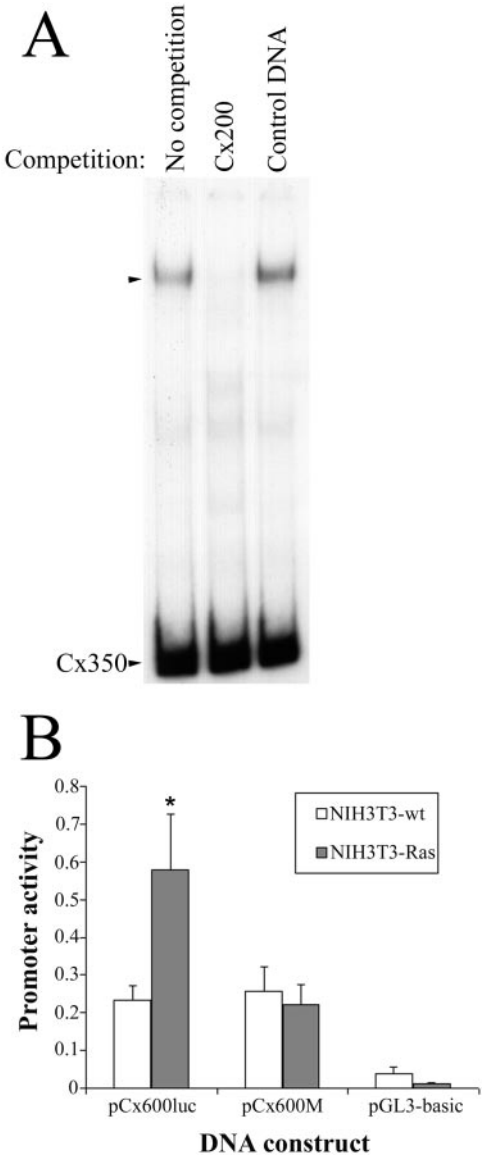


Fig. 5. Study of the involvement of Cx200 in the Ras-mediated induction of the human Cx43 promoter. A, EMSA was performed using the labeled Cx43 promoter fragment Cx350 and nuclear extracts (5 μg per lane) from NIH3T3-Ras cells. Binding to Cx350 was competed with Cx200 (100-fold in excess) or a control DNA (negative control, pGL3-basic empty vector). Top arrows indicate shift, and bottom arrows indicate free radiolabeled Cx350. B, transient promoter assays were performed on NIH3T3-wt and NIH3T3-Ras cells. Cells were cotransfected with 1 μg of promoter DNA and 0.2 μg of pRL-0 and were lysed 2 days after transfection. Luciferase assays were carried out. Promoter activity is a measure of firefly luciferase divided by *R. reniformis* luciferase (pRL-0) activities. Error bars represent S.D.

with Cx200 for binding. We then designed FR3 mutations (Table 2A) to further localize the sequence required for complex binding to Cx200. Of the oligonucleotides tested, only FR3-M3, FR3-M4, and FR3-M6 could not compete to Cx200 (Fig. 6A), indicating that the regulatory element is located between positions +149 and +158 of the human Cx43 promoter. The putative regulatory element, hCx + 149, consists of the sequence 5′-AGTTCAATCA-3′and was named Ras-responsive Cx43 element (RRCxE). This sequence is not homologous to any other known consensus sequence. To examine whether it is a noncanonical sequence of a known *cis*-element, competition-EMSA studies were performed using known consensus *cis*-elements as competitors to Cx200. Figure 6B shows that the complex could not be competed with elements to Myc-Max, Ets1/PEA3, Ets, Sp1, or AP2. The RRCxE sequence was further compared with its homologs in the mouse and rat Cx43 (Table 3) and was found to be at the same position (starting at approximately +144 to +149) from the transcription start site, within the nontranslated region of exon 1, and before the intron sequence (starting at positions +191 and +190 in human and mouse Cx43, respectively). Combining the three RRCxE sequences provided the putative consensus sequence 5′-AGTTC(A/C)A(T/C)CA-3′, as shown in Table 3.

Identification of the Components of the Protein Complex Binding to the Cx43 Promoter. Supershift assays were performed using antibodies that recognize factors involved in the protein complex that recognizes Cx200. As shown in Fig. 7, the complex binding to RRCxE was competed by antibodies against c-Myc and HSP90, but not by antibodies to AhR (negative control) or Sp1, suggesting that HSP90 and c-Myc are present in the Cx43 DNA binding complex. The c-Myc involvement in the protein complex is unusual, because the complex itself was not recognized by the Myc-Max element (E-Box), which is the predominant *cis*-element recognized by c-Myc (Fig. 6B). Also, the absence of Sp1 transcription factor has been confirmed both by supershift assays (Fig. 7) and by competition studies (Fig. 6B).

HSP90 in Cx43 Regulation. The HSP90 involvement was further confirmed by the use of the HSP90-specific inhibitor geldanamycin. The concentration of geldanamycin (2 μM) used to block the interaction of HSP90 with other proteins in NIH3T3 cells was described previously (Grenert et al., 1997; Schulte et al., 1997). Transient assays demonstrated that the activity of the human Cx43 promoter was reduced after treatment of NIH3T3 cells with 2 μM geldanamycin for 24 h, suggesting the involvement of HSP90 in Cx43 promoter regulation (Fig. 8A). Furthermore, EMSA studies indicated that NIH3T3 cells treated with geldanamycin (2 μM for 24 h) showed reduced complex binding to the Cx200 (Fig. 8B).

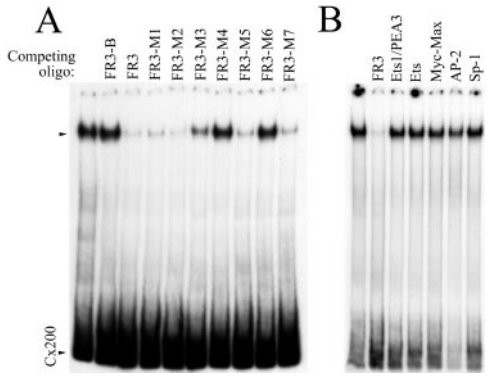


Fig. 6. Identification of the Cx200-sequence recognized by the protein complex. Competition-EMSA studies were performed using the synthesized DNA oligonucleotides (200-fold in excess of Cx200) described in Table 2A (A) or DNA oligonucleotides of known consensus sequences (200-fold in excess of Cx200) described under *Materials and Methods* (B) as competitors to Cx200. Nuclear extracts (5 μg per lane) from NIH3T3-Ras cells were combined with radiolabeled Cx200 and the indicated oligonucleotides. Top arrows indicate shift, and bottom arrows indicate free radiolabeled Cx200.

Interaction between HSP90 and c-Myc. There is no evidence in the literature that HSP90 interacts with c-Myc. Coimmunoprecipitation studies were therefore performed to prove this interaction. As shown in Fig. 9A, c-Myc-containing complexes from NIH3T3-wt and NIH3T3-Ras protein extracts were immunoprecipitated with the mouse monoclonal antibody against c-Myc and probed with a mouse antibody against HSP90. HSP90 was detected in c-Myc-containing protein complexes from NIH3T3-Ras cells (Fig. 9A). Immunoprecipitation of HSP90-containing complexes using a mouse HSP90 antibody coupled to Western blotting using a mouse monoclonal c-Myc antibody revealed the presence of c-Myc; the level of the unphosphorylated c-Myc in the complexes was greater in NIH3T3-Ras than in NIH3T3-wt cells, whereas the phosphorylated form of c-Myc was approximately equal for both cell lines.

Discussion

The human Cx43 promoter contains many putative *cis*-elements, and among the most studied are AP1 and Sp1 (Geimonen et al., 1996; Echetebu et al., 1999). An SP1 sequence is thought to be involved in at least one *trans*-element interacting with the human Cx43 promoter (Echetebu et al., 1999), and it is located upstream of +148 and does not include our putative promoter RRCxE. Other studies indicate that a downstream promoter element may be very important for Cx43 regulation. For example, the downstream region in exon 1 of the Cx43 sequence (+1 to +191), which also contains RRCxE, is important for promoter activity, and its removal reduces Cx43 promoter activity by 70% (Schiavi et al., 1999). Another study suggested that although Cx43 RNA and promoter activity are induced in response to mechanical stimulation, the responsible *cis*-element is located outside the area between −1686 and +162 of the Cx43 transcription start site (Cowan et al., 1998). Although most promoter studies have focused on the sequence upstream of the transcription start site, DPEs have been shown to be active in a number of other genes, where it allows for the docking of the transcription initiation machinery (Knutson et al., 2000; Kutach and Kadonaga, 2000; Veenstra and Wolffe, 2001).

The Ras proto-oncogenes have been implicated in many cellular pathways; both H- and K- Ras can transform cells, although K-Ras is most often found to be mutated and constitutively activated in human tumors (Lundberg et al., 2002). Although all Ras genes are targeting common downstream pathways, the differential signaling seems to be regulated by localization in the plasma membrane and such functions as endocytosis (Roy et al., 2002). The H-Ras overexpression in the cells used in this study provides a model in which to examine downstream gene targets of the important Ras signaling pathway, which has a number of different

TABLE 3

Comparison of the human *cis*-element RRCxE with the homologous sequence of the mouse and rat Cx43 promoter.

The RRCxE element of each sequence is shown in bold. The consensus sequence of RRCxE consists of the combination of the *cis*-elements from the three species. The nonhomologous bases are shown in parentheses. Putative consensus sequence, AGTTC(A/C)A(T/C)CA.

Sequence	Species	GenBank Accession No.	Position Relative to Transcription Start Site (+1)
CCTCCAAGG**AGTTCAATCA**CTTGGCGTGACTTCACTA	Human	U64573	+149 to +158
CCTCCAAGG**AGTTCCACCA**CTTTGGCGTGCCGGCTTC	Mouse	L10387	+144 to +153
CCTCCAAGG**AGTTCCACCAA**CTTTGGCGCGCCGGCTT	Rat	AH003191	+148 to +157

initiating signals. Specific affinity to different effectors could vary resulting in selective downstream responses. For example, Ras has been implicated in the stimulation of both pro-apoptotic and antiapoptotic pathways. In fibroblast cells, Ras mediates apoptosis via the Raf-1 pathway (Lin et al., 1998; Zhu et al., 1998), whereas it also mediates cell survival and proliferation via the phosphatidyl inositol 3-kinase pathway (Kauffmann-Zeh et al., 1997; Gire et al., 2000). Our data using the MEK1-inhibitor PD98059 indicate that the Cx43 promoter is stimulated via the MEK-ERK pathway, which is downstream of Raf-1. This is in agreement with previous studies associating MEK1 activity with Cx43 RNA and protein up-regulation (Hossain et al., 1999a; Bao et al., 2000). Other studies suggest that it may regulate Cx43 at a variety of different steps in its transcription, and even post-translational processing, because Ras is shown to lead to a decrease in GJIC, even as it up-regulates Cx43 protein in primary mouse keratinocytes and mouse 10T1/2 fibroblasts (Brissette et al., 1991; Nagy et al., 1996). It is noteworthy that the Wnt signaling pathway, associated with oncogenesis, has also been shown to induce Cx43 (van der Heyden et al., 1998).

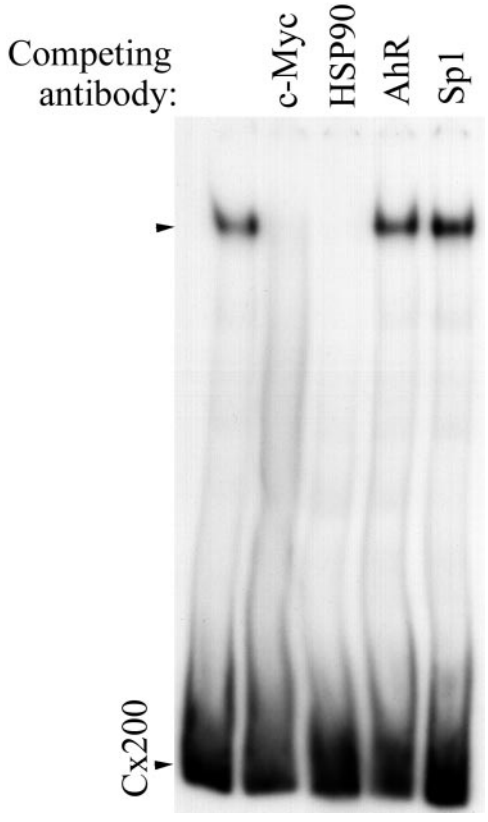
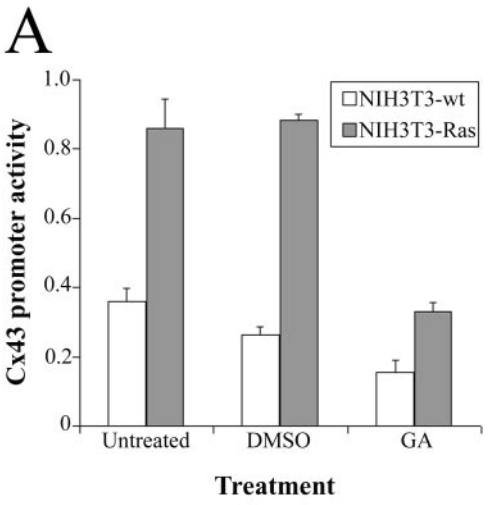


Fig. 7. Analysis of the protein complex recognizing Cx200. Supershift assays were performed using antibodies against known proteins that could be involved in the complex that recognizes Cx200. NIH3T3-Ras nuclear extracts (10 μg per lane) were incubated with radiolabeled Cx200 and antibodies to c-Myc, HSP90, AhR (negative control), and Sp1. Top arrows indicate shift, and bottom arrows indicate free radiolabeled Cx200.

We have confirmed and added to previous data that showed that the neu oncogene can inhibit connexins (Jou et al., 1995) by demonstrating that ErbB2 overexpression inhibits the human Cx43 promoter activity. Because ErbB2 (neu) receptor is known to activate proximal steps of the Ras-raf signaling pathway, the different effects of ErbB2 and Ras on Cx43 expression we observed are probably related to a distal effector, perhaps at the DNA binding complex level. Our data indicate that the protein complex binding to the Cx43 promoter contains HSP90 and c-Myc, in addition to other proteins. The Cx43 *cis*-element reported in this study does not resemble any other known *cis*-elements studied, including noncanonical and canonical (5′-CACGTG-3′) E-box sequences, which are recognized by the Myc-Max heterodimer, and we showed that an E-box sequence does not compete with the protein complex under study here, so it is unlikely that a Myc/Max complex is a component of the binding. In fact there are reports showing that Myc can act in association with other proteins, such as YY-1, AP-2, BRCA-1, Miz-1, and TFII-I (reviewed in Sakamuro and Prendergast, 1999), but so far not with HSP90. Further studies are required to examine the other components of the transcriptional complex binding this putative regulatory sequence, as well as the nature of their interaction.

Although the Myc-HSP90 interaction has not previously been described, one of the Ras effectors, Raf-1, was one of the first proteins shown to associate with HSP90. Removal of Raf-1 from the HSP90 complex leads to Raf-1 depletion (Schulte et al., 1997; Stancato et al., 1997). The association of HSP90 with Raf-1 aids in the translocation of Raf-1 within the cytoplasm. Upon Raf-1 stimulation, Raf-1 is associated with $p50^{cdc37}$-HSP90 (Silverstein et al., 1998; Grammatikakis et al., 1999). Activated Raf-1 directly associates with and activates MEKs (Huang et al., 1993; van Aelst et al., 1993), which in turn activate ERKs. Because the activated ERKs dissociate from the complex before translocating to the nucleus, HSP90 is not likely to exist in association with the entire Ras-Raf-MEK complex. However, HSP90 has been involved in protein trafficking within the cytoplasm and the interior of the nucleus (DeFranco et al., 1998; Pratt et al., 1999); we have shown previously that in this role and as part of a protein complex, it may result in regulation of carcinogen-responsive genes (Caruso et al., 1999). Geldanamycin has been shown to inhibit interaction of HSP90 with other proteins (Schulte et al., 1997; Stancato et al., 1997; Vasilevskaya and O'Dwyer, 1999), such as Raf-1, and in this report, we showed that it also inhibits Cx43 promoter activity. Our data indicate that Geldamycin inhibits Cx43 pro-



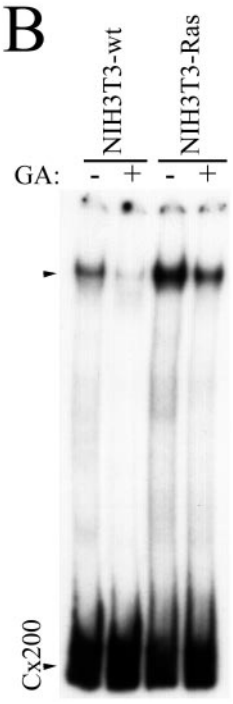


Fig. 8. Study of the involvement of HSP90 in the regulation of the Cx43 promoter. A, NIH3T3-wt and NIH3T3-Ras cells were transfected with 1 μg of pCx2400luc and 0.2 μg of pRL-0. On the next day, DMSO (control) or 2 μM geldanamycin (GA) were added in the appropriate wells. Cell lysis and luciferase assays were performed 24 h after treatment. Promoter activity is a measure of firefly luciferase (pCx2400luc) divided by *R. reniformis* luciferase (pRL-0) activities. Error bars represent S.D. B, EMSAs were performed using radiolabeled Cx200 and nuclear extracts from NIH3T3-wt and NIH3T3-Ras cells (10 μg per lane) treated with DMSO (control) or 2 μM geldanamycin (GA) for 24 h. Top arrows indicate shift, and bottom arrows indicate free radiolabeled Cx200.

moter activity as well as nuclear protein binding to the putative promoter.

Raf activation leads to c-Myc promoter induction and protein expression within 2 to 6 h after stimulation and also increases myc protein stability by inhibiting myc-degradation by the 26S proteasome (Sears et al., 1999). In the present study, the MEK1 inhibitor PD98059 led to a decrease in Cx43

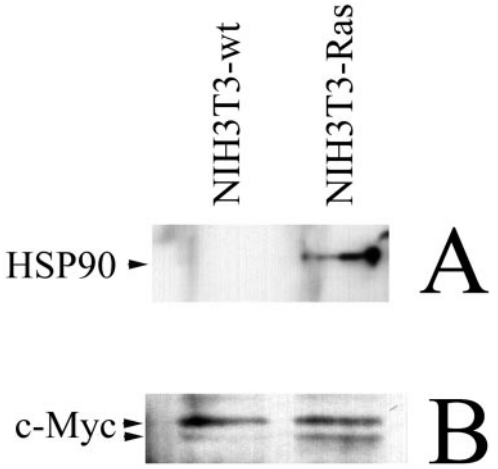


Fig. 9. Study of the interaction between HSP90 and c-Myc. Protein extracts (500 μg per lane) from NIH3T3-wt and NIH3T3-Ras cells were incubated with protein G Sepharose and either 5 μg of a mouse antibody against c-Myc (A) or 3 μl of mouse ascites of a mouse antibody against HSP90 (B). Immunoprecipitated complexes were size-fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. Western Blotting was performed using antibodies against HSP90 (A) or c-Myc (B), respectively.

promoter activity. Because it is previously established that MEK1 activity is important for c-Myc expression (Kerkhoff and Rapp, 1998), our data suggest that Ras induces Cx43 at least in part via MEK-ERK pathway induction of c-Myc.

No previous work shows a physical interaction between c-Myc and HSP90, although they are known to participate in common pathways. c-Myc is important in DNA sequence recognition and binding, and the Myc phosphoprotein contains a nuclear localization signal and its subcellular localization is tightly controlled, whereas the nature of its transport remains less defined (Lemaitre et al., 1995; Saphire et al., 1998). Because HSP90 is implicated in protein folding and trafficking (Pratt, 1993), it is possible that in this setting, HSP-90 is important in the subcellular trafficking of c-Myc and the other cofactors of the protein complex.

In summary, H-Ras overexpression leads to an increase in Cx43 protein level. This induction is caused by an increase in Cx43 promoter activity, which is mediated by a novel *cis*-element located between positions +149 and +158 downstream of the Cx43 transcription start site and is named RRCxE. This element is recognized by a protein complex that includes c-Myc and HSP90. The explanation for the apparent paradox of Cx43 promoter stimulation by a proto-oncogene signaling pathway may only be explained as we determine the nature of the promoter-binding complex.

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