Modulation of Gap Junction Transcript and Protein Expression during Pregnancy in the Rat

Boris Risek, Sarah Guthrie, Nalin Kumar, and Norton B. Gilula

Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, California 92037

Abstract. The expression of three different gap junction transcripts, α1 (Cx43), β1 (Cx40), and β2 (Cx26) was examined in several organs during pregnancy in the rat. In all of the organs that were examined—uterus, ovary, heart, and liver—there was a strong correlation between levels of gap junction mRNA and gap junction antigens that were detected at different stages of pregnancy. A striking change in α1 transcript levels (a 5.5-fold increase) was detected in the uterine myometrium on the day before parturition. This elevation of the α1 transcript is thought to be associated with the formation of gap junctions that are required for synchronizing the contractility of the myometrial cells during parturition. 2 d before parturition, there was a detectable elevation of β2 transcripts and protein in the endometrial epithelium, which was then followed by a dramatic decrease in β2 gap junctional protein on the day before parturition. There was also a substantial elevation of α1 transcripts (a 6.7-fold increase) in the stromal regions of the ovary on the day before parturition that was identical to the temporal pattern of α1 expression in the myometrium. In all three instances—the α1 transcripts in the myometrium, β2 transcripts in the endometrium, and α1 transcripts in the ovary—the transcript modulation appeared to be cell specific, because the changes in transcript levels of these three gene products occurred independently of the poly(A)+RNA concentrations at the same pregnancy stages in the respective organs. There were no specific changes detected in gap junction transcript levels in the heart and liver during pregnancy. These observations indicate that a cell-specific modulation of gap junction expression occurs in two regions of the uterus and the ovary during pregnancy. Further, it appears that the same gap junction gene in different organs, such as the α1 gene in the uterine myometrium and the heart, can be differentially regulated.

It is now well established that cell-to-cell communication in various tissues is mediated by specialized membrane regions known as gap junctions (Gilula et al., 1972; Loewenstein, 1981). Recent molecular characterizations have provided direct evidence for a family of related gap junction (GJ) proteins that form intercellular channels. This includes the identification of cDNA clones coding for three different GJ proteins with deduced molecular masses of 32 kD from mammalian liver (Paul, 1986; Kumar and Gilula, 1986), 43 kD from mammalian heart (Beyer et al., 1987), and a 26-kD protein from mammalian liver (Nicholson and Zhang, 1988). A fourth candidate is a 70-kD junction protein component of the lens fiber junctions that has an amino terminus that is homologous to those of the liver and heart GJ proteins (Kistler et al., 1988; Beyer et al., 1989). Because all of these coding sequences have a conserved region of ~200 amino-terminal amino acid residues containing four putative transmembrane stretches with the amino- and carboxy-terminal residues likely to be located on the cytoplasmic side of the junction membrane, it has been possible to deduce a generalized structure for junctional membrane protein topology (Zimmer et al., 1987; Beyer et al., 1987; Milks et al., 1988; Goodenough et al., 1988; Nicholson and Zhang, 1988; Hertzberg et al., 1988; Yancey et al., 1989).

It has been known for some time that there are significant changes in GJ populations in various female reproductive organs during hormone-dependent events. For example, the mammalian preovulatory ovarian follicle contains a large population of GJs that connect granulosa cells to each other, as well as to the oocyte itself (Gilula et al., 1978; Larsen et al., 1981). The ovarian follicle undergoes dramatic changes in structure and activity during the menstrual cycle and pregnancy. An apparent downregulation of GJs has been reported during early and late preovulatory periods for the follicular cells and the cumulus-oophorus in response to an ovulatory stimulus (Larsen et al., 1981; Larsen et al., 1986), indicating that GJ structures can be regulated differentially within the same follicular unit.

Address correspondence to Dr. Norton B. Gilula, Department of Molecular Biology, Research Institute of Scripps Clinic, 10666 N. Torrey Pines Road, La Jolla, CA 92037.

1. Abbreviations used in this paper: GJ, gap junction; KLH, keyhole limpet hemocyanin.
GJs have been also documented to provide low-resistance pathways for electrical coupling between neighboring cells in several smooth muscle cell systems (for review see Daniel, 1987). This includes the uterine myometrium, which has been shown to dramatically increase its GJ content at parturition (term) (Garfield et al., 1977, 1978, 1982; Dahl and Berger, 1978; Saito et al., 1985; Ikeda et al., 1987) or after estrogen treatment of immature animals (Burghardt et al., 1984; Garfield et al., 1978). In addition to these morphological observations, a GJ transcript has been detected in term uterus (Beyer et al., 1987) and a major 43-kD protein has been detected in uterine samples using peptide antibodies prepared to a region of the rat heart GJ protein (Dupont et al., 1988; Beyer et al., 1989).

The present system that has been used to distinguish the different GJ gene products is based principally on the molecular weights of the deduced protein products (Beyer et al., 1987). Unfortunately, this approach has limitations because protein products of many different sizes have been reported (Table I), and similar genes in different organisms can produce protein products of slightly different sizes; i.e., the mammalian liver gene product is 32 kD (Paul, 1986; Kumar and Gilula, 1986), whereas the Xenopus liver gene product is 30 kD (Gimlich et al., 1988). Consequently, we have applied a Greek nomenclature that emphasizes genetic origin rather than protein product size. For this purpose, the gene for the 43-kD protein from the heart (Beyer et al., 1987) has been designated as the α1 GJ gene, because the sequence for this protein is significantly different from those for the 32- and 26-kD proteins from the liver (Paul, 1986; Kumar and Gilula, 1986; Nicholson and Zhang, 1988). The sequences for the other two proteins are quite similar, and therefore have been designated as members of the β class of GJ genes: the 32-kD gene is referred to as the β1 gene and the 26-kD gene is referred to as the β2 gene (see Table I).

In this study we have examined the expression of three different GJ transcripts (α1, β1, β2) and their related protein products in various tissues during different stages of pregnancy to determine if there is a modulation of GJ expression in response to hormonal stimuli during pregnancy.

Materials and Methods

I. Animals and Tissue Collection
Timed pregnant Wistar rats (220–250 g body weight), with a gestational period of 22 d, were obtained from Simonsen Laboratories (Gilroy, CA). Day 0 of pregnancy was defined by the presence of a uterine plug.

Table I. Gap Junction Genes and Related Products

| Gene | mRNA size (kb) | Deduced protein product size (kD) | Reported protein product sizes (Cx) | Reference* |
|------|----------------|----------------------------------|-----------------------------------|------------|
| α1   | 3.6            | 43                               | 44-47, 43, 30                     | Beyer et al., 1987 |
| β1   | 1.6            | 32                               | 32, 28, 27, 26                    | Paul, 1986; Kumar and Gilula, 1986 |
| β2   | 2.8            | 26                               | 26, 22, 21                        | Nicholson and Zhang, 1988 |

* Denotes reference to deduced protein product sizes.

II. Immunoblot Analysis and Immunohistochemistry
Affinity-purified antibodies prepared against synthetic peptides corresponding to a postulated cytoplasmic domain of the human β1 liver (Milks et al., 1988), the mouse β2 liver (Nishi, M., N. Kumar, and N. B. Gilula, manuscript in preparation), and rat α1 heart GJ protein (Beyer et al., 1987) were used for the immunohistochemistry. This region of protein sequence has been designated the J region (Milks et al., 1988). The J epitope lies in a similar cytoplasmic surface location for these three proteins (α1, β1, β2) according to current models of GJ topology (Zimmer et al., 1987; Beyer et al., 1987; Milks et al., 1988; Nicholson and Zhang, 1988), but is completely divergent in sequence for the α and β GJ proteins. The amino acid sequence for the J region in the α1 protein extends from residues 131 to 142, from residues 111 to 125 for the β1 protein, and from residues 112 to 125 for the β2 protein (KNEFKDEIEKTQK). These three peptide antibodies are designated in this study as αJ, β1J, and β2J, respectively. Another peptide antibody that was prepared to a portion of the carboxy-terminal domain of the β1 protein sequence has also been used in this study. This domain extends from residues 252 to 280 and has been designated as region S (Milks et al., 1988), and the antibody is identified as β1S. All antibodies were prepared and characterized as described previously (Milks et al., 1988). The β1J, β2J, and αJ antibodies were affinity-purified using corresponding synthetic peptide matrices. The β1S antibody was affinity-purified using the purified native β1 GJ protein.

βJ and αJ peptide antibodies were analyzed for reactivity and specificity by immunoblot analysis using purified GJ protein isolated from rat liver or rat heart, respectively. Rat liver GJs were isolated by using the alkaline extraction procedure of Herzberg (1984) as modified by Zimmer et al. (1987). Purified GJs isolated from rat heart according to the procedure of Manjunath and Page (1986) were kindly provided by Dr. Mark Yeager (Research Institute of Scripps Clinic, La Jolla, CA). βJ peptide antibodies were characterized by immunoblot analysis of NaOH-insoluble homogenates prepared from nonpregnant liver and d-2 endometria. Tissues were alkali extracted (Hertzberg, 1984) and applied for analysis without further purification after protein determination (Lowry et al., 1951). SDS-PAGE and immunoblots were performed essentially as described by Milks et al. (1988), using the procedures of Laemmli (1970) and Towbin et al. (1979).

For indirect immunohistochemistry, small pieces of rapidly frozen fresh tissue were sectioned on a cryostat (Mimotome; International Equipment Co., Boston, MA). Sections (3 to 5 μm) were collected on gelatinized slides and incubated with 5% BSA, 3% normal goat serum (Vector Laboratories, Burlingame, CA) in PBS (10 mM Na phosphate, pH 7.5, 0.9% NaCl) for 1 h at room temperature to reduce nonspecific binding. Incubation with peptide antibodies or preimmune rabbit IgG (5-10 μg/ml in 3% normal goat serum in PBS) was performed overnight at 4°C, followed by washing in PBS (three times for 15 min each). FITC-conjugated Fab’2 goat anti-rabbit IgG (Cappel Laboratories, West Chester, PA) diluted 1:100 in PBS was then added and incubated at room temperature for 1 h. After incubation with the secondary antibody, the slides were washed (three times for 15 min each) in PBS and mounted in PBS containing 0.1% N, N, N’, N’-tetramethyl-p-phenylenediamine, 90% glycerol. Immunolabeling was analyzed using a Zeiss Axiophot microscope with epifluorescence. All photographs were taken with Kodak T-MAX 400 black-and-white film (Eastman Kodak Co., Rochester, NY).

III. Extraction of Nucleic Acids and Normalization of poly(A)+ RNA
Total RNA was isolated by pulverization and homogenization of quick fro-
zen tissues in guanidine isothiocyanate (Chirgwin et al., 1979) followed by removal of DNA by overnight precipitation in 2 M LiCl (Palmiter, 1973). RNA was quantified by absorbance at 260 nm, and normalized for poly (A)+. RNA content by oligo(dT) hybridization as described by Harley (1987). In brief, total RNA was denatured by 5 × SSC (5 × SSC contains 0.15 M NaCl, 0.015 M Na citrate, pH 7.0) and 7.4% formaldehyde for 15 min at 65°C, and then applied to a nitrocellulose membrane (1 μg/100 μl) using a Miniifold apparatus (Schleicher & Schuell, Keene, NH). A poly(A)+ standard (Pharmacia LKB Biotechnology, Inc., Pleasant Hill, CA) was quantified by absorbance at 260 nm and spotted on nitrocellulose as described above. After baking at 80°C under vacuum for 2 h, the nitrocellulose-bound samples were prehybridized in 5 × Denhardt's (5 × Denhardt's contains 0.02% BSA, 0.02% Ficol, 0.02% polyvinylpyrrolidone), 5 × SSC, 100 μg/ml tRNA at 37°C for 2 h, followed by hybridization with 32P end-labeled oligo (dT) (Pharmacia Fine Chemicals, Piscataway, NJ) overnight at 37°C. Finally, the nitrocellulose was washed four times for 15 min each in 2 × SSC at room temperature, prior to exposure to Kodak XAR-5 film at -70°C. Single spots (duplicates) were cut and counted using Cerenkov radiation to quantitate the bound radioactivity. The concentrations of total RNA from all of the tissues were adjusted, based on the results of the oligo(dT) hybridization assay, in order to utilize equal amounts of poly(A)+RNA for Northern blot analysis. The normalization of total RNA was verified by a second oligo(dT) hybridization.

IV. Northern Blot Analysis

Total RNA aliquots, normalized to equivalent amounts of poly(A)+RNA, were separated by electrophoresis on 1% agarose gels containing 0.6 M formaldehyde, and transferred to nylon membranes (Micron Separations, Inc., Westboro, MA) for hybridization with three different GJ cDNAs. (a) Human β1-GJ cDNA: the human liver cDNA that codes for a 32 kD GJ protein (Kumar and Gilula, 1986). (b) Rat α1-GJ cDNA: this is the cDNA clone isolated from a rat granulosa cell cDNA library, and it codes for a 43 kD GJ protein. (c) Mouse β2-GJ cDNA: this cDNA clone was isolated from a mouse liver cDNA library (Nishi, M., N. M. Kumar, and N. B. Gilula, manuscript in preparation) and it codes for a 26-kD GJ protein. Hybridizations with the α1- and β2-GJ cDNAs were performed in 50% formamide, 5 × SSPE (5 × SSPE contains 150 mM NaCl, 10 mM NaH2PO4, 1 mM EDTA-Na2), 5 ×× Denhardt's, 100 μg/ml tRNA, followed by washing in 0.1× SSC at 65°C. Hybridization conditions for the human β1-GJ cDNA were similar, except that 40% formamide was used, followed by washing in 2 × SSC at 55°C. Blots were processed by autoradiography at -70°C using Kodak XAR-5 film with an intensifying screen for 3 d. Signal intensities were quantified by densitometry using a LKB laser scanner (UltroScan IL; Bromma, Sweden). Multiple exposures were taken of the blot to allow for linearity in x-ray film response.

V. Screening of cDNA Libraries

A cDNA library constructed in lambda gt11 (Huynh et al., 1984) from rat granulosa cell mRNA was generously provided by Dr. Joanne Richards (Baylor College of Medicine, Houston, TX). Approximately 5 × 105 phages were screened at low stringency (20% formamide, 5 ×× Denhardt's, 5 × SSC, 100 μg/ml tRNA, 37°C; washing in 5 × SSC at 45°C) using a synthetic oligonucleotide corresponding to the amino acid residues 201–215 of the endogenous α1-GJ protein sequence (Beyer et al., 1987). Five hybridizing plaques were isolated, and then subcloned into the M13mpl9 vector for DNA sequencing. The cDNA clone coding for the mouse β2-GJ protein was isolated from a mouse liver cDNA library (Nishi, M., N. M. Kumar, and N. B. Gilula, manuscript in preparation) using a synthetic oligonucleotide corresponding to amino acid residues 1–15 of the endogenous rat liver protein (Nicholson et al., 1987). The cDNA library was constructed from mouse liver mRNA according to the procedure of Gubler and Hoffman (1983). Sequence from the endogenous 3'-end of the α1-GJ protein sequence (Beyer et al., 1987) was used to screen and select an α1-GJ cDNA from a rat granulosa cDNA library. The cDNA has been sequenced (Risek, B., N. M. Kumar, and N. B. Gilula, manuscript in preparation), and it is identical to the reported rat heart α1-GJ cDNA (Beyer et al., 1987) in the 5'-untranslated and coding regions at the nucleotide level. However, the rat granulosa α1-GJ cDNA differs in sequence from the heart α1-GJ clone in the 3'-untranslated region. The rat granulosa cDNA codes for a protein with a predicted molecular mass of 43 kD that is identical to the size and amino acid sequence predicted from the heart cDNA (Beyer et al., 1987).

VI. DNA Sequence Analysis

α1-GJ cDNA clones were excised from the bacteriophage vector and inserted into the Eco RI cloning site of M13mpl9 for DNA sequencing by the modified chain termination method using the USB Sequenase kit (Tabor and Richardson, 1987). Internal sequence data were obtained by using synthetic oligonucleotides that were synthesized with a synthesizer (model 380A; Applied Biosystems, Inc., Foster City, CA). Sequence data were analyzed using PCGene software from IntelliGenetics (Palo Alto, CA).

VII. SI Nuclelease Protection Analysis

For detection of low abundance rat β1-GJ transcripts, total RNA was hybridized in solution with a single-stranded 269-nt Kpn I fragment from a rat β1-GJ cDNA (Kumar and Gilula, 1986). The probe was biotinylated from a (+)-strand M13mpl9 subclone using the large fragment of DNA polymerase I as described by Gilmich et al. (1988). The double-stranded extension product was digested with restriction endonuclease Kpn I (New England BioLabs, Beverly, MA) followed by electrophoretic purification of the antisense fragment on a 4% polyacrylamide gel (containing 7 M urea), and subsequently localized by autoradiography. After hybridization with total RNA (1 ng/ml) in 80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM Pipes (pH 6.4), at 50°C, residual single-stranded probe was digested at 37°C with SI nuclease (Bethesda Research Laboratories, Gaithersburg, MD) as described by Berk and Sharp (1977). DNA fragments protected by annealing with RNA were separated by electrophoresis in a 6% polyacrylamide gel containing 7 M urea. Bound radioactivity was detected by autoradiography at -70°C using Kodak XAR-5 film with an intensifying screen.

VIII. Thin-Section Electron Microscopy

Immediately after decapitation, the middle part of the d-2 and d 0 uterine horns were initially fixed in situ by injection of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) into the muscle layer for 10 min. The tissue was then excised, cut into 1 × 2-mm pieces, and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate for 60 min. at room temperature. After fixation, tissues were rinsed several times in 0.1 M cacodylate buffer, and then postfixed and embedded in epoxy resin as described previously (Gilula et al., 1978). Thin sections were cut with a diamond knife on a LKB Ultratome III, mounted on copper grids, post-stained with uranyl acetate and lead citrate, and examined with a Philips CM-12 electron microscope.

Results

Cloning a Rat Granulosa Cell α1 GJ cDNA

A synthetic oligonucleotide probe corresponding to amino acid residues 210–215 of the rat α1-GJ protein (Beyer et al., 1987) was used to screen and select an α1-GJ cDNA from a rat granulosa cDNA library. The cDNA has been sequenced (Risek, B., N. M. Kumar, and N. B. Gilula, manuscript in preparation), and it is identical to the reported rat heart α1-GJ cDNA (Beyer et al., 1987) in the 5'-untranslated and coding regions at the nucleotide level. However, the rat granulosa α1-GJ cDNA differs in sequence from the heart α1-GJ clone in the 3'-untranslated region. The rat granulosa cDNA codes for a protein with a predicted molecular mass of 43 kD that is identical to the size and amino acid sequence predicted from the heart cDNA (Beyer et al., 1987).

RNA Normalization and Peptide Antibody Specificities

For Northern blot analysis, the total RNA from each sample was "normalized" to a constant poly(A)+ RNA content using an oligo(dT) dot blot hybridization technique (see Materials and Methods and legend to Fig. 1 for details). An example of the RNA normalization for myometrial RNA samples is presented in Fig. 1.

The two peptide antibodies used for most experiments, termed βJ and αJ, were prepared against portions of the cytoplasmic loop of the βJ- and αJ-GJ proteins. The specificity of these antibodies was determined by immunoblot analysis (Fig. 2). Neither of the preimmune IgGs reacted with the native βJ- or αJ-GJ proteins. The βJ antibody bound specifically to the 32-kD βJ-GJ protein that is present in rat liver, as well as to related multimers or large proteolytic fragments (>20 kD). The αJ antibodies reacted specifically with the 43-kD GJ protein prepared from rat heart, and to its related multimers and proteolytic fragments. No cross-
Figure 1. Normalization of total poly(A)+RNA by oligo(dT) dot blot hybridization. 1 µg of total RNA isolated from the myometrium at different stages of pregnancy was bound to a nitrocellulose membrane and hybridized with end-labeled oligo(dT)12-18. After autoradiography, the bound radioactivity was determined for individual samples (dots) by Cerenkov counting. (A) (from top to bottom) 0.3, 1, 3, 10, 30, and 100 µg yeast RNA. (B) (from top to bottom) 0.03, 0.1, 0.3, 1, 3, and 10 ng synthetic poly(A). (C and D) Total RNA was analyzed from the myometrium of nonpregnant (n.p.), d-4, d-2, d-1, d0, d+1, d+2, and d+4 pregnancy staged animals. In C, each sample represents 1 µg total RNA as determined by 260 nm absorbance, and the samples in D contain a normalized amount of total poly(A)+RNA.

Figure 2. Immunoblot of isolated rat liver (β1) and rat heart (α1) gap junction proteins with β1J and α1J peptide antibodies. (A) Coomassie blue-stained SDS-PAGE of isolated GJ proteins from liver (L) and heart (H). (B) Immunoblot of a gel identical to that in A, incubated with affinity-purified preimmune IgG obtained from rabbit before immunization with β1J/KLH conjugate. (C) Identical immunoblot incubated with affinity-purified β1J peptide antibodies. These antibodies strongly react with the 32-kD β1 protein (small arrowheads) and its related multimers. There is no detectable cross-reaction with the α1J protein. (D) Immunoblot treated with affinity-purified IgG obtained from rabbit before immunization with α1J/KLH conjugate. (E) Immunoblot incubated with affinity-purified α1J antibodies. These antibodies react with the 43-kD α1 protein (large arrowheads) as well as with the proteolytically related products of 34 and 32 kD. α1J antibodies do not cross-react with the β1J-GJ protein.

Figure 3. Northern blot analysis of β1- and α1-GJ transcripts in total RNA isolated from the rat heart, liver and ovary at different stages of pregnancy. Total RNA samples containing equal amounts of poly(A)+RNA were transferred to a nylon membrane and probed at high stringency (50% formamide, 37°C, washing in 0.1x SSC at 65°C) with the rat granulosa cell α1-GJ cDNA (top and bottom), or the human liver β1-GJ cDNA (middle). A transcript of 3.6 kb was detected in cardiac (top) and ovarian (bottom) samples with the α1-GJ probe. In the liver (middle), a transcript of 1.6 kb was detected with the β1-GJ probe. Signal intensities were analyzed by densitometry and are represented in Fig. 13.

Gap Junction Expression in the Heart

A 3.6-kb transcript was detected in rat heart RNA by hybridization with the α1-GJ cDNA probe (Fig. 3). The abundance of this transcript was not altered during different stages of pregnancy as determined by densitometric analysis. Also, no β1 or β2 transcripts were detected in the heart RNA samples after hybridization with the β1- or β2-GJ cDNAs (data not shown). These observations were confirmed by indirect immunofluorescence analysis using α1J antibodies.

Gap Junction Expression in the Liver

An analysis of β1-GJ transcripts in liver during different stages of pregnancy using the human liver β1-GJ cDNA probe is shown in Fig. 3. The size of the detected transcript was 1.6 kb. Relative to the transcript level in nonpregnant animals, which was defined as "1," the transcript abundance was significantly reduced in pregnant animals during the prepartum period (d-4, -2, and -1 d before delivery), reaching the lowest value of 0.42 at d-1. On the day of parturition (d0), 3–6 h after delivery, the abundance was decreased to 0.8 of the nonpregnant level. In postpartum animals at d+1, the β1-GJ transcript abundance reached the nonpregnant sam-
ple level. A slight decrease was consistently observed at d+4. With the same hybridization conditions, no $\alpha$ or $\beta_2$ transcripts were detected in the liver RNA after treatment with the $\alpha_\infty$ or $\beta_2$-GJ cDNAs (data not shown).

The observed pre- and postpartum changes in liver $\beta_1$-GJ transcripts were supported by indirect immunofluorescence using the $\beta_1$ peptide antibodies. Fig. 4 (right) contains sections of liver from nonpregnant, d-2, d0, and d+2 animals treated with the $\beta_1$ antibodies. There was prominent punctate staining at the cell borders with comparable intensity and distribution in the nonpregnant, d0, and d+2 specimens. In contrast to nonpregnant and postpartum liver, in d-2 samples the staining was patchy, with only a few regions showing intense pericellular fluorescence (Fig. 4 h). In other regions, the amount of staining was much lower; the immunofluorescence was detectable as small faint spots. Similar results were obtained in samples from d-4 and d-1, in agreement with the decrease in junctional transcripts observed at these stages using Northern blot analysis. Thus, both the $\beta_1$-GJ transcripts and the corresponding protein appeared to be downregulated in the liver of prepartum rats, relative to nonpregnant and postpartum animals.

**Gap Junction Expression in the Ovary**

Hybridization of total RNA from ovaries using the $\alpha_\infty$-GJ cDNA resulted in detection of a 3.6 kb transcript (Fig. 3). Using the same stringency conditions, hybridization with the $\beta_1$-GJ and $\beta_2$-GJ cDNAs failed to detect any transcripts. The abundance of the 3.6-kb transcript was significantly altered during pregnancy, increasing from essentially nonpregnant levels at d-4 and d-2 to peak at d-1 (6.7-fold over the nonpregnant level). The level declined rapidly at d0 (2.5 times), reached the non-pregnant level again at d+2, and remained constant at d+4.

These observations were correlated with changes in the immunostaining of GJ protein in the ovaries. Immunofluorescence analysis on the nonpregnant ovary using the $\alpha_1$ antibodies indicated extensive staining between granulosa cells of the preovulatory ovarian follicles (Fig. 5 a). In some regions this staining was punctate, and in other areas it formed a continuous cell surface rim, consistent with very high levels of GJ protein. In addition, the theca externa surrounding the follicles contained some punctate staining, as well as the stromal cells of the postovulatory corpora lutea (Fig. 5 b). In ovaries from animals at different stages of pregnancy, there was no detectable alteration in the intensity or density of GJ staining in the theca externa or in the preovulatory follicles. In contrast, there were considerable changes in the staining of the stromal regions. In the nonpregnant specimen (Fig. 5 c), there was a low level of immunolabeling. At stages d-4 and d-2, the staining pattern was more prominent (Fig. 5 d), and by d0 many of the stromal cells contained intense fluorescent spots (Fig. 5 e). The staining density decreased subsequently to reach almost the nonpregnant level at d+2 (Fig. 5 f). Thus, the temporal modulation of ovarian stroma $\alpha_\infty$-GJ protein is consistent with the results obtained by Northern blot analysis.

**Gap Junction Expression in the Uterus**

The Myometrium. Hybridization of myometrial RNA with the $\alpha_\infty$-GJ cDNA identified a single 3.6-kb band (Fig. 6), identical to that found in the heart and ovary RNA. The abundance of this transcript varied markedly during pregnancy. In addition, the temporal pattern of expression was strikingly similar to that previously observed in the ovary. At d4 before birth (d-4), the transcript level was comparable to that in nonpregnant animals. By d2, the level had increased 2.3 times over the nonpregnant level, reaching a maximal value at d-1 where transcripts were 5.5 times that found in nonpregnant animals. At d0, the transcript abundance rapidly declined (3.6-fold over nonpregnant level), and, at d+1, was 2-fold greater than the nonpregnant sample. The level increased slightly at d+2 relative to d+1 (data from three independent experiments) and declined at d+4 to the nonpregnant state. No $\beta_1$-GJ transcripts were detected in the myometrial RNA samples by Northern blot analysis.

The sizable enhancement of $\alpha_1$-GJ transcripts in the myometrium at d-1 was paralleled by immunofluorescence observations on $\alpha_\infty$-GJ protein in uterine sections. With the $\alpha_1$ antibodies, the nonpregnant myometrium contained occasional small fluorescent spots between the smooth muscle cells (Fig. 7 b). This staining pattern was similar in the d-1 and d+1 samples, and there was fluorescence between connective tissue cells and around the periphery of blood vessels, which are located between the circular and longitudinal muscle layers. However, a dramatic change was observed in tissue sections taken from animals 3 h after parturition (d0) (Fig. 7, c and f). Myometrial cells contained large numbers of brightly fluorescent spots along their borders, easily visible even at low magnification. This staining was distributed between the cells in the inner circular muscle layer, the outer longitudinal muscle layer, and in the decidual cells of the endometrium. (After implantation, stromal cells are transformed to decidual cells.) In the d0 specimen the fluorescent spots were frequently aligned in rows along the long axis of muscle fibers in the circular muscle layer (Fig. 7 f). Some fluorescence was also detected at the serosal surface. However, the staining was, by far, most prominent in the circular muscle layer. These findings suggested that an increase in the $\alpha_\infty$-GJ transcript level on d-1 was followed by a rapid increase in the $\alpha_\infty$-GJ protein on the day of parturition. No staining was observed in the endometrial epithelium using the $\alpha_1$ antibodies.

The presence of myometrial GJs at d0 localized in the inner circular smooth muscle layer by indirect immunofluorescence was confirmed by thin-section EM. Myometrial GJs, with a typical septilaminar structure, were found predominantly between cells in the circular smooth muscle layer (Fig. 7 g). Immediately after delivery, GJs were increased in number and in size, compared with nonpregnant uterus (data not shown).

The Endometrium. Total RNA was isolated from the endometrium at four different stages of gestation. The endometrial samples also varied in total RNA content but were normalized to contain the same amount of poly(A)+RNA. Although there were no detectable signals for $\beta_1$-GJ transcripts on Northern blots of endometrial RNA, $\beta_1$-transcripts were detected by S1 nuclease protection (Fig. 8, lane 4). Hybridization with the $\alpha_\infty$-GJ probe resulted in the detection of a 3.6-kb transcript (data not shown). Because the temporal distribution was comparable to that found in the myometrium at the same stages of pregnancy, the $\alpha_\infty$-GJ transcripts that were detected in the endometrium may repri-
Figure 5. Immunohistochemical localization of \( \alpha_1 \) antigen in frozen sections of ovary with \( \alpha_1 \)J antibodies. Bound antibody was detected with FITC-goat anti–rabbit IgG. (a) Preovulatory ovarian follicle from nonpregnant animal with extensive cell surface fluorescence outlining the follicular granulosa cells. Bar, 100 \( \mu \)m. (b) Three regions of nonpregnant ovarian tissue, follicle cells (top left corner), theca externa (below follicle), and stroma of postovulatory corpus luteum (bottom right). At this magnification, \( \alpha_1 \) antigen is detectable in the follicle and theca externa, but not in the stromal region. Bar, 50 \( \mu \)m. (c–f) Stromal regions of postovulatory corpora lutea in ovaries from animals at different stages of pregnancy (c, nonpregnant; d, d–2; e, d0; f, d+2). Bar in f, for c–f, 20 \( \mu \)m.

Figure 4. Immunohistochemical localization of \( \alpha_1 \) and \( \beta_1 \) antigens in frozen sections of heart and liver with \( \alpha_1 \)J and \( \beta_1 \)J antibodies during pregnancy. Heart and liver sections were treated with \( \alpha_1 \)J (heart), \( \beta_1 \)J (liver), or corresponding preimmune antibodies. Antibody binding to gap junction antigens was detected with FITC-goat anti–rabbit IgG. Bar, 50 \( \mu \)m. (a–e) Heart sections treated with preimmune (a) and with \( \alpha_1 \)J antibodies at different stages of pregnancy (b, nonpregnant; c, d–2; d, d0; e, d+2). (f–j) Liver sections treated with preimmune (f) and with \( \beta_1 \)J antibodies at different stages of pregnancy (g, nonpregnant; h, d–2; i, d0; j, d+2).
cence are consistent with the results obtained by EM.

much larger in size (Fig. 10 e), as compared with nonpregnant tissue where only small GJs were found (data not shown). Thus, the observations made by immunofluorescence suggested that the \( \beta \)1-GJ protein, raised against an epitope on the rat liver \( \beta \)1-GJ protein, cross-reacted primarily with \( \beta \)1-GJ protein in the endometrial epithelium. To explore this possibility, material from the liver and uterus was treated with two additional antibodies. One of these, \( \beta \)IS was prepared to a synthetic peptide sequence in the carboxy-terminal region of the \( \beta \)1 protein (Milks et al., 1988). Because the \( \beta \)2-GJ protein (26 kD) has a much shorter carboxy terminus than the \( \beta \)1-protein (32 kD), it does not include a region homologous to the S region, and consequently should not bind the \( \beta \)S antibodies. The second one, \( \beta \)JS, is an antibody prepared against a synthetic peptide to the cytoplasmic loop region of the \( \beta \)2-GJ protein. Although there is some potential antigenic homology between the \( \beta \)J and \( \beta \)JS sequences, the two antibodies clearly discriminated between the \( \beta \)1 and \( \beta \)S gene products by immunoblotting of SDS-denatured proteins (Fig. 11). Under these conditions, the \( \beta \)1 protein was detectable in nonpregnant liver, but not in the d-2 endometrial epithelium (Fig. 11 B). However, the \( \beta \)2 protein was detected in both liver (low levels) and endometrial epithelium samples (Fig. 11 C). Based on the densitometric analysis of bands shown in Fig. 11 C (lanes L and E), \( \beta \)2 protein in the d-2 endometrium was 3.5-fold greater than in liver. Because the amount of liver material applied was eight times the amount of d-2 endometrial sample (50 vs. 6.25 \( \mu \)g), the relative abundance of endometrial \( \beta \)2 protein can be estimated to be 28-fold greater than that present in nonpregnant liver.

When d-2 frozen sections of uterus were treated with the \( \beta \)JS antibodies, the level of staining was significantly reduced (Fig. 12 d), compared with the \( \beta \)J antibodies (Fig. 12 b). There was also a slight decrease in labeling density in rat liver frozen sections with the \( \beta \)JS antibodies, compared with the \( \beta \)J antibodies (Fig. 12, a and c). This may indicate that the \( \beta \)J antibodies were interacting with two types of liver GJ protein (\( \beta \)1 and \( \beta \)2), whereas the \( \beta \)JS antibodies reacted only with the \( \beta \)2-GJ protein. Analysis by immunofluorescence of liver sections from animals at different stages of pregnancy showed similar changes using both the \( \beta \)J and the \( \beta \)JS antibodies, except that the degree of labeling with the \( \beta \)JS antibodies was consistently lower (data not shown). Finally, efforts to discriminate between the \( \beta \)1 and \( \beta \)2 proteins by immunofluorescence using the \( \beta \)J and \( \beta \)JS antibodies have been ambiguous. For example, both \( \beta \)J and \( \beta \)JS antibodies detected high levels of antigen in both liver and endometrial epithelium sections (Fig. 12, a, b, e, and f). However, all of the data combined (mRNA, protein, and immunofluorescence) indicated that most of the antigen detected by the \( \beta \)J antibody in the endometrium was, in fact, the \( \beta \)1 protein, and that this was the most abundant GJ protein in the endometrial epithelium.

**Gap Junction Transcript Abundance Relative to Poly(A)+ RNA Levels during Pregnancy**

To determine if the alterations in GJ mRNA levels during pregnancy were unique for the junction mRNAs or were related to a general fluctuation of poly (A)+ RNA, the two values were determined in different organs during pregnancy and compared directly (Fig. 13).

Although the level of cardiac \( \alpha \)1-GJ mRNA was not altered during pregnancy (the observed changes were within experimental error), there was a slight increase in the steady-state level of total poly(A)+ RNA at d-1 and d0, reaching a
Figure 7. Immunohistochemical localization of α1-GJ antigen in frozen sections and thin-section EM of uterine myometrium. (a–d) Histo-
logical sections through the circular muscle layer of the uterine myometrium at different stages of pregnancy treated with α1 antibodies.
Bound antibodies were detected with FITC goat anti-rabbit IgG. (a), nonpregnant; b, d–l; c, d0 (3 h after birth); d, d+1). Bar, 20 μm.
(e) Section through the circular muscle layer of the myometrium and stromal layer of the endometrium in a nonpregnant uterus. (f) Section
through the circular muscle layer of the myometrium, decidua and epithelium of the endometrium on d0. Note staining with the αJ anti-
body in smooth muscle and endometrial decidua region, but not in the endometrial epithelium (bottom left). Bar for e and f, 50 μm.
(g) Thin-section electron micrograph of a gap junction between two smooth muscle cells in the circular muscle layer on d0 (3 h after
birth). Bar, 0.1 μm.
Figure 8. Detection of \( \alpha_1 \)-GJ transcripts in total RNA isolated from different tissues by SI nuclease protection assay. A 268-base single-stranded rat liver \( \alpha_1 \)-GJ cDNA fragment was annealed to completion with 30 \( \mu \)g total RNA. After SI nuclease digestion, a protected fragment of 224 nucleotides was detected in RNA from the endometrium (lane 4), myometrium (lane 5) and liver (lane 7), but not in the heart (lane 3) and ovarian (lane 6) samples. Lane 1 contains the probe; lane 2, 30 \( \mu \)g of tRNA.

Figure 9. Northern blot analysis of the \( \beta_2 \)-GJ transcript in total RNA isolated from the endometrium at different stages of pregnancy. Samples of total RNA containing equal amounts of poly(A)+RNA were hybridized at high stringency (50% formamide, 25°C) using the liver \( \beta_2 \)-GJ cDNA and visualized by autoradiography. Ethidium bromide staining of total RNA (18S rRNA) (top). Varying amounts of a 2.8 kb \( \beta_2 \)-transcript were detected in the RNA samples with the \( \beta_2 \)-GJ cDNA probe (bottom). Signal intensities of 2.8-kb transcript were analyzed by densitometry and are presented in Fig. 13.

maximum at d+1 (1.45-fold increase over nonpregnant sample level). At d+2, the RNA returned to the nonpregnant level, and remained constant at d+4. Thus, the \( \alpha_1 \)-GJ mRNA level was not detectably influenced by changes in the poly(A)+RNA abundance in the heart during pregnancy. In liver, the profile of total poly(A)+ RNA was essentially the same as observed for \( \beta_2 \)-GJ mRNA (Fig. 13). Therefore, the change in liver \( \beta_2 \)-GJ transcripts was most likely due to a general alteration of poly(A)+ RNA levels in the liver during pregnancy.

In contrast to liver, the observed modulation of ovarian \( \alpha_1 \)-GJ mRNA was probably not due to changes in poly(A)+ RNA content. The steady-state level of ovarian poly(A)+ RNA gradually increased from d-4, reaching a high value of 1.7 at d+4, whereas the \( \alpha_1 \)-mRNA level reached a maximum at d-1 (Fig. 13). Thus, this significant difference in temporal profiles between \( \alpha_1 \)-GJ mRNA and total poly(A)+ RNA indicates that there was a specific modulation of ovarian \( \alpha_1 \)-GJ transcripts during different stages of pregnancy.

The temporal expression of myometrial \( \alpha_1 \)-GJ transcripts also appeared to be modulated in a specific manner, as the profile differed markedly from that obtained with total poly(A)+ RNA (Fig. 13). During all three prepartum stages examined, the concentration of total poly(A)+ RNA was \( \sim \)50% less than the pregnant level. After parturition, the abundance increased gradually, reaching the nonpregnant level at d+4. Conversely, the poly(A)+ RNA concentration in the endometrium decreased during the prepartum period to a minimum of 0.4 times at d-1; a decrease at d-1 was also observed for the \( \beta_2 \)-GJ mRNA. However, comparison of the \( \beta_2 \)-GJ mRNA profile with that for endometrial total poly(A)+RNA indicated that there was a specific modulation of the \( \beta_2 \)-GJ transcript. Whereas the relative concentration of \( \beta_2 \)-GJ mRNA increased about twice over the value at d0, the abundance of poly(A)+RNA was 0.7 times at the same time.

Discussion

In this study, the expression and distribution of three different GJ gene products has been examined in various tissues using specific cDNA probes and peptide antibodies during different stages of pregnancy. Several relevant observations have resulted from this analysis. First, a cell-specific modulation of the \( \alpha_1 \)-GJ transcripts has been observed in the myometrium and ovary during pregnancy. Whereas the expression of \( \alpha_1 \)-GJs in the myometrium and ovary was modulated in a similar manner, the heart \( \alpha_1 \)-GJs were expressed constitutively. Second, the \( \alpha_1 \)-GJ antigen was localized in the circular and longitudinal smooth muscle layer of the myometrium, in the endometrial decidua, in the ovarian stroma, and in the heart. Third, identification, transcript modulation, and cellular localization of the \( \beta_2 \)-GJs were described in the endometrial epithelium. Fourth, a modulation of \( \beta_2 \)-GJ transcripts and protein was observed in the liver during the pre- and postpartum period of pregnancy. Finally, it was possible to determine temporal differences for maximal myometrial \( \alpha_1 \)- and endometrial \( \beta_2 \)-GJ expression during pregnancy.

In pregnant animals, the highest concentration of \( \alpha_1 \)-GJ mRNA was found in the myometrium and ovaries 1 d before delivery (d-1), resulting in a maximal increase of \( \alpha_1 \)-GJ structures at d0 as determined by immunofluorescence. Because there was no detectable alteration of \( \alpha_1 \)-GJ expression in the heart, these findings indicate that there was a tissuespecific modulation of the \( \alpha_1 \)-GJ transcript in the myometrium and ovary. In the myometrium, both the outer longitudinal and inner circular smooth muscle layers contained \( \alpha_1 \)-GJ protein, with a more prominent immunostaining in the circular muscle layer. No \( \beta_2 \)-GJ transcripts were detected in endometrial RNA by Northern blot analysis, but...
they were detectable using the more sensitive S1 nuclease protection assay. Clearly, the most abundant transcript in the endometrium was 2.8 kb, and it was detected using the \( \beta_2 \)-GJ cDNA probe.

The immunofluorescence observations with \( \beta_1 \) and \( \beta_2 \) antibodies indicated that both \( \beta_1 \) - and \( \beta_2 \)-GJ proteins were exclusively localized in the endometrial epithelium. This observation was supported by both the Northern blot analysis and the S1 nuclease protection assay. However, application of \( \beta_1 \), \( \beta_3 \), and \( \beta_1 \) antibodies provided a unique discrimination between the \( \beta \)-related antigens in the endometrium. These observations suggested that the \( \beta_1 \) antibodies also interacted with the cytoplasmic J loop of the \( \beta_2 \)-GJ protein, where 5 of 15 amino acids are identical in the two proteins.
Figure 11. Immunoblot of NaOH-insoluble rat liver and rat endometrium homogenates with \( \beta_1 \) and \( \beta_2 \) peptide antibodies. (a) Coomassie blue-stained SDS-PAGE of nonpregnant liver (L, 50 \( \mu g \)) and d-2 endometrium samples (E, 25 \( \mu g \)). (b) Immunoblot of a gel identical to that in A, incubated with affinity-purified \( \beta_1 \) peptide antibodies. A polypeptide of 32 kD (large arrowhead), as well as the 46-kD dimeric form, was detected in rat liver, but not in the endometrial sample. (c) Immunoblot of a gel identical to that in A, with different amounts of endometrial material (E1, 6.2 \( \mu g \); E2, 12.5 \( \mu g \); E3, 25 \( \mu g \)), treated with affinity-purified \( \beta_2 \) peptide antibodies. These antibodies bound to a polypeptide of 26 kD (small arrowhead) present in rat liver (L) and d-2 endometrium (E). In addition, an aggregated or dimeric form of the protein was detected in liver (43 kD) and endometrium (38 kD) (d) Immunoblot treated with affinity-purified IgG obtained from rabbit before immunization.

The apparent cross-reactivity was most likely due to the presence of a conformationally similar epitope in these two proteins in frozen sections, because the \( \beta_2 \)-GJ protein was not detected on immunoblots of d-2 endometrial samples using \( \beta_1 \) antibodies. Thus, even though peptide antibodies may provide exquisite sequence specificity on SDS-denatured antigens (by immunoblotting), the same antibodies may be capable of binding to conformationally related epitopes in other proteins under immunohistochemical conditions where the antigens are not SDS denatured. Consequently, stringent criteria and appropriate controls must be applied when determining the specificity of “sequence-specific” peptide antibodies under the conditions applied for immunohistochemistry. This potential cross-reactivity is important to consider in the context of two recent studies that have reported the immunofluorescence localization of \( \beta_2 \)-GJ protein in the endometrial epithelium of the rabbit (Winterhager et al., 1988) and the rat (Beyer et al., 1989).

In contrast to the elevation of \( \alpha_1 \)-GJ transcripts in the myometrium, the increase in endometrial \( \beta_2 \)-GJ transcripts occurred one day earlier, reaching a maximal concentration at d-2. The maximal immunostaining of myometrial \( \alpha_1 \)-GJ protein was obtained immediately after delivery at d0, whereas the peak of \( \beta_2 \)-GJ structures in the endometrium was observed 2 d before birth. At birth, the \( \beta_2 \)-GJ antigen was barely detectable in the endometrium by immunofluorescence using \( \beta_1 \) antibodies. These results not only document that two different GJ proteins are abundant in the pregnant uterus, but they also demonstrate that they are regulated differently.

The differential modulation of the \( \alpha_1 \) and \( \beta_2 \) gene products that has been reported here is interesting to consider in the context of circulating ovarian steroid hormone concentrations in the pregnant rat. At the onset of pregnancy, the progesterone concentration is high and begins to fall progressively between day 15 of gestation and term (Puri and Garfield, 1982; Saito et al., 1985). During this period, \( \beta_2 \)-GJ antigen is expressed in the endometrial epithelium with an absence of \( \alpha_1 \)-GJ antigen in the smooth muscle layer of the myometrium. At the same time, the estrogen level is low.

Figure 12. Immunohistochemical localization of \( \beta_1 \) and \( \beta_2 \) gap junction and antigens in frozen sections of non-pregnant liver and d-2 uterine endometrial epithelium. Bound antibodies were detected with FITC-goat anti-rabbit IgG. Sections of non-pregnant liver (top) and d-2 endometrial epithelium (bottom) were treated with \( \beta_1 \)J (a and b), \( \beta_1 \)S (c and d) and \( \beta_2 \)J (e and f) peptide antibodies. Bar in top, 50 \( \mu m \); in bottom, 20 \( \mu m \).
in maternal tissues. Approaching delivery, progesterone declines to an undetectable level resulting in an increase in the estrogen/progesterone ratio, and one observes during this period the formation of α₁-GJs in the myometrium and ova-

dies with concomitant regression of β₂-GJs in the endometrial epithelium. Thus, it appears that the synthesis of endometrial β₂-GJs may be at least partly under the control of ovarian progesterone (progesterone from fetal origin may be additionally involved), whereas the formation of myometrial and ovarian α₁-GJs appears to be an estrogen-dependent process. Although the physiological significance of the ovarian α₁-GJ changes observed in corpora lutea is not known, it may be related to the process of luteolysis, i.e., regression of the corpus luteum after pregnancy. In contrast to the ovary, much more is known about the primary target organ, the myometrium, where formation of α₁-GJs appears to be a necessary prerequisite for synchronized muscle contraction during delivery (Garfield et al., 1977). Conversely, the preceding loss of endometrial β₂-GJs on d-1 may be, in part, involved in the process of uncoupling the fetal-maternal communication to facilitate birth at the same time that the myometrial α₁-GJs reach a maximum to permit a synchronized contraction of the uterine wall.

Although a specific modulation of GJ transcripts in steroid hormone responsive tissues has been shown in this study, the mechanisms whereby these effects are exerted in a temporal and cell-specific manner are not known. Because transcript analysis and cell-specific localization of assembled GJ proteins are restricted to steady-state analysis of corresponding mRNA concentrations, the sites of action of these regulatory controls remain to be determined. A priori, the observed transcript modulation may be a consequence of an altered rate of gene transcription, altered stability of the primary transcript or processing intermediates, or differential mRNA turnover (Darnell, 1982). However, based on current concepts of steroid hormone action (for reviews, see Yamamoto, 1985; Evans, 1988), it is likely that an increase in GJ transcripts results, in part, from a hormonally induced increase in the rate of transcription of the specific gene(s). Therefore, to understand the potential hormonal regulation of the GJ genes, it will clearly be important to identify the potential hormone-responsive elements in these genes.

This study of the pregnant animal model has provided evidence for the differential modulation of three members of the GJ gene family. Consequently, this biological model offers a unique opportunity to study processes underlying hormonal, developmental, and cell-specific gene expression of a group of important membrane proteins.

The authors are grateful for the outstanding technical assistance of Jessica Van Leeuwen, Matt Haynes, Venetia Collier, and Dan Jones, the excellent secretarial support from Cheryl Negus, and the constructive suggestions and criticisms from Dr. Bill Beers during the course of this study. We are grateful to Dr. Joanne Richards for providing the rat granulosa library. We also appreciate the opportunity to utilize peptide antibodies from Dr. Linda Milks, the β₁ cDNA clone from Dr. Miyuki Nishi, and heart gap junction protein from Dr. Mark Yeager.

This work was supported by National Institutes of Health grants GM-37904 (to N. B. Gilula), EY-06884 (to N. M. Kumar), and GM-37907 (to N. B. Gilula and N. M. Kumar).

Received for publication 12 July 1989 and in revised form 17 October 1989.

References
Berk, A. J., and P. A. Sharp. 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease digested hybrids. Cell. 12:721-732.
Beyer, E. C., L. Paul, and D. A. Goodenough. 1987. Molecular cloning of
