Preparation of a Gap Junction Fraction from Uteri of Pregnant Rats: The 28-kD Polypeptides of Uterus, Liver, and Heart Gap Junctions Are Homologous

ANTONIOS S. ZERVOS, JAMES HOPE, and W. HOWARD EVANS
National Institute for Medical Research, Mill Hill, London, N.W. 7, England

ABSTRACT A procedure for the preparation of a gap junction fraction from the uteri of pregnant rats is described. The uterine gap junctions, when examined by electron microscopy of thin sections and in negatively stained preparations, were similar to gap junctions isolated from heart and liver. Major proteins of similar apparent molecular weight (M_r 28,000) were found in gap junction fractions isolated from the uterus, heart, and liver, and were shown to have highly homologous structures by two-dimensional mapping of their tryptic peptides. An M_r 10,000 polypeptide, previously deduced to be a proteolytic product of the M_r 28,000 polypeptide of rat liver (Nicholson, B. J., L. J. Takemoto, M. W. Hunkapiller, L. E. Hood, and J.-P. Revel, 1983, *Cell*, 32:967–978), was also studied and shown by chymotryptic mapping to be homologous in the uterine, heart, and liver gap junction fractions. An antibody raised in rabbits to a synthetic peptide corresponding to an amino-terminal sequence of the liver gap junction protein recognized M_r 28,000 proteins in the three tissues studied, showing that the proteins shared common antigenic determinants. These results indicate that gap junctions are biochemically conserved plasma membrane specializations. The view that gap junctions are tissue-specific plasma membrane organelles based on previous comparisons of M_r 26,000–30,000 polypeptides is not sustained by the present results.

Gap junctions are specialized regions of the plasma membrane where proteins are specifically oriented to construct a direct hydrophilic pathway providing continuity between the cytoplasms of contacting cells (1, 2). These junctional complexes have been studied mainly by morphology (3–5) and they are now regarded as providing the channels that allow direct intercellular exchange between cells of small ions and molecules, a process extensively documented by electrical coupling, dye transfer, and metabolic cooperation studies (1, 6). The role of gap junctions in coordinating the behavior of groups of cells is well illustrated in heart tissue, where they allow synchronized beating (7), and in uterine myometrium, where their increased incidence at term is believed to be linked to a requirement for coordinated contractions during delivery (8).

Gap junction fractions have been isolated and analyzed in biochemical detail mainly from liver (9–11), lens (12, 13), and heart (14, 15). With one apparent exception (16), gap junction fractions analyzed by PAGE appear to be relatively simple membrane specializations constructed of a major polypeptide with an apparent molecular weight in the 26,000 to 30,000 range; in some instances, dimers (M_r 47,000–54,000) were observed (10). Comparative analysis of the eye lens fiber M_r 26,000 polypeptide and the liver M_r 28,000 gap junction polypeptide has shown that they differ by peptide mapping (17) and amino acid sequencing (13, 18, 19). Furthermore, peptide maping of the liver M_r 28,000 and heart M_r 30,000 polypeptides are interpreted as showing that gap junction proteins differ and are thus tissue specific (18, 29). In the present paper we describe the isolation of a gap junction fraction from uteri of pregnant rats and show by two-dimensional peptide mapping that M_r 28,000 polypeptides in gap junctions prepared from rat liver, heart, and uteri of pregnant animals are similar. The results support the conclusion that there is unlikely to be a high degree of tissue specificity of gap junction proteins.

MATERIALS AND METHODS

Preparation of Gap Junctions: Female Sprague-Dawley rats (150–200 g wt) were used to prepare the subcellular fractions. Gap junctions were prepared from the uteri of 10–15 pregnant rats at term (days 21–22 after

fertilization) by modifying methods used to obtain them from heart tissue (14, 15, 21). Uteri, separated from placentae, fetuses, connective tissue, and fat, were homogenized using an Ultraturrax (setting 5; 30 s) in 1 mM $NaHCO_3$, pH 8.2, 1 mM phenylmethylsulfonyl fluoride (PMSF),[1] ~50 ml/uterus (6–8 g wt). The homogenate was filtered through eight layers of cheese cloth and the filtrate centrifuged at 33,000 *g* for 15 min. After repeating the centrifugation, the resultant pellet was suspended and stirred for 16 h at 4°C in 100 ml of 0.6 M KI, 6 mM $Na_2S_2O_3$, 1 mM PMSF, 1 mM $NaHCO_3$, pH 8.2. The KI-insoluble pellet was washed by centrifugation (33,000 g for 15 min) in 0.6 M KI, 6 mM $Na_2S_2O_3$, 1 mM PMF, 1 mM $NaHCO_3$, pH 8.2, and then in 5 mM Tris-HCl, pH 9. The final pellet, suspended in 60 ml of 5 mM Tris-HCl, pH 10, 0.3% Na-sarcosinate, 1 mM PMSF, was layered onto four discontinuous gradients constructed of 44.5% (10 ml) and 35% (wt/vol) (10 ml) sucrose solutions containing 0.3% deoxycholate, 5 mM Tris-HCl, pH 10, and centrifuged at 108,000 *g* for 1 h (Beckman SW.28 rotor) (Beckman Instruments, Inc., Palo Alto, CA). Gap junctions were recovered at the interface of the two sucrose solutions, and then washed by repeated low speed centrifugation when suspended in 1 mM $NaHCO_3$, pH 8.2. In some preparations, aprotinin (1 mM) and leupeptin (1 mM) were also included in media used for preparation. Gap junctions were prepared from 10–12 rat hearts as described in references 14, 15. Liver plasma membranes (22) were used to prepare gap junctions by a procedure (23) involving extraction of the membranes with deoxycholate followed by fractionation in Lubrol-containing sucrose gradients. Hepatic gap junctions were mainly located at the 48–54% (wt/vol) sucrose interface.

Electron Microscopy: Samples were routinely examined by negative staining using 1% Na silico tungstate, pH 7. Gap junction pellets were processed and examined in a Philips 300 electron microscope (21).

Electrophoresis: SDS PAGE was carried out in 15% acrylamide, 0.086% bis-acrylamide, using samples dissolved by boiling for 30 s in 2% SDS, 1 mM dithiothreitol, 100 mM Tris-HCl, pH 6.8 (24). The molecular weight markers used were as follows: myosin, 200,000; phosphorylase a, 94,000; catalase, 60,000; creatine kinase, 40,000; trypsin inhibitor, 21,000; and lysozyme, 14,000, purchased from Sigma Chemical Co., St. Louis, MO.

Peptide Mapping: This was carried out essentially as described by Elder et al. (25). The Coomassie Blue-stained polypeptide bands in the polyacrylamide gels were excised and iodinated with ^{125}I (300 μCi per slice) using chloramine T (26), and digested overnight at 37°C using 50 μg diphenylcarbamyl chloride trypsin or chymotrypsin (Sigma Chemical Co.) dissolved in 0.05 M NH_4HCO_3, pH 8. The gel slices were removed and the digests lyophilized. In the mixing experiments, approximately equal amounts of radioiodinated peptides of the heart, liver, and uterus M_r 28,000 polypeptide were pooled and then applied to the plates. Samples were dissolved in electrophoresis buffer: acetic acid, formic acid:H_2O (15:5:80) (vol/vol/vol) and electrophoresed at 600 V in cellulose sheets (Eastman Kodak No. 13255); progress was assessed using 2% orange G and 1% acid fuchsin as marker dyes. After drying, chromatography was carried out in butanol:pyridine:acetic acid:H_2O (32.5:25:5:20) (vol/vol/vol/vol). The dried plates were analyzed by autoradiography overnight at −70°C using Kodak XRP-1 X-ray film.

Immunoblotting: Partially purified gap junction fractions isolated from the rat liver, heart, and uterus were resolved in 15% SDS polyacrylamide gels and electrophoretically transferred to nitrocellulose (0.1 μm) (Schleicher & Schuell Co., Dassel, Germany) in a BioRad apparatus at 10 V for 16 h (27). The efficacy of transfer and M_r of the immunoreactive proteins was monitored by simultaneous transfer of a series of pre-stained marker proteins (Bethesda Research Laboratories, Gaithersberg, MD). Nitrocellulose sheets were washed in 2% bovine serum albumin in a Tris-buffered saline (pH 7.5) and then incubated with an IgG fraction prepared from serum of rabbits immunized with a synthetic peptide corresponding to amino acid residues 7–21 of the amino-terminal sequence of the rat liver M_r 27,000 gap junction protein reported by Nicholson et al. (31). The peptide was coupled to porcine thyroglobulin. Full details of the preparation and properties of this antiserum will be reported elsewhere. After several washes using 0.1% Nonidet P-40 in Tris-buffered saline, affinity-purified ^{125}I-protein A (2×10^6 cpm/ml) (Amersham International, U.K.) in Tris-buffered saline was added, and after 1 h at 20°C the nitrocellulose was washed twice with 0.1% Nonidet P-40. Nitrocellulose sheets were autoradiographed using Kodak XAR-5 film.

RESULTS

Morphology and Biochemical Composition of Uterus Gap Junctions

In the fractionation of uteri removed from pregnant rats, material banding at the 33–44.5% interface was found to contain large numbers of gap junctions together with amorphous material when examined in the electron microscope (Fig. 1). The features of the intact gap junctions isolated from uterus were indistinguishable from those of gap junctions isolated from liver (9, 10, 18) and heart (14, 15, 29) tissues. In negatively stained preparations (Fig. 2) hexagonal arrays were evident, and the center-to-center spacing of 80–84 Å measured was similar to that in liver and heart gap junctions. While attempting to modify conditions to increase the morphological purity of the uterine gap junctions, it was found that the junctions showed a tendency to change into amorphous structures. Addition of proteolytic inhibitors failed to prevent or reduce this, and often it was possible to observe gap junction structures in the process of breakdown (Fig. 3). The high susceptibility of uterine gap junctions to breakdown during isolation may reflect the presence of large amounts of tissue proteases in the uterus during parturition. The yield of the gap junctions obtained from pregnant uteri was about 1.5 μg protein/g wet tissue weight. When the same procedure was used with a similar number of nonpregnant uteri, a very low amount of material was recovered and examination of this material by electron microscopy indicated that gap junctions were present, with a similar morphology to those prepared from pregnant animals.

Fig. 4, A–C, shows that the gap junction fractions prepared from the three tissues contained a major M_r 28,000 polypeptide that increased in intensity relative to other polypeptides present as the junctions were purified. An M_r 10,000 polypeptide, previously deduced to be a degradation product of the M_r 28,000 peptide of liver gap junctions treated with trypsin (18, 30, 31) was present in varying amounts in the gap junction fractions isolated from the three tissues. This M_r 10,000 polypeptide appeared spontaneously during the preparation, handling, and storage of the subcellular fractions. Indeed, breakdown of the M_r 28,000 polypeptide of the heart and uterine gap junction fraction occurred rapidly during handling and it was noted that the intensity of low molecular weight components, including the M_r 10,000 polypeptide, increased with time.

Peptide Mapping

The M_r 28,000 and M_r 10,000 polypeptide bands of gap junction fractions isolated from the liver, heart, and uterus were excised from polyacrylamide gels, radioiodinated, digested with trypsin or chymotrypsin, and the products then analyzed by two-dimensional mapping. Fig. 5 compares the tryptic maps of the M_r 28,000 and M_r 10,000 polypeptides. Two conclusions emerge. First, the tryptic maps were similar, indicating that the M_r 28,000 polypeptides in liver, heart, and the uterus of pregnant rats were highly homologous. This homology was reinforced in an experiment in which the tryptic peptides of the M_r 28,000 of the liver, heart, and uterus were mixed (Fig. 6, panel *A*). Tryptic digestion of other polypeptides from the gels gave dissimilar maps (Fig. 6, panels *B* and *C*). Secondly, comparison by peptide mapping of an M_r 10,000 polypeptide present in the fractions prepared from the three tissues also indicated a high degree of homology. An M_r 10,000 polypeptide, shown by Nicholson et al. (31) to be produced when intact liver gap junctions are treated with trypsin, contained an amino-terminal amino acid sequence identical to the M_r 28,000 polypeptide. It is considered that

[1] *Abbreviation used in this paper:* PMSF, phenylmethylsulfonyl fluoride.

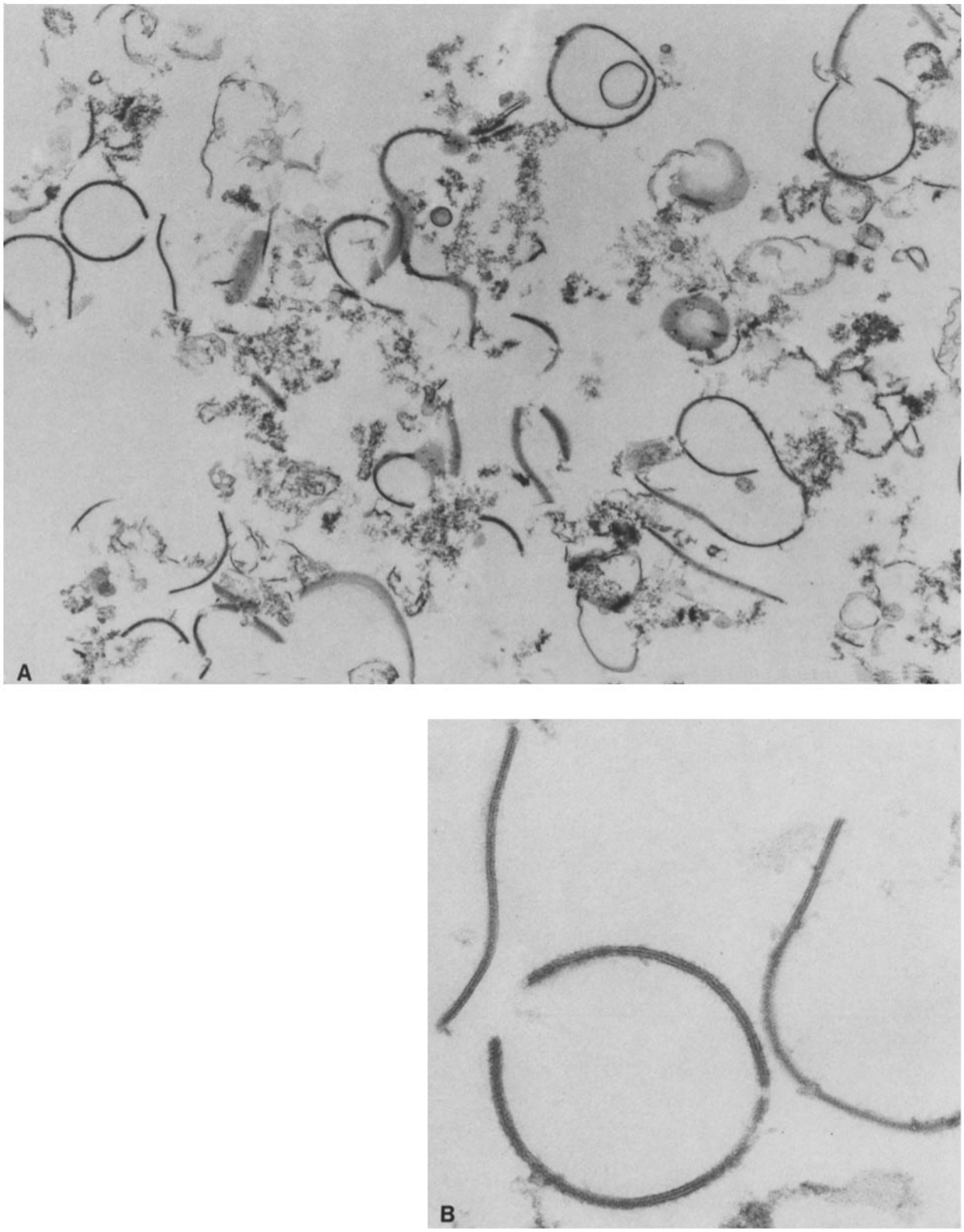

FIGURE 1 (*A*) Electron micrograph of a thin-sectioned pellet of gap junction fraction prepared from uteri of pregnant rats. × 19,500. (*B*) Higher magnification (× 103,350) shows the heptalaminar appearance of the uterine gap junctions.

the M_r 10,000 polypeptide now studied may correspond to a proteolytic fragment (31). Fig. 7 compares the peptide maps produced by chymotryptic digestion of the M_r 10,000 polypeptide in the gap junction fractions. Compared to the tryptic maps, a greater number of iodinated peptides were separated and the results again indicated the similarities of the chymotryptic digests of the M_r 10,000 polypeptide of liver, heart, and uterus. However, the chymotryptic maps of the heart M_r 10,000 polypeptide also showed one and possibly more further peptides to be present (Fig. 7, panel *B*), suggesting that minor differences may indeed exist between the gap junction polypeptides. Within the limits of the method, however, the results

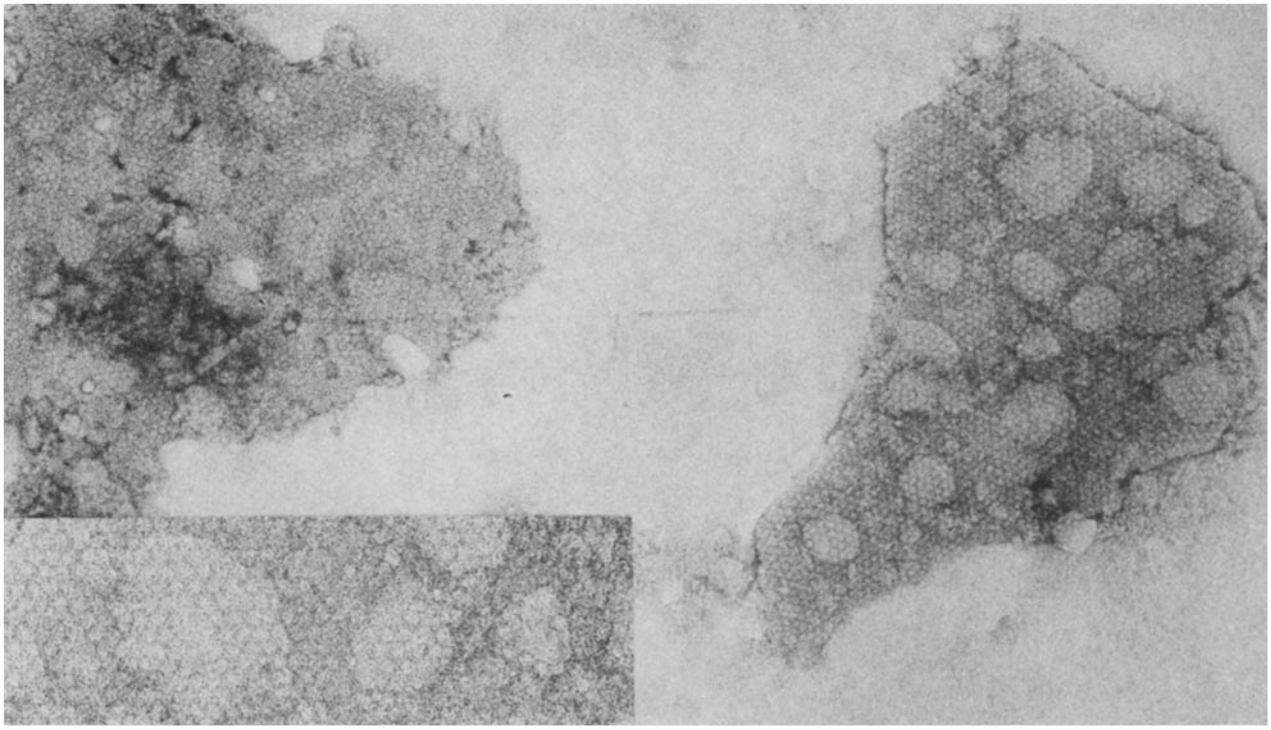

FIGURE 2 Gap junction fraction prepared from rat uterus, negatively stained with 1% Na silicotungstate, pH 7.0. × 150,000. (*Inset*) Higher magnification (× 300,000) showing hexagonal lattice.

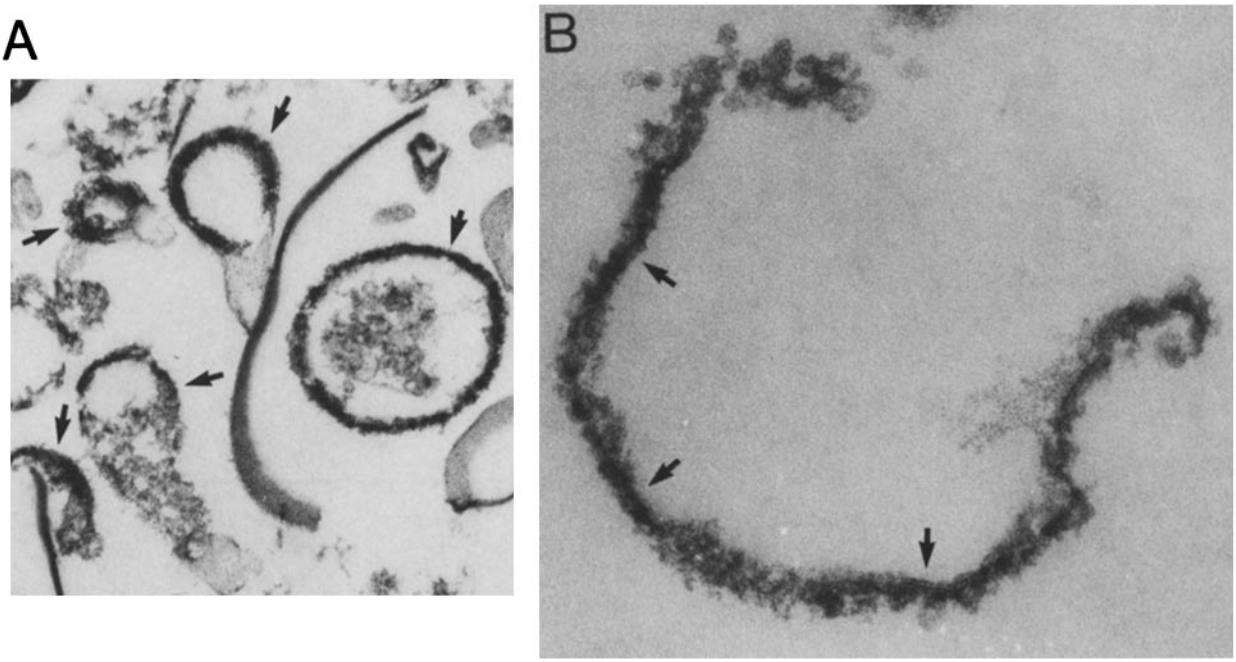

FIGURE 3 Electron micrograph of a thin-sectioned pellet of uterine gap junction fraction showing gap junction structures in the process of breakdown. (*A*) An intact gap junction in center view surrounded by others in the process of breakdown (arrows). × 64,750. (*B*) Higher magnification (× 135,500) of a uterine gap junction. Arrows point to the heptalaminar structure still recognizable between areas where degradation is evident.

indicate that the M_r 28,000 polypeptide and a possible M_r 10,000 degradative product in liver, heart, and uterine gap junction fractions are highly homologous polypeptides.

Immunological Similarities

To show that the M_r 28,000 polypeptide analyzed in the present work by peptide mapping was indeed the same gap junction polypeptide as that used by other investigators (9, 11, 31), we used an antibody raised against a peptide corresponding to an amino-terminal region of the sequence determined on the M_r 27,000 gap junction protein of rat liver (31). Fig. 8 shows that the antibodies bound specifically to the M_r 28,000 polypeptide in fractions isolated from liver, heart, and

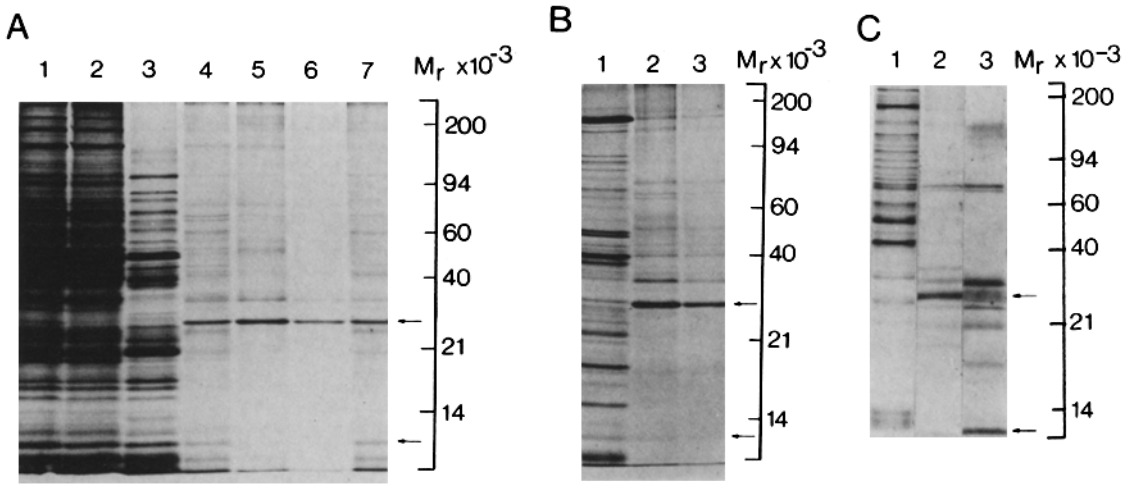

FIGURE 4 (a) SDS PAGE of the various subcellular fractions leading to the isolation of liver gap junction fractions. Lane *1*, liver plasma membrane fraction; *2*, plasma membrane proteins soluble in sodium deoxycholate; *3*, liver homogenate; *4*, liver plasma membrane components insoluble in sodium deoxycholate; *5* and *6*, gap junction fractions; *7*, a gap junction fraction showing endogenous proteolysis of the M_r 28,000 polypeptide and the appearance of an M_r 10,000 polypeptide. (*b*) Heart. Lane *1*, homogenate; *2* and *3*, heart gap junction fractions (arrows point to the M_r 28,000 and M_r 10,000 polypeptides). (c) Uterus. The uteri were removed from pregnant rats on day 21. Lane *1*, homogenate; *2* and *3*, separate preparations of uterine gap junction fractions showing the M_r 28,000 and M_r 10,000 polypeptide.

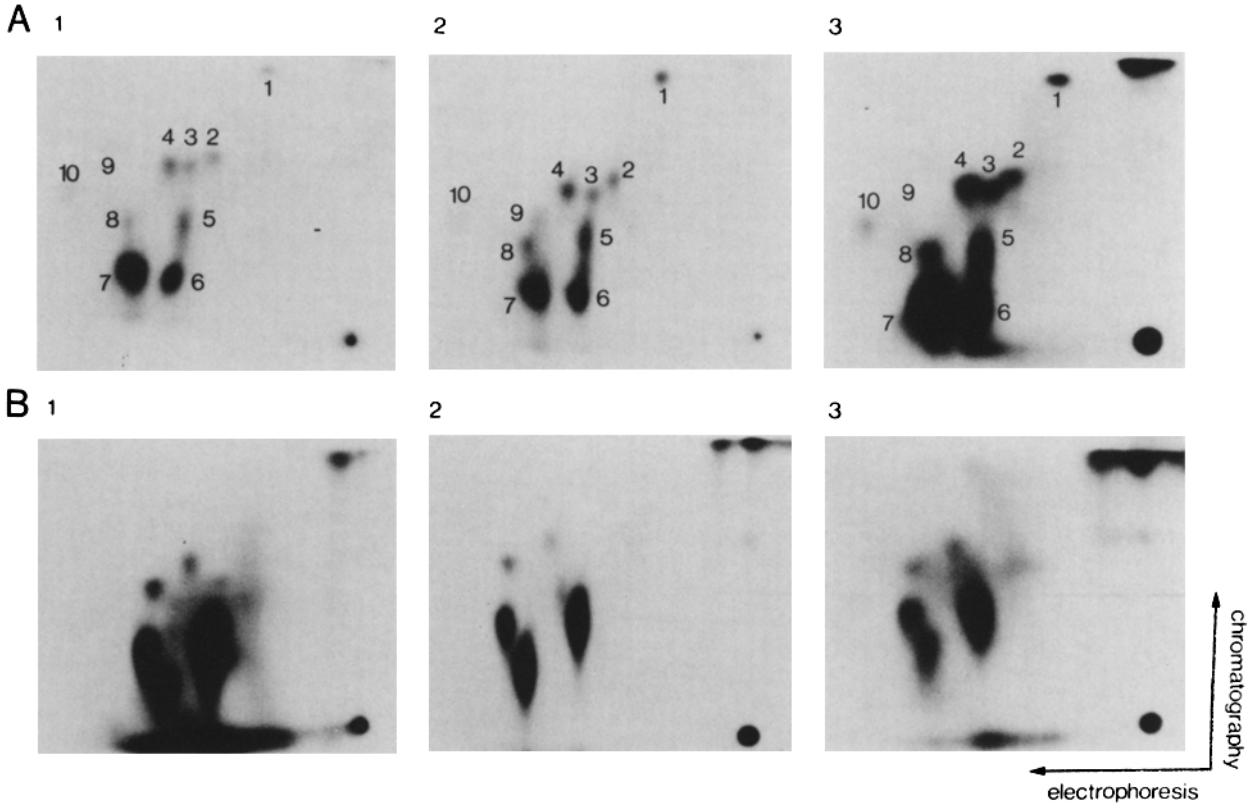

FIGURE 5 Autoradiographs of the two-dimensional tryptic peptide maps of the M_r 28,000 (*A*) and M_r 10,000 (*B*) gap junction proteins from uterus (*1*), heart (*2*), and liver (*3*).

uteri. The results confirm the cross-reactivity of this antibody with the rat liver gap junction polypeptides (20) and show that these polypeptides are immunologically homologous in the rat heart and uterus.

DISCUSSION

The morphological properties of the gap junctions prepared from the uterus of pregnant rats were similar to those of junctions obtained from cardiac (14, 15, 29) and liver tissue (9, 10, 18). In thin sections and in negatively stained preparations, no differences were discerned between gap junctions in the fractions isolated from rat liver, heart, and uteri of pregnant animals. Compared to the extensive morphological studies on junctional complexes in liver and heart tissue, the limited studies carried out with the smooth muscle of the

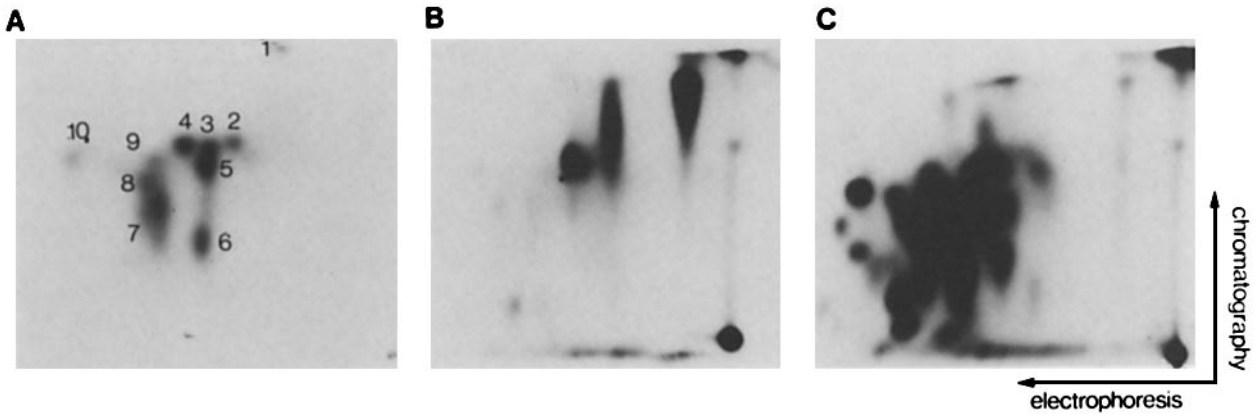

FIGURE 6 Autoradiographs of two-dimensional tryptic peptide maps of (*A*) uterus, heart, and liver M_r 28,000 polypeptide mixed in equal amounts (peptides are numbered to allow comparison with those in Fig. 5); (*B*) M_r 18,000 polypeptide of uterus; and (*C*) M_r 32,000 polypeptide of uterus.

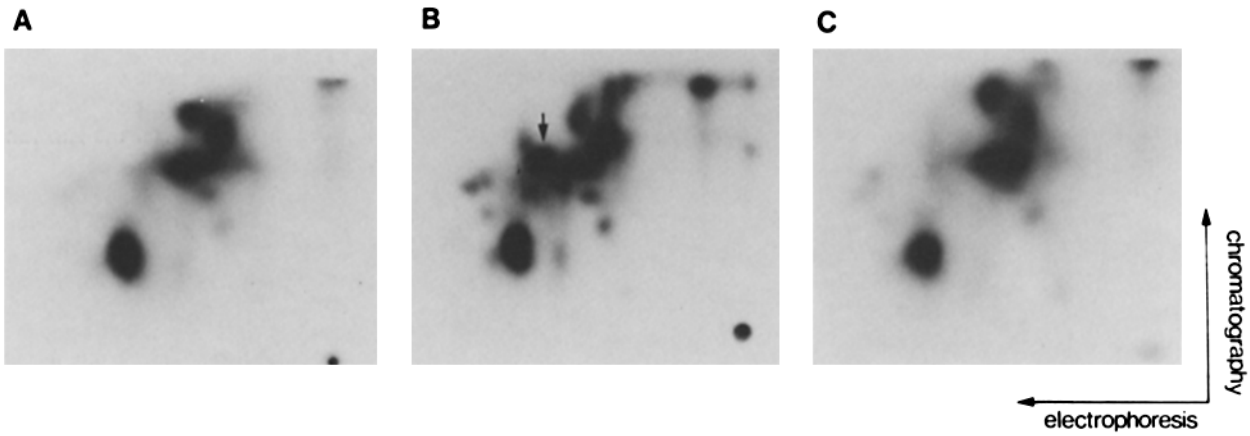

FIGURE 7 Autoradiographs of the two-dimensional chymotryptic peptide maps of the M_r 10,000 polypeptide from (*A*) uterus, (*B*) heart, and (*C*) liver. The arrow points to a peptide which is present in heart but apparently not in liver and uterus.

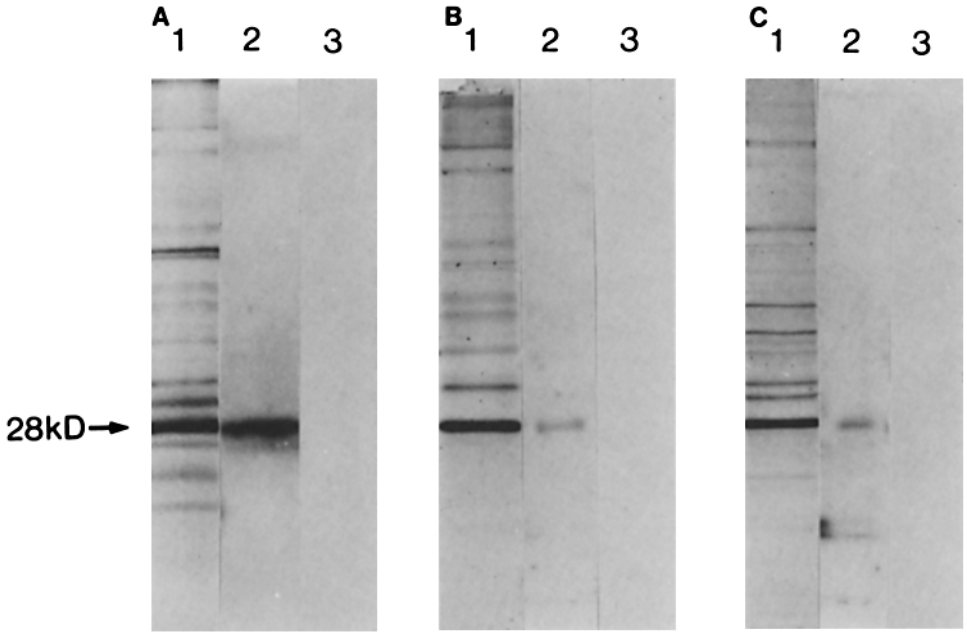

FIGURE 8 Immunoblots of partially purified gap junction fractions prepared from uterus (*A*), liver (*B*), and heart (*C*). Lanes *1* show Coomassie Blue staining of polypeptides separated by PAGE in 15% (wt/vol) gels. Lanes *2* show autoradiographs of the electrophoretically transferred polypeptides exposed to a rabbit anti-peptide antibody. Lanes *3* show antibody binding in the presence of excess peptide.

uterus indicated that although normally relatively small numbers of gap junctions were present, there was a sixfold increase in the gap junction area prior to parturition (8, 34), and this was accompanied by an increase in electrical conductivity (35). The present work used uteri removed at term and shows that the isolated junctions were morphologically similar to those investigated in other tissues (5). Minute amounts of gap junctions were also recovered from uteri removed from nonpregnant rats; these had a similar morphology, suggesting no differences between those gap junctions induced during preg-

nancy and gap junctions present in normal myometrium.

Analysis of the polypeptide composition of the uterine gap junctions showed that a major M_r 28,000 component was present. A polypeptide of similar electrophoretic mobility was also a major component in liver and heart gap junctions. Other polypeptides identified in minor amounts were possibly contaminants, especially in uterine gap junction fractions that were partially purified, but the presence of proteolytic degradation products as well as polymeric aggregates must also be considered. The inclusion of proteolytic inhibitors during the preparation of the gap junction fractions helped to minimize breakdown, but persistent action of endogenous proteases was still evident. It was noted that the gap junction polypeptide in heart and uterus fractions was extremely susceptible to breakdown, with a diffusely staining M_r 26,000–28,000 band preceding conversion into lower molecular weight components, with the M_r 10,000 polypeptide being especially prominent. The proclivity of the M_r 28,000 polypeptide to aggregate (10) may also complicate the situation. Morphological analysis of uterine gap junctions also indicated that deterioration of the characteristic gap junction heptalaminar structure was another likely consequence of proteolysis.

There is a large body of evidence showing that an M_r 26,000–29,000 polypeptide is present in gap junction fractions prepared by detergent extraction of rodent liver plasma membranes (9–11, 31). An alternative method for the preparation of liver gap junctions, involving extraction of liver plasma membranes with 20 mM NaOH, also showed an M_r 27,000 polyeptide to be a major component (32). Further supporting the candidacy of the M_r 26,000–28,000 polypeptides as major gap junction constituents are studies showing that antibodies raised against an M_r 26,000 polypeptide of mouse liver gap junctions immunolocalized to gap junction plaques (36). In contrast is a report that an M_r 16,000 polypeptide, often coexisting with an M_r 28,000 polypeptide, comprises gap junctions in mammalian liver and other tissues (16). The relationship of this component with the M_r 26,000–28,000 components awaits clarification.

Analysis of heart gap junction fractions showed a major M_r 28,000–30,000 component to be present (14, 15). Studies by Manjunath et al. (37) claim that an M_r 29,500 polypeptide comprises the intramembrane component of rat heart gap junctions, with an associated M_r 14,500–17,500 polypeptide that may correspond to a fuzzy layer, removable by serine proteases, on their gap junctions. The present results indicate that an M_r 28,000 polypeptide is the major component.

Comparative analyses of the M_r 26,000–29,000 gap junction polypeptide have been carried out with liver and lens, and liver and heart. Comparison of lens and liver gap junctions by tryptic mapping (2), analysis of cyanogen bromide peptides (13), and amino-terminal analysis (19) indicated that the major polypeptides were not homologous, despite variable results on the immunological cross-reactivity of the junctions (38, 39). These results, combined with the documentation of major morphological differences between eye lens fiber junctions and gap junctions in other vertebrate tissues (5, 40–42) have led to the view that lens fiber junctions comprise a different class of intercellular contact not necessarily involved in intercellular communication. Gros et al. (29) compared the composition of rat liver and heart gap junctions and showed that an M_r 30,000 cardiac gap junction polypeptide and an M_r 28,000 liver gap junction polypeptide were not homologous on the basis of two-dimensional tryptic maps, but they reported that a short amino-terminal sequence of amino acids of heart and liver M_r 28,000 polypeptides were similar, a result more in line with the present report.

Homology between the M_r 28,000 liver gap junction investigated in the present work and by others (9, 11, 31) was evident by showing that antibodies raised against a synthetic peptide corresponding to an amino-terminal amino acid sequence of this protein also cross-reacted, by immunoblotting with this protein (20). The present results also show that the anti-peptide antibodies also cross-reacted with an M_r 28,000 polypeptide present in heart and uterus gap junctions. The overall results now show that the major gap junction polypeptide of at least three rat tissues comprise a family of homologous proteins. However, the extent of amino acid sequence homology will only be clarified when the complete primary amino acid sequence is determined.

In conclusion, the present study describes the isolation of gap junction fractions from uteri of pregnant rats, and compares an M_r 28,000 polypeptide with a polypeptide of similar electrophoretic mobility that comprises the major component of liver and heart junctions. This study shows that gap junctions are highly conserved plasma membrane organelles from tissue to tissue. Immunoreactivity between the gap junction polypeptides of these tissues has also been shown. The results are in accord with (*a*) the demonstration that co-culture of heterologous cells resulted in gap junction–mediated intercellular communication (43–45) and (*b*) studies which show that gap junctions in several tissues shared common antigenic determinants (28, 33, 39).

We thank Kate Sullivan and Elaine Brown for electron microscopy.

A. S. Zervos thanks the Brain Research Fund for support and J. Hope thanks the Medical Research Council for a postdoctoral fellowship.

Received for publication 29 November 1984, and in revised form 18 March 1985.

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