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Tight Junction Development Between Cultured Hepatoma Cells: Possible Stages in Assembly and Enhancement With Dexamethasone

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Freeze-fracture and thin-section methods were used to study tight junction formation between confluent H4-II-E hepatoma cells that were plated in monolayer culture in media with and without dexamethasone, a synthetic glucocorticoid. Three presumptive stages in the genesis of tight junctions were suggested by these studies: 1) "formation zones" (smooth P-fracture face ridges deficient in intramembranous particles), apparently matched across a partially reduced extracellular space, develop between adjacent cells; 2) linear strands and aggregates of 9–11 nm particles collect along the ridges of the formation zones. The extracellular space was always reduced when these structures were found matched with pits in gentle E-face depressions; 3) the linear arrays of particles on the ridges associate within the membranes to form the fibrils characteristic of mature tight junctions. The formation zones resemble tight junctions in terms of size, complexity and the patterns of membrane ridges. Although some of the beaded particle specializations may actually be gap junctions, it is unlikely that all can be interpreted in this way. No other membrane structures were detected that could represent developmental stages of tight junctions. Dexamethasone (at 2×10^{-6} M) apparently stimulated formation of tight junctions. Treated cultures had a greater number of formation zones and mature tight junctions, although no differences in qualitative features of the junctions were noted.

Key words: hepatoma, cell culture, tight junctions, gap junctions, freeze-fracture, dexamethasone

Tight junctions act primarily to prevent small molecules from moving across epithelia by way of the extracellular space [1–3]. They are distributed widely in vertebrate epithelia where they are similar in basic structure though differing in complexity [2, see also 4, 5]. Their function depends on arrays of anastomosing, intramembranous fibrils which link the membranes of adjacent cells in continuous belts near the apical surface [6–9].

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To fully understand the nature of the junctional sealing elements, one must determine the precise arrangement of junctional components within the membranes and the means of interaction between junctional membranes. Studies of tight junction formation represent a useful approach to these issues. It has been suggested that formation involves rows of closely spaced particles in each membrane that make head-to-head contact [8]. Support for such a model has come from recent investigations of fetal liver that show closely spaced linear arrays of particles prior to the appearance of tight junctional fibrils [10]. Other studies report similar findings [11–14].

Experiments are reported here on the hepatoma-derived H4-II-E cell line, which we have previously shown to be capable of forming tight junctions [15]. In our current study, besides demonstrating structures much like those in fetal liver, we have observed membrane ridges remarkably free of intramembranous particles. These less differentiated structures may represent earlier stages in formation. Consequently, a series of developmental stages in the assembly of H4 tight junctions is suggested and discussed.

Furthermore, tight junction development was investigated in the presence of dexamethasone, a synthetic glucocorticoid known to influence H4 cells [16]. From our results it appears that dexamethasone enhances the frequency of H4 tight junction assembly. These findings are most interesting in view of the hypothesis that glucocorticoids modulate the differentiation of bile canaliculi [17].

MATERIALS AND METHODS

Dissociation and Culture Conditions

Cultures of H4-II-E hepatoma cells were grown in MEM plus 10% fetal calf serum (as previously described) [15]. Under these conditions the cells have a doubling time of approximately 24 h. To obtain single-cell populations, confluent cultures were dissociated with 0.05% trypsin and 1.5 mM EDTA in saline at pH 7.4 for 15 min. Following this treatment, cells were pooled and vigorously pipetted several times to ensure disruption of established cell junctions. Samples of the dissociated cell population (95–97% singles) were taken immediately after dissociation for freeze-fracturing (zero time), for cell counting, and for determining cell viability (3–8% trypan blue positive after dissociation). The remaining cells were centrifuged and resuspended in fresh growth medium (MEM plus serum). These were untreated controls. Experimentally treated cultures were plated in growth medium containing 2×10^{-6} M dexamethasone (Sigma Chemical, St. Louis, Missouri). Ethanol controls (0.1% absolute ethanol) were also run since stock solutions of free dexamethasone were prepared in absolute ethanol at a 2 mM concentration. Although detailed studies were not done, dexamethasone did appear to slow cell growth. For all the studies reported here, cells were plated at confluency ($4–5 \times 10^5$ cells/cm^2) and cultured for 4 h to 4 days after which they were fixed for electron microscopy. During the longer culture periods, cells formed multilayers and piled up in certain regions of the cultures.

Prior to carrying out these experiments on cells plated at confluency, other approaches were pursued to select the most useful method for analyzing H4 tight junction development. We first studied cultures of different ages (up to one week) and varying cell densities (up to confluency). We also dissociated cells with different combinations and concentrations of EDTA and trypsin with varying amounts of mechanical agitation. Finally, we dissociated cells, placed them in fresh growth medium with serum and spun them into a loose cell pellet to maximize cell contact [as in 18]. We found the procedure of plating cells at confluency to be most effective in maximizing the extent and synchrony of formation. Consequently, all of the data reported here come from such preparations.

Electron Microscopy

H4-II-E cultures were fixed in situ with 2.5% glutaraldehyde in 0.1 M cacodylate, pH 7.4 for 1 h at room temperature. For freeze-fracturing, after treatment in 30% glycerol buffered with 0.1 M cacodylate, the cells were gently scraped from the culture dishes with a "rubber policeman," pooled at each time sampled, frozen in gold holders and fractured by reported procedures for the Balzers BAF 301 freeze-etch device [19]. Freeze-fracture nomenclature was adopted from Branton et al [20]. All freeze-fracture micrographs are oriented with the direction of shadow approximately from the bottom, unless otherwise indicated by an encircled arrow. For thin sectioning, H4-II-E cultures were prepared in situ using previously described methods [15].

EM Quantitation

Replicas were scanned for apparent cell-to-cell interfaces. An interface was defined as a membrane fracture face 16 μm^2 or greater with a breakthrough revealing both a P-fracture face and an apposed E fracture face. (A field of view of 16 μm^2 corresponds to a magnification of 20,000 × on the AEI-801 EM.) Initial studies [15] suggested that membrane faces smaller than 16 μm^2 were unlikely to contain either breakthroughs or tight junctions.

Formation zones on membrane P-faces were distinguished from the surrounding membrane areas by the presence of membrane ridges having a deficiency of intramembranous particles (see Results and Figs. 1, 5). Even when chains and aggregates of 9–11 nm particles were found on the ridges, the overall particle density appeared sparse (Fig. 6). An isolated ridge was scored as a single formation zone, while adjacent ridges were scored as separate formation zones only if the ridges did not make direct contact and the particle-free areas around each ridge were separated by a region with a higher particle density. As a result, formation zones with concertina-like patterns resembling those of mature junctions were frequently scored as single zones. Although formation zones were more difficult to identify on E-faces, the membrane depressions (and often pits as well) made identification possible (Fig. 2).

Using a compensating polar planimeter (Lasico, Los Angeles), the areas (μm^2) of all formation zones were determined by the same individual to minimize subjective variations. The boundary of a P-face formation zone was taken as the outer perimeter or base of the ridge complex, where the normal distribution of intramembranous particles abutted on the smooth ridge. On E-faces the edge of the depression was used as the boundary, after a careful evaluation of the platinum shadow.

The diameters of particles in the linear strands and aggregates of the formation zones routinely measured 9–11 nm. Thus, numbers of 9–11 nm particles were determined for each zone and expressed as particle densities (particles/μm^2) to provide an indication of this aspect of formation zone development. The counting process was greatly facilitated because 9–11 nm particles tended to occur in groups and were usually found along the tops of the formation zone ridges.

A continuous tight junctional fibril was scored as a single tight junction, regardless of its length or complexity. Adjacent fibrils were scored as separate junctions only if they were separated by an area of nonjunctional particles or it was clear that the fibrils were part of two separate fibril patterns. No attempt was made to quantitate the areas of tight junctions.

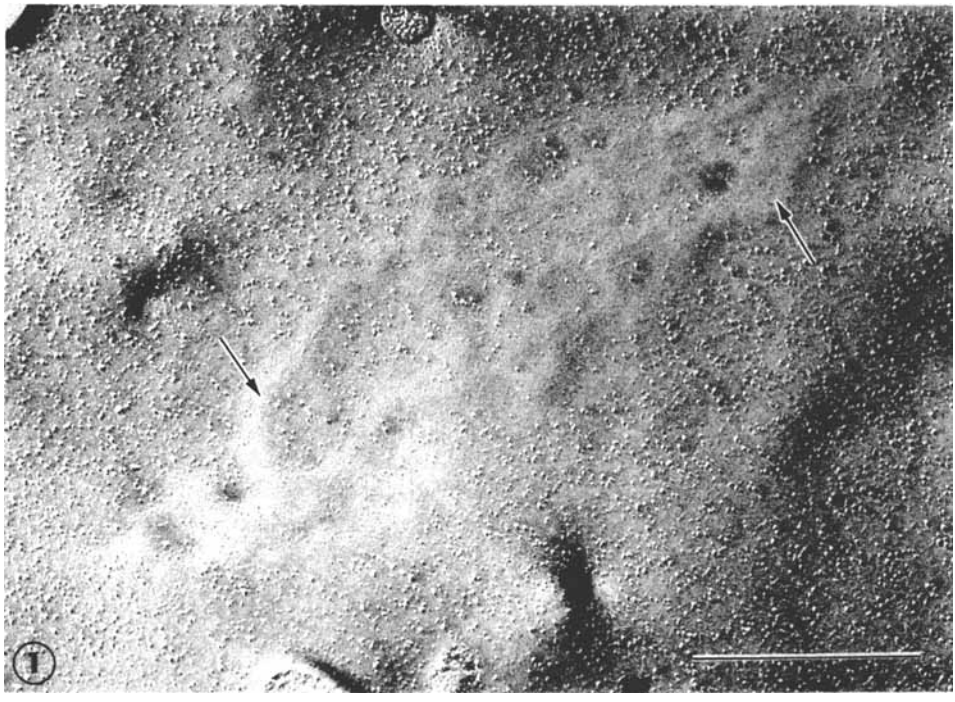

Fig. 1. A tight junction formation zone is characterized in its earliest form by smooth, prominent ridges (arrows) on membrane P-fracture faces. This figure illustrates well the very small number of particles found in some formation zones. × 66,000, scale indicates 0.5 μm.

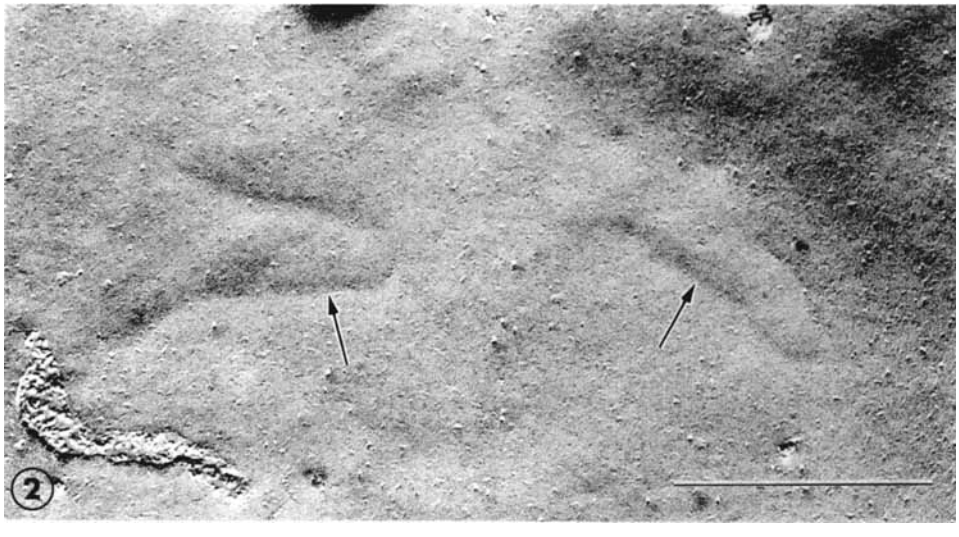

Fig. 2. Early formation zones appear as depressions (arrows) on membrane E-fracture faces. A few pits can be detected in the two depressions shown here. × 74,800, scale indicates 0.5 μm.

RESULTS

During the course of evaluating the influence of dexamethasone on the development of tight junctions between H4-II-E cells, several membrane specializations were studied in detail. They appear to represent stages in junctional differentiation. We will first report these qualitative observations which relate to both control cultures and those treated with dexamethasone. We will then present our quantitative results on mature and developing junctions in the dexamethasone experiments.

Dissociated Cells

The membranes of dissociated cells contained a few tight junctions at zero time (approximately 3 macular tight junctions/100 interfaces), but by 4–12 h no junctions were observed (a total of 300 interfaces sampled at 4, 6, and 12 h). Thus, it is likely that the few junctions existing at the time of dissociation were internalized and/or disassembled. In fact, some of the junctions observed at zero time were found on convex or concave membrane fracture faces rather than flat ones, and may have been already in the process of degradation.

Development of Tight Junction "Formation Zones"

A prominent feature of replicas from early preparations (15–20 h) was the appearance of smooth ridges (50–100 nm wide) which occurred on P-faces and which had few, if any, intramembranous particles (Fig. 1). These ridges were either short and straight or longer and arranged in an intersecting network covering a large area. On E-fracture faces, gentle depressions, apparently complementing the ridges, were noted (Fig. 2). These regions, which we have termed "formation zones" for reasons discussed below, were well defined and easily recognized 15 h after replating although none were observed at earlier times (4, 6, and 12 h). Infrequent fracture breakthroughs within early formation zones suggested that the smooth ridges and depressions were matched across a partially reduced extracellular space (Fig. 3).

In thin sections of early preparations (15–20 h), we observed regions possibly corresponding to cross sections of the formation zones. In such situations, the apposed cell membranes had small convexities with increased curvature (radius of curvature approximately 20–30 nm) and they appeared to be matched (see arrows in Fig. 4) across the extracellular space. The space itself was reduced to 3–5 nm from the normal space of 10–20 nm. Serial thin sections showed that the outer membrane leaflets of the matched areas of curvature did not touch and remained separated by at least 3 nm. In addition, a filamentous material was observed beneath each membrane (Fig. 4).

9–11 nm Particles in Formation Zones

While smooth formation zones were most common in the early samples (15–20 h), these preparations and later ones contained numerous zones with 9–11 nm particles distributed preferentially along the ridges (Fig. 5). The particles were organized primarily as closely-aligned, linear arrays (10–12 nm center-to-center spacing) but were also found as tightly packed aggregates (Figs. 5–7). The linear segments of closely spaced particles were observed at multiple locations in the formation zone complex. Often these linear segments of 9–11 nm particles were contiguous with the tightly packed aggregates. These patterns of 9–11 nm particles on P-fracture faces were also observed on membrane E-faces as linear and "aggregated" arrays of pits (10–12 nm center-to-center spacing; see Figs. 8a and 9b).

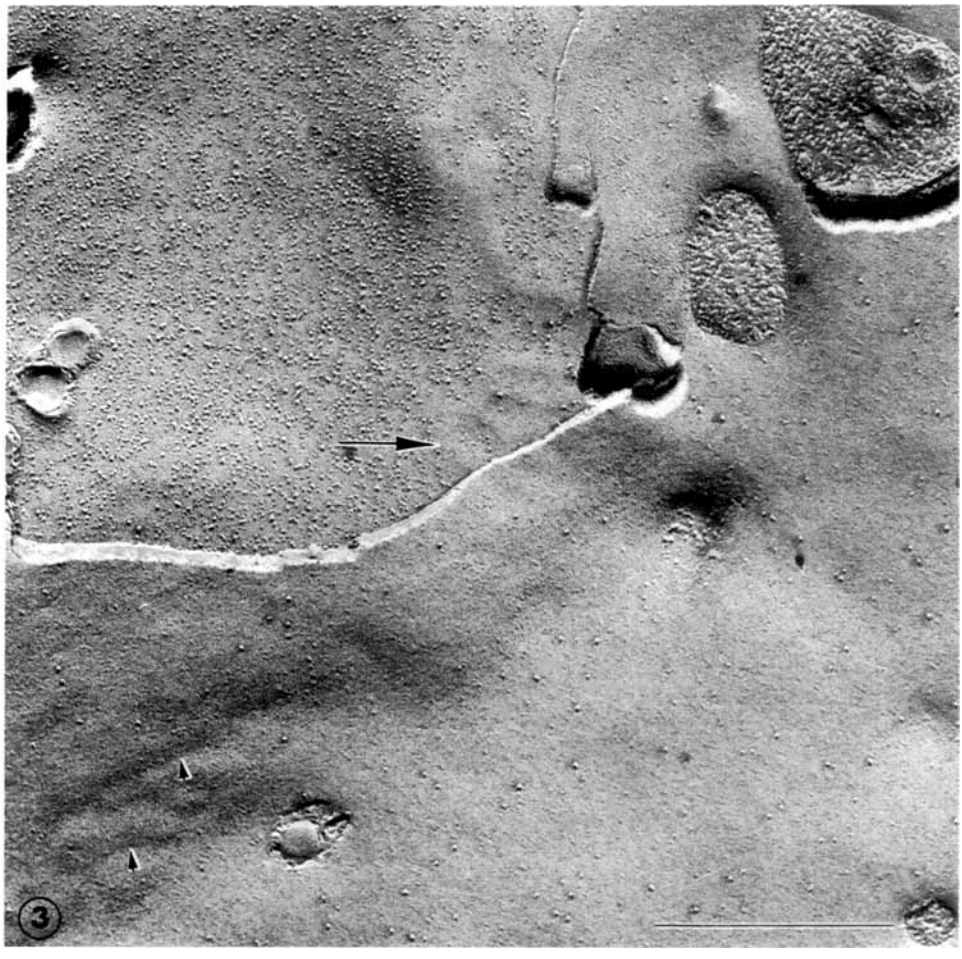

Fig. 3. A breakthrough within an early formation zone (to the right of the large arrow) suggests that the P-fracture face ridges may be matched with apposed E-fracture face depressions. The extracellular space is only partially reduced at this stage. Steps within early formation zones from one membrane face to another were rare in this investigation. Numerous other formation zone depressions (arrow heads) are present on the E-fracture face. × 68,400, scale indicates 0.5 μm.

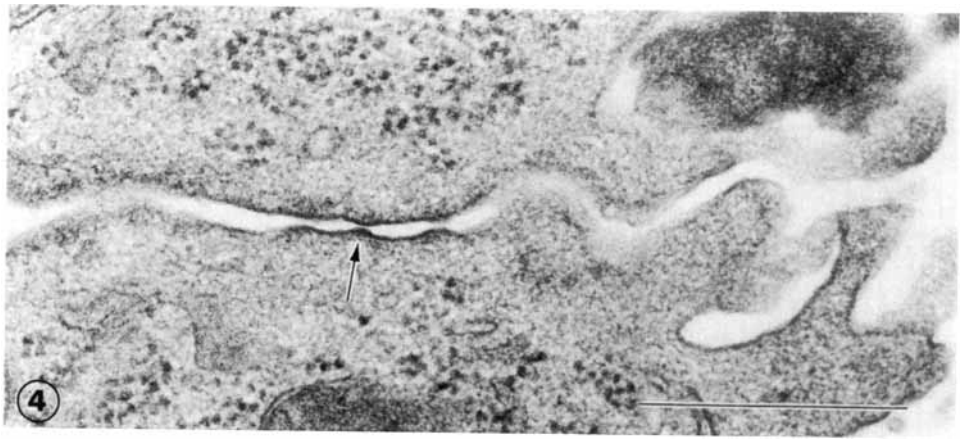

Fig. 4. A presumptive, early formation zone from one of 10 serial sections is shown here. The matched ridges on each apposed cell membrane characteristically display an increased curvature and staining density (arrow). Such ridges were not observed to touch within these serial sections. Microvilli project from the apical surface of the culture. × 77,000, scale indicates 0.5 μm.

Fig. 5. Closely spaced, 9–11 nm particles typically align and aggregate along the formation zone ridges at several locations (arrows). Note that very few 9–11 nm particles are found elsewhere in the formation zone.

Fig. 6. Development of the formation zone is believed to progress with an increased incorporation and linear alignment of the 9–11 nm particles along the formation zone ridges. × 76,700, scale indicates 0.5 μm.

Particle Matching in Formation Zones

Fracture breakthroughs within more complex formation zones showed that the linearly aligned particles on the P-fracture faces were continuous with linearly aligned pits on the apposed E-fracture faces (Fig. 8a). Furthermore, the extracellular space (ECS) was reduced at the point where the 9–11 nm particles met with linearly aligned pits (Figs. 8a, 8b). Although the dimensions of the ECS are often difficult to assess in replicas, we consistently found the reduction of the ECS in instances of particle-pit matching to be comparable to that of fully developed tight junctions. This is in contrast to the observations made on particle-free formation zones in which the ECS was only partially reduced (see above).

Fig. 7. More elaborate patterns of particles are generated as the 9–11 nm particles appear to fill in spaces along the membrane ridges. × 81,700, scale indicates 0.5 μm.

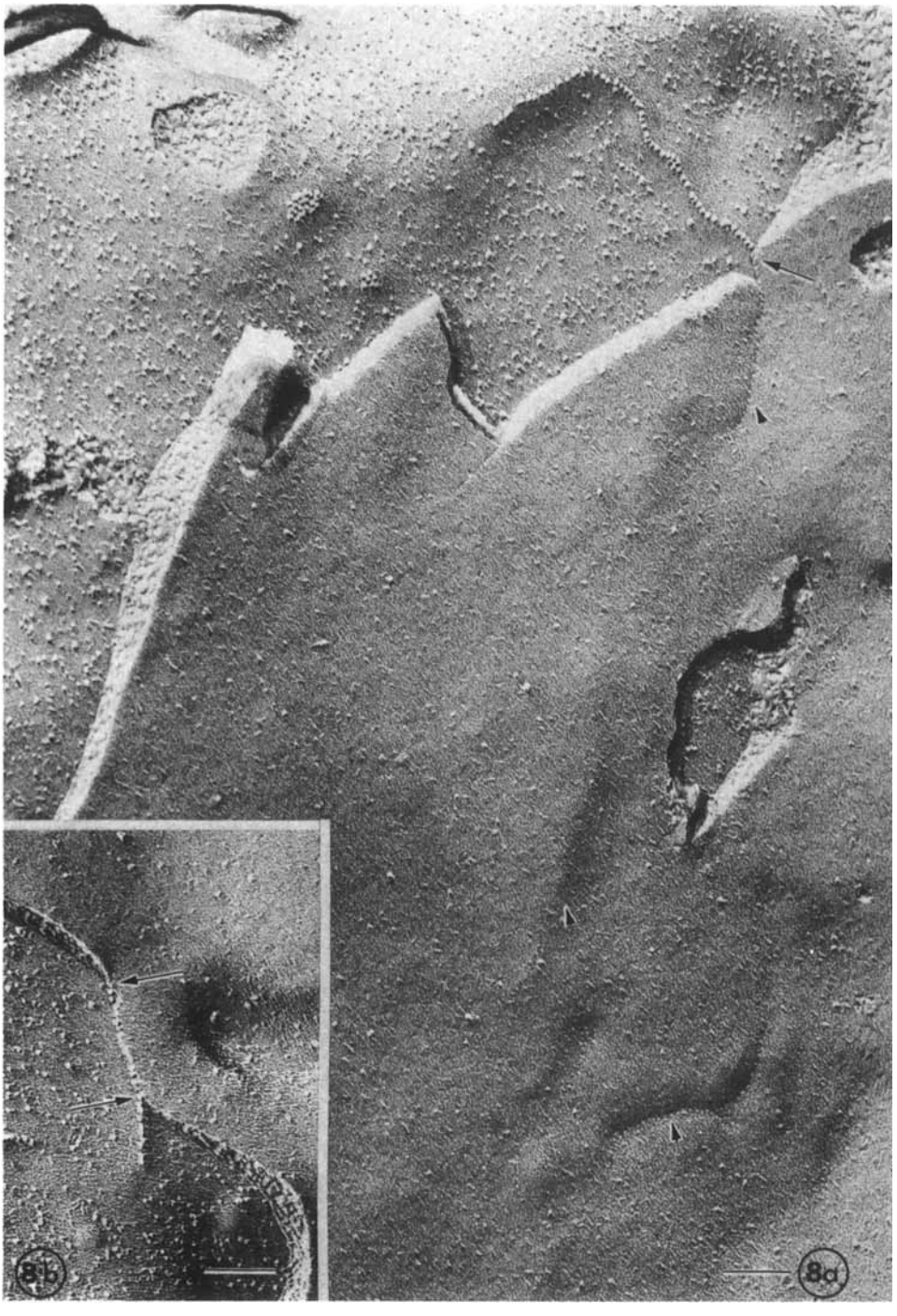

Fig. 8 (a). Linearly aligned P-fracture face particles make contact at this stage with another set of 9–11 nm particles in the contiguous cell membrane as is shown by the continuity of P-fracture face particles with the E-fracture face pits (arrow). It is not clear whether this is precise "head-to-head" contact. Other closely-spaced pits and depressions (arrowheads) are also evident on the E-fracture face. × 104,000, scale indicates 0.1 μm. (b). The extracellular space at the point of the apparent 9–11 nm particle matching (arrows) is reduced as in a mature junction. Also see Figure 8a.

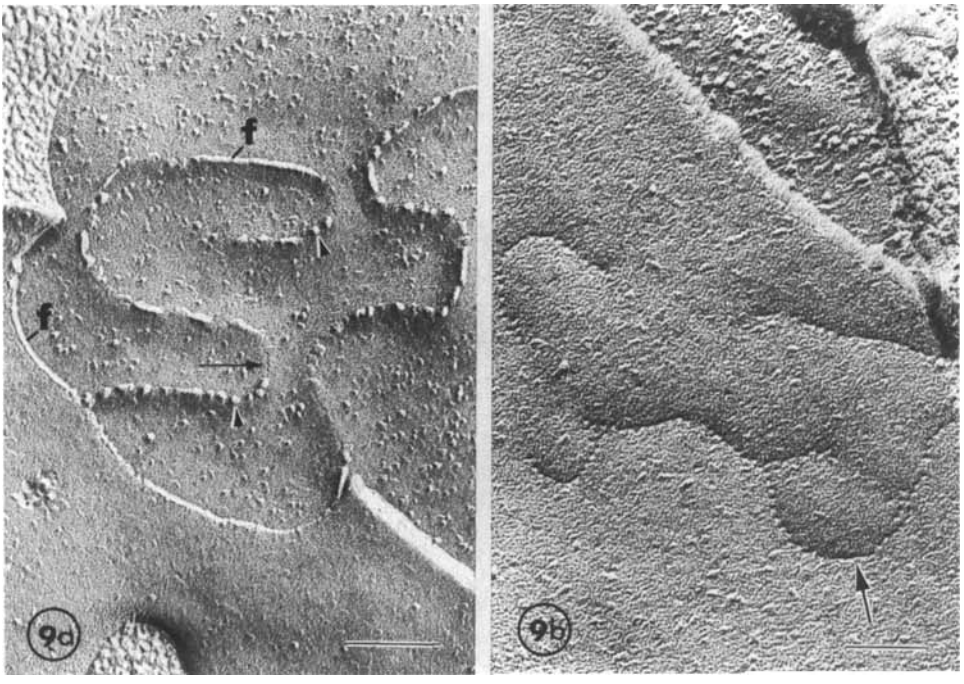

Fig. 9 (a). This micrograph illustrates a macular tight junction similar to those seen in standard H4 cultures. It contains 9–11 nm particles (arrowheads), short junctional fibrils (f) and closely spaced P-face pits (black arrow). These pits are observed only rarely. × 103,000, scale indicates 0.1 μm. (b). This E-face displays a clear array of pits with center-to-center dimensions of 9–11 nm. A short, subtle groove (arrow) is shown in continuity with the string of pits. Compare this possible groove with that shown in Fig. 10b. × 134,000, scale indicates 0.1 μm.

Junctional Maturation

The mature H4 tight junction is composed of intersecting fibrils arranged in networks similar to those produced by the smooth ridges and by their associated chains of particle (compare Figs. 10 and 11). The similarity of these patterns is consistent with the idea that the fibrils are somehow formed from the chains of particles, as suggested by others [10]. This idea is also consistent with our observations of limited numbers of possible hybrid structures involving both fibrils and beaded strands of particles or pits (Fig. 12a, 12b). If such structures do represent a transition from particles to fibrils, the fact that they occur infrequently may indicate that the process is a very rapid one.

(We also observed irregular structures resembling those seen in undissociated H4 cultures and reported in other systems, which involved discontinuous fibrils associated with particles or closely spaced pits on P-faces, Fig. 9a. We believe these structures reflect variability in fracturing patterns rather than developmental or transitional forms.)

In a situation where formation zones give rise to mature tight junctions, we might expect to find an increase in the number of formation zones followed by an increase in the number of mature tight junctions. Recently, a trend of this type was reported in an in vivo study of mouse fetal mesothelium [14]. Our quantitative analysis of formation

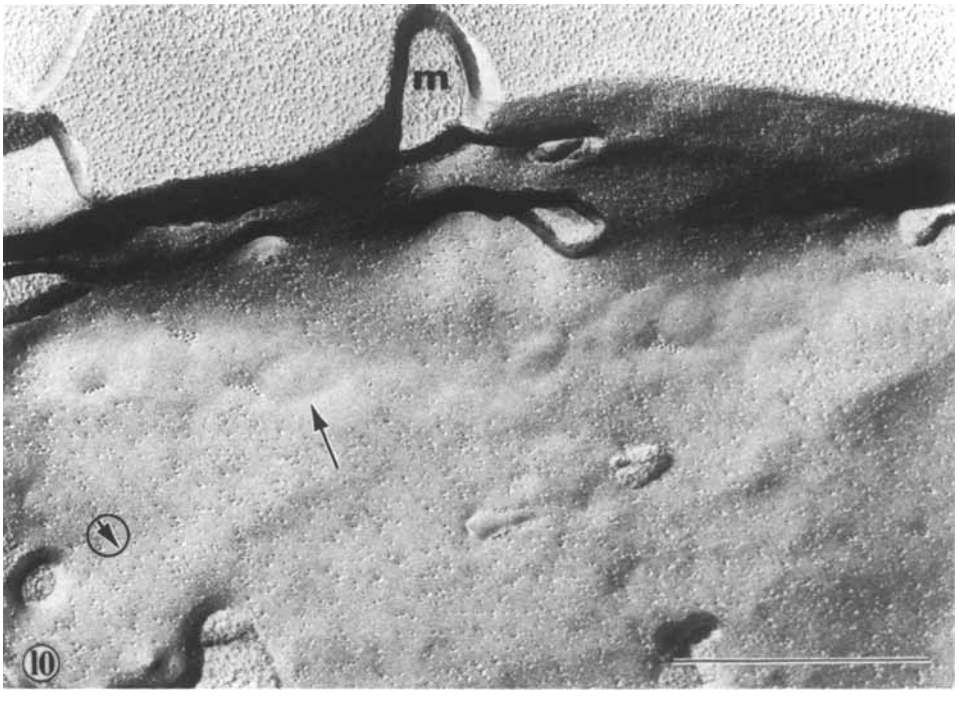

Fig. 10. This figure and Fig. 12 depict the similarities in size, complexity and location of formation zones and mature tight junctions. Here an early formation zone (arrow) is observed near the apical surface of the culture. The direction of shadow is indicated by an encircled arrowhead. (m) large apical microvillus. × 73,900, scale indicates 0.5 μm.

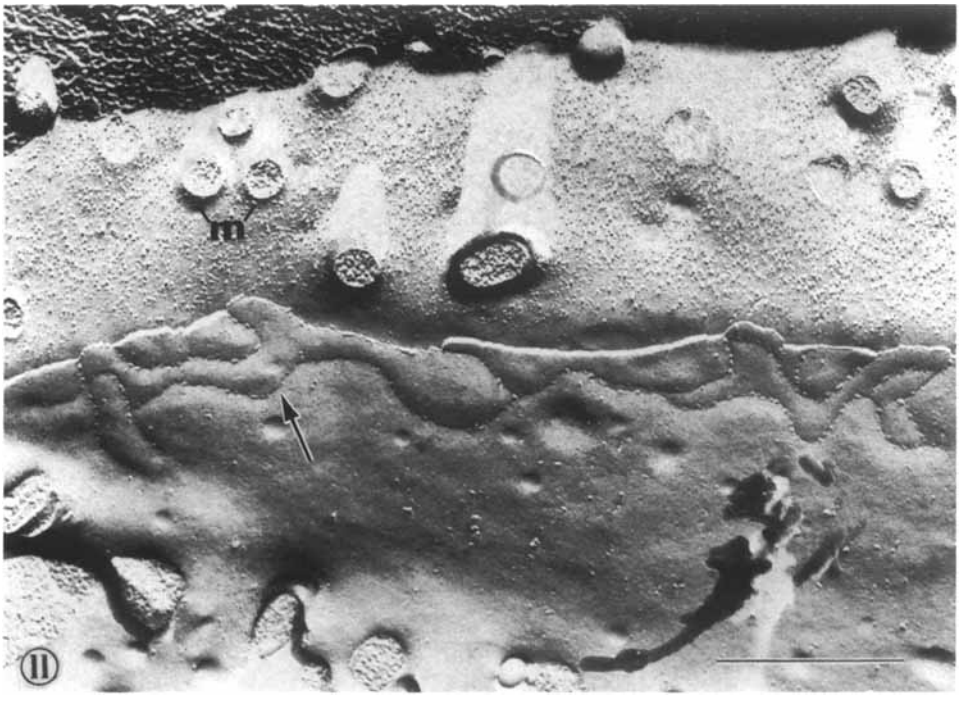

Fig. 11. This mature tight junction (arrow) on an E-fracture face resembles the formation zone in Fig. 10. (m) apical surface microvilli. × 54,100, scale indicates 0.5 μm.

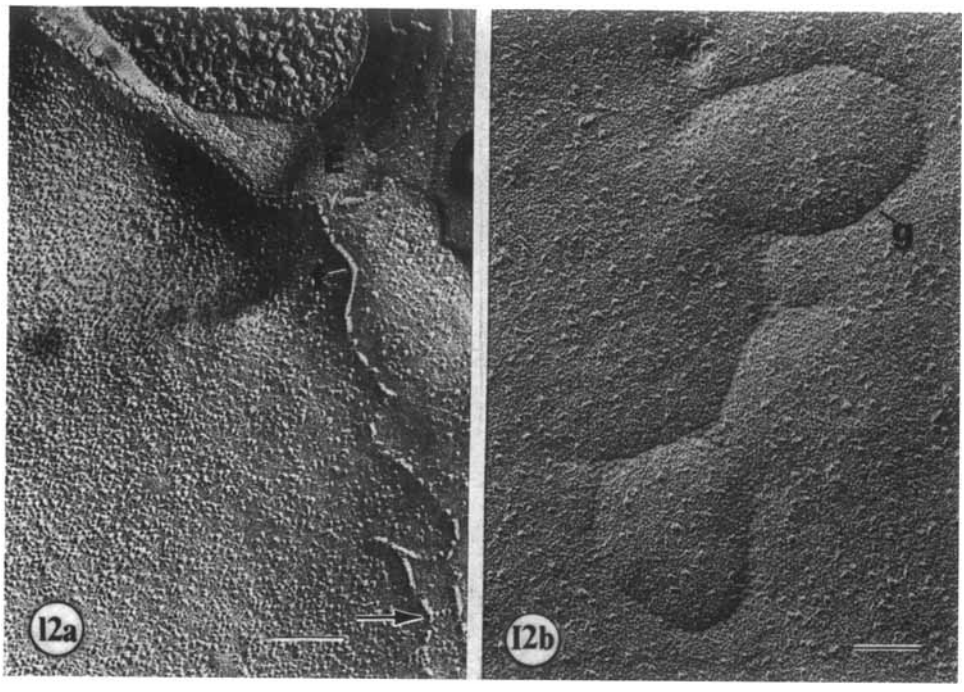

Fig. 12 (a). A rare situation involving a row of P-face particles (p) included close to a tight junctional fibril (f). Three particles also connect two short fibrils (arrow) found above a small aggregate. An apposed E-face (E) bears tight junctional grooves. × 100,000, scale indicates 0.1 μm. (b). This irregular arrangement of E-face pits is continuous with a groove (g) resembling that of a mature tight junction. Infrequent micrographs such as this suggest that the 9–11 nm particles fuse to form the tight junctional fibril. × 100,000, scale indicates 0.1 μm.

zones and tight junctions, however, was inconclusive. As shown in Table I, both formation zones and tight junctions appear first at 15 h. There may be a decrease in the number of formation zones with time, but the small sample size and variability leave us with an equivocal result. The number of mature tight junctions, on the other hand, apparently fails to change.

Gap Junctions and "Formation Plaques"

Apparent sites of gap junction formation, ie, "formation plaques" [18], were also observed between H4 cells during this study (see Fig. 13a, 13b). As defined in other systems [18, 21, 22], these flat, often circular plaques were deficient in small intramembranous particles, but contained clusters of 9–11 nm particles and particle aggregates. The gap junction formation plaques occurred either alone (Fig. 13a), or in conjunction with a tight junction formation zone (Fig. 13b). Based upon particle diameters, it was not possible to differentiate the particles of the tight junction formation zones from those of the gap junction formation plaques. Moreover, aggregates within the formation zones appeared indistinguishable from structures that were presumably isolated gap junctions outside the formation zone complex (Fig. 14).

TABLE I. Quantitation of Junctional Features.*

Time after plating	Number of interfaces examined	Membrane area examined (μm^2)	Number of formation zones	Formation zone areas (mean μm^2 ± SE)	9–11 nm particle (or pit) density (mean number ± SE per μm^2)	Number of mature tight junctions	Number of isolated gap junctions
0 h	100	2,400	0	0	0	3	0
15 h E	100	2,896	15	0.16 ± 0.04	269 ± 85	27	2
15 h C	100	2,416	8	0.18 ± 0.04	93 ± 29	17	3
20 h E	100	2,384	23	0.13 ± 0.02	374 ± 137	32	7
20 h C	100	2,480	14	0.08 ± 0.03	453 ± 211	20	5
25 h E	100	2,560	16	0.13 ± 0.02	413 ± 206	21	11
25 h C	100	2,544	1	0.03	343	9	2
30 h E	100	2,640	9	0.15 ± 0.04	136 ± 86	19	3
30 h C	100	2,832	12	0.30 ± 0.14	328 ± 174	6	1
34 h E	100	2,480	9	0.14 ± 0.04	964 ± 553	25	4
34 h C	100	2,544	4	0.18 ± 0.08	536 ± 462	15	1
40 h E	100	1,984	12	0.11 ± 0.03	1,649 ± 556	31	7
40 h C	100	2,048	2	0.14 ± 0.13	1,538 ± 1,461	15	5

*Cells were plated in the presence of 2×10^{-6} M dexamethasone with 0.1% ethanol (experimental, E) or 0.1% ethanol (ethanol control, C). Samples were fixed at the above times and at 4, 6 and 12 hours, where neither formation zones nor mature junctions were detected. Interfaces were defined under Methods. Membrane areas were determined with a polar planimeter. A two-way analysis of variance was used to evaluate the numbers of junctions observed in this study with and without dexamethasone. The "P" values for total numbers of formation zones ($P < 0.05$) and total numbers of mature tight junctions ($P < 0.0001$) were significant, but data on total gap junctions yielded a value ($P < 0.07$) that is probably not significant. Furthermore, significant differences were not obtained when a number of statistical tests were used to evaluate data on formation zone areas and particle/pit densities.

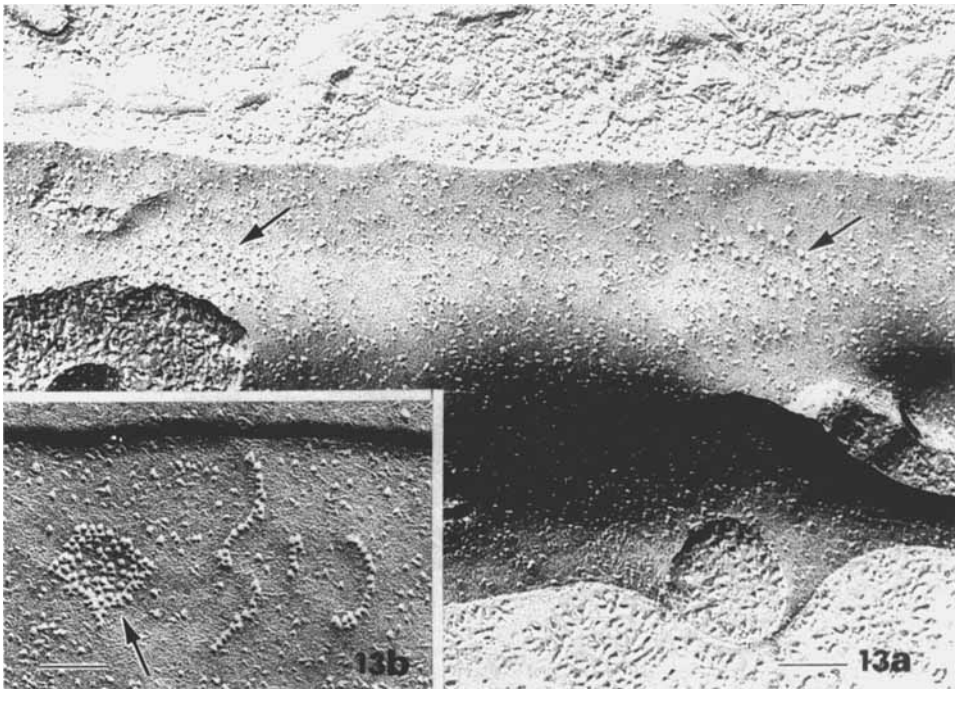

Fig. 13 (a). Gap junction "formation plaques" (arrows) are found in H4-II-E cultures. The 9–11 nm particles are characteristic of these specializations. These plaques were not associated with a formation zone. × 99,700, scale indicates 0.1 μm. (b). This apparent gap junction (arrow) is adjacent to several linear particle arrays. × 98,700, scale indicates 0.1 μm.

Dexamethasone

Our initial experiments with dexamethasone were aimed at determining if drug levels known to influence canalicular differentiation (2×10^{-6} M) had any effect on tight junction development. While we found no difference in the structure of mature or developing tight junctions, there appeared to be a quantitative enhancement of formation.

To pursue this observation we carried out a detailed experiment, sampling nine time points over a 40-h period. Samples treated with 2×10^{-6} M dexamethasone (plus 0.1% ethanol) were compared with samples treated only with 0.1% ethanol (the solvent for the stock solution of dexamethasone). As shown in Table I, at nearly all time points, there were small increases in developmental forms as indicated by numbers of formation zones/100 interfaces. Furthermore, the numbers of mature tight junctions clearly were increased in a statistically significant way, as described in the legend to Table I. Gap junction differences were probably not significant.

To be certain that the observed effects were not due to the ethanol treatment, further experiments were run over a three-day period. These included untreated controls, ethanol controls and dexamethasone preparations. The two control groups, which gave very comparable results, contained few formation zones and the dexamethasone group displayed again an enhanced development of formation zones. As a result, the observed modification of formation zones appears to be associated with the presence of dexamethasone.

Fig. 14. Isolated aggregates (arrows, apparent gap junctions) appear identical to the aggregated particles in the extensive formation zone shown here. Notice that in the breakthrough regions (arrowheads) the extracellular space is completely reduced. This micrograph illustrates a greater development of aggregates than what was typically encountered. × 77,900, scale indicates 0.1 μm.

DISCUSSION

This study is a direct outgrowth of our earlier description of the forms of tight junctions seen in long term cultures of H4-II-E cultures [15]. In that earlier report we suggested that tight junctions of increasing complexity represented intermediates in the formation process. However, the cells in those early studies had been plated at low density and were studied only after reaching confluency following a substantial growth period (several days). Consequently, early formation stages may have been missed and variations due to cell migration and junction degradation were likely.

In the current study we plated cells at confluency and identified more primitive structures that appear to represent the initial phases of tight junction formation. We did not achieve complete synchrony of formation and thus, the staging remains speculative, but it is consistent with our data and those of others. We suggest the following stages: 1) Formation zones are produced as matched areas on adjacent cells. The earliest formation zones have smooth, particle-free ridges on P-faces (or depressions on E-faces). The ECS is reduced but not obliterated. 2) The formation zones gradually accumulate 9–11 nm particles which are primarily arranged in linear, beaded strands. The ECS is further reduced at this stage. 3) The particles in the linear strands associate, probably in a rapid fashion, to form fibrils. The fibril networks grow more complex either by addition of particles or consolidation of several simpler fibril networks.

The entire formation process can occur over a short time period. Samples from 12 h cultures showed no mature or developing tight junctions, while all formation stages were seen at 15 h. Thus, some junctions can form completely in three hours. This is an upper estimate and quite likely formation can and does occur more rapidly.

We consider this sequence to be the most economical interpretation of our observations. However, two complicating possibilities need to be considered. This first is that the formation zones and particle strands result from degradation of tight junctions carried intact throught the dissociation procedure or formed early and degraded later [23, 24]. Degradation of carried-over junctions is unlikely considering the long period after replating (4–12 h), during which no tight junctions or formation zones were seen. The second view that formation zones result from degradation of newly formed junctions cannot be excluded. However, continued breakdown of mature junctions to formation zones should give rise to more numerous formation zones at later times than were observed in this study. Furthermore, other studies [10–13], in particular a recent quantitative effort [14], suggest that tight junctional fibrils may be preceded in development by particle-free ridges and linear strands of particles. Therefore, we also favor this interpretation rather than the idea that these structures represent degradation products.

The second possible interpretation is that the beaded strands of particles are gap junctions, since both structures are composed of intramembranous particles measuring 9–11 nm in diameter. These particles are arranged in complementary arrays in adjacent membranes and are separated by a reduced ECS. Although gap junctions usually contain more extensive aggregates of particles, many cells have very small gap junctions [4, 5, 25] and even linear gap junctions have been reported (eg, in retinae [26]; and, in amphibian heart, [27]). However, in older cultures [15] we found no beaded strands, only tight junction fibrils often not associated with particle aggregates. Unequivocal gap junctions were seen infrequently and often some distance from the tight junctions. Thus, if the linear strands were some form of gap junctions, they would have to be relatively transient structures, possibly unrelated to the gap junctions seen in older cultures. It seems that the transient nature of the strands is more consistent with their involvement in tight junction formation and we have adopted this as our provisional conclusion.

Others have also concluded that beaded strands of particles occur as intermediates in tight junction development. Recent reports have described the gradual development of tight junctions in several embryonic tissues. A revealing investigation of fetal liver has demonstrated linear, beaded arrays and aggregates of particles on P-face membrane ridges adjacent to developing bile canaliculi [10]. As in our system, the extensive ridges, which were generally free of smaller intramembranous particles, created patterns on the membrane faces that strongly resembled those of mature tight junctions. Fully formed tight junctions were also identified adjacent to the bile canaliculi. The developing tight junctions in fetal liver, however, differed in one important respect from those in H4 cultures. In the development of a complete tight junction adjacent to bile canaliculi in the liver, the linear arrays of P-face particles, which were common, were accompanied by E-face grooves rather than pits. The lack of pits in fetal liver was interpreted as evidence for a differential maturation of the tight junctional fibril, with maturation occurring first on the E-face and then the P-face [10]. In contrast, we routinely found linear arrays of E-face pits in H4 formation zones. It seems to us more likely that the lack of visualization of pits in the liver material was a technical matter.

In the developing thyroid [12] and choroid plexus [11], beaded particle strands are also present, but not as easily recognized. In these cases, the tight junction complexity

increased with time (on the order of days) and, in the thyroid, the transepithelial barrier to lanthanum was shown to increase with time as well [12]. Observations on the differentiating epithelium of the choroid plexus, including the presence of particulate strands, led investigators to theorize that such beaded strands, as well as tight junctional fibrils, grew by the end-on addition of individual particles [11]. They further suggested that the latter process was important for tight junctional development when trypsinized plexus epithelium was allowed to reassemble in vitro. Other studies of junctional development in the testis and in mesothelia also suggest that tight junctions form by fusion of membrane particles initially found in linear array [13 and 14].

Two features of formation zones, which offer clues to the underlying mechanisms, deserve emphasis. First, the structures we have interpreted as the earliest formation zones are free of intramembranous particles, even those measuring 9–11 nm in diameter. Secondly, as noted previously [10], the particles accumulating along the bare membrane ridges of the formation zones are not uniformly distributed. Instead, short linear arrays and aggregates appear at multiple sites, often separated by fair distances, with essentially all 9–11 nm particles in contact with other 9–11 nm particles (Figs. 5, 6, and 11). These and related observations raise some interesting possibilities for the mechanisms of ridge development and matching between cell membranes. It is possible that cell contacts are required for the development of formation zones. These contacts could lead to localized Ca^{++} influxes and subsequent reorganization of microfilaments underlying the membrane [see 28]. Membrane-filament interactions could be an integral part of generating the particle-free ridge and/or introducing the 9–11 nm particles to this region. Alternatively, the filamentous material we see in sections under putative formation zones may serve only to anchor or stabilize junctional components. It is also possible that the most essential feature of formation zone development is the adhesion between cell surfaces. The deficiency of intramembranous particles in early formation zones could implicate peripheral proteins or membrane lipids as important components in this association. Once adhesion developed between matched formation zones, nonjunctional particles could be excluded from the region, eg, due to charge repulsion [29] or membrane curvature [30]. With time the 9–11 nm particles could partition out along the ridges for a number of reasons, possibly as a result of interactions with similar complexes in the apposed membrane or an affinity for membrane lipids found in the formation zone.

Since tight junctions clearly form compartments in adult organisms [2] and since they appear to form developmentally significant compartments as well [31], the control of junctional assembly deserves careful scrutiny. One aspect of control involves the triggering of assembly by agents such as vitamins and hormones. During the course of mucous metaplasia, Vitamin A has been shown to modify the differentiation of both tight and gap junctions [32]. Furthermore, a number of hormones appear to influence gap junction development [33–35], suggesting a more general role for hormones in controlling features of the cell surface.

In our studies, dexamethasone enhanced the development of H4 formation zones over the course of several days. The enhancement was reflected in the frequencies of formation zones, and also in the number of mature tight junctions (Table I). These findings are particularly interesting in view of the suggestion that glucocorticoids regulate the development of the bile canaliculi [17]. Clearly, the formation of hepatocyte tight junctions is an integral part of canalicular differentiation [10]. Thus, it is appealing to think that one action of glucocorticoids, including dexamethasone, is the induction of the synthesis of tight junctional components and/or their subsequent assembly. The induction by

glucocorticoids of another membrane protein in a hepatocyte-derived cell line has already been demonstrated [36]. The interesting influence of dexamethasone on tight junctions certainly warrants further investigation. Perhaps, the use of primary hepatocyte cultures, known to possess canalicular structures and tight junctions [37], would provide a meaningful approach to the issue.

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