Induction of Early Stages of Kidney Tubule Differentiation by Lithium Ions

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Kidney tubules develop by a mesenchyme–epithelium transition, normally induced by ureteric bud through a mechanism that remains obscure. Murine nephrogenesis in vitro has always required heterologous inducing cells. We have discovered that Li+ can elicit the early stages of epithelial differentiation in isolated nephrogenic mesenchyme. We have made detailed comparisons of the timing of morphoregulatory molecule expression between Li+-mediated induction and the traditional in vitro method using induction by spinal cord. Both followed the same program of early morphoregulatory molecule expression, though Li+-induced samples failed to progress into the later parts of the nephrogenic process. Mesenchymes induced by Li+ showed more DNA synthesis than controls, though less than those induced by spinal cord. Discovery of a chemical means to activate differentiation in the absence of heterologous tissue offers a new basis for studying molecular mechanisms regulating the early events of nephrogenesis, as well as for investigating transduction of inductive signals that initiate the process. © 1996 Academic Press, Inc.

INTRODUCTION

More than 60% of vertebrate cell types are epithelial, yet genetic mechanisms regulating epithelial differentiation are unknown and effective model systems for study of de novo epithelogenesis are rare. Of the models available, nephron development is one of the most promising because it involves a mesenchyme–epithelium transition, and must therefore include the most fundamental events in acquisition of an epithelial phenotype. The process is normally induced by growing tips of the invading ureteric bud (prospective collecting duct system), though other tissues such as embryonic spinal cord can act as surrogate inducers in culture (Grobstein, 1955). Murine nephrogenesis has been the subject of decades of study (reviewed by Saxén, 1987; Davies, 1993), but the nature of the inductive signal continues to elude investigators.

As well as frustrating understanding of the signaling phenomenon, lack of a purified inducing factor hinders investigation of nephron differentiation in general. Without such a factor, nephrogenesis can be induced only in the presence of other tissues such as ureteric bud or spinal cord. Even in “transfilter” induction, cell processes cross the filter and mingle with nephrogenic mesenchyme (Wartiovaara et al., 1974; Saxén et al., 1976). Presence of another tissue complicates analysis of gene expression by PCR or subtractive hybridization because of the inducing tissue’s own transcription. Similarly, research into the effects of pharmacological agents on development is obfuscated by possible effects on the inducing, rather than the induced, tissue.

We have taken a new approach to the problem. Whatever signal initiates nephrogenesis, it is likely to be received at the cell membrane and conducted to the nucleus by a second messenger system. Many of these are amenable to pharmacological manipulation, so we have screened a variety of second messenger modulators for their ability to activate differentiation in isolated nephrogenic mesenchyme. One of these, the Li+ ion, proved to be capable of inducing the early phases of nephrogenesis, including expression of characteristic epithelial markers such as keratins and desmocollins. Study of morphological change, expression of morphoregulatory molecules, and DNA synthesis showed that Li+-induced differentiation followed a program broadly similar to that initiated by living inducers, although it failed to extend beyond the “comma” stage of nephron development.

The discovery that Li+ ions can induce differentiation in the absence of other tissues provides a new basis for study of regulatory mechanisms underlying kidney tubule development and a novel approach for investigating the mechanism of signal transduction involved in natural nephron induction.

MATERIALS AND METHODS

Tissue Culture

MF1 mice were allowed to mate ad libitum and checked for vaginal plugs each morning (day of plug discovery = E1). Metanephrogenic mesenchyme fragments
were isolated from E11 embryo kidney rudiments by microdissection in Eagle’s MEM (no enzymes were added to aid dissection, as preliminary experiments had shown that their use was unnecessary and that enzyme-treated mesenchymes showed more variable timing of spinal cord-induced development than those isolated by purely mechanical means). Isolated mesenchyme fragments from all kidneys dissected on a particular day were pooled, divided randomly into control and experimental groups, placed on 1-μm Nuclepore filters at the surface of culture medium, and cultured in 5% CO₂ at 37°C for 1–168 hr. Control culture medium consisted of Eagle’s MEM with Earle’s salts and 10% fetal calf serum, while experimental culture media consisted of the control medium supplemented with one of the following; 1–40 mM LiCl; 0.5–5 mM dibutyryl cyclic AMP; 0.25–1 mM dibutyryl cyclic GMP; 40–80 ng/ml staurosporine; 10–100 ng/ml phorbol 12-myristate acetate; 10–40 mM KCl; 1–20 mM CaCl₂.

For tissue-mediated induction, dorsal fragments of E11 embryonic spinal cord (cervical/thoracic) were juxtaposed directly to the mesenchyme as described previously (Davies, 1994) and cultured in the medium described above for 1–168 hr. α-Amanitin (final concentration 11 μg/ml; Johnson et al., 1977) was added to the medium of some cultures at 26, 26, or 28 hr to prevent new gene transcription (Johnson et al., 1977). Complete developing kidneys for sectioning were removed from E11 embryos and cultured intact, on Nuclepore filters as above, for 48 hr.

Immunohistochemistry

Cultures were fixed in −20°C methanol, washed in PBS, and incubated in primary antibody overnight at 4°C or 6 hr at room temperature. Antibody dilutions in PBS were: NCAM (Mab OBI1) 1/100; syndecan (Mab 281-2) 1/5–1/1200; laminin (σL9399) 1/100; laminin A (Mab 201) 1/200; E-cadherin (Mab DECMA1) 1/800; cytokeratin (ΣK4252) 1/60; α6-integrin (Mab GoH3) 1/3; CD15 (Mab C8D-1) 1/100; fibronectin (Mab FN-362) 1/100; vimentin (Mab V1118) 1/10; desmocollin (Mab 523) neat. Specimens were washed in PBS, incubated in 1/100–1/200 FITC-conjugated secondary antibody 4 hr at room temperature, washed in PBS, mounted in glycerol, and viewed under a Zeiss Axioshot fluorescence microscope. Controls which received only secondary antibody or irrelevant primary antibody showed no fluorescence.

Quantitation of Response to Inductive Stimuli

Grobstein (1955) introduced two parameters for quantification of response of nephrogenic mesenchymes to inductive stimuli; (i) the fraction of mesenchymes showing any response and (ii) the number of tubulogenic areas per mesenchyme. We have used a similar method, modified to yield additional information on the timing of expression of morphoregulatory molecules. Samples induced for 1–168 hr by dorsal spinal cord or lithium (and controls incubated without inducers for the same periods) were fixed and stained for particular antigens as above and examined intact by low-power epifluorescence microscopy (the technique of whole-mount examination was chosen so that no responding areas would be missed, as could be the case if sections were examined). The fraction of mesenchymes expressing the antigen in at least one cell aggregate/tubule was recorded, as was the number of cell aggregates/tubules expressing the antigen in each mesenchyme.

Sectioning

Cultures of whole kidneys and Li⁺-induced mesenchymes were detached from their filters, fixed overnight in Bouin’s fixative, stained briefly in Mayer’s hematoxylin (to make them visible during subsequent processing), dehydrated in ethanol, and embedded in methylmethacrylate resin. Then, 3.5-μm sections were stained in Mayer’s hematoxylin and eosin and viewed on a Zeiss Axioshot microscope.

BrdU Incorporation

Fragments of nephrogenic mesenchyme were assigned randomly to the three conditions of culture described above; standard medium, 15 mM LiCl, and contact with dorsal-half spinal cord in standard medium. During the period 0–6 hr, or 6–18 hr, BrdU (final concentration 100 μM) was added to the medium, and afterward samples were fixed in 10% formalin (4°C, overnight). They were then washed in PBS, treated with proteinase K (10 μg/ml, 15 min, 37°C), lightly refixed in 4% formalin (15 min, room temperature), washed in PBS, and incubated in 1/1000 anti BrdU (MAB Bu-33, Sigma B2531) overnight at 4°C. Following a 30-min PBS wash, samples were incubated in FITC anti-mouse IgG (2 hr), washed again in PBS, and incubated in 10 μg/ml propidium iodide in PBS (10 min, room temperature), washed again in PBS (10 min, room temperature), mounted in glycerol, and viewed using a Biorad MRC 600 confocal imaging unit attached to a Zeiss Photomicroscope. For each field examined, the total number of nuclei (indicated by propidium iodide staining) and the number staining for BrdU were recorded. In the case of spinal cord-induced cultures, only the nephrogenic mesenchyme, which was easily distinguished from the spinal cord by size and structure, was examined.
Atomic Absorption Spectroscopy Analysis

Standard LiCl-mediated induction was performed using medium containing 15 mM LiCl and trace quantities of [3H]inulin (final concentration 490,000 dpm/ml), a marker for extracellular space. Cultures were incubated for 18 hr, removed from their filters, washed three times in approximately 5 ml PBS, and transferred to 50 µl 0.1% Triton X-100, to which 10 µl 10 N HNO3 was added after 5 min. The mix was frozen and thawed repeatedly and left at room temperature for 6 hr. After that, 5 µl of the lysate was removed for 3H assay (scintillation counting), while the rest was tested for Li⁺ using a Perkin-Elmer atomic absorption spectrometer calibrated against known concentrations of LiCl.

RESULTS

Effects of Second Messenger Modulators on Metanephric Mesenchyme

In an attempt to activate epithelial differentiation in isolated nephrogenic mesenchyme, uninduced mesenchymal fragments from E11 mouse metanephros were treated with different signaling effectors. The substances (0.5–5 mM dibutyl cyclic AMP, 0.25–1 mM dibutyl cyclic GMP, 40–80 ng/ml staurosporine, 10–100 ng/ml phorbol 12-myristate acetate, 10–40 mM KCl, 1–20 mM CaCl2, 1–40 mM LiCl) were applied for 6–48 hr, and whole-mount immunocytochemistry was used to detect changes in protein expression. Mesenchymes left untreated, or treated with any of the above agents except LiCl, showed no differentiation and began to degenerate slowly (Figs. 1A, 2A, and 2B), a process that has been described in detail elsewhere (Koseki et al., 1992).

Within LiCl-treated mesenchymes, small aggregates of cells began to acquire an epithelial phenotype (Figs. 1 and 2). The average number of responding aggregates per culture (a form of quantitation introduced by Grobstein, 1955), assessed using either bright-field optics or anti-NCAM staining of whole mounts, was 2.9 (σ = 2.3) for 15 mM LiCl. It was essential that all changes of gene expression observed were initiated by Li⁺ treatment and were not due to receipt of an inductive signal from ureteric bud prior to dissection of mesenchyme. In order to establish this, dissected mesenchymes were pooled and then selected randomly from the pool for use in Li⁺ treatment or as controls. Control mesenchymes were cultured for identical periods of time to Li⁺-treated specimens and were then stained for expression of the same markers. In the very rare event of expression being detected in any controls, indicating that mesenchymes had been dissected too late and after receipt of the natural inducing stimulus, the results of the entire experiment were discarded. Thus, inductions recorded here are supported by completely negative controls.

The dose-response curve for Li⁺ induction is shown in Fig. 3; all mesenchymes responded to 15 mM Li⁺, while concentrations below 5 mM and above 30 mM had no effect. The active concentration of Li⁺, 15 mM, is an order of magnitude higher than that used clinically against manic depression, but is much less than that needed for some other developmental effects such as sea urchin exogastrulation (>0.1 M, Herbst, 1883). To compare further the doses of lithium in nephron induction and other biological systems, we measured its average concentration in the mesenchyme by atomic absorption spectroscopy. It was no higher than 6 µM even after 18 hr in 15 mM Li⁺, a concentration comparable to those reported in other systems (for review see Birch, 1991).

Comparison of Morphoregulatory Molecule Expression Induced by Li⁺ and Living Inducers

Lithium-induced epithelia never produced the long tubular morphology characteristic of maturing nephrons; most epithelializing aggregates were small compared with those in intact developing kidneys (compare Figs. 2C, and 2D) and the most advanced reached only the comma-shaped stage (Fig. 1H). It seemed, however, that the differentiating cells might follow a normal early developmental program even if they failed to complete its later features. To compare Li⁺-induced differentiation to that induced by living tissues, both processes were studied in detail. This was essential, because only a general idea of the sequence of events following tissue-mediated induction may be obtained from the numerous published descriptive studies of morphoregulatory molecule expression in kidney development (for example Ekblom et al., 1980, 1981, 1983; Holthofer et al., 1984; Vestweber et al., 1985; Felming and Symes, 1987; Vainio et al., 1989; Garrod and Fleming, 1990; Lackie et al., 1990; Bard and Ross, 1991; Korhonen et al., 1992).

Development Induced by Spinal Cord

Whole-mount immunocytochemistry, for morphoregulatory molecules known to be regulated during nephrogenesis (see references above), was used to establish the timing of protein expression in mesenchymes placed next to a surrogate inducer, dorsal-half spinal cord. Response was quantified by recording the fraction of cultures that expressed each antigen in at least one cell aggregate/developing tubule, after a given period of incubation. The method of placing the inducing and responding tissues next to each other was chosen in preference to the more commonly used transfilter system because it gave much greater reproducibility of timing (±1 hr over the first 2 days) in our preliminary experiments. The method of whole-mount microscopy, though sacrificing spatial resolution, enabled the entire cultures to
Fig. 1. Epithelial differentiation induced by 15 mM Li⁺; images lack fine spatial resolution because specimens were mounted unsectioned to ensure that small areas of differentiating cells could not be overlooked. (A) Control mesenchyme cultured for 48 hr in the absence of Li⁺ and stained for NCAM, which is expressed at a uniformly low level. Controls also expressed laminin B at low levels but were negative for all epithelial antigens. (B) Bright-field image of Li⁺-induced local condensation of cells. (C) Early Li⁺-induced condensate expressing NCAM at greatly elevated levels. (D) Expression of desmocollins (desmosomal glycoproteins) in a large condensate; the surrounding mesenchyme is completely unstained. (E) Virtual exclusion of fibronectin from an area of tightly aggregated differentiating cells (arrow). (F) Cytokeratin expression in Li⁺-induced condensates. (G) Basement membrane forming around an epithelial sphere (arrow), revealed by laminin A immunofluorescence. (H) Example of a culture induced to the comma-shaped body stage of nephrogenesis (CB), stained for laminin. Developing glomerular cleft is marked G. Scale bar, 50 μm.
be examined to ensure that no responding area could be missed and that the earliest expression of each antigen was accurately recorded.

Contact with spinal cord induced nephrogenesis typical of that described elsewhere (for review see Saxén, 1987; Davies, 1993). The process is illustrated in Fig. 4, which shows samples representative of the 255 mesenchymes used to determine the time course summarized in Table 1a. The examples in Fig. 4 have been chosen to emphasize changing morphology as well as differentiation and thus enable a clear comparison with Li⁺ induction (Fig. 1, Table 1b). The results show that the developmental program can be divided into four phases:

**Quiescence.** During the first 18 hr, there were neither morphological changes nor alterations in the expression of morphoregulatory molecules studied.

**Epithelogenesis.** During the next phase, 18–36 hr, groups of cells aggregated and became epithelial. The average number of differentiating aggregates per culture was 6.2 (σ = 2.2), comparable with the average figure of 6 reported by Grobstein (1955). (Saxén, 1987, quotes much larger numbers (>20), probably because
the area of contact between the tissues in his transfilter system is much greater than that in the lateral apposition system we describe here). Differentiation began with greatly elevated NCAM and syndecan expression by groups of cells (Fig. 4a), which later increased their expression of laminin B (22 hr), then laminin A (26 hr) (Fig. 4e). Soon afterward they acquired α6-integrin, a laminin receptor, and the calcium dependent adhesion molecules, E-cadherin and desmocollins (Figs. 4g and 4j). Laminin became concentrated in a basement-membrane-like distribution (Fig. 4f), and between 30 and 33 hr after induction, the cells acquired keratin and lost vimentin (Table 1a).

Onset of tubule morphogenesis. During the next 36 hr, cells that had established their basic epithelial phenotype began the complex morphogenesis of an elongated excretory tubule (Figs. 4g~4j). They continued to express antigens seen at the 36-hr stage, although the intensity of staining for NCAM and syndecan reduced steadily and α6-integrin, which was originally on lateral as well as basal surfaces, acquired a basement-membrane-type distribution (Fig. 4j).

Tubule maturation. The last phase of development was characterized by differentiation of the specialized segments of the excretory tubule. By 120 hr CD15, a marker for proximal segments (Bard and Ross, 1991), was detectable in most cultures (Fig. 4i). The distal marker calbindin-D$_{28k}$ is expressed from 144 hr of this sequence, provided 1,25-dihydroxy vitamin D3 is added to the culture medium (Davies, 1994). By this stage long epithelial tubules dominated the explant (Fig. 4k).

To check that the expression sequence in Table 1a was a genuine developmental program and that it did not simply arise through different sensitivities of the various antibodies used, we applied α-amanitin (11 μg/ml; Johnson et al., 1977) to inhibit RNA synthesis at various times and assessed the state of differentiation reached at 40 hr. Continuous presence of the drug from 0 hr abolished all signs of tubulogenesis; neither increased staining for antigens such as laminin nor decreased staining for ones such as fibronectin took place. Application of α-amanitin at 20 hr prevented laminin and α6-integrin synthesis, while treatment at 26 hr resulted in laminin-rich aggregates of cells which were unpolarized and expressed no keratin. Though not every antigen was tested in these experiments, the results show that application of α-amanitin rapidly halts differentiation and suggest that the program is, at least partially, regulated at the transcriptional level.

Induction by Lithium

The timing of Li$^+$-induced development was studied using the same immunocytochemical techniques that were used for spinal cord-induced development; representative samples are shown in Fig. 1. The results of approximately 350 experimental cultures are shown in Table 1b. Comparison of Tables 1a and 1b reveals a striking similarity in timing of early responses to spinal cord and lithium, but some differences in later development.

Both methods of induction featured an initial period of quiescence followed by upregulation of NCAM and syndecan, then cell aggregation (Figs. 1B, 1C, and 1D). After this, the pace of development of Li$^+$-induced cultures slowed, though the order of events was generally the same. An important exception was laminin A expression which was surprisingly late, as was polarization. The delay in polarization presumably reflects its dependence on interactions of laminin A and α6-integrin (Sorokin et al., 1990). The response to Li$^+$ became increasingly variable at later stages of the differentiation program; while all cultures upregulated NCAM, fewer went on to express epithelial markers such as desmocollins and cytokeratins (Figs. 1C and 1D; Table 1b). Only very few (<5%; Table 1b) produced a comma shape (Fig. 1H) and none progressed beyond this to form elongated tubules. Again, 11 μg/ml α-amanitin halted differentiation whenever it was applied.

DNA Synthesis Following Induction

The fact that development of Li$^+$-induced cultures always halted, albeit at a variable stage, implied that there must have been an important difference between responses to Li$^+$ and spinal cord. Examination of sectioned differentiating aggregates revealed that those induced by lithium (Fig. 2C) were much smaller than com-
parable ones in intact kidneys and that they had unusually condensed nuclei (Fig. 2D). A possible explanation for the small size of Li⁺-induced aggregates, and perhaps their ultimate failure to mature, could be that Li⁺ failed to stimulate the cell multiplication elicited by living inducers (Saxén et al., 1983).

To compare the effect of Li⁺-mediated induction on DNA synthesis with that of spinal cord-mediated induction, mesenchyme fragments from a common pool were assigned randomly to control, lithium, or spinal cord cultures, and the thymidine analogue, BrdU, was added to the medium. Two intervals of BrdU labeling (0–6 hr and 6–24 hr) were studied, corresponding to periods before and during the massive increase in mesenchymal DNA synthesis that takes place in response to spinal cord (Saxén et al., 1983; Ekblom et al., 1983). Confocal microscopy was used to compare the number of BrdU-labeled nuclei with the total number of nuclei (visualized using propidium iodide staining) in optical sections of each mesenchyme; results are shown in Table 2.

In the period 0–6 hr, there was no significant difference between the fraction of BrdU-positive nuclei in control and Li⁺-treated cultures (both approximately 1 ± 0.6%). At the end of the 6- to 24-hr labeling period, the
TABLE 1
SEQUENCE OF PROTEIN EXPRESSION DURING EPITHELIAL DIFFERENTIATION INDUCED BY DORSAL-HALF SPINAL CORD (a) AND LITHIUM (b)

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Note: Each entry represents the fraction of cultures expressing detectable levels of an antigen at a particular time, periods of positive expression being emphasised by a solid black bar above the data. In total, 255 spinal cord-induced cultures and 314 Li⁺-induced cultures were examined (excluding controls). The programs of differentiation activated by the different induction systems are essentially identical, though there are minor differences in absolute timing, and a lower proportion of Li⁺-induced cultures express markers of late differentiation. Abbreviations used: Synd, syndecan; LN, laminin; o6, o6-integrin; Deol, desmocollins; E-cad, E-cadherin; Polar, polarized expression of laminin (i.e., basement membrane present around aggregates of differentiating cells); ker, cytokeratins; FN, fibronectin; ND, not determined.

fraction of BrdU-positive nuclei in controls was very similar (0.8 ± 1%). If DNA synthesis had continued at a constant rate, one would expect approximately three times as many nuclei to have become labeled in the 18-hr period (6-24 hr) as had become labeled in the earlier 0- to 6-hr period. That this did not occur indicates that
average DNA synthesis had fallen with time, an effect that has been described elsewhere (Saxén et al., 1983; Ekhblom et al., 1983). During the 6- to 24-hr period, lithium induced 5 ± 2% of nuclei to become BrdU-positive, 6× more than controls, while spinal cord induced 36 ± 5% to do so, 40× more than controls. Lithium induction therefore enhances proliferation, but to a lower extent than the strong inducer, dorsal spinal cord.

Combined Treatment with Lithium and Other Signal Effectors

In an attempt to antagonize the effects of lithium, and thus to identify the signaling system involved in Li⁺-mediated induction, mesenchymal fragments from E11 mouse metanephros were treated for 18-48 hr with Li⁺ in the presence of other signaling effectors (0.5-5 mM dibutyryl cyclic AMP, 0.25-1 mM dibutyryl cyclic GMP, 40-80 ng/ml staurosporine, 10-100 ng/ml phorbol 12-myristate acetate, 10-40 mM KCl). None prevented Li⁺-induced differentiation (assessed by immunohistochemistry for NCAM).

DISCUSSION

We have discovered that lithium treatment induces nephrogenic mesenchyme to begin a program of differentiation similar to that initiated by classical living inducers.

The natural mediator of induction seems to operate via cell-cell contact rather than a small diffusible factor (Wartiovaara et al., 1974; Saxén et al., 1976). Contact presumably activates a second messenger pathway leading to activation of specific genes. We suggest that Li⁺ operates by intervening in this second messenger pathway. Li⁺ can act via the inositol phosphate pathway (e.g., sea urchin development; Forer and Sillers, 1986), by altering cyclic nucleotide metabolism (e.g., brain; Ebstein et al., 1978), or by changing membrane potential (e.g., pituitary; Kato et al., 1991). Our attempts to alter the effects of lithium on kidney mesenchyme with modulators of these pathways were unsuccessful, and thus we have no indication of what the critical pathway might be. This is unfortunately quite common; the mechanism of lithium action remains stubbornly obscure in several important systems, such as control of human leukopoiesis and manic depression (reviewed in Birch, 1991).

Lithium-mediated induction of differentiation is “weaker” than that mediated by dorsal spinal cord in two respects. First, the average number of lithium-induced differentiating cell aggregates per mesenchymal fragment (2.9) was low compared with induction by dorsal spinal cord (6.2). Grobstein (1955) compared several candidate embryonic tissues for their ability to induce differentiation and found a great difference between those he characterized as “strong” inducers such as dorsal spinal cord (6 tubules per rudiment) and “weak” inducers such as the ventral-most part of spinal cord (2 tubules/rudiment). In this context, Li⁺ is comparable to one of Grobstein’s weak inducing tissues. The analogy may go further; Grobstein found that while strong inducers gave a uniform developmental response, the effect of weak ones was much more variable in terms of the structures induced.

The second respect in which Li⁺-mediated induction is weak is the failure of Li⁺-induced epithelia to complete their developmental program; they stall at or usually before the comma-shaped stage. This seems not to be due to Li⁺ overaccumulation during prolonged culture, as Li⁺ removal after 15 hr did not prevent it. It is also not due to a fault with the tissue used, as mesenchyme fragments taken from the same pool but used for spinal cord induction formed normal tubules. One possible explanation could be that while lithium treatment is efficient at eliciting the earliest responses to induction, it may fail to mimic other signals that are needed for progression along the developmental program. Indirect evidence exists for additional signals and “checkpoints” in nephron development (reviewed by Bard et al., 1994); for example, addition of leukemia inhibitory factor (LIF) to developing kidneys inhibits nephron development beyond the condensation stage, suggesting the existence of a LIF-sensitive “checkpoint” at this stage of development (Bard and Ross, 1991).

Cells differentiating in response to lithium had unusually condensed nuclei which are generally associated with a terminal fate rather than with continuing development; this may reflect lack of survival factors that normally save differentiating kidney cells from apoptosis (Koseki et al., 1992; Coles et al., 1993). Thus,
while Li⁺ undoubtedly activates a pathway leading to epithelial gene expression, it may fail to activate, or even inhibit, another pathway required for full tubular maturation. Failure of maturation may be connected with inadequate cell cycling; mesenchymes incubated in Li⁺ show more DNA synthesis than controls, but much less than those induced by dorsal-half spinal cord. This provides a simple explanation for the small size of lithium-induced differentiating aggregates. Nephrogenic aggregates induced by lithium may fail to mature simply because they have too few cells to do so.

While comparing the development of lithium- and tissue-induced mesenchymes, we studied the precise timing of nephron differentiation in detail. Its most striking feature is sequential change; alterations in gene expression take place according to a definite program rather than simultaneously. While progressive development has been suggested by previous work (Ekbloem et al., 1980, 1981, 1983; Holthrofer et al., 1984; Vestweber et al., 1985; Fleming and Symes, 1987; Vainio et al., 1989; Garrod and Fleming, 1990; Lackie et al., 1990; Bard and Ross, 1991; Korhonen et al., 1992), our results yield sufficiently high resolution to demonstrate many distinct stages of differentiation (Table 1). In particular, we confirm an observation suggested by our previous work, that desmosomal components (desmocollins) precede the expression of keratin intermediate filaments (Garrod and Fleming, 1990). As well as illustrating the similarity between Li⁺- and tissue-mediated induction, the timetable of morphoregulatory molecule expression will allow: (i) the precise roles of these proteins in renal morphogenesis to be determined more clearly, (ii) the significance of sequential expression to be discovered, and (iii) the mechanisms regulating sequential expression to be studied.

Li⁺- and tissue-mediated induction both show a substantial delay between inductive stimulus and morphogenetic response. In other systems, such delays conceal cascades of regulatory gene transcription (e.g., first Mix1 and Xbra, then XmyOD and XMyF, then muscle actin in Xenopus mesoderm induction; Smith et al., 1991). The delay in kidney epithelogenesis may contain an analogous cascade. It may also reflect processes that sort out cell fates; although all mesenchymal cells are exposed to Li⁺, only some aggregate and become epithelial, implying either initial heterogeneity or a self-organizing property of induced mesenchyme.

Discovery of a chemical means to activate epithelial gene expression in the kidney will greatly facilitate studies of regulatory mechanisms previously hampered by the presence of contaminating inducing tis-sues. Such investigations may hold the key to the control of the earliest events in nephric development and may contribute to our understanding of the basic mechanisms of epithelial differentiation.

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