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Actions of Extracellular Matrix on Sertoli Cell Morphology and Function[1]

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ABSTRACT

Sertoli cells were isolated and cultured in the absence or presence of extracellular matrix (ECM) to determine whether ECM may influence Sertoli cell function on a molecular level. As previously described, a morphological analysis of the cells indicated that ECM allows the expression of a columnar histotype and the formation of functional complexes. The combined actions of ECM and hormones were found to have a profound effect in promoting the expression of a polarized Sertoli cell morphology. In our investigation of the effects of ECM on Sertoli cells, we used transferrin and androgen-binding protein (ABP) production as biochemical markers of Sertoli cell function. The presence of ECM was found to cause a 25% increase in the basal level of transferrin production; however, ECM had no effect on the basal level of ABP production by Sertoli cells. Regulatory agents such as follicle-stimulating hormone (FSH) and a combination of FSH, insulin, retinol, and testosterone stimulated the production of both transferrin and ABP. The ability of hormones to stimulate these Sertoli cell functions was not influenced by the presence of ECM. Similar results were obtained with 2-μm- or 50-μm-thick ECM and with a seminiferous tubule biomatrix preparation. ECM was found to increase the maintenance of long-term Sertoli cell cultures; however, the decline in Sertoli cell functional integrity, which occurs during cell culture, was not affected by the presence of ECM. An additional functional parameter examined was the radiolabeled proteins secreted by Sertoli cells. ECM did not promote the production or affect the electrophoretic profile of Sertoli cell-secreted proteins under basal or hormonally stimulated conditions. Combined results indicated that although ECM allowed the expression of a normal Sertoli cell histotype, ECM had no major effects on the Sertoli cell functions analyzed nor on the hormonal regulation of these functions. The inability of ECM to affect Sertoli cell function on a molecular level is discussed with regard to environmental as opposed to regulatory cellular interactions. Our observations imply that dramatic effects of ECM on cell morphology do not necessarily correlate to subsequent effects on cellular function.

INTRODUCTION

The organization of a tissue and separation of different cell types generally requires the formation of a complex extracellular matrix (ECM) (for review, Hay, 1981). An example of this is the separation of epithelial cells and mesenchymal cells by a basement membrane. ECM provides the proper extracellular environment for a cell, as well as structural integrity for a tissue. Interactions between different cell types that are mediated by ECM which involve cell attachment and cell recognition are examples of environmental cellular interactions. For these reasons, ECM components have been used in the in vitro culture of cells to elucidate the functions and importance of ECM. Observations have indicated that the use of ECM in vitro often provides a more appropriate environment for cell attachment and growth which results in a more natural morphology (Hay, 1981; Kleinman et al., 1981). Suggestions also have been made that ECM may promote or maintain a more normal differentiated state of the cell (Hay, 1981; Gospodarowicz, 1984).

Sertoli cells are the epithelial cells that form the seminiferous tubule and play a major role in the control and maintenance of spermatogenesis. Peritubular myoid cells are the predominant mesenchymal cell type that surround the seminiferous epithelium and are separated from Sertoli cells by a basement membrane (Dym and Fawcett, 1970). Sertoli cells and peritubular cells cooperate in the production and formation of this complex ECM (Skinner et al., 1985;

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Skinner and Fritz, 1985a). The morphology of Sertoli cells in vivo has been shown to be very complex (Russell et al., 1983) and closely associated with the basement membrane of the seminiferous tubule (Dym and Fawcett, 1970). The importance of this basement membrane has been emphasized by the observation that isolated Sertoli cells cultured on a plastic substratum do not have a normal morphology, whereas cells cultured on ECM express a more normal histotype (Tung and Fritz, 1984; Hadley et al., 1985).

Sertoli cell cultures have also been used to elucidate cellular functions through the identification of secreted products. The iron-binding protein testicular transferrin (Skinner and Griswold, 1980) and androgen-binding protein (ABP) (Fritz et al., 1976) are two proteins secreted by Sertoli cells. Hormonal regulation of Sertoli cell function has been investigated through an analysis of the regulation of transferrin and ABP production (Skinner and Griswold, 1982; Mather et al., 1983; Skinner and Fritz, 1985b). Both transferrin and ABP provide biochemical markers of Sertoli cell function. Agents that influence the production of these proteins affect Sertoli cell function on a molecular level. A previous study suggested that ECM may have a potential direct trophic effect on Sertoli cells (Hadley et al., 1985). The current study was designed to investigate the effect of ECM on Sertoli cell function and on the hormonal regulation of these functions. The potential actions of ECM on cell morphology versus cellular function was examined.

MATERIALS AND METHODS

Cell Preparation and Culture

Sertoli cells were isolated from the testis of 20-day-old rats by sequential enzymatic digestion (Dorrington et al., 1975) according to a modified procedure previously described (Tung et al., 1984). Decapsulated testis fragments were digested with trypsin (2.5 mg/ml) (Gibco, Grand Island, NY) to remove Leydig cells, then with collagenase (1 mg/ml Type I, Sigma Chemical Co., St. Louis, MO) and finally with hyaluronidase (1 mg/ml, Sigma). Sertoli cells were then plated in 24-well (1 ml per well) Linbro plates (Linbro, McLean, VA) at approximately 5×10^5 cells per well. Cells were maintained at 32°C in a 5% CO_2 atmosphere in Ham's F-12 medium (Gibco). Sertoli cultures were treated at the time of plating as described in the *Results* section and re-treated after 48 h of culture when the medium was replenished. Unless otherwise stated, medium was collected for analysis after a further 72 h, on Day 5 of culture, and the cells were harvested for DNA assay. Sertoli cell cultures were treated as outlined in the *Results* section with 100 ng/ml follicle-stimulating hormone (FSH; NIADDK, Pituitary Agency, Baltimore, MD), 5 μg/ml insulin, 0.35 μM retinol, and 1 μM testosterone. Peritubular cells were obtained from the collagenase digestion supernatant after tubule segments had been obtained by gravity sedimentation as previously described (Skinner and Fritz, 1985c).

Preparation of Extracellular Matrix-Coated Plates

Half of a 24-well Linbro plate (Falcon Plastics, Los Angeles, CA) was coated with a ECM (Matrigel, Collaborative Research Inc., Waltham, MA) the other half was left uncoated and was designated plastic. The Matrigel was diluted (1:1) with cold medium; a small amount was added to each well and the excess was removed. When indicated, a thicker (increased) matrix layer was used. For these experiments, a 100-μl aliquot of diluted Matrigel was applied to the 2-cm^2 plastic surface. The plates were pre-cooled prior to coating and kept over ice during the coating process to prevent premature gelation. Coated plates were either incubated in the culture chamber for 30 min to promote gelation or stored at 4°C. Gel formation was initiated in the stored plates by incubation in the culture chamber just prior to use.

As an alternate ECM, a seminifeorus tubule biomatrix was isolated as previously described (Tung and Fritz, 1984; Meezan et al., 1978), with the following modifications. The tunica was removed from adult rat testes and the tubules were minced and washed with gravity sedimentations in Hanks' Balanced Salt solution plus 10 μg/ml DNAase. The pellet was washed with 10 volumes of water with agitation for 1 h and centrifuged. The resulting pellet was washed, with agitation, in three successive buffers for 2 h, each wash followed by centrifugation: 1) in 3% (v/v) Triton X-100, 2) in 1 M NaCl and 10 μg/ml DNAase (Sigma), and finally 3), in 4% (w/v) deoxycholate. The final pellet was washed four times with water, frozen in liquid nitrogen, and placed in a plastic capsule with plastic pestle (L. D. Caulk Co., Milford, DE) that had been cooled in liquid nitrogen. The cold capsules and contents were agitated

in a Wig-L-Bug dental amalgamator (Crescent Dental Manufacturing Co., Nashville, TN). The resulting powder was then suspended in culture medium and allowed to settle over Matrigel ECM-coated plates or suspended in 95% ethanol and placed on a plastic surface and allowed to evaporate before use (Tung and Fritz, 1984).

Radioimmunoassays

Transferrin production by Sertoli cells was assayed by a radioimmunoassay described previously (Skinner and Griswold, 1982). An aliquot of the culture medium was incubated with rabbit anti-rat transferrin antibody (Cooper Biomedical, Melvern, PA) and iodinated transferrin for 1 h at 37°C followed by a 1-h incubation with goat anti-rabbit immunoglobulin G (IgG) antibody (Sigma). Complexed antibody was then precipitated with polyethylyene glycol (Sigma) and pelleted by centrifugation, and radioactivity in the pellets was determined.

ABP was assayed by radioimmunoassay as described previously (Skinner and Fritz, 1985b). An aliquot of the culture medium was incubated with rabbit anti-rat ABP (National Hormone and Pituitary Program, NIH, Bethesda, MD) and iodinated ABP at 4°C for 18 h. Goat anti-rabbit IgG and polyethylene glycol were added and the samples were incubated an additional 18 h at 4°C. The antibody complex was then pelleted by centrifugation and the radioactivity was determined.

DNA and Protein Assay

DNA was measured fluorometrically with ethidium bromide (Karsten and Wollenberger, 1977). At the end of the culture period, the medium was removed, ethidium bromide buffer (EBB: 20 mM sodium chloride, 5 mM ethylenediaminetetracetic acid, 10 mM tris (hydroxymethyl)aminomethane, pH 7.8; Sigma) was added to the wells and the cells were sonicated. An aliquot of the sonicated cell suspension was added to an equal volume of ethidium bromide solution (0.25 mM ethidium bromide, 100 units/ml heparin in EBB) and diluted 1:2 with EBB buffer and allowed to incubate at room temperature for 30 min. Fluorescent emission at 585 nm with 350 nm excitation was then monitored. A standard curve with calf thymus DNA was used to quantify DNA levels in the culture wells. This assay has a sensitivity of approximately 0.1 μg DNA and is linear up to 2.5 μg DNA. Total protein concentration was measured according to the method of Bradford (Bradford, 1976).

Gel Electrophoresis and Fluorography

Starting on Day 5 of culture, Sertoli cell and peritubular cell cultures were maintained for 48 h in glycine-free and methionine-free medium containing 5 μCi/ml [^{35}S]methionine and 5 μCi/ml [^{3}H]glycine. The medium was collected, centrifuged, and used for analysis. The amount of radiolabeled proteins analyzed was obtained from the same number of cells measured as μg DNA. Radiolabeled proteins were electrophoretically analyzed on sodium dodecyl sulfate 5–15% polyacrylamide gradient slab gels under reducing conditions with the Laemmli (Laemmli, 1970) buffer system. The gels were fluorographed with diphenyloxazole in acetic acid as previously described (Skinner and Griswold, 1983).

Microscopy

Sertoli cells obtained from 20- and 10-day-old rats were plated over a plastic or matrix-coated substratum and cultured for 5 or 14 days. To obtain a true representation of cell-cell interactions, wells that contained isolated cells or very small groups of cells were not accepted for morphological analysis.

Cells maintained on plastic or on ECM-coated surfaces were rinsed with phosphate-buffered saline (PBS), then fixed for 2 h in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). After rinsing with the buffer, cells were post-fixed in 1% osmium tetroxide in the same buffer solution. After dehydration in a graded series of ethanol solutions, propylene oxide was added to the culture vessels to release the monolayers from plastic. Samples were retrieved immediately from the vessels, transferred through a second change of propylene oxide, then infiltrated and embedded in epoxy resin (Embed 812, Electron Microscopy Sciences, Ft. Washington, PA). Sections 1 μm thick were stained with alkaline toluidine blue for light microscopic examination and selection of appropriate areas for electron microscopic examination. Variable toluidine blue staining intensity is attributed to the processing of sections at different times and altered staining duration. After blocks were trimmed, sections showing silver interference color were collected on copper grids, stained with uranyl acetate and lead citrate, and examined at 75 kv in a Hitachi H-600 electron microscope. This analysis was

done as previously described (Olson et al., 1983) by Dr. Loren Hoffman, Director of the Vanderbilt University Reproductive Biology Research Center, Electron Microscopy Core Laboratory.

RESULTS

Morphological Analysis

A morphological analysis was performed to validate the ECM culture system used in the current study. Light micrographs of vertical sections from cultures of Sertoli cells plated on plastic and on ECM substratum are shown in Figure 1. Cells cultured on ECM or plastic were maintained in the presence of a combination of regulatory agents including FSH, insulin, retinol, and testosterone (FIRT). Hormonal stimulation of Sertoli cells on plastic substratum resulted in contraction and flattening of the cell, as previously described (Tung et al., 1975). Cells cultured on ECM in the presence of hormones had a dramatic increase, 4- to 5-fold, in cell height and polarization (Fig. 1). More than 90% of the nuclei were localized on the basal surface of the Sertoli cells plated on ECM (Fig. 1). Cells cultured on ECM in the absence of hormones showed a decreased ability to polarize in culture. Our observations indicated that a combination of ECM and hormones had a more dramatic effect on the thickness of the polarized cell layer than the presence of ECM alone (data not shown). Sertoli cells cultured for 5 days after a similar morphological analysis showed a reduced effect of ECM on the thickness of the polarized cell layer. Most previous studies on Sertoli cell morphology used cells cultured for 14–20 days (Tung and Fritz, 1984; Hadley et al., 1985). For these reasons,

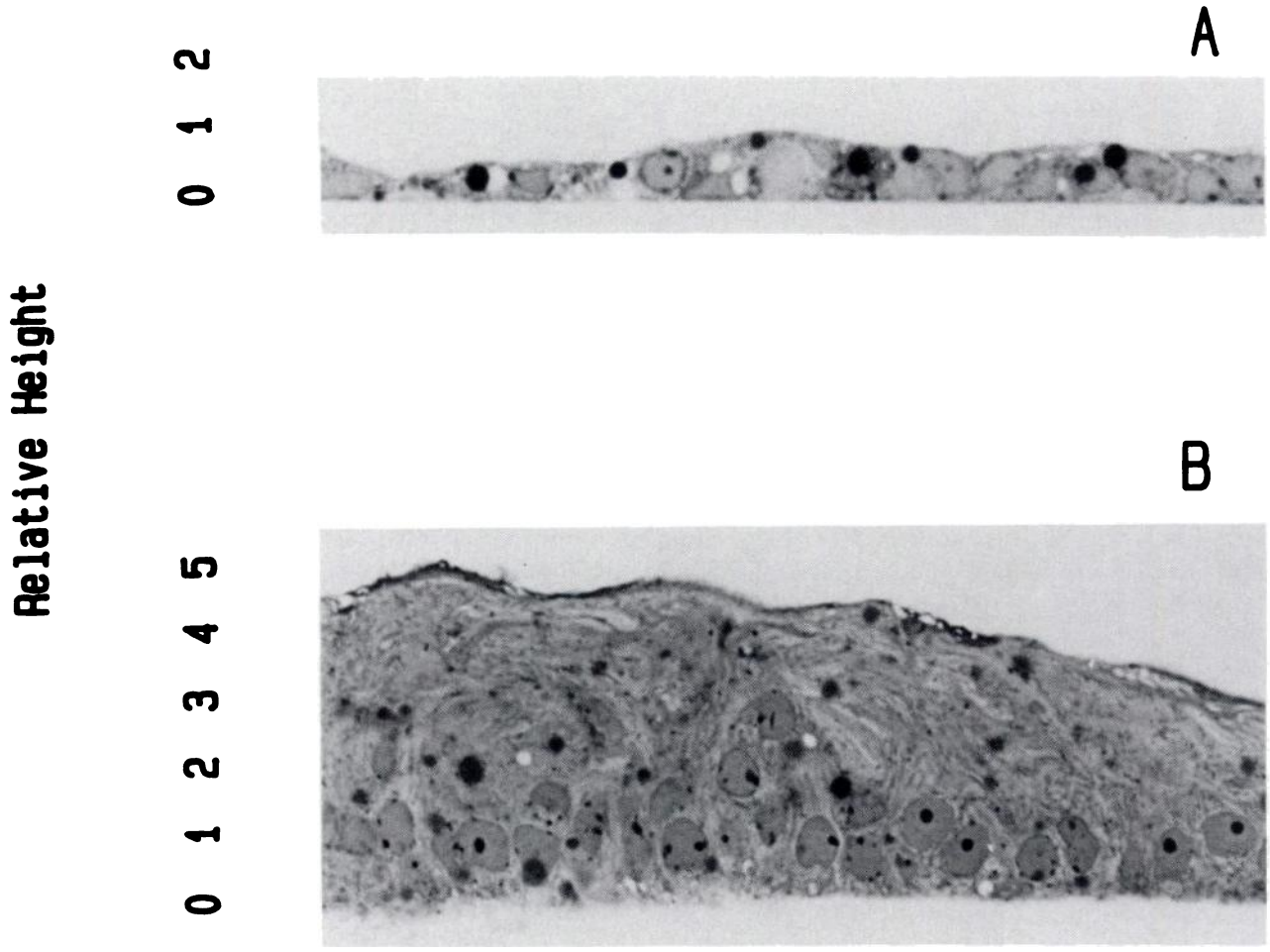

FIG. 1. Light micrographs (×800) of Sertoli cells isolated from 20-day-old animals and plated over a plastic substratum *(A)* or over a matrix-coated substratum *(B)*. Sertoli cells were cultured in the presence of regulatory agents (combination of follicle-stimulating hormone, insulin, retinol, testosterone) for 14 days. The cells were then fixed and processed as thick sections as described in *Materials and Methods*.

the micrographs obtained at 14 days of culture are presented for comparison.

Figure 2, a high-power electron micrograph of a section representative of those shown in Figure 1, demonstrates that the ECM completely coated the culture dish and provided a uniform surface for cell attachment. This matrix coating was evident in all micrographs of matrix-coated plates examined with an approximate thickness of 1–2 μm. No cells were exposed to the plastic surface in any of the matrix-coated plates examined.

Cells cultured on both plastic and ECM substrata were viable and contained an appropriate assemblage of organnelles, including irregularly shaped nuclei, abundant mitochondria, and large lipid droplets. Less than 1% of the cells examined with either light or electron microscopy exhibited abnormal morphologies, regardless of the absence or presence of ECM. Although no morphometric analysis was done, no obvious differences in the size or number of organelles was observed whether cells were cultured on plastic or ECM. Junctional complexes were more often observed when cells were cultured on ECM. Results of a morphological analysis on Sertoli cells isolated from 10-day-old animals were essentially the same as those obtained from 20-day-old animals, with the possible occurrence of more junctional complexes with the 10-day-old cells (unpublished observation). Morphological analysis of Sertoli cells on a 50-μm ECM generated similar results, with the exception of the occurrence of some cellular inclusions within the matrix.

Functional Analysis

Transferrin and ABP production by Sertoli cells were used as biochemical markers of Sertoli cell function. Transferrin production was significantly increased by treatment with FSH and with FIRT (Fig. 3). The presence of an ECM did not have any major effects on transferrin production or on the ability of hormones to stimulate transferrin production. A consistantly small increase in transferrin secretion was seen when cells were cultured on ECM, but the increase was not statistically significant (Fig. 3). When the data from 13 different experiments on different cell preparations were combined (giving an n value of 70) ECM was found to increase the basal level of transferrin production a small amount, from 25 to 30 ng/μg DNA, that was statistically significant ($p<0.01$, Student's t-test). Therefore, our results indicated that ECM had a minor influence on the basal level of transferrin production when a large number of experiments were combined for analysis.

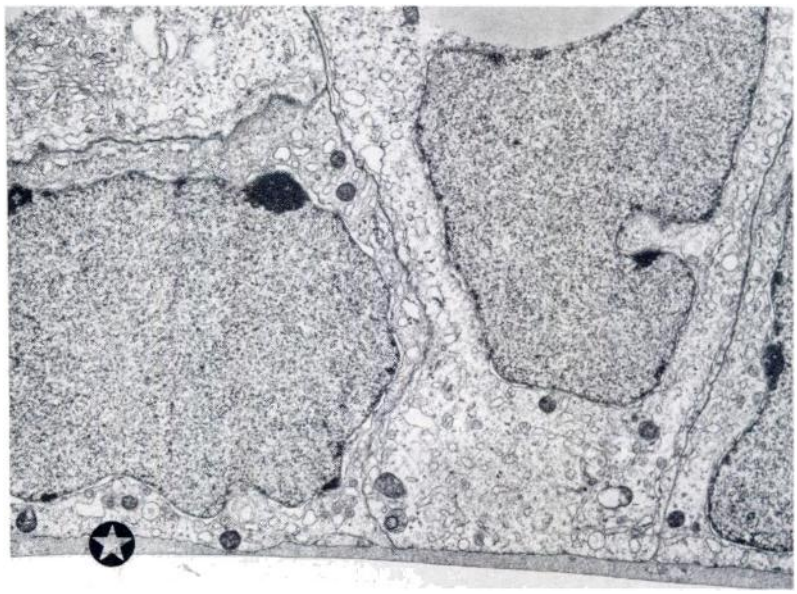

FIG. 2. Electron micrograph (×8000) of Sertoli cells plated over a matrix-coated substratum, designated with a *star*. Cells were isolated from 20-day-old animals and cultured for 14 days in the presence of regulatory agents (combination of follicle-stimulating hormone, insulin, retinol, testosterone).

ABP was produced by Sertoli cells at approximately one-tenth the level that transferrin was produced. Both FSH and FIRT treatment of Sertoli cell cultures stimulated ABP production (Fig. 4). FSH stimulated both ABP and transferrin production approximately 2-fold (Figs. 3, 4). The presence of ECM did not significantly influence ABP production or the ability of hormones to stimulate ABP production (Fig. 4). Similar results were obtained when Sertoli cells isolated from 10-day-old animals were used (data not shown).

ECM coatings 1–2 μm thick and 30–50 μm thick, as determined with electron microscopy, were prepared to determine if the thickness of the ECM would alter the results obtained. As found with the 1- to 2-μm-thick ECM, the 50-μm-thick ECM also had no effect on the basal level of transferrin production (Table 1). The data obtained with the 50-μm-thick ECM had a larger degree of uncertainty, which, in part, was due to the variability in plating efficiency and cell density observed. Results indicate that the 50-μm-thick ECM did not have a significant effect on the hormonal regulation of transferrin production.

TABLE 1. The effect of variable thicknesses of extracellular matrix (ECM), as determined by electron microscopy, on transferrin production by Sertoli cells.*

Treatment	Plastic	ECM	
		1–2 μm thick	30–50 μm thick
Control	23 ± 1	29 ± 1	25 ± 2
FSH**	53 ± 5	57 ± 3	54 ± 7
FIRT***	101 ± 7	106 ± 7	122 ± 5

*Values are represented as ng transferrin/μg Sertoli cell DNA and are presented as the mean ± SEM of a minimum of three experiments done in triplicate. Values within treatment groups were not statistically different ($p<0.01$, Student's t-test.)

**FSH = follicle-stimulating hormone.

***FIRT = combination of FSH, insulin, retinol, and testosterone.

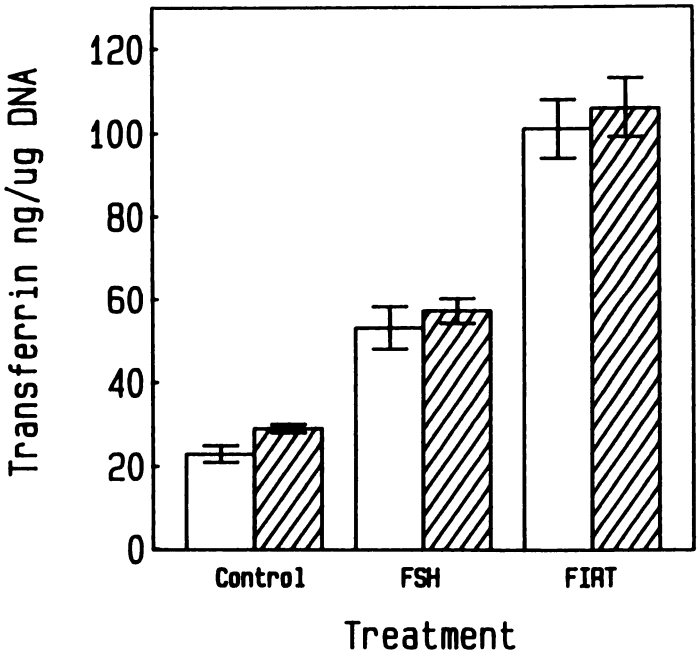

FIG. 3. The effect of extracellular matrix on transferrin secretion by Sertoli cells. Sertoli cells isolated from 20-day-old rats were plated over a plastic substratum *(open bars)* or over a matrix-coated substratum *(hatched bars)*. The cells were stimulated with follicle-stimulating hormone *(FSH)*, a combination of regulatory agents designated *FIRT* (FSH, insulin, retinol, testosterone) or were left untreated *(control)*. Hormones, when present, were added at the time of plating and replenished after a 48-h culture period. Transferrin accumulated during Days 2–5 of culture was determined and the data were normalized to μg DNA/well from cells harvested on Day 5 of culture. Data are averages ± SEM from triplicate wells from at least 9 experiments. Differences between values obtained for plastic and matrix-coated plates were compared by Student's t-test, and no statistical differences were noted ($p>0.01$).

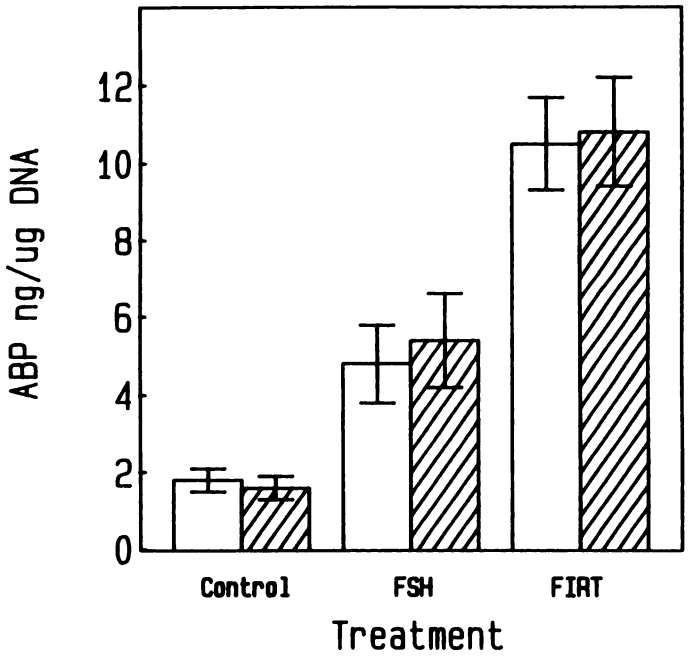

FIG. 4. The effect of extracellular matrix on androgen-binding protein (ABP) secretion by Sertoli cells. Sertoli cells isolated from 20-day-rats were plated over a plastic substratum *(open bars)* or over a matrix-coated substratum *(hatched bars)*. The cells were stimulated with follicle-stimulating hormone *(FSH)*, a combination of regulatory agents designated *FIRT* (FSH, insulin, retinol, testosterone) or untreated *(Control)*. Hormones, when present, were added at the time of plating and replenished after a 48-h culture period. ABP accumulated during Days 2–5 of culture was determined and the data were normalized to μg DNA/well from cells harvested on Day 5 of culture. Data are averages ± SEM from triplicate wells from at least 7 experiments. Differences between values obtained for plastic and matrix-coated plates were compared by using Student's t-test, and no statistical differences were found ($p>0.01$).

TABLE 2. The effect of a seminiferous tubule biomatrix preparation on transferrin production by Sertoli cells.*

Treatment	Plastic	Biomatrix	Biomatrix on matrigel
Control	23 ± 1	22 ± 2	23 ± 2
FSH**	53 ± 5	45 ± 3	51 ± 5
FIRT**	101 ± 7	106 ± 10	124 ± 16

*Values are represented as ng transferrin/μg Sertoli cell DNA and are presented as the mean ± SEM of a minimum of three experiments done in triplicate. Values within treatment groups were not statistically different ($p<0.01$, Student's t-test.)

**FSH = follicle-stimulating hormone.

***FIRT = combination of FSH, insulin, retinol, and testosterone.

Our observations indicated that similar results were obtained with ECM either 1–2 μm thick or 30–50 μm thick (Table 1).

A seminiferous tubule biomatrix was prepared as an alternate ECM to the Matrigel. Electrophoretic analysis of this revealed the presence of major protein components between 100 and 400 kDa. Proteins that comigrated with collagen, fibronectin, and proteoglycans could be identified. Sertoli cells plated on this biomatrix preferentially attached to the matrix and formed large cell clumps. This confirmed previous observations that the Sertoli cells adhere readily to the biomatrix (Tung and Fritz, 1984). Transferrin production by Sertoli cells was not altered by the presence of the biomatrix (Table 2). These results were similar whether the biomatrix was coated over a plastic substratum or over a 1- to 2-μm-thick Matrigel-coated substratum. Our observations indiated that the data obtained with the Matrigel ECM were similar to those obtained with a biomatrix isolated from seminiferous tubules (Table 2).

Transferrin production by Sertoli cells was measured over an 8-day period to determine if the presence of ECM would extend the period of maximal secretion. In this study, the Sertoli cells were maximally stimulated with regulatory agents, then, every 48-h culture period, selected wells were terminated, culture medium was collected for assay and the cells were sonicated to assess DNA content. For Sertoli cells plated over a plastic substratum, transferrin production rose steadily from Day 2 to Day 6 in culture and dropped from Day 6 to Day 8 (Fig. 5). When the Sertoli cells were plated over a matrix-coated plate, transferrin production rose from Day 2 to Day 4 and decreased gradually from Day 4 to Day 8 of culture. When the two sets of data were compared, the differences between plastic and matrix-coated plates were not statistically significant at any time point. Similar analysis of ABP production also demonstrated that ECM did not effect ABP production as a function of time in culture (data not shown).

The DNA levels for cells plated over ECM were compared with those plated over a plastic substratum for each time point. These data were taken as an indicator of the cell number at the time the medium was removed for assay and were used to determine if the presence of ECM would enhance the longevity of cell attachment in culture. The data show that DNA levels decreased steadily from Day 2 to Day 10 for cells cultured on a plastic substratum (Fig. 6). However, the DNA levels for cells plated on an ECM were relatively stable from Day 2 through Day 6 in culture, then dropped from Day 6 to Day 10 (Fig. 6). Therefore, ECM was found to improve the maintenance of long-term Sertoli cell cultures.

To examine a more general effect of ECM on Sertoli cell function, radiolabeled secreted proteins were analyzed. Proteins previously identified as Sertoli cell-secreted products include transferrin (Skinner and

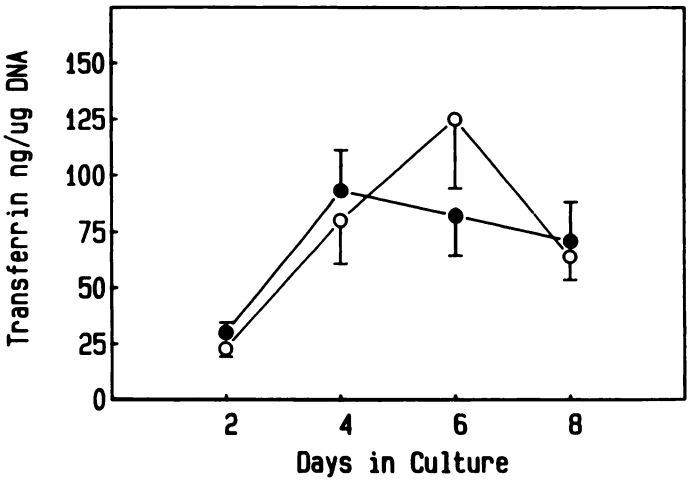

FIG. 5. Effect of extracellular matrix on the time course of transferrin secretion by Sertoli cells. Sertoli cells isolated from 20-day-old rats were plated over a plastic (○) or a matrix-coated substratum (●). The cells were stimulated with a combination of regulatory agents designated *FIRT* (follicle-stimulating hormone, insulin, retinol, testosterone). Hormones were added at the time of plating and maintained throughout the culture period. The medium was changed every 48 h, but on Days 2, 4, 6, and 8 of culture, selected wells were terminated, the medium was removed for assay, and the cells were harvested for DNA assay. Data are averages ± SEM of duplicate wells from 3 experiments. Differences between values obtained for plastic and matrix-coated plates on a specific day of culture were compared by Student's t-test,and no statistical differences were noted ($p>0.01$).

Griswold, 1980), dimeric acidic glycoprotein (Sylvester et al., 1984), and ceruloplasmin (Skinner and Griswold, 1983b). Production of all these proteins increased when the cells were hormonally stimulated (Fig. 7). ABP is a minor secretory product of Sertoli cells and cannot be visualized with this procedure. The presence of ECM did not appear to enhance the synthesis and secretion of any protein in either control or hormonally stimulated Sertoli cell cultures (Fig. 7). As an alternate cell type, radiolabeled proteins secreted by testicular peritubular cells were also analyzed electrophoretically. Fibronectin is a protein that has been shown to be produced by peritubular cells and is not produced in detectable amounts by Sertoli cells (Tung et al., 1984) (Fig. 7). The absence of a 200 kDa fibronectin protein in the Sertoli cell-secreted protein profile indicated a minimal contamination of the Sertoli cell preparation with peritubular cells. The presence of ECM did appear to alter the pattern of proteins synthesized and secreted by peritubular cells. Serum treatment of the cells also appeared to stimulate the production of the same proteins as those stimulated by ECM (Fig. 7). Therefore, although ECM had no effect on the synthesis of Sertoli cell-secreted proteins, effects were observed on the synthesis and/or presence of peritubular cell-secreted proteins.

The effect of ECM on the culture environment was also determined by examining the release of proteins from ECM-coated culture plates. Cell-free ECM-coated and plastic culture plates were incubated for 7 days with changes of medium on Days 2 and 5. The amount of protein released by a 2-cm² surface area into 1 ml of medium was determined (Table 3). As expected, nondetectable levels of protein were found in medium collected from plastic culture plates. Detectable levels of protein were present in medium collected from ECM-coated plates. The amount of protein released by ECM decreased with time in culture such that nondetectable levels were present after 7 days of incubation. An increase in the thickness of the ECM coating resulted in a corresponding increase in the amount of protein released into the medium (Table 3). Electrophoretic analysis of the proteins released by the ECM demonstrated the presence of proteins with molecular weights between 20,000 and 400,000, with the majority being greater than 60,000 (unpublished observation). Therefore, in addition to providing a substratum for cell attachment, the extracellular matrix also released proteins into the culture environment in the early stages of incubation.

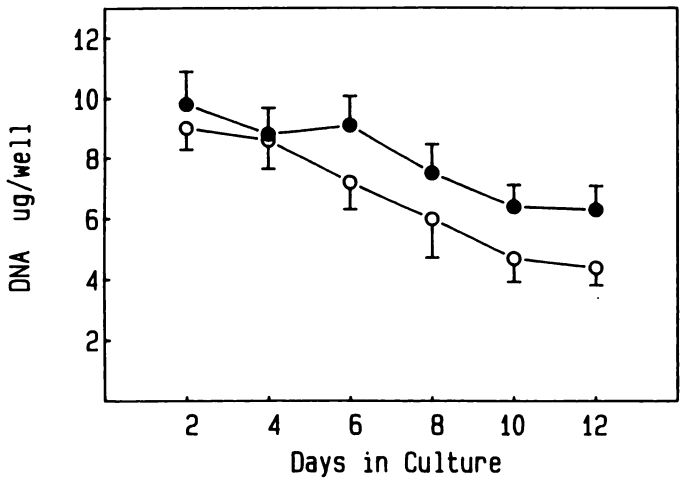

FIG. 6. The effect of extracellular matrix on DNA levels obtained from cells harvested at various time points after plating. Data are the average of DNA values obtained from plates used in *Figure 7*. Sertoli cells were plated on a plastic (○) or matrix-coated (●) substratum.

TABLE 3. The amount of total protein released from cell-free plastic and from culture plates coated with different thicknesses of extracellular matrix was determined (μg protein/2 cm²) from medium collected on Days 2, 5, and 7.*

Collection (day)	Plastic	Matrigel matrix (1–2 μm thick)	Matrix (30–50 μm thick)
0–2	ND	6 ± 0.4	50 ± 1.7
2–5	ND	1 ± 1.0	20 ± 3.8
5–7	ND	ND	6 ± 1.1

*Values are the mean ± SEM of two experiments done in replicate; ND represents nondetectable levels of protein.

DISCUSSION

Morphological analysis of Sertoli cells cultured on plastic and ECM substrata was performed on cells isolated from 10- and 20-day-old rats. The presence of ECM significantly increased the height of the polarized cell layer and improved the cytoarchitecture of the cultured cells. Micrographs indicate that cells cultured on ECM and treated with hormones form a polarized columnar shape. This confirms previous observations that ECM allows the Sertoli cell to express a more normal histotype (Tung and Fritz, 1984; Hadley et al., 1985, 1987; Janecki and Steinberger, 1987). Studies examining the effects of ECM on Sertoli cell morphology have generally utilized hormone-supplemented medium (Tung and Fritz, 1984; Hadley et al., 1985, 1987; Janecki and Stein-

berger, 1987). Observations from the current study indicate that a combination of ECM and hormones appears to have a more significant effect on Sertoli cell morphology than the presence of ECM alone. Therefore, ECM appears to provide the proper extracellular environment required for the cells to remain attached and in close contact, such that ECM and hormones can promote the expression of a unique morphology. Morphologic analysis also indicated that cell viability was not a major variable in the current study. Observations confirm previous reports on the dramatic effects of ECM on Sertoli cell structure and indicate that the cell culture system used is adequate to examine the effects of ECM on Sertoli cell function.

Secreted protein such as testicular transferrin and ABP are used as biochemical markers of Sertoli cell function. Agents that regulate the production of these proteins reflect a modulation of Sertoli cell function on a molecular level. Previous investigations have demonstrated that a combination of FSH, insulin, retinol, and testosterone is required to obtain an apparent maximum stimulation of both transferrin (Skinner and Griswold, 1982) and ABP (Karl and Griswold, 1980) production. This combination of regulatory agents stimulated the production of transferrin and ABP approximately 4-fold above basal levels in

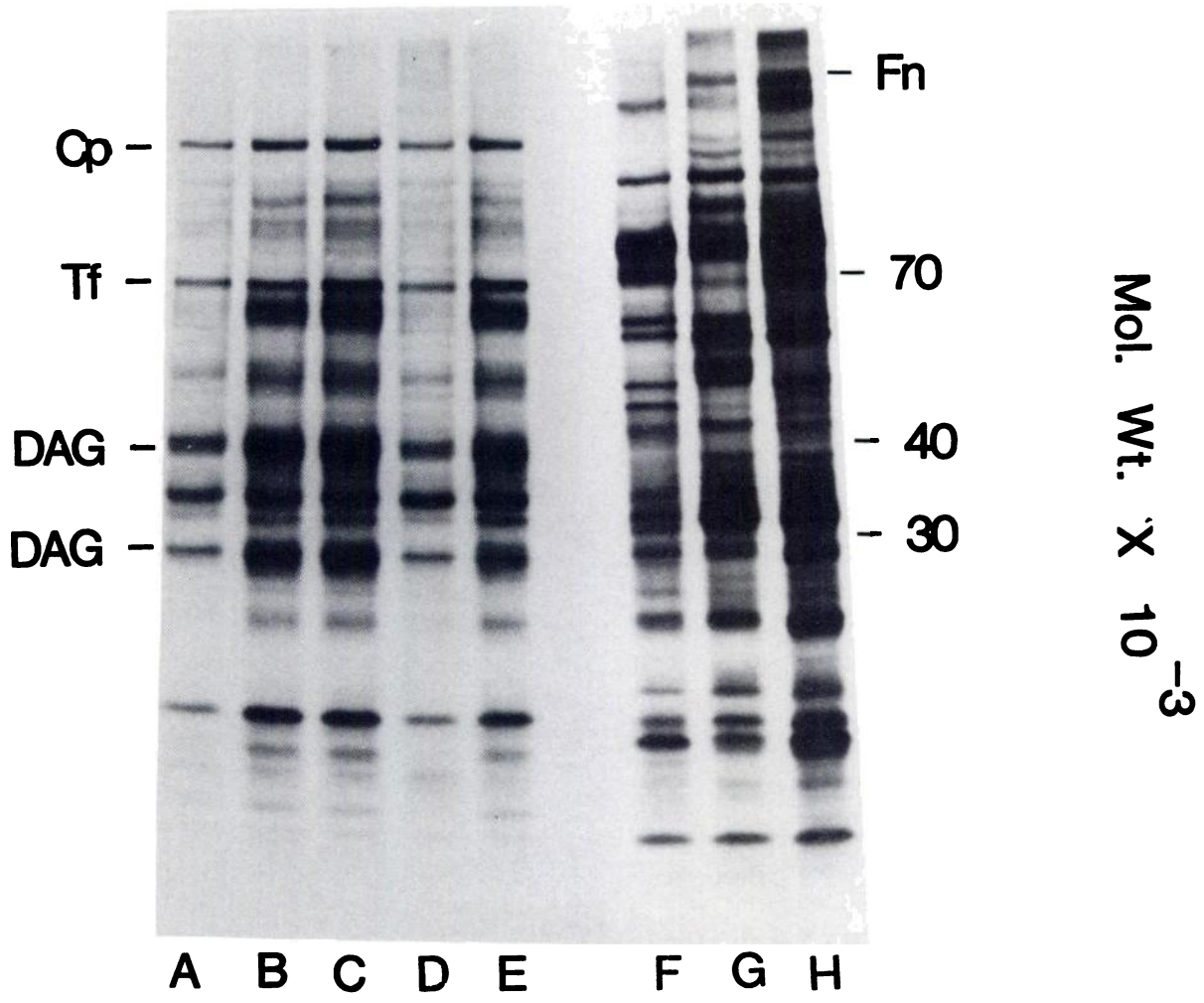

FIG. 7. The effect of extracellular matrix on the synthesis and secretion of radiolabeled proteins by cultured Sertoli cells and peritubular cells. Sertoli cells were plated over a plastic *(Lanes A–C)* or matrix-coated substratum *(Lanes D* and *E)* and cultured either with or withoutregulatory agents (follicle-stimulating hormone [FSH]or a combination of FSH, insulin, retinol, testosterone [FIRT]). Peritubular cells were plated over a plastic *(Lanes F* and *G)* or matrix-coated *(Lane H)* substratum and cultured in a serum-free environment. As a control, some peritubular cells were cultured with serum for 2 days to enhance cell attachment and then changed to a serum-free environment. The medium was removed, centrifuged, and analyzed by SDS gel electrophoresis and fluorography as described in *Materials and Methods (A, D,* Control; *B,* FSH; *C, E,* FIRT; *F, H,* no serum; *G,* serum Days 1 and 2). Previously identified Sertoli cell products are transferrin *(Tf)* ceruloplasmin *(Cp),* and dimeric acidic glycoprotein *(DAG);* a peritubular cell product is fibronectin *(Fn).*

the current study. These results are consistent with previous observations on the hormonal regulation of Sertoli cell function (Skinner and Griswold, 1982; Skinner and Fritz, 1985b). The presence of ECM had no dramatic effect on the basal level of transferrin or ABP production. Similar results were obtained whether a 1- to 2-μm-thick or 30- to 50-μm-thick ECM was present. Using the combined data from 13 different experiments and 70 samples, we observed 25% increase in the basal level of transferrin production when the Sertoli cells were cultured on ECM. Neither the sensitivity of the Sertoli cells to hormones nor the magnitude of stimulation by hormones was affected by the presence of ECM. Therefore, the current study indicates that ECM does not appear to have major effects on the hormonal regulation of Sertoli cell function. These observations are in contrast to a previous report which implied that ECM increases both the basal and hormone-stimulated levels of transferrin and ABP production (Hadley et al., 1985). A complete explanation for these apparent discrepancies is not available at this time. One of the major technical differences between the study of Hadley and coworkers (1985) and the current investigation is the method of data normalization. All the data obtained in the current study were normalized per μg DNA at the time of medium collection. Normalization of data with a hemocytometer or automated cell counter was found not to be as accurate as the DNA assay, because a single cell suspension of Sertoli cells could not be obtained without a severe loss of cells due to lysis. The inability to obtain a single cell suspension may be due, in part, to the increased junctional complex formation between Sertoli cells on ECM. Therefore, data normalized with cell counts will have an artificially low cell number, particularly for cells cultured on ECM, and thus increase the value of the final data. In addition, as shown in Figure 6, cell number decreased with time in culture. For this reason, normalization of data at the time the medium is collected is more accurate than normalization of data at a later time in the culture period. When a DNA assay was used to normalize the data in the current study, we observed no major effects of ECM on transferrin or ABP production by Sertoli cells.

Total secreted proteins were examined to investigate qualitative effects of ECM on Sertoli cell function. The radiolabeled Sertoli cell-secreted protein profile shown in Figure 7 is similar to those reported previously (Skinner and Griswold, 1980; Tung et al., 1984; Skinner and Griswold, 1983a,b). Hormones significantly stimulated the production of a number of the secreted proteins; however, ECM had no apparent influence on the basal or hormone-stimulated secreted protein profiles. In contrast, ECM did promote the production of a number of radiolabeled secreted proteins by testicular peritubular cells. Collagen synthesis by Sertoli cells previously has been shown to be stimulated by the presence of ECM (Hadley et al., 1985). We found that ECM did not stimulate the production of proteins corresponding to the molecular weights of collagen by Sertoli cells, but did with peritubular cells. A more sensitive procedure to detect effects on collagen synthesis would be to radiolabel proteins with [^{3}H]proline. The method used in the current study may not have been sensitive enough to detect small effects on collagen synthesis. Our observations imply that ECM has no major effects on the proteins secreted by Sertoli cells. These data support the observations regarding the inability of ECM to influence transferrin production by Sertoli cells.

The ECM associated with a given tissue often has unique characteristics that may be tissue-specific (Hay, 1981). Preliminary results indicate that Sertoli cells may respond differently to different types of ECM (Mather et al., 1984). The commercially available ECM used was derived from a transformed cell line. Therefore, a seminiferous tubule biomatrix was prepared, as previously described (Tung and Fritz, 1984; Meezan et al., 1978), and used to assess possible tissue-specific effects of a more normal biomatrix on Sertoli cells. The isolation of this biomatrix required detergent and water washes that might modify its normal, in vivo biological activites; therefore, this is a technical limitation of the current study that must be considered in any data interpretation. This biomatrix was found to promote cell attachment and clumping as previously described (Tung and Fritz, 1984), but had no effect on transferrin production or on the ability of hormones to influence transferrin production. Similar results were obtained with both the seminiferous tubule biomatrix and the commercially available ECM. Therefore, the lack of effect of ECM on Sertoli cell function and on the hormonal regulation of Sertoli cell function does not appear to be due to the type of ECM used.

ECM is a valuable tool for cell culture because it promotes increased plating efficiencies, cell attach-

ment, and a more normal extracellular environment. Our observations indicate that ECM improves cell attachment and increases the longevity of Sertoli cells in culture. However, functional parameters of Sertoli cells, including transferrin and ABP production, are lost with time in culture at the same rate regardless of the presence or absence of ECM. This decrease in functional integrity is similar to previous reports regarding Sertoli cell cultures (Skinner and Griswold, 1982; Skinner and Fritz, 1985b). Therefore, although ECM improved the long-term maintenance of the Sertoli cell cultures, no effect on the maintenance of functional integrity was observed. ECM also was shown to release proteins into the medium in the early periods of culture, which may indicate improved cell viability. The presence of these components released from the ECM coating must be considered when conditioned medium obtained from ECM cell cultures is used.

The current study supports the interpretation that ECM plays an important role in promoting and maintaining the structural differentiation of the Sertoli cell. However, our data indicate that ECM does not alter Sertoli cell functions on a molecular level or influence the hormonal regulation of Sertoli cell function. The ability of ECM to alter the cytoarachitecture of the cell is an example of an important environmental interaction that involves the binding of ECM to the cell surface. The inability of ECM to affect Sertoli cell function indicates that ECM does not promote regulatory events on a molecular level. Therefore, ECM would appear to provide a type of environmental cellular interaction for Sertoli cells and not a regulatory type of interaction that requires a receptor-mediated signal transduction. Both environmental interactions and regulatory interactions are crutial in maintaining cell viability and identity; however, the respective mechanisms and physiological functions of these two types of interactions are distinct (Skinner, 1987). A direct correlation of the effect of an ECM on cell morphology versus function may be dependent on the specific cell type and probably will involve different mechanisms of action. As demonstrated in the current study, a dramatic effect of ECM on cell morphology may not necessarily reflect a subsequent influence on cellular function. The speculation is made that cellular interactions mediated by ECM will be important for the structural differentiation of a cell; however, cell-cell interactions mediated via soluble hormones and paracrine factors may have a more important role in actively regulating cell function, growth, and differentiation on a molecular level.

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