Proc. Nat. Acad. Sci. USA
Vol. 71, No. 12, pp. 4747–4751, December 1974

Does RNA Pass from Mesenchyme to Epithelium During an Embryonic Tissue Interaction?

(RNA density labeling/equilibrium centrifugation)

ROBERT M. GRAINGER*† AND NORMAN K. WESSELLS‡

*Department of Zoology, University of California, Berkeley, Calif. 94720; and ‡Department of Biological Sciences, Stanford University, Stanford, California 94305

Communicated by Curt Stern, August 12, 1974

ABSTRACT **Chick and mouse embryonic lung mesenchyme were incubated *in vitro* with an equimolar mixture of ^{13}C- and ^{15}N-labeled adenosine, guanosine, cytidine, and uridine, and trace amounts of the four tritiated ribonucleosides. This permits an unambiguous method for detecting transfer of macromolecular RNA from such a preincubated mesenchyme to responding lung epithelium across a Millipore filter, and for purifying any RNA transferred for further characterization. During the important period of mesenchymal support of epithelial morphogenesis, no detectable transfer of RNA was found. The level of sensitivity of these experiments was such that less than 0.01% of the labeled RNA in the mesenchyme could have been detected in the epithelium, corresponding to less than 75 labeled RNA molecules transferred to each epithelial cell. No detectable transfer of DNA from mesenchyme to epithelium was found during the inductive period, using the same approach, although with much lower sensitivity.**

The classical experiments of Spemann (1) and many later studies on developmental inductions have indicated a very specific role for some embryonic territories and tissues in commanding the fate of others, and have suggested that one might find informational molecules passing between interacting tissues during epigenesis (2). For example, a transplanted optic cup is capable of inducing lens formation in epithelium which would never otherwise form lens, which may be interpreted to indicate the transfer of some instructive molecules to the epithelium (3). More recent studies of a variety of tissue interactions, including ones in embryonic lens (4), pancreas (5), and notochord (6), suggest that the responding tissues may be determined prior to the interaction, and that information supplied during the interaction stimulates cells in the determined state at the right time and place (7).

We have investigated the interaction of mesenchyme and epithelium in the developing chick and mouse lung; we have asked whether a significant transfer of RNA occurs from mesenchyme to epithelium during the interaction. The embryonic lung was chosen because the lung bronchial epithelium branches and differentiates normally only when stimulated by bronchial mesenchyme; it does not respond well to other mesenchymal stimuli (8). Furthermore, bronchial mesenchyme will induce tracheal epithelium, which normally never branches, to do so (9). In this respect the interaction may be like that of optic cup and ectoderm during lens formation (3); i.e., an "instructive" tissue interaction (10). Finally we wanted to select a responding tissue, such as the lung, which ultimately produces identifiably differentiated cell types [e.g., cells that secrete lung surfactant (11)] and one in which the interaction between epithelium and mesenchyme will occur in organ culture, with the mesenchyme and epithelium separated by a Millipore filter (12).

Labeling with stable heavy isotopes has been a powerful tool permitting the physical separation of labeled and unlabeled nucleic acids in bacterial systems (13) and the demonstration of *de novo* synthesis of proteins in some plants (14, 15). These studies have utilized ^2H_2O or $H_2{}^{18}O$ as solvents, simple ^{13}C-labeled carbon sources, and ^{15}N-labeled salts, which are not readily utilized by eukaryotic animal cells. For the experiments presented here we have prepared density labeled ribonucleosides, precursors that are extensively incorporated by eukaryotic cells at high concentrations (R. Grainger and F. Wilt, in preparation), with no apparent toxic effects. We present here the rationale and experiments that have allowed us to distinguish whether RNA precursor or macromolecular RNA is transferred between tissues during the lung tissue interaction. Using density labeling, it would be possible to physically isolate macromolecular RNA transferred to the epithelium if such transfer occurred.

In these experiments lung mesenchyme is cultured alone, *in vitro*, in order to density label its RNA. Trace amounts of radiolabeled nucleosides are present at the same time to follow incorporation levels. After extensive washing, the mesenchyme is placed on one side of a Millipore filter and fresh unlabeled epithelium is placed on the other side. A culture period follows, during which the lung mesenchyme is essential for epithelial morphogenesis. Subsequent equilibrium centrifugation of RNA from epithelial tissue permits one to distinguish transfer of dense macromolecular RNA between tissues from transfer of precursors which might be incorporated by epithelium and utilized for synthesis of RNA. Thus, density labeled nucleotides that might be transferred would be diluted by precursor pools in the epithelium, and the RNA made by this indirect route should have a density much closer to normal.

MATERIALS AND METHODS

Density and Radioactive Isotopes; Labeling Conditions. ^{13}C-^{15}N-Labeled nucleosides were prepared with a density isotopic substitution of 82–86% ^{13}C for ^{12}C and 92–96% ^{15}N

† Present address: Department of Biology, Yale University, New Haven, Conn. 06520.

for ^{14}N, yielding a 3.3% intrinsic density increase (R. Grainger and F. Wilt, in preparation). When completely density labeled nucleic acids are banded in equilibrium salt gradients, under conditions described below, the density increase is reduced to about 2.7%, presumably due to interactions with cesium ions and water (16). During incubations all four density labeled ribonucleosides were present at 1 mM concentration with each of the four tritiated ribonucleosides added at 500 μCi/ml to follow incorporation, so that the final specific activity was 1.1×10^9 dpm/μmol. [^{3}H]adenosine (15 Ci/mmol), [^{3}H]cytidine (26 Ci/mmol), [^{3}H]guanosine (7.4 Ci/mmol), and [^{3}H]uridine (24.3 Ci/mmol) were obtained from Schwarz/Mann. The levels of tritiated nucleosides employed contribute about 5% of the total amount of nucleoside in the medium, and thus only slightly decrease the expected density shifts. [^{14}C]Uridine (54 Ci/mol), used for preparation of RNA standards, was also supplied by Schwarz/Mann.

Tissue Incubations and Nucleic Acid Extractions. Embryos were obtained from either 11-day pregnant female mice (Balb/c ♀ × C3H ♂, or C57Bl ♀ × DBA-6 ♂) or from 5-day White Leghorn chick embryos (Kimber Farms, Niles, Calif.). Whole lungs were dissected from the embryos and cleaned of adhering cells and nerves. Bronchial lung mesenchyme was dissected from the epithelium so that the mesenchyme exposed to density label was not subjected to enzyme treatment. Epithelia were cleaned of all mesenchyme using trypsin–pancreatin as described elsewhere (17). Except during experimental manipulations, tissues were kept in a humidified 37° incubator in a 5% CO_2–95% air atmosphere. Lung mesenchyme was labeled in 0.2 ml of modified Ham's F-12 medium (18), containing 10% (v/v) dialyzed fetal calf serum with density and radioactive labels present in the concentrations described above. After incubation, mesenchyme was washed extensively to remove remaining labeled nucleosides. Millipore filter assemblies were prepared as described for earlier experiments (17), using Millipore TH filters (0.45 μm porosity, 25 μm thickness). Fresh lung epithelium was held to the bottom of the filter apparatus by a plasma clot (17) and prelabeled mesenchyme was then placed on top of the filter assembly. Great care was taken at all stages to prevent mesenchymal contamination of epithelium. Living cultures were photographed with a Zeiss photomicroscope through a light optical system; techniques for electron microscopy have been described (19). RNA was extracted by a phenol–chloroform procedure described elsewhere (20, 21). DNA was purified by the same procedure, omitting DNase treatment.

Determination of Cell Number per Bronchial Epithelium. Because epithelia are very resistant to enzymatic dissociation, it was impossible to count cells by dissociation. The DNA content of single epithelia was measured by the method of Hinegardner (22) and the number of cells calculated from the known amounts of DNA per diploid nucleus (23).

Equilibrium Gradient Analysis of RNA Preparations. Samples were added to a cesium formate solution (Harshaw Chemical Co., optical grade), made up in 0.05 M Tris·HCl, pH 7.6, 0.05 M EDTA, and 1% HCHO, to give a final volume of 1.5 ml with a density of 2.05 g/cm^3. Gradients were centrifuged in polyallomer tubes overlaid with mineral oil in an SB-405 International rotor at 55,000 rpm. After centrifugation for 48 hr at 20°, gradients were collected in 5-drop fractions dripped through a 22-gauge needle. Refractive indices were immediately measured for selected fractions. H_2O (0.5 ml) was added to all fractions, which were then carefully transferred to scintillation vials for counting directly in 5 ml of Instagel (Packard and Co.). Maximum counting efficiency for tritium in a Nuclear Chicago Mark I Liquid Scintillation Counter under these conditions is about 25%.

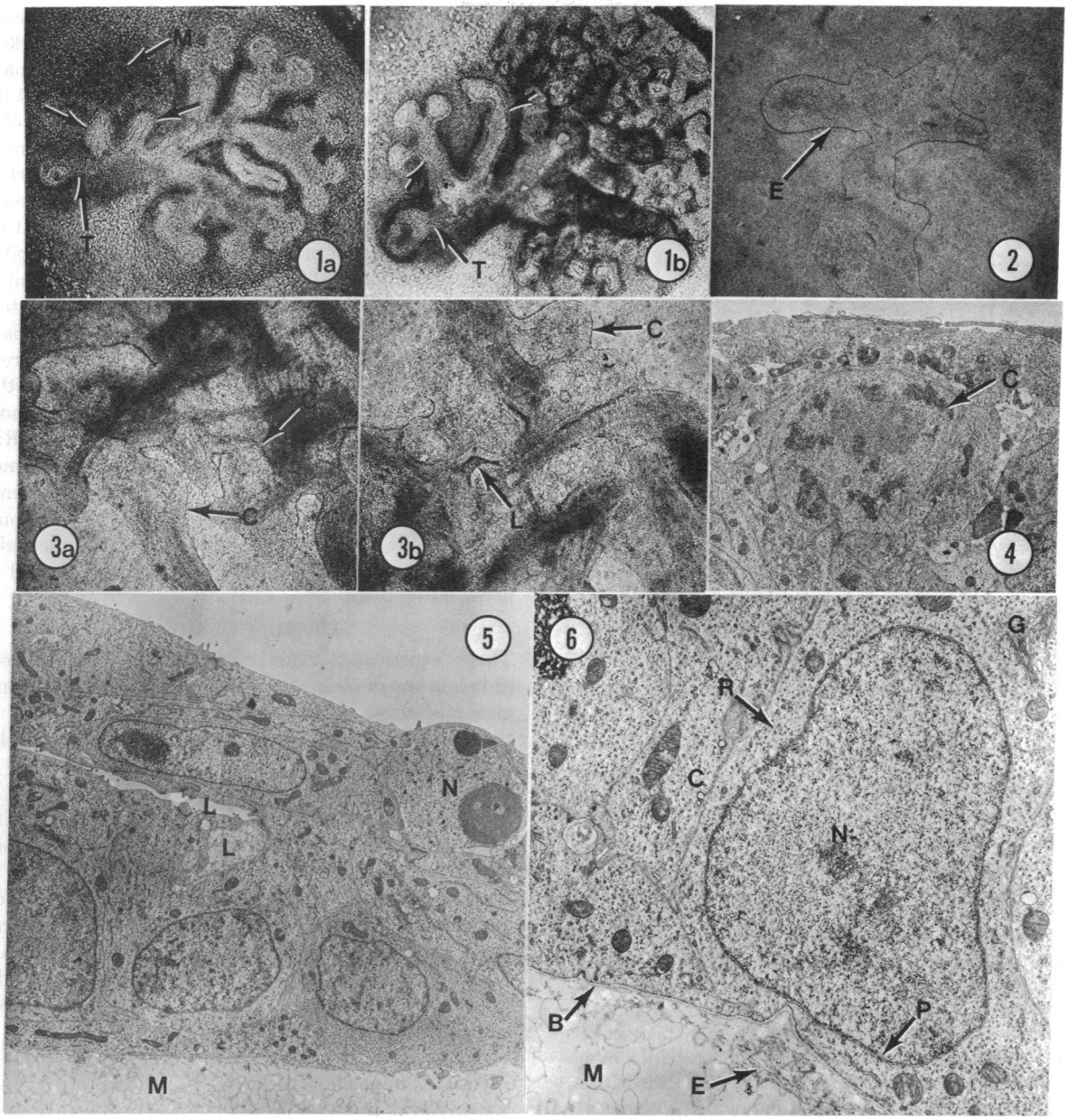
RESULTS

Preincubation of Lung Mesenchyme In Vitro with Density Labeled Nucleosides. Many embryonic tissues are only able to interact over short periods of time and under specific incubation conditions (24). We wanted to determine if preincubation of mesenchyme with density label interfered with its ability to later induce morphogenesis in fresh lung epithelium. Mouse epithelium was used in these experiments with chick mesenchyme because it shows a similar but more striking response than chick epithelium (12). Eleven-day mouse lung corresponds to 5-day chick in developmental stage (12).

Pieces of bronchial mesenchyme were removed from 5-day chick embryos and incubated for 24 hr in the density labeling medium, and then were thoroughly washed. Subsequently, fresh lungs were dissected from 11-day mouse embryos and small areas of tracheal mesenchyme were removed (25). The density labeled chick mesenchyme was then placed next to the bare wall of the trachea. During subsequent culturing, so-called "supernumerary" lung buds grew forth from the trachea [Figs. 1a and b; as described by Alescio *et al.* (9) and Wessells (25)]. The results show that the preincubation with density label does not prevent the mesenchyme from eliciting the novel morphogenetic response from the tracheal epithelium.

A transfilter control was performed with chick mesenchyme preincubated for 24 hr in density labeling medium placed across Millipore filters from single 11-day mouse epithelia. As shown in Fig. 2, preincubation does not inhibit the ability of mesenchyme to support bud formation and branching transfilter, though as previously reported (19), morphogenesis in a transfilter culture is never identical to that seen when tissues are in direct contact. Electron microscopic analysis of the cultured epithelium, shown in Figs. 4–6, indicates normal cell morphology, dividing cells (a response to mesenchyme, see refs. 5 and 10), even basal lamina and junctional complexes, all expected in a normal interaction (26). We conclude that preincubation of lung mesenchyme with density labeled medium does not interfere with its capacity to interact with epithelium.

Analysis of RNA Transfer During Induction. Bronchial mesenchyme was surgically dissected from fourteen 5-day chick embryos and incubated for 24 hr in 0.2 ml of medium containing density labeled and tritiated nucleosides. At the end of this incubation period, following extensive washing, 10–20% of the tissue was removed to be used for nucleic acid extraction. Fresh lung rudiments from eighteen 5-day chick embryos were then dissected and clean bronchial epithelia were prepared (tracheal and syringeal epithelia were discarded). The epithelia were grouped into two portions, and each group was attached to the bottom of a Millipore filter apparatus by a plasma clot. The remaining prelabeled mesenchyme was split into two approximately equal portions and placed on top of each filter apparatus across from the group of fresh epithelia. Transfilter assemblies were then



FIGS. 1a and b. A mouse embryonic lung growing in organ culture. A piece of chick embryonic lung mesoderm (M) that had been subjected to labeling in medium containing density labeled nucleosides was placed next to the trachea at time zero. Here, at 48 and 72 hr, two "supernumerary" lung buds (arrows) protrude from the wall of the trachea (T); the one on the left is carrying out morphogenetic branching. ×29.

FIG. 2. A piece of chick embryonic lung epithelium (E) photographed through the Millipore filter platform after chick mesoderm was removed from the opposite side of the filter to improve visibility. The sharp contours and rounded surfaces are characteristic of epithelium grown transfilter to mesoderm and unlike the extreme flattened condition seen in controls cultured in the absence of mesoderm [as in Fig. 3 of ref. (19)]. The mesoderm used in this experiment was exposed to medium with density labeled nucleosides prior to the transfilter culture period. ×36.

FIGS. 3a and b. Small areas of massed cultures of chick lung epithelium (as in Fig. 2) photographed on the lower surface of a filter after density labeled mesoderm was removed for assay (nucleic acids from these epithelia were subsequently extracted and banded in cesium formate gradients shown in Fig. 7). The rounded contours (C) and lumina (L) are seen in these cultures and are evidence of interaction with the mesoderm. ×38.

FIG. 4. A portion of chick lung epithelium cultured transfilter to density labeled mesoderm for about 18 hr prior to fixation for electron microscopy. Chromosomes (C) of a mitotic figure are seen in one epithelial cell. ×5710.

FIG. 5. Lung epithelium on a Millipore filter (M) after about 18-hr culture transfilter to medium with density labeled nucleosides. Most cells appear quite healthy; lumina (L) surrounded by cells connected with junctional complexes and containing abundant microtubules and microfilaments are seen. A possible necrotic (N) cell is also present. ×4450.

FIG. 6. A higher power view of a typical cell in epithelium cultures as in Fig. 5. A basal lamina (B), extracellular deposits (E), and normal appearing intracellular organelles are seen [Golgi (G); nucleoli (N); coated vesicles (C); nuclear pores (P); and ribosomal aggregates (R); Millipore filter (M)]. ×8,820.

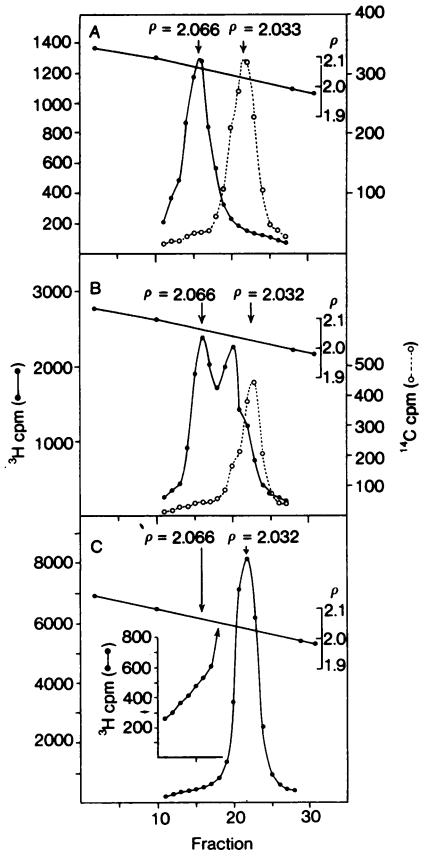


FIG. 7. Cesium formate equilibrium density gradients of RNA extracted from density labeled and control chick lung tissues. Five-day chick lung mesenchyme was incubated 24 hr in medium containing density labeled and tritiated nucleosides. Most of the labeled mesenchyme was placed transfilter to fresh 5-day lung bronchial epithelia in unlabeled medium for a second 24-hr incubation during which there was induction of epithelium. (A) RNA from a small portion of lung mesenchyme after the first 24-hr incubation shows extensive density labeling as followed by concomitant tritium incorporation. Chick lung RNA labeled for 24 hr with [^{14}C]uridine is shown here as a normal density marker. (B) RNA from mesenchyme after the second 24-hr incubation in unlabeled medium shows a heterogeneity of density labeled RNA. (C) RNA from epithelia after the 24-hr interaction with density labeled mesenchyme shows that there has been no transfer of intact density labeled RNA (inset is expanded scale on ordinate). Incorporation of tritium label in RNA near the normal density is most likely due to transfer of small amounts of precursor from mesenchyme. All gradients were centrifuged in 1.5-ml gradients at 300,000 × g for 48 hr. Densities, ρ, are in g/cm^3.

incubated for 24 hr in modified Ham's F-12 medium with 10% fetal calf serum, during which time the tissue interaction occurred. After this second 24-hr incubation the tissues were carefully removed to prevent mesenchymal contamination of epithelium. Figs. 3a and 3b show that epithelial tissue, although very densely packed, has retained normal tubular morphology and undergone branching typical of this kind of interaction in transfilter culture *in vitro*. On top of the filter, the numerous pieces of mesenchyme had fused to form a smooth, thick sheet of cells firmly attached to the top of the filter assemblies, an indication of healthy tissue.

RNA was then extracted independently from both mesenchyme and epithelia and banded in cesium formate equilibrium gradients. RNA extracted from 5-day chick lung labeled with [^{14}C]uridine for 24 hr was used as a marker. Fig. 7A shows that after 24 hr the newly made mesenchymal RNA is extensively density labeled with a distinct buoyant density 1.6% greater than normal. Following the next 24 hr of transfilter incubation in unlabeled medium (Fig. 7B) some of the unstable RNA in the mesenchyme has been recycled into slightly less dense molecules, yielding a heterogeneous pattern of density labeled RNA molecules. Fig. 7C shows that there is extensive transfer of precursor radioactivity to the epithelium during induction (4–6% of the radioactivity present in the mesenchyme at the beginning of the tissue interaction), as shown by incorporation of radioactivity into epithelial RNA displaying a barely detectable density shift. There is no detectable transfer of intact dense RNA to the epithelium, and the distribution of tritium in the density gradient is virtually identical to the distribution of ^{14}C control RNA (in Fig. 7B). These experiments were performed three times with 5-day chick embryos and once with 11-day mouse embryos, and gave identical results. Since mouse lung is somewhat smaller at the same developmental stage as the chick, about 30 embryos were dissected to prepare mesenchyme and 50 embryos for epithelia.

DISCUSSION

These experiments show very clearly that conventional radiolabeling of mesenchyme prior to tissue interaction would give misleading results about the transfer of RNA to epithelium. In the experiments presented here 4–6% of the total radioactive label present in mesenchyme after 24 hr of density labeling appears in epithelial RNA after interaction, while the density labeling confirms that none of the radioactivity present is due to transfer of macromolecular RNA. Although the tissues were thoroughly washed, label remained in mesenchymal precursor pools and probably persisted for long periods due to turnover of unstable RNA. This extensive transfer of precursor from mesenchyme to epithelium and subsequent incorporation into epithelial RNA would be indistinguishable from transfer of macromolecular RNA in a radiolabeling experiment.

Since we did not find transfer of intact RNA, it becomes especially important to have quantitative estimates of the sensitivity of the experiments. It was possible to detect transfer to the epithelium of less than 0.01% of the RNA in the mesenchyme and we believe this is the first time clear limits can be placed on the amount of RNA transfer that might occur. Each experiment yielded slightly over 2 × 10^6 cpm in density labeled mesenchymal RNA. Half of the epithelial RNA from one incubation was centrifuged in a single cesium formate equilibrium gradient in which it was possible to detect 100 cpm of RNA above background levels in fractions at the high density. The low level of radioactivity seen in Fig. 7C at 2.066 g/cm^3 represents noise due to diffusion from the main band, which is comprised of a heterogeneous RNA population, including some small RNAs, and is expected to show this kind of distribution. This "noise" level is indistinguishable in authentic ^{14}C control RNA (Fig. 7B) and [^{3}H]RNA recovered from epithelia. This implies a lower limit of sensitivity of 200 cpm for the whole sample, or about 0.01%. Sucrose density gradients of RNA prepared from 24-hr prelabeled mesenchyme showed that the labeling pattern corresponds with steady-state optical density traces, with ribosomal RNA predominating. Assuming a few percent of

the labeled RNA is in messenger RNA, as is typical in many cells (27), we could have detected transfer of less than 1% of the labeled messenger RNA of the mesenchyme.

One useful aspect of density labeling is that it permits a very simple determination of the minimal number of RNA molecules that would have been detectable in a transfer between tissues. As discussed in *Materials and Methods*, the density shift of a completely ^{13}C-^{15}N-labeled RNA in a cesium formate gradient is 2.7%. The experimental shift was 1.6%, indicating that precursor pools in the mesenchyme were 59% saturated, and that the specific activity of the newly made RNA is thus 59% of the value for external nucleosides ($0.59 \times 1.1 \times 10^9$ dpm/μmol; see *Materials and Methods*). Since it was possible to detect 100 cpm in the cesium formate gradient in Fig. 7C, using the 59% value for the specific activity of the labeled RNA, we could have detected transfer of a total of 2×10^8 labeled RNA molecules (assuming average length of 1500 nucleotides). Lung epithelia from about 20 embryos were used in chick lung experiments, comprised of 2.5 to 3×10^6 cells (see *Materials and Methods*) yielding a lower limit of sensitivity of about 75 labeled molecules per epithelial cell.

Numerous studies indicate that cells take up and transfer RNA under a variety of conditions. However, there are experimental aspects which often make results difficult to interpret so that there is as yet no compelling theory to explain such transfer (28). Cells in culture may normally exchange RNA (29), but it is difficult to eliminate the possibility of transfer of precursors, rather than macromolecular RNA. Substantial evidence has accrued for the transfer of RNA between different kinds of cells during the immune response (30). These experimental systems would be amenable to density labeling experiments which could resolve currently ambiguous interpretations. Many attempts have been made to determine if RNA is involved in developmental inductions (28, 31). While there is evidence that tissues cultured *in vitro* will utilize exogenous RNA (31), it is difficult to prove that such reactions occur *in vivo* or that they have biological significance. One cannot unambiguously exclude a role for RNA at the epithelial cell surface, or utilization and rapid breakdown of transferred RNA. However, stable RNA associated with cell surface or extracellular components of the epithelium (as in Fig. 6) would have been assayed along with epithelia.

Density labeled precursors can also be utilized to determine if DNA and proteins are transferred during tissue interactions. Since ribonucleotides are normally converted to deoxyribonucleotides for DNA synthesis, we were able to analyze DNA transfer in the experiments presented above. DNA, as well as RNA, was extracted in some of our experiments, and samples of DNA of both mesenchyme and epithelium were centrifuged to equilibrium in alkaline cesium chloride gradients. DNA in the mesenchyme was extensively density labeled (1.5%); no detectable amounts of DNA were transferred from mesenchyme to the epithelia at this high density, but, as with the RNA gradients, incorporated radiolabel was near the normal buoyant density. The amount of material recovered in labeled DNA was only a few percent of that in RNA and we could only have detected transfer of about 0.5% of the density labeled DNA.

Transfer of proteins during tissue interactions has been well documented, particularly the deposition of collagen between tissues in several mesenchymal–epithelial interactions (32). Since proteins in lung tissues can be density labeled with ^{13}C-^{15}N-labeled amino acids, we can investigate whether there is a transfer of protein during the lung interaction.

We thank Drs. Michael Anderson, Chung-Shu Kung, and Fred Wilt for helpful consultations, Michael Coughlin for aiding with some of the tissue dissections, and Merton Bernfield for pregnant mice. This work was supported by grants from the National Institutes of Health GM-13382 and HD-04708. R.M.G. was the recipient of an NIH Predoctoral Fellowship (5 F01 GM48856).

1. Spemann, H. (1938) *Embryonic Development and Induction* (Yale University Press; reprinted in 1967 by Hafner Publishing Co., New York).
2. Yamada, T. (1961) in *Advances in Morphogenesis*, eds. Abercrombie, M. & Brachet, J. (Academic Press, New York), Vol. 1, pp. 1–53.
3. Mangold, O. (1931) *Ergeb. Biol.* **7**, 193–403.
4. Mizuno, T. (1972) *J. Embryol. Exp. Morphol.* **28**, 117–132.
5. Levine, S., Pictet R. & Rutter, W. J. (1973) *Nature New Biol.* **246**, 49–52.
6. Ellison, M. L. & Lash, J. W. (1971) *Develop. Biol.* **26**, 486–496.
7. Holtzer, H. (1968) in *Epithelial-Mesenchymal Interactions*, eds. Fleischmajer, R. & Billingham, R. E. (Williams and Wilkins, Baltimore, Md.), pp. 152–164.
8. Spooner, B. S. & Wessells, N. K. (1970) *J. Exp. Zool.* **175**, 445–454.
9. Alescio, T. & Cassini, A. (1962) *J. Exp. Zool.* **150**, 83–94.
10. Wessells, N. K. (1973) "Tissue interactions in development," in *Addison-Wesley Module* (Addison-Wesley Publ., Reading, Mass.), Vol. 9, pp. 1–43.
11. Sorokin, S. (1965) in *Organogenesis*, eds. DeHaan, R. L. & Ursprung, H. (Holt, Reinhart and Winston, New York), pp. 467–491.
12. Taderera, J. V. (1967) *Develop. Biol.* **16**, 489–512.
13. Meselson, M. & Stahl, F. W. (1958) *Proc. Nat. Acad. Sci. USA* **44**, 671–682.
14. Anstine, W., Jacobsen, J. V., Scandalios, J. G. & Varner, J. E. (1970) *Plant Physiol.* **45**, 148–152.
15. Filner, P. & Varner, J. E. (1967) *Proc. Nat. Acad. Sci. USA* **58**, 1520–1526.
16. Vinograd, J. & Hearst, J. E. (1962) *Fortschr. Chem. Org. Naturst.* **20**, 372–422.
17. Wessells, N. K. (1967) in *Methods in Developmental Biology*, eds. Wilt, F. H. & Wessells, N. K. (Thomas Y. Crowell Co., New York), pp. 445–456.
18. Cahn, R. D., Coon, H. G. & Cahn, M. B. (1967) in *Methods in Developmental Biology*, eds. Wilt, F. H. & Wessells, N. K. (Thomas Y. Crowell Co., New York), pp. 493–530.
19. Wessells, N. K. & Cohen, J. H. (1968) *Develop. Biol.* **18**, 294–309.
20. Kung, C. S. (1974) *Develop. Biol.* **36**, 343–356.
21. Wilt, F. H. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 2345–2349.
22. Hinegardner, R. T. (1971) *Anal. Biochem.* **39**, 197–201.
23. Mirsky, A. E. & Ris, H. (1949) *Nature* **163**, 666–667.
24. Wessells, N. K. (1968) in *Epithelial Mesenchymal Interactions*, eds. Fleischmajer, R. & Billingham, R. E. (Williams and Wilkins, Baltimore, Md.), pp. 132–151.
25. Wessells, N. K. (1970) *J. Exp. Zool.* **175**, 455–466.
26. Hay, E. D. (1968) in *Epithelial-Mesenchymal Interactions*, eds. Fleischmajer, R. & Billingham, R. E. (Williams and Wilkins, Baltimore, Md.), pp. 31–55.
27. Darnell, J. E. (1968) *Bacteriol. Rev.* **32**, 262–290.
28. Bhargava, P. M. & Shanmugan, G. (1971) in *Progress in Nucleic Acid Research and Molecular Biology*, eds. Davidson, J. N. & Cohn, W. E. (Academic Press, New York), Vol. 11, pp. 103–192.
29. Kolodny, G. M. (1971) *Exp. Cell Res.* **65**, 313–324.
30. Bell, C. & Dray, S. (1974) *Ann. N.Y. Acad. Sci.* **227**, 200–224.
31. Niu, M. C. & Deshpande, A. K. (1973) *J. Embryol. Exp. Morphol.* **29**, 485–491.
32. Bernfield, M. R. & Wessells, N. K. (1970) *Develop. Biol., Suppl.* **4**, 195–249.