nels could be made available for activation by phosphorylation during the oscillations in kinase activity that are an integral part of the cell cycle (*1, 14*). Alternatively, the oscillations in Cl^- current density could result from insertion and removal of channels from the plasma membrane with each cell cycle. This mechanism is consistent with the small changes in surface area we recorded (Figs. 3 and 4) (*15, 16*), although it does not account for the time lag between the maximum Cl^- current and maximum capacitance.

Our experiments indicate a close association between the electrical properties of the plasma membrane and the endogenous cell cycle clock of the early embryo. It may also be true of other embryos that electrical properties recorded in a given blastomere depend critically on the state of the cell relative to its cleavage cycle. Thus in the interpretation of such experiments, permanent developmental changes in ion channel properties should be distinguished from transient ones. The significance of the oscillations in functional Cl^- channel density in the ascidian embryo may involve their role in promoting increased fluxes of HCO_3^- (*17*) and water across the plasma membrane, which could contribute to changes in intracellular *p*H and the maintenance of osmotic balance that occur at cleavage (*18, 19*). These changes may be important to maintain the intracellular environment in a state that permits the completion of mitosis.

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9. General methods of animal collection and maintenance, gamete preparation, and whole-cell recording were as described (*8*). In experiments in which extracellular Cl^- concentration was changed, bath potentials were recorded with a KCl microelectrode and subtracted from the patch pipette voltage. Artificial seawater (ASW) had the following composition: 400 m*M* NaCl, 10 m*M* KCl, 10 m*M* $CaCl_2$, 50 m*M* $MgCl_2$, and 10 m*M* Hepes, *p*H 8.1. To block the K^+ inward rectifier, Ba^{2+} was substituted for Ca^{2+} (10 Ba ASW). For Cl^- replacement, gluconate was the substitute. To measure tail currents of the Cl^- current, Na^+-free ASW (choline substitution) was used to eliminate the Na^+ current. Cytochalasin B (Sigma) was dissolved in ethanol at 1 mg/ml and diluted for use at a final concentration of 2 to 4 μg/ml. A23187 (Sigma) stock solution (4.5 m*M* in dimethyl sulfoxide) was diluted to a final concentration of 2.7×10^{-5} *M* in ASW for activation. Pipette (internal) solution contained 400 m*M* KCl, 10 m*M* NaCl, 10 m*M* EGTA, and 20 m*M* Hepes, *p*H 7.3. All experiments were performed at 10° to 12°C.
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13. Several control experiments were done to ensure that the cyclical changes in Cl^- current were not due to prolonged whole-cell recording or exposure to Ba^{2+} ions. Prolonged whole-cell recordings from unfertilized oocytes showed no significant change in the Cl^- current; brief whole-cell recordings initiated at various times after fertilization or prolonged two-microelectrode recordings showed the same increase in Cl^- current as cleavage approached. Similar results were obtained without Ba^{2+} in standard artificial seawater, in which the Cl^- current could be resolved as a slowly activating component superimposed on the K^+ inward rectifier. The oscillatory changes in current and capacitance did not result from changes in access resistance. Series resistance compensation was adjusted before each measurement, and usually only small adjustments were needed to maximize clamp speed. The small changes observed were steady increases in access resistance during the experiment, probably caused by clogging of the pipette tip by yolk droplets in the cytoplasm.
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15. Although many cell properties differentiate in apparently normal fashion in cytochalasin-arrested ascidian embryos (*12*), capacitance and ultrastructural measurements indicate that the addition of new membrane is substantially disrupted [T. Hirano and K. Takahashi, *J. Physiol. (London)* **386**, 113 (1987)]. The small oscillations in membrane capacitance are superimposed on a large ramplike increase as cleavage approaches. With cytochalasin treatment, the ramp increase disappears, leaving the small oscillation. The ramplike increase may be responsible for the continuous addition of functional Ca^{2+} and inwardly rectifying K^+ channels to the early embryo, so that the density of these currents remains constant through the eight-cell stage, despite a threefold increase in total embryo membrane area (*8*). Scanning electron micrographs of ascidian embryos show a cyclical extension and retraction of microvilli with each cleavage cycle, which may represent the ultrastructural correlate of our cytochalasin-resistant capacitance oscillations. The timing of these cannot be explained by a model based on the advanced insertion of membrane to be used later in furrow formation [N. Satoh and T. Deno, *Dev. Biol.* **102**, 488 1984)], and the function of this membrane, which seems more closely associated with the cell cycle oscillator, is not known. In *Xenopus* embryos, permanent changes in K^+ permeability occur during early cleavages. These changes are cytochalasin resistant, but unlike the changes in ascidian embryos, the cytochalasin treatment does not appear to block the addition of new membrane in *Xenopus* (*4*).
16. Several experiments indicate that newly added membrane in cleaving embryos can have different populations of functional ion channels or pumps than the old membrane [P. J. Woodward, *J. Gen. Physiol.* **52**, 509 (1968); (*5*); A. Ohara, K. Murayama, Y. Doida, Y. Marunaka, *Med. Sci. Res.* **15**, 1401 (1987)].
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21. Supported by NIH postdoctoral fellowship NS 07775 to M.L.B., and by NIH grant HD17486 and a Research Career Development Award to W.J.M. We thank M. Kyte for collecting animals, and M. Bosma and L. Bernheim for reading the manuscript.

6 September 1989; accepted 11 December 1989

Induction by Soluble Factors of Organized Axial Structures in Chick Epiblasts

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Inductive action of soluble factors was tested on isolated chick epiblasts. An assay was developed wherein conditioned medium derived from the *Xenopus* XTC cell line induced the formation of a full-length notochord and rows of bilaterally symmetric somites. Basic fibroblast growth factor, epidermal growth factor, retinoic acid, and transforming growth factor type β1 and β2 were not capable of inducing axial structures. Thus, soluble factors can elicit the development of polarity stored in the epiblast and behave as true morphogens since they can induce the formation of the organized complex structures that constitute the embryonic axis.

THE CHICK BLASTULA [STAGE XIII OF Eyal-Giladi and Kochav (*1*)] is formed of two physically distinct layers. The upper layer consists of the primary ectoderm or epiblast, from which all embryonic structures develop (*2*), plus the peripheral area opaca and marginal zone regions. The lower layer consists in its entirety of the primary endoderm or hypoblast. Interaction between the epiblast and the polarized hypoblast (*3*) results in the formation of axial mesodermal structures. In the absence of the hypoblast and the marginal zone, cells from the competent epiblast proceed to form nonaxial mesoderm, such as blood islands, but are not capable of generating any type of

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axial structures, such as somites and notochord (*4*).

The chick blastoderm has been a convenient system to study early development in higher vertebrates, mainly because it is easily accessible and can be manipulated in vitro (*5*). However, a limitation of this system has been the need to culture the blastoderm on the natural vitelline membrane through which the explant obtains nutrients from the underlying egg yolk–albumin culture medium (*6*). We have devised an alternative defined culture medium (DCM) that allows early chick blastoderms or explants derived from blastoderms to grow in vitro. DCM is prepared by allowing a 1-ml solution of 1.8% agarose in Roswell Park Memorial Institute (RPMI) medium to gel in a 35-mm petri dish. The blastoderm explant is removed from the egg, layered onto DCM, and cultured (37°C, 100% humidity, and 5% CO_2) for periods of up to 2 weeks. Whole stage XI–XIII blastoderms, when grown in DCM for 48 hours, develop into normal embryos. The rate of development roughly corresponds to that of normal blastoderms grown in ovo. We now use this system to test soluble factors for possible inductive action in isolated epiblast explants.

When grown in DCM for 48 hours, central disks from stage XIII blastoderms, containing only the isolated epiblast and the underlying hypoblast, generated axial structures in 75% of the cases (Table 1). When the hypoblast was removed from the central disks and the isolated epiblasts were explanted onto DCM, and cultured for 48 to 72 hours (Fig. 1, A and B), axial structures were not observed in 91% of the cases (see Table 1). In agreement with previous findings (*4*), isolated epiblasts formed nonaxial types of mesoderm, such as disorganized mesenchyme and blood islands. In 9% of cases, axial structures did appear from isolated epiblasts, probably as a result of technical errors either in staging the blastoderms or in isolating the central epiblastic disks (*4, 5*).

Isolated epiblast explants grown in DCM containing basic fibroblast growth factor (bFGF), transforming growth factor type β1 and β2 (TGF-β1 and TGF-β2), epidermal growth factor (EGF), retinoic acid (RA) (*7*), or combinations of them were not capable of forming axial structures and were indistinguishable from untreated epiblasts (see Table 1). Similarly, serum-free conditioned medium obtained by culturing cells derived from either whole stage X blastoderms (st-X CM) or from the peripheral area opaca and marginal zone of stage XIII blastoderms (*8*) (AO-MZ CM) could not induce axial structures in isolated epiblasts (Table 1).

In contrast, 70.1% of stage XIII–isolated epiblasts grown in DCM in the presence of 25% serum-free conditioned medium derived from the *Xenopus* XTC cell line (XTC CM) (*9*) developed normal axial structures, including a full-length notochord and several pairs of somites (Table 1 and Fig. 1C). Occasionally, a neural plate (Fig. 1D) and, more rarely, a neural tube also developed, presumably as a result of a secondary induction by the axial mesoderm on the ectoderm.

The high degree of organization displayed by the XTC CM–treated epiblasts can be understood in view of previous experiments showing that the epiblast itself is a polarized structure (*10*). In such experiments, the in-

Table 1. Axial structures, including notochord and somites, in isolated chick epiblasts.

Culture medium	Stage XIII epiblasts (no.)	Axial structures (%)
RPMI	122	9.0
RPMI + st-X CM* (chick)	13	0.0
RPMI + AO-MZ CM* (chick)	59	6.7
RPMI + bFGF (10 to 400 ng/ml)	14	7.1
RPMI + TGF-β2†	43	7.0
RPMI + XTC CM‡ (*Xenopus*)	31	70.1
(Normal chick hypoblast)	12	75.0

*St-X CM was obtained by plating cells derived from whole stage X (*1*) chick blastoderms at a concentration of 2×10^5 per milliliter in RPMI containing 10% fetal calf serum (*8*). After 24 hours the cells were rinsed five times with RPMI, fresh RPMI without serum was added, and the cells were incubated for a further 24 to 48 hours. The medium was collected and stored at −20°C until used. AO-MZ CM was obtained in an identical manner except that cells were derived from the area opaca and the marginal zone of stage XIII blastoderms. Conditioned media were used at concentrations ranging from 1 to 100% of the total culture medium. †TGF-β2 was added to the DCM either alone or in combination with one or several of the following factors: bovine bFGF, TGF-β1, EGF, and RA. The following combinations were tested: TGF-β2 and TGF-β1; TGF-β1 and bFGF; TGF-β2 and bFGF; TGF-β2, TGF-β1, and bFGF; TGF-β2 and EGF; TGF-β2, EGF, and bFGF; and TGF-β2, EGF, bFGF, and RA. Factor concentration in the DCM ranged from 1 to 300 ng/ml. None of the factors when tested alone gave inductive values above control. Porcine TGF-β1 and TGF-β2 were purchased (R&D Systems, Minneapolis). ‡Epiblast explants were grown in DMC in RPMI in the presence of 25% serum-free XTC CM (*12*). When XTC CM was less than 15% of the culture medium, no axial structures were observed, 25% XTC CM in RPMI gave the more consistent results and only those are shown . In about 40% of the cases XTC CM was boiled for 5 min before it was applied to the DCM. No difference was observed between the heated and unheated XTC CM in their capacity to induce axial structures.

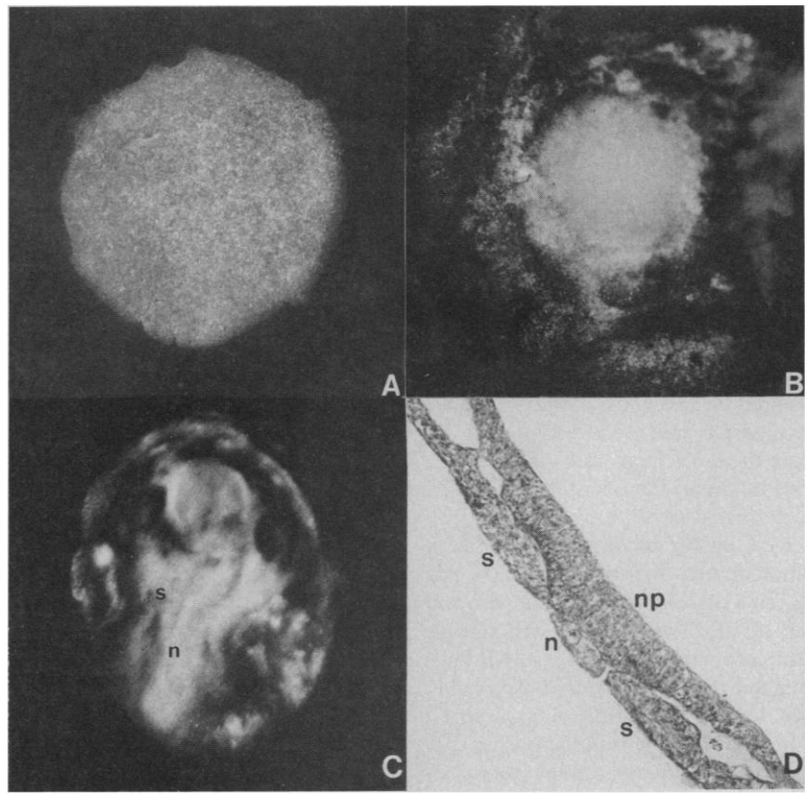

Fig. 1. (**A**) Isolated epiblast 1 hour after being explanted into DCM (×90). (**B**) Isolated epiblast 48 hours after being explanted into DCM in the absence of soluble factors. No axial structures developed (×90). (**C**) Isolated epiblast 48 hours after being explanted in DCM in the presence of 25% serum-free XTC CM. Organized axial structures such as notochord (n) and several pairs of somites (s) developed (×90). (**D**) Transverse section through the mid-region of the epiblast explant shown in (C). The notochord (n), neural plate (np), and bilaterally symmetric somites (s) can be seen (×600).

ductive hypoblastic layer was artificially depolarized by dissociating and reaggregating the hypoblastic cells. This layer retained the capacity to induce axial mesodermal structures, that developed according to a weaker polarity stored in the epiblast (*10*).

Soluble growth factors have been shown to induce mesoderm formation in *Xenopus*. Slack *et al.* (*11*) reported that bFGF can induce the formation of non-axial mesoderm in ectodermal animal caps of *Xenopus* blastulae. Later, Smith (*12*) showed that XTC CM can induce animal cap cells to differentiate into patches of disorganized muscle and notochord. In the *Xenopus* assay system, a number of growth factors have now been identified which can induce mesoderm in animal caps (*13, 14*). In that system, factors from the FGF family can induce mesoderm mainly of the more ventral or nonaxial type. Factors of the TGF-β family can, to a certain extent, potentiate this effect and induce differentiation of more dorsal types of mesoderm (*14, 15*). In the amphibian system, there have been no reports of induction of fully organized mesodermal tissue structures by soluble factors. Secondary axes, however, have been obtained when animal caps, treated with soluble factors, are grafted into normal *Xenopus* blastulae (*16*). In such cases the axial structures arise as a secondary interaction with the induced grafted tissue, although the type of structures that develop depend on the factor previously applied to the graft (*16*).

Our experiments show that bFGF is not sufficient to induce axial structures in the chick, but do not rule out the possibility that it could be involved in the induction of nonaxial mesoderm. Recent experiments indicate that in the chick, both bFGF RNA and protein are already expressed in the epiblastic region, before stage XII (*17*). Direct experiments on induction of nonaxial mesoderm cannot be performed in the chick since by the time the epiblast and hypoblast tissues sort themselves out into two separate entities, nonaxial mesoderm has already been determined (*4, 8*).

We were unable to obtain induction of axial mesodermal structures by TGF-β1, TGF-β2 or a combination of both, with and without bFGF. These results, although in apparent contradiction to the *Xenopus* findings, suggest that other factors are probably required for the formation of axial structures. In *Xenopus*, it is becoming clear that the mesoderm-inducing activity found in XTC CM (*18*) (XTC MIF) is probably due to a factor related to but distinct from TGF-β2 (*15, 19*). We have no evidence whether the same factor is operating in the chick or more than one factor is necessary in the avian system. Boiling XTC CM does not remove the inducing activity, although we have not been able to enhance the activity by this treatment (see Table 1) as is the case in *Xenopus* (*12*).

Our results show that soluble factors applied in a nonpolar manner to a polarized competent tissue can act as true morphogens, since they can induce the formation of complex organized axial structures.

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20. We thank J. Smith for providing the XTC conditioned medium, I. Vlodavsky for providing bovine bFGF, and Y. Gruenbaum for reading and commenting on the manuscript. This work was supported by the U.S.–Israel Binational Science Foundation grants 8500296 and 8800081.

12 October 1989; accepted 16 January 1990

Stable Carbon Isotopic Evidence for Carbon Limitation in Hydrothermal Vent Vestimentiferans

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Stable carbon isotope composition ($\delta^{13}C$ values) can be used to evaluate an animal's source of nutritional carbon. Most animals with chemoautotrophic endosymbionts have quite negative tissue $\delta^{13}C$ values due to discrimination against ^{13}C associated with chemoautotrophic assimilation of inorganic carbon. However, the $\delta^{13}C$ values of hydrothermal vent (HTV) vestimentiferans are significantly higher than the values reported for non-HTV vestimentiferans or other invertebrates with chemoautotrophic endosymbionts. Tissue $\delta^{13}C$ values of two species of HTV vestimentiferans increase with increasing size of the animals. This relation supports the hypothesis that the relatively high $\delta^{13}C$ values are the result of inorganic carbon limitation during carbon fixation. A more favorable relation between gas exchange and carbon fixation in the smaller individuals is expected, due to differences in the geometric scaling of gas-exchange surfaces and trophosome volume.

THE STABLE C ISOTOPE CONTENT OF HTV animals was one of the first indications that nonphotosynthetic food sources were utilized by these communities (*1*). Measurements have now been made of over 30 symbioses between marine invertebrates and chemoautotrophic or methanotrophic bacteria (*2*). The basis for the utility of stable C isotopes in investigations of these symbioses is twofold. First, chemoautotrophic bacteria discriminate significantly (~25 per mil) against ^{13}C during the incorporation of inorganic C into organic compounds (*3*), and this partitioning results in organic compounds that have more negative $\delta^{13}C$ values than those originating from photosynthetically fixed C. Second, methanotrophs discriminate only slightly during incorporation of methane C into cellular components, and methane in the marine environment is highly enriched in ^{12}C ($\delta^{13}C = -35$ to -90 per mil) (*4*). As a result, tissues from most invertebrates with chemoautotrophic endosymbionts have $\delta^{13}C$ values that range from -23 to -50 per mil and invertebrates with confirmed methanotrophic symbionts have values that range from -40 to -76 per mil, whereas $\delta^{13}C$ values of other marine organisms normally range from -12 to -22 per mil (*2*).

Unlike $\delta^{13}C$ values from other animals

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