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Evidence of a role for endogenous electrical fields in chick embryo development

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Summary

We have tested directly the hypothesis that the endogenous electrical field in the chick embryo plays a causal role in development. Conductive implants, which shunt currents out of the embryo and thus alter the internal field, were placed under the dorsal skin at the mid-trunk level of stage 11-15 embryos. Currents leaving the posterior intestinal portal (p.i.p.) of these embryos were reduced by an average of 30%. Control embryos receiving non-conductive implants showed no change in p.i.p. currents. In the group receiving current shunts, 92% of the embryos exhibited some developmental abnormality. Only 11% of the control group displayed defects. The most common defect in the experimental group (81%) was in tail development. Tail defects ranged from complete absence to the formation of a normal length, but morphologically abnormal tail. Internally, tail structures (neural tube, notochord and somites) were frequently absent or aberrantly formed. In 33% of the experimental embryos, the notochord continued lengthening in the absence of any other tail development. This led to the formation of ourenteric outgrowths from the hindgut. Defects in limb bud and head development were also found in experimentally treated embryos, but at a much lower frequency than tail defects.

The abnormalities observed in experimental embryos were very similar to those produced naturally in *rumpless* mutant chicks. A vibrating probe analysis of these mutants (from both dominant and recessive strains) showed that currents leaving the p.i.p. were significantly lower in phenotypically abnormal mutants than in wild-type and phenotypically normal mutant embryos from both strains. There was no apparent correlation between the average transepithelial potential (TEP) of these mutants and the development of tail abnormalities. The possible role of endogenous electrical fields in chick tail development is discussed.

Key words: endogenous currents, electrical fields, chick development, tail development, rumplessness, rumpless mutant.

Introduction

In order for embryogenesis to progress normally, a tremendous amount of information must be generated, transmitted, received and interpreted by the cells and tissues of the embryo. This may be information specifying cell fate, directing axis formation, guiding cell movements or orchestrating any one of many other processes. The mechanisms by which developmental signals are transferred are not known, although a number of putative carriers have been proposed. For example, retinoic acid may act as a patterning agent during chick limb development (Thaller and Eichele, 1987; Summerbell and Maden, 1990), in chick antero-posterior axis formation (Mitrani and Shimoni, 1989), and in the developing *Xenopus* central nervous system (Durston et al., 1989; Ruiz i Altaba and Jessel, 1991). There is also considerable evidence that various peptide growth factors (e.g., members of the FGF and the TGF-β families) play a role in mesodermal induction and axis formation in amphibians (Ruiz i Altaba and Melton, 1990; Melton, 1991) and chicks (Mitrani et al., 1990). Proposed directional growth/migration controls include chemical gradients (chemotaxis), substratum adhesive gradients (haptotaxis) cell-to-cell contacts and endogenous electrical fields (galvanotaxis). These and other possible factors influencing directional cell movements have been reviewed by Trinkaus (1985) and Erickson (1990).

One agent that long has been hypothesized to carry developmentally relevant information is an endogenous electrical field (see e.g., Burr and Northrop, 1935; Nuccitelli, 1984), which, due to its inherent polarity, is a natural candidate for such a function. There have been numerous studies on endogenous steady electrical currents and their relationship to the development of multicellular animal embryos, and nearly all of this work has focused on their possible role in guiding cell

growth and/or migration. Many embryonic systems have been shown to generate electric currents (measured extraembryonically with the vibrating probe) that can often be correlated, both spatially and temporally, with some specific developmental event (Jaffe and Stern, 1979; Borgens et al., 1983; Robinson, 1983; Robinson and Stump, 1984; Winkel and Nuccitelli, 1989). In addition, many embryonic cell types have been shown to respond to applied electric fields *in vitro* with directed growth or movement (Hinkle et al., 1981; Erickson and Nuccitelli, 1984; Stump and Robinson, 1984; McCaig and Dover, 1991). There is presently very little evidence in support of other roles for bioelectric fields in embryogenesis.

In vertebrates, steady endogenous electric currents are generated by the transepithelial potential (TEP) which arises due to electrogenic Na^+ transport by the electrically tight skin epithelium (Vanable, 1989). Any disruption of the integrity of the epithelium, either natural (e.g., via programmed cell death) or artificial (e.g., by wounding) will create a low-resistance pathway for current flow out of an animal that maintains an internally positive TEP. This current flow through the animal will generate an internal electric field, the magnitude of which will be proportional to the resistance of the tissues through which it flows. It is this internal field that is presumably important for directing some developmental processes, although any causal role for an endogenous electrical field has heretofore not been demonstrated.

We have shown previously that stage 15 to 22 chick embryos produce large (> 100 $\mu A/cm^2$) ionic currents that leave the embryo via the posterior intestinal portal (p.i.p.; the opening into the hindgut from the yolk sac) during the period of tail gut reduction when there is extensive cell death in the caudal end of the embryo (Boyden, 1922). These currents enter through the intact ectodermal epithelium over the rest of the embryo (Hotary and Robinson, 1990). The current flow through the embryo was found to generate a caudally negative intraembryonic voltage gradient that averaged 21 mV/mm. This is a level well above the minimum 7 - 10 mV/mm generally needed to affect cells *in vitro* (Hinkle et al., 1981; Erickson and Nuccitelli, 1984; Stump and Robinson, 1984; Nuccitelli and Smart, 1989). We hypothesized that if this voltage gradient plays a necessary role in embryogenesis, its disruption should alter development in some manner. We tested this hypothesis by creating an alternative pathway for current flow out of the embryo via a second low-resistance region (i.e., a non-healing wound), thus altering both the magnitude and the pattern of the natural field.

In this paper we describe the effects on chick embryo development of shunting the endogenous electrical field through an artificially created, non-healing wound. We found that this treatment had profound effects on tail development, producing fairly severe abnormalities. Defects in head and limb bud formation were also induced, but at a considerably lower frequency. The induced abnormalities in tail development were very similar to those appearing in "rumpless" mutant chick embryos (Zwilling, 1942; 1945a). This led us to investigate some of the electrophysiological properties of these mutants at the same stages of development. Portions of this work have appeared elsewhere in preliminary form (Hotary and Robinson, 1991).

Materials and methods

Embryos

Wild-type white leghorn chicken embryos obtained from the Purdue Poultry Farm were used for all of the implant experiments. *Rumpless* mutant chicken embryos from both a dominant (*Rp*) and a recessive (*rp-2*) stock were obtained by overnight express from the University of Connecticut Agricultural Experiment Station (Storrs, CT). Dominant mutants were obtained by crossing a heterozygote with a homozygous wild-type white leghorn (*Rp* + × + +). Therefore, 50% of the embryos would be expected to be genotypically wild-type. Recessive mutants were obtained through a heterozygote cross (*rp-2* + × *rp-2* +). Approximately 25% of the embryos from this cross would be homozygous for (and thus express) the rumpless phenotype. Expression of the mutant phenotype by both genotypes is highly variable, and the penetrance is about 30 - 40% (see Landauer, 1955). Before any measurements were made on these embryos, they were classified as phenotypically normal or abnormal based on their external morphology. All embryos were incubated at 38°C until use. Staging of the embryos was done according to the criteria of Hamburger and Hamilton (1951).

Implant procedure

Implant experiments were performed primarily on stage 11 - 14 wild-type embryos. These are the stages immediately preceding the onset of outward currents at the p.i.p. (Hotary and Robinson, 1990) and were used so that the alteration of the endogenous electrical field would begin immediately with its generation. Implant operations were also performed on nine stage 15 embryos. This is a transition stage in which about 50% of the embryos begin to show outward currents at the p.i.p. Six of these operations (three experimentals and three controls) were considered successful based on the criteria given in the results section.

Several ml of albumen were withdrawn from the egg in order to lower the level of the embryo, and a window was cut in the shell using the cutting wheel of a Dremel tool. The embryos were exposed by cutting a small hole in the overlying vitelline membrane. One to two drops of nile blue was added to aid in visualizing the embryos. A slit 100 - 250 μm long was made in the flank ectoderm perpendicular to the neural tube at the level of somite 16 to 20 with a flame-sharpened tungsten microneedle. For stage 11 embryos, the slit was made just caudal to the final somite. Implants (see below) were placed in the slit and were slid 200 - 600 μm under the ectodermal epithelium roughly parallel to the neural axis using jeweler's forceps. If the implant penetrated the neural tube to any extent, the embryo was discarded. Since the implants inevitably entered at some angle relative to the longitudinal body axis, they would sometimes penetrate the ventral side of the embryo. Those embryos in which such a ventral side penetration was found were discarded, as were those that showed any blood loss with the operation. A few drops of warm (38°C) Hanks Balanced Salts Solution (HBS: 1.2 mM $CaCl_2$; 0.8 mM $MgSO_4$; 5.4 mM KCl; 0.4 mM KH_2PO_4; 137 mM NaCl; 0.3 mM Na_2HPO_4; 5.5 mM glucose; 10 mM

Hepes; pH 7.2) supplemented with 1% antibiotic-antimycotic (10,000 units/ml penicillin, 10,000 μg/ml streptomycin and 25 μg/ml fungizone: Gibco) together with 1 - 2 ml warm albumen were added to the egg and the window was sealed using cellophane tape. The eggs were then incubated at 38°C until examination 1 - 3 days later.

Current shunts were made from Pasteur pipettes that had been heated in a Bunsen burner and hand-pulled to produce thin, hollow fibers. These fibers were then suction-filled with HBS or HBS gelled with 2% agarose (Sigma type IX) and broken to a length of 0.9 - 1.1 mm. Agarose-gelled HBS was used to fill some shunts as a control against bulk fluid transfer between the embryo and the surroundings. The outside diameter of these shunts was 75 - 100 μm. Solid glass rods for control experiments were made in the same manner and to the same dimensions from solid glass stirring rods.

Two-dimensional vibrating probe

For vibrating probe measurements, eggs were broken into a bowl of warm HBS and the embryos were lifted out after cutting around the outside circumference of the area vasculosa. The embryos were then rinsed several times in warm HBS to remove adherent yolk granules. They were then secured into glass Petri dishes with insect pins inserted outside the area vasculosa into an underlying layer of Sylgard (Dow Corning, Midland, MI). When necessary, extraembryonic membranes were removed using fine jeweler's forceps. Any embryos injured during this process were not used for probe measurements. All measurements were performed in HBS (resistivity=90 Ω · cm).

The two-dimensional vibrating probe used for this study is based on the design of Nuccitelli (1986). A detailed description of this system is forthcoming (Hotary, Nuccitelli and Robinson, submitted). The probe itself is a parylene-insulated stainless steel electrode (Microprobe, Inc., Clarksburg, MD) electroplated at the exposed tip with a 20 μm diameter platinum black ball. The probe is vibrated in a circle so that both orthogonal components of a current in the horizontal plane can be measured simultaneously. This circular vibration is achieved by using two perpendicularly mounted piezoelectric elements connected to a titanium frontpiece to form a parallelogram. The probe is mounted at the vertex of the parallelogram and two sine waves of the same frequency but 90° out of phase with one another are used to power the bender elements. The probe and a platinum/platinum black reference electrode are connected to a 100×-gain preamplifier (Applicable Electrotechnics, W. Yarmouth, MA) whose output is routed to a two-phase lock-in amplifier (model 5204, Princeton Applied Research, Princeton, NJ). The two signal components are digitized by a Labmaster TM40-PGL A/D converter (Tekmar Co., Solon, OH) and analyzed on an IBM PCXT computer using modified software originally supplied by the Vibrating Probe Co. (Davis, CA). The computer calculates the average vector from the two signal components and displays it on a video monitor superimposed on an image of the experimental preparation. Data were stored on a hard disk and the experiments were recorded by taking 35-mm photographs of the monitor at various times during the course of an experiment. All average current densities are presented as the mean ± the standard error of the mean (s.e.m.).

TEP measurements

Embryos were prepared for TEP measurements in the same manner as that described for vibrating probe measurements. All TEP measurements were done from the dorsal side using glass microelectrodes filled with 100 mM NaCl. These electrodes had outside tip diameters of 1 - 2 μm and typically had resistances between 5 and 20 MΩ. Relatively large-tipped electrodes were used for this study in order to minimize the chances of impaling individual cells and adding the membrane potential to the TEP. Since some leakage would be expected from these electrodes, they were filled with 100 mM NaCl, as this should approximate the Na^+ concentration in the extracellular fluid (see Grabowski, 1963). Electrodes were made on a vertical puller (model 700C, David Kopf Instruments, Tujunga, CA) from 1.5 mm O.D. thin-walled borosilicate capillaries with an internal filament (TW150F, WPI, New Haven, CT). The recording electrode and a Ag/AgCl pellet bath ground were connected to a model M701 amplifier (WPI) and the measured potentials were displayed on a chart recorder. Only those measurements that stabilized within 10 min of impalement and showed a baseline drift of 3 mV or less after electrode withdrawal were considered acceptable. All potentials are given as the mean ± the s.e.m.

Scanning electron microscopy and histology

Embryos to be examined by scanning electron microscopy (SEM) were fixed in 3% glutaraldehyde in phosphate buffer (pH 7.2) for 24 hours and then postfixed in 1% OsO_4 for 1 hour. Dehydration was performed in a graded ethanol series and the embryos were critical point dried from liquid CO_2. They were then mounted on aluminum stubs with copper tape and were sputter coated with gold. Examinations were done at 10 kV on a JEOL 840-I scanning electron microscope.

For histological sections, the embryos were fixed in Bouin's solution for 24 - 48 hours, dehydrated through a graded ethanol series and embedded in paraffin. Serial sections were cut at 7 - 10 μm and stained with hematoxylin and Beibrich's scarlet.

Results

The implant procedure was performed on a total of 154 embryos, with 45 (27 experimentals and 18 controls) of them considered successful. Fifty-eight embryos were dead when the eggs were opened for examination. These deaths were due to a number of factors, including dehydration, puncturing of the yolk sac, and damage to the embryos that was not detected at the time of the operation. Of the 96 living embryos, 28 had lost their implants at some time during the incubation period and another six were destroyed before they could be thoroughly examined. Of the remaining embryos, 17 had retained their implants, but these were found to have penetrated through the ventral side. These embryos were not included in the analysis. The six stage 15 embryos that were successfully operated on were pooled with the others, as there were no apparent differences in the effects of the implants in either the experimental or control group. Likewise, there were no differences in the effects between shunts filled with HBS alone and those filled with agarose-gelled HBS.

Shunt effects on endogenous currents

Vibrating probe measurements were performed on some embryos in order to confirm that the shunt implants did carry current out of the embryo and that the solid glass rods did not. When properly implanted under the skin, current left the shunts (Fig. 1) with an

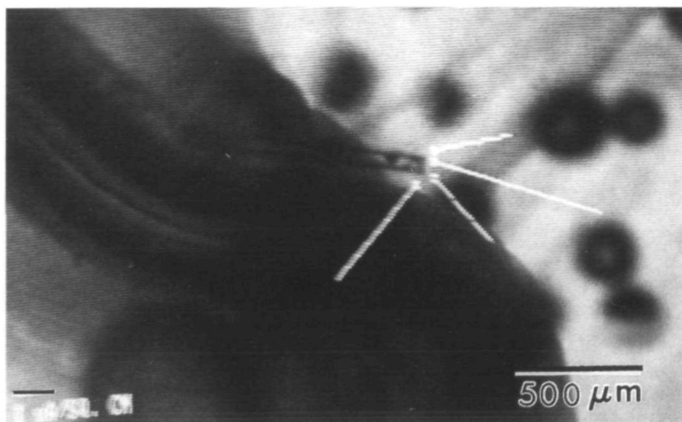

Fig. 1. Two-dimensional vibrating probe measurements of currents leaving an implanted shunt. Current vectors are represented by lines originating at a dot, which indicates the probe's position when the measurement was made. The direction of the line away from the dot indicates the direction of current flow at that position and the line's length is proportional to the current density (left-hand scale bar=1 μA/cm^2). For this embryo, measurements were made approximately 30 hours after implanting the shunt.

Table 1. *Summary of defects in current-shunted and control embryos*

	Experimental (n=27)	Solid rod control (n=18)
	Abnormal	Abnormal
Tail	22 (81%)	2 (11%)
Gut (ourentery)	9 (33%)	1 (6%)
Leg buds		
Ipsilateral	11 (41%)	1 (6%)
Contralateral	8 (30%)	1 (6%)
Wing buds		
Ipsilateral	6 (22%)	0 (0%)
Contralateral	5 (19%)	1 (6%)
Head	4 (15%)	0 (0%)

average maximum density of 18 ± 4 μA/cm^2. The net effect of this treatment on the maximum outward current at the p.i.p. was to reduce it by an average of 30 ± 6% (n=6) in comparison to untreated embryos at the same developmental stage. There were no detectable currents leaving the solid glass rods of the control embryos and the presence of these implants had a negligible effect on the maximum p.i.p. current, as it changed by only 1 ± 7% (n=4). The difference in the effect on the maximum outward current density at the p.i.p. between the experimental and control groups was significant ($0.01 < P < 0.02$; *t-test*).

General effects of shunts on development

In the experimental group, 25/27 (92%) of the embryos exhibited some form of aberrant development. Only 2/18 (11%) of the control embryos exhibited similar abnormalities, with the remainder developing normally. The difference between these two groups is highly significant ($P < 0.001$; *chi-square*). The most common defect in the experimental group was in tail development (Table 1). Several other types of abnormalities were also found with a fairly high frequency in the experimental group. These included defects in limb development and head formation and the formation of caudal ourenteric outgrowths (aberrant tissue growths from the hindgut). There was a clear tendency for abnormalities to occur most frequently in caudal structures. The frequency of defects progressively decreased in the rostral direction (Table 1). Twelve (44%) embryos from the experimental group displayed multiple defects, as did the two abnormal control embryos.

The defects in limb bud and head development observed in some experimental embryos were not analyzed in any detail and will be only briefly described here. In three of the embryos, one of the limb buds was bilobed in appearance (Fig. 2A), and in four others one or more of the limb buds was completely absent (Fig. 2B). In the other cases the aberrant limb buds were irregularly shaped and were sometimes flattened distally (Fig. 2B). For both the leg and the wing buds in the experimental group, defects on the side contralateral to the shunt were always coincident with ipsilateral defects; however, some embryos displayed ipsilateral limb bud defects while the contralateral side was normal.

Abnormalities in the development of head structures were seen in 15% of the experimental embryos. All of these embryos displayed some retardation in the formation of one or more of the brain divisions (Fig. 2C), and two of them showed defective eye development. In these embryos the eyes were much smaller than normal, appearing as small, pigmented spots or slits. One embryo displayed a protuberance of unknown origin emanating from between the cerebral hemispheres.

Shunt effects on tail development

The most common defect detected in current shunted embryos was in tail development, with 81% showing some abnormality. In contrast, only 11% of the control embryos into which non-conductive solid glass rods had been implanted exhibited similar tail abnormalities. The difference between these two groups in the frequency of tail defects is highly significant ($P < 0.001$; *chi-square*).

The tail abnormalities found in the experimental group showed some variation. Just over half of these embryos (12/22) possessed tails that were nearly normal in length but were clearly abnormal morphologically (Fig. 3A and 3B; see also Fig. 2A). In these embryos, the tail was normal for a short distance (generally 1/2 or less of the total tail length), but the distal portion lacked any apparent structure and terminated in a sac-like appendage. Sagittal sections of these embryos (Fig. 4A) showed that the structural components of the tail (i.e., the neural tube, notochord and somites) ended abruptly, causing the caudal end to appear blunted and raised, with an attached sac of loosely-packed mesenchymal cells. Often this region contained remnants of

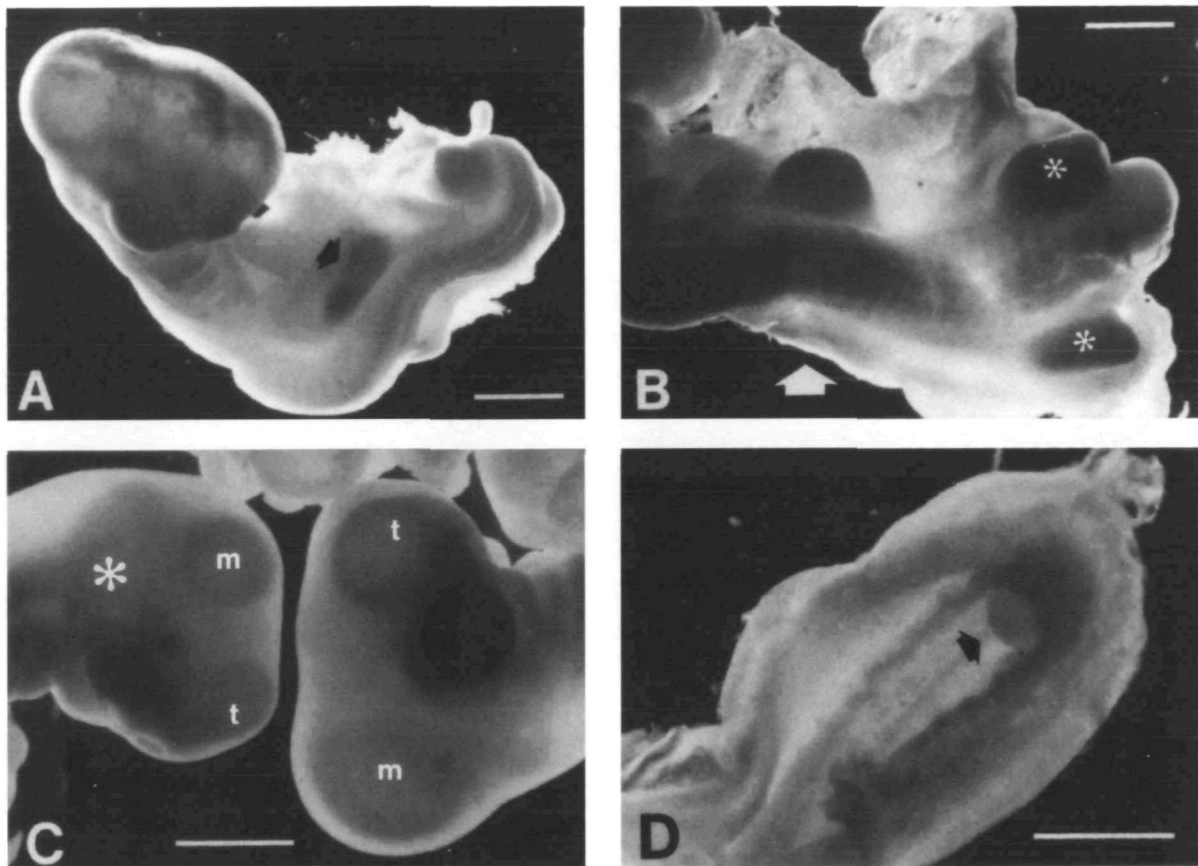

Fig. 2. Abnormalities in limb, head and gut development in current shunted embryos. (A) Bilobed wing bud (arrow) on the side contralateral to the implant. Note also the abnormal tail. (B) The ipsilateral wing bud is completely absent (arrow) and both leg buds are enlarged and flattened distally (asterisks). The tail of this embryo is also misshapen. (C) Brain development was retarded in the current shunted embryo on the left (asterisk). Only the mesencephalon (m) and the telencephalon (t) are labeled, but all of the brain divisions were abnormal. The other embryo (which also contained a current shunt) displayed normal brain development but abnormal tail formation (not shown). Both embryos were incubated for the same amount of time and both were at stage 20 when examined. (D) Ventral side of an experimental embryo showing an ourenteric outgrowth (arrow) from the p.i.p. Scale bars: A=1 mm; B, C, D=500 μm.

the neural tube and/or notochord and areas that appeared to have undergone a form of aberrant somite formation.

The remaining 10 embryos either lacked tails altogether, with the caudal end terminating in a blunt stump distal to the leg buds, or possessed only very short, nipple-like projections. These projections completely lacked any structural components and in sections were similar to those described above containing loosely-packed mesenchymal cells. Embryos in this group frequently exhibited ourenteric outgrowths (Figs 2D and 4B). Externally, these outgrowths appeared as structureless balls of cells emanating from the p.i.p. (Fig. 2D). This defect was always associated with abnormal tail formation and was most pronounced in those embryos that lacked tails altogether. Histological sections (Fig. 4B) revealed that in some of these embryos the notochord would turn approximately 180° and extend in the rostral direction into the cloaca. Serial sections revealed that the outgrowths contained primarily notochordal tissue with some associated mesenchymal cells. It appears in these sections that the notochord continued to lengthen normally while the rest of the tail tissue did not. Thus, the notochord was forced to extend along the probable path of least resistance into the hindgut. The neural tube did not appear to develop along with this tissue mass, but rather ended abruptly in the tail stump, as did somite formation.

Rumpless mutants

The tail abnormalities induced in chick embryos by shunting the endogenous current were very similar to those appearing naturally in *rumpless* mutant chicks. This suggested to us the possibility that these mutants might also exhibit differences from wild-type embryos in their electrophysiological characteristics, particularly in the pattern and/or magnitude of their endogenous currents. We therefore performed vibrating probe and TEP measurements on both dominant and recessive *rumpless* mutants at the same developmental stages as those examined previously in wild-type embryos (Hotary and Robinson, 1990).

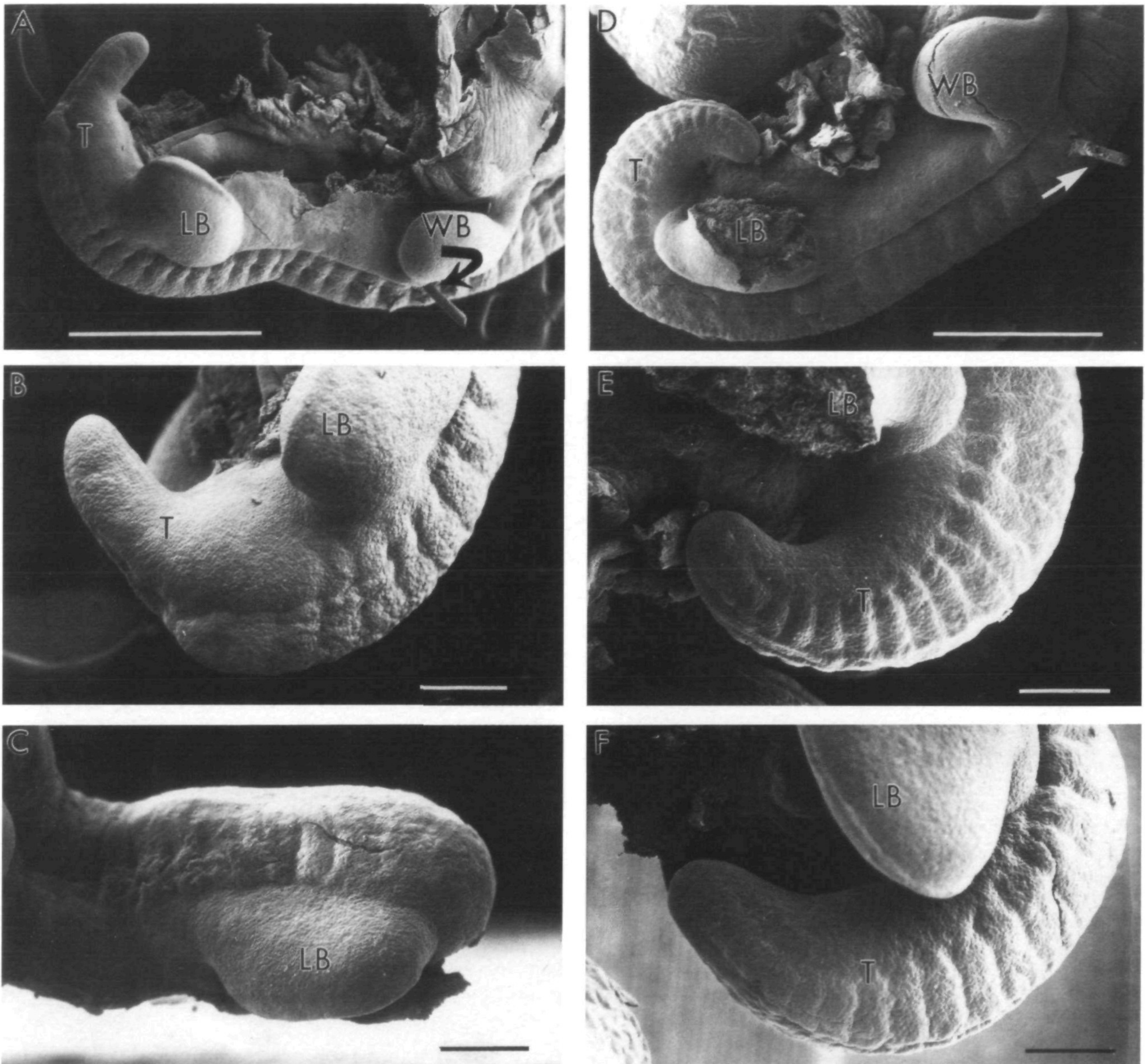

Fig. 3. Scanning electron micrographs of experimental and control embryos. A and D show low magnification views of a current shunted (A) and a solid glass rod control (D) embryo. The arrows in each indicate the position of the implants near the level of the wing buds (WB). (B) Higher magnification of the tail region of the embryo shown in A. The tail (T) is nearly normal in length, but the distal half appears unstructured, lacking any somites or extension of the neural tube. (C) Experimental embryo that failed to develop a tail, terminating in a blunt stump just distal to the leg buds (LB). (E) Tail region of the solid rod control embryo shown in D. The leg bud has been partially removed in order to clearly visualize the normally formed tail. (F) Unoperated control embryo showing a normally formed tail. Scale bars: A, D=1 mm; B, C, E and F=250 μm.

Examples of the rumpless mutant phenotypes are shown in Fig. 5. Both the dominant and the recessive mutants have been described previously in some detail by Zwilling (1942; 1945a), and the external morphological characteristics of the embryos used in the present study matched his descriptions. For both mutant stocks, tail development ranged from being completely normal to the complete absence of all caudal structures. Abnormalities were generally much more severe in the dominant stock, and in some cases the normal development of axial structures ceased at the trunk level. Both mutants often exhibited ourenteric outgrowths from the p.i.p. In some cases these outgrowths were so large as to completely obscure the p.i.p., thus rendering these embryos useless for vibrating probe measurements. Limb bud abnormalities were rare in both stocks, and in fact limb development usually appeared to proceed normally in embryos displaying severe tail abnormalities.

p.i.p. currents in rumpless mutants

The two-dimensional vibrating probe was used to

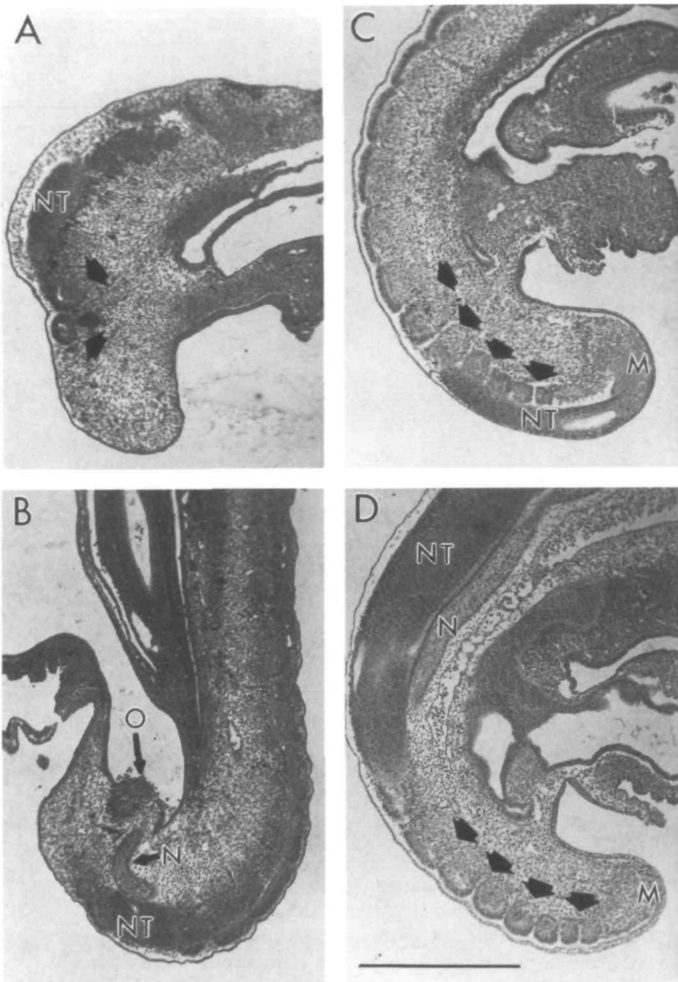

Fig. 4. Sagittal sections through the tail regions of experimental (A and B) and control (C and D) embryos. (A) In this experimental embryo, the tail extends for some distance caudally, but lacks any normal structural components, containing mostly loosely packed mesenchymal cells and abortive somites (arrows). (B) The embryo failed to form a tail, and the neural tube (NT) and somite development ended abruptly in the caudal stump. The notochord (N), which continued to lengthen, turned ventrally and then rostrally, eventually passing into the cloaca (O=ourenteric outgrowth). C and D show different sections through the same control embryo. The somites (arrows) and neural tube formed normally and extend to near the tip of the tail, which terminates in an identifiable mass of undifferentiated mesenchymal cells (M). This demarcated tissue mass was not visible in experimental embryos with abnormally formed tails. Scale bar=500 μm.

determine the maximum outward current density at the p.i.p. of stage 15 - 21 *rumpless* mutant chick embryos. Fig. 6 shows representative measurements around the p.i.p. of a phenotypically normal (Fig. 6A) and an abnormal (Fig. 6B) recessive mutant. These embryos were both at stage 18, were from the same batch of eggs and were examined consecutively. The maximum current density shown leaving the p.i.p. of the abnormal embryo (44 $\mu A/cm^2$) was 41% of the maximum in the phenotypically normal embryo (108 $\mu A/cm^2$), and was 47% of the average maximum current density (93 $\mu A/cm^2$) at the p.i.p. at this stage in wild-type embryos.

In wild-type embryos, there is a clear relationship between the magnitude of the p.i.p. current and the stage of development (Hotary and Robinson, 1990). Due to the low fertility rates (<50%) and the low rate of mutant embryos in fertile eggs, a stage-by-stage analysis of the p.i.p. currents was not feasible. Instead, the vibrating probe data were pooled for each mutant stock. The maximum p.i.p. current density for each embryo was taken as a percentage of the average maximum p.i.p. current density in wild-type embryos at the same developmental stage. These percentages were then pooled into "normal" and "abnormal" groups for each mutant stock based on the classifications of each embryo made before measurements were started. The pooled data are shown in Fig. 7A. Statistical tests on the data were done using the Mann-Whitney *U*-test.

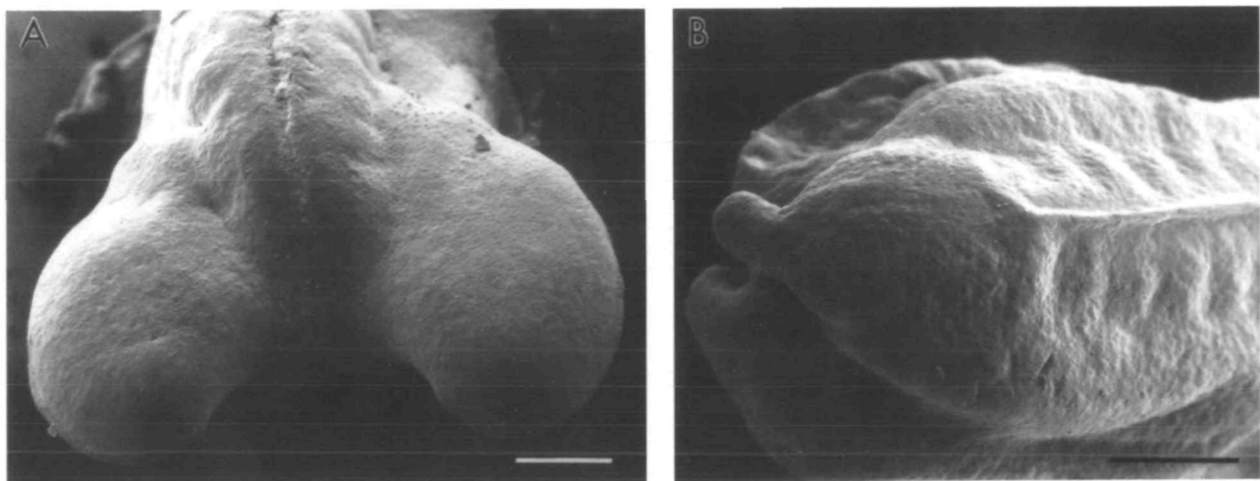

Fig. 5. Scanning electron micrographs of the tail regions of a dominant (A) and a recessive (B) *rumpless* mutant chick embryo. In A, no tail structures formed and the caudal end terminated at the level of the leg buds (compare to Fig. 3C). In B, a nipple-like tail lacking any structural components developed (compare to Fig. 3A, B). Scale bars=250 μm.

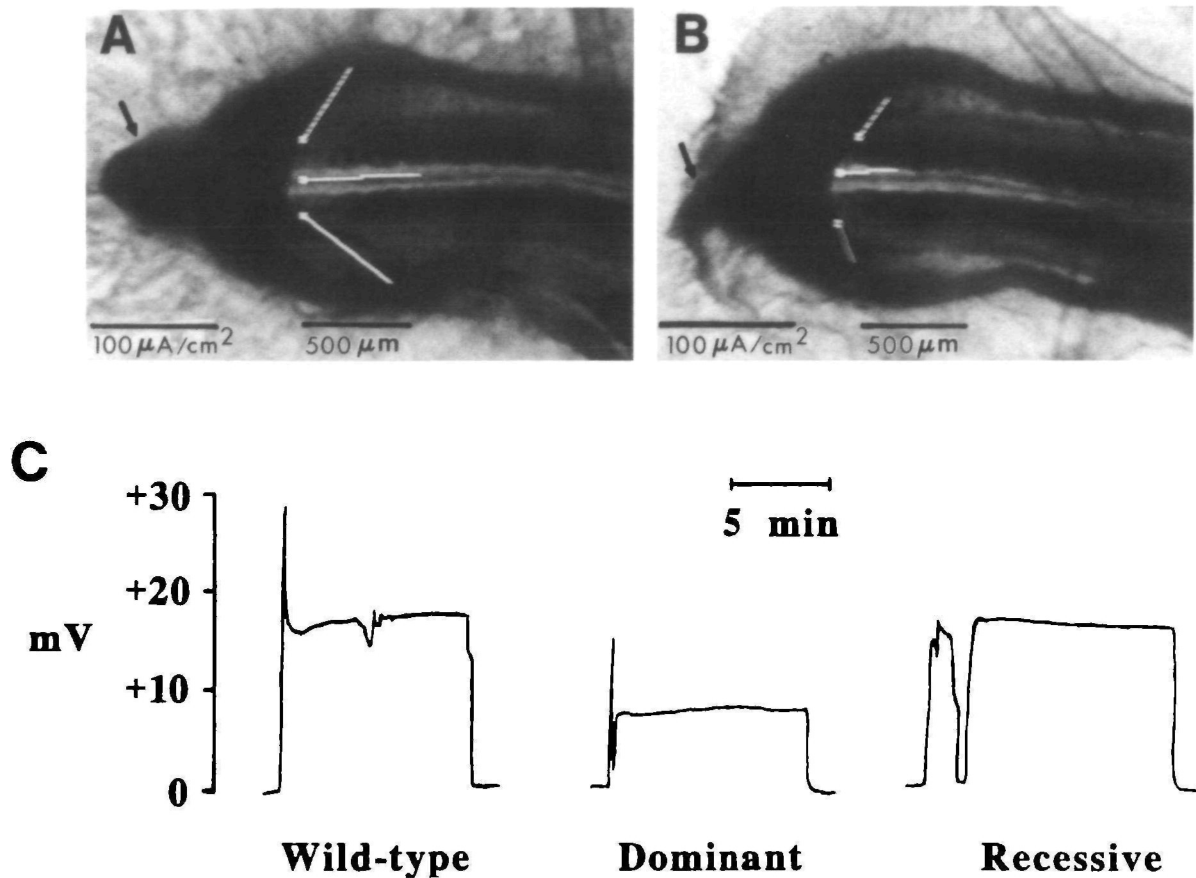

Fig. 6. Examples of current and voltage measurements in *rumpless* mutants. (A) Currents measured leaving the p.i.p. of a phenotypically normal stage 18 embryo from a batch of recessive mutant eggs. The genotype of this embryo could not be determined (see text). The currents leaving the p.i.p. of this embryo were comparable in magnitude to those which leave the p.i.p. of wild-type embryos of the same stage. The arrow indicates the normally formed tail. (B) Currents leaving the p.i.p. of a phenotypically abnormal stage 18 recessive mutant from the same batch as the embryo in A. The currents in B were much lower than in A and in comparison to wild-type embryos of the same stage. Note the abnormally formed tail (arrow). (C) Chart recording tracings of TEP measurements made in wild-type, dominant and recessive rumpless mutants. Stable, positive potentials were measured in embryos from all three groups. The TEP of the dominant mutant was much lower than both the wild-type and the recessive mutant, which were of about the same magnitude.

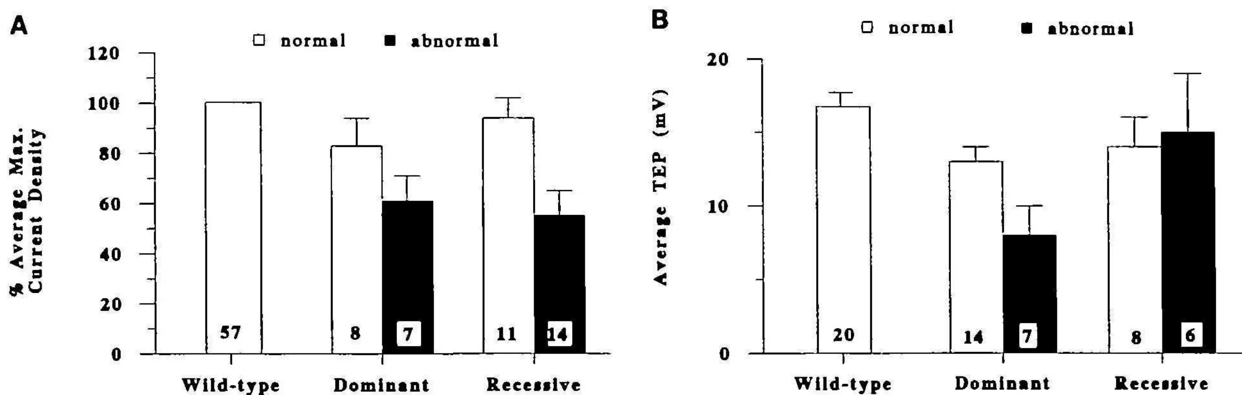

Fig. 7. Summary of p.i.p. currents (A) and TEP measurements (B) in *rumpless* mutant chick embryos. In A, the numbers within each bar indicate the number of embryos examined, while in B the numbers indicate the total number of TEP measurements made in each group. No more than two measurements from any single embryo were included. The error bars for both A and B represent the s.e.m. Statistical differences between the groups and the tests used to determine them are described in the text.

Neither phenotypically normal group showed a significant difference in p.i.p. currents from the wild-type embryos; however, the p.i.p. currents for both abnormal groups were significantly different ($0.001 < P < 0.005$ for the dominants; $P < 0.001$ for the recessives) from the wild-type. The difference between the phenotypically normal and abnormal dominant mutants was not significant, while the two groups from the recessive stock were significantly different from one another ($P < 0.01$). Outward currents were consistently found only at the p.i.p. of stage 15 to 21 embryos. Inward currents of a pattern and magnitude (1 - 7 $\mu A/cm^2$ range) similar to those detected previously in wild-type embryos (Hotary and Robinson, 1990) were found on the dorsal and dorsolateral surfaces of mutant embryos. Likewise, only inward currents were detected at the p.i.p. of stage 14 mutant embryos.

The TEP of rumpless mutants

Measurements of the TEP were performed in stage 15 - 20 wild-type, dominant and recessive mutant embryos. Representative TEP measurements from each of these groups are shown in Fig. 6C. The measurement characteristics were the same for all three genotypes and were similar to those described in an earlier study (Hotary and Robinson, 1990). When possible, measurements were made on the dorsal side at two different positions along the rostral-caudal axis in each embryo: (1) 500 - 1000 μm caudal to the otic vesicle and (2) lateral to the neural tube around the level of entry of the vitelline vessels. Since chick embryos at the stages used in the present study are known to generate a caudally negative voltage gradient that is steepest in the tail region, no measurements made caudal to the mid-trunk level are included in this analysis.

A summary of the TEP measurements is shown in Fig. 7B. The average TEP in wild-type embryos was 17 $\pm$ 1 mV. Neither the phenotypically normal nor the abnormal recessive mutants showed a significantly different TEP from one another or from wild-type embryos. Both the normal and abnormal phenotypes in the dominant group did, however, show a significantly lower TEP than the wild-type ($P < 0.01$ for the normal phenotype and $P < 0.001$ for the abnormal phenotype; *t-test*). The average TEPs of these two groups were also significantly different from one another ($P < 0.05$).

Discussion

Bioelectric fields have long been suspected to play a causal role in embryonic development. This supposition has persisted primarily because of the existence of a large body of mostly circumstantial evidence which shows: (1) the presence of endogenous electric currents in a number of developing systems from a variety of phylogenetic classes (reviewed by Nuccitelli, 1990); (2) that endogenous current patterns can often be correlated with a specific morphogenetic event, such as limb bud formation and outgrowth (Borgens et al., 1983; Robinson, 1983) or tissue involution (Jaffe and Stern, 1979; Robinson and Stump, 1984; Winkel and Nuccitelli, 1989), and (3) the clear directional influence of an applied electrical field on motile cells *in vitro* (reviewed by Robinson, 1985; Nuccitelli, 1988). Despite these suggestive results, a clear link between bioelectric fields and any process during embryonic development has never been demonstrated.

The results presented in this paper suggest that the endogenous electrical field does play a functional role in chick embryo development. This suggestion is supported primarily by the current shunt experiments; the creation of a permanent site of current leakage in the dorsal trunk of the chick embryo during a period when an intraembryonic voltage gradient is known to exist (Hotary and Robinson, 1990) resulted in a high frequency of developmental abnormalities. That this treatment had an effect on the endogenous field is indicated by a reduction in currents measured at the p.i.p. in treated embryos. Interestingly, the highest frequency of abnormalities occurred in the tail region, where the endogenous voltage gradient is steepest. It was surprising to us that such a relatively small change in the endogenous currents in both the experimentally treated embryos and in the *rumpless* mutants could produce such dramatic defects. We do not presently know the basis for this effect.

Developmental abnormalities were also detected in the limb buds and the heads of some current-shunted embryos. Limb bud defects, like those of the tail, ranged from the complete absence of a particular bud to the formation of malformed buds. Head abnormalities were generally in the formation of the brain and the eyes. For reasons that are presently unclear, the appearance of these defects was considerably less common than were those in tail formation. There was no apparent correlation between the stage at which the shunts were implanted and the development of these defects, and since the shunts were always implanted in the same region (within 3 to 4 somites) from embryo to embryo, it is unlikely that these defects were due to its position along the rostral-caudal axis. One concern was that the wing bud ipsilateral to the implant would develop abnormally due to physical constraints imposed by the implant. This does not seem to be the case, however, as no control embryos showed ipsilateral wing bud defects while six experimental embryos did.

The placement of the shunt in a region several mm from where it had its main effect, and the fact that non-conductive implants produced very few abnormalities would seem to rule out the possibility that any defects were due merely to the physical presence of the shunt. Any potential toxic effects of the agarose used to fill the shunts can also be discounted, as any such reaction would likely be localized to the area immediately surrounding the implant and not to any area several mm distant. Furthermore, shunts filled with ungelled HBS alone produced identical defects. Finally, the use of agarose-gelled HBS filled shunts makes it unlikely that any abnormalities were caused by fluid transfer between the embryo and the surroundings, assuming that

agarose toxicity and fluid transfer would not cause identical abnormalities at remote locations.

In the control group, 11% of the embryos displayed defects in tail development. While the difference in the frequencies of tail defects between this group and the experimental group is highly significant, this incidence in controls is still higher than the approximately 1% "rumplessness" that would be expected to occur spontaneously in normal embryos (Landauer and Baumann, 1943). It may be that this was caused by current leakage around the implant site of these few embryos. Such leakage would occur if the implant did not completely heal in place, as could be caused by the flexion process developing chicks normally undergo. Outward currents were detected at the implant site in a few of the examined embryos. These cases were relatively rare, however, and in most instances the shunt or rod was tightly held in place and there were no detectable outward currents around the insertion site.

The possibility that the electrical field in the chick embryo plays a causal role in tail development is further supported by the vibrating probe measurements on *rumpless* mutant embryos. The tail defects seen in many of these embryos, from both the dominant and the recessive strain, were strikingly similar to those induced in wild-type embryos receiving current shunts. Currents leaving the p.i.p. of phenotypically abnormal mutants were significantly lower than in wild-types, while currents in phenotypically normal mutants (which may or may not carry the mutant allele) are essentially the same as those in wild-types. The reduced currents measured in abnormal embryos are an indication of an altered (from wild-type) internal electrical field, and there is a clear correlation between reduced currents at the p.i.p. and abnormal tail development. Obviously, this correlation alone says nothing about cause and effect; however, together with the current shunt experiments it does suggest a causal role for the electrical field in the development of the embryonic chick tail. Furthermore, the expression of this abnormality seems to be independent of any direct effect on the TEP alone, as TEPs significantly lower than wild-type were measured in both normal and abnormal dominant mutants. Conversely, the TEP in both recessive phenotypes was not significantly different from the wild-type.

A variety of seemingly disparate treatments have been found to induce rumplessness in chick embryos. The mechanisms of this induction are not clear. Landauer and Baumann (1943) found that mechanical shaking of eggs caused tail abnormalities, and Zwilling (1945) induced tail defects that "satisfy all of the requisites for phenocopies" of genetically rumpless mutants by transecting the bodies of two day old embryos. A disruption of the integrity of the ectodermal epithelium likely occurs with the former treatment, and unquestionably does occur with the latter. In both cases then, a leakage pathway for electric currents leaving the embryo would be created. Presumably, the internal electrical field would then also be altered in a manner analogous to that described in the present study.

Tail abnormalities in chick embryos can also be produced by treatment with the vital dye trypan blue (Kaplan and Grabowski, 1967; Jelinek and Rychter, 1972; Nadeau and Mun, 1978), subjection to hypoxia (Grabowski and Schroeder, 1968) and by the sub-blastodermal injection of retinoic acid (Griffith and Wiley, 1989; 1991). All of these agents have been found to cause the formation of axial blisters and hematomas prior to the development of malformed tails. These lesions have been suggested to cause the abnormalities through a mechanical effect (Grabowski, 1963); however, both trypan blue (Nadeau and Mun, 1978) and retinoic acid (Griffith and Wiley, 1989) have been shown to have direct effects on tail development not related to blister and/or hematoma formation. Furthermore, rumplessness occurs without the formation of lesions in both dominant and recessive mutants and with the administration of some other teratogens, including insulin (Landauer, 1945; Mosely, 1947) and wheat germ agglutinin (Griffith and Wiley, 1990). The formation of edematous blisters indicates an electrolyte imbalance and a disruption of the osmoregulatory mechanisms of the embryo. Such imbalances have been demonstrated with some of these treatments by Grabowski (1963). One can speculate then that these treatments alter the functioning of the ion-transporting epithelium, thus disrupting the ionic regulatory mechanisms of the embryo and altering the endogenous electrical field. This alteration of the field results in aberrant tail formation. It should be noted here that neither blisters nor hematomas were observed in current shunted embryos in the present study.

The underlying mechanism by which an endogenous electrical field may exert an influence on development remains to be discovered. Most prevailing hypotheses suggest that a field acts to directionally guide the growth and/or migration of some embryonic cells (see reviews by Robinson, 1985; Nuccitelli, 1988). The electrical field in the chick embryo may indeed perform such a function, but it is difficult to explain our results by this mechanism. Rather, the defects in tail development induced by shunting the endogenous field suggest some possible additional or alternative functions.

The electrical field may directly affect the differentiation of some tail structures, in particular those derived from the tail bud. There is some evidence that applied fields can affect the differentiation of cells *in vitro* (Hinkle et al., 1981). In many cases when shunt implants were made, the neural tube and somites (which are derived from the tail bud; Schoenwolf, 1977) failed to form; however, the caudal notochord (which is not derived from the tail bud but from a more rostral region called the prospective notochordal region; Schoenwolf, 1978) often continued to grow along the presumed path of least resistance into the hindgut, resulting in ourentery. This effect is very similar to that seen with extirpation of the tail bud during early development (Criley, 1969; Schoenwolf, 1978).

The endogenous electrical field in the chick embryo

may also act indirectly by regulating the distribution of chemical morphogens. Woodruff and Telfer (1980) showed that an endogenous voltage gradient acts to electrophoretically distribute charged proteins in the oocyte-nurse cell syncytium of the saturniid moth, *Hyalophora cecropia*. More recently it has been shown that an applied electrical field of only 2 mV/mm can significantly alter the distribution of Ca^{2+} in the cytoplasm of injured axons (Strautman et al., 1990). Similarly, Cooper et al. (1989) have shown that an applied electrical field as low as 20 mV/mm can cause the electromigration of carboxyfluorescein in gap-junction coupled tissue. It seems possible that relatively large, slowly-diffusing molecules could be redistributed in the extracellular spaces of an embryo by an endogenous electrical field that is even larger than those artificially imposed in the above studies. In this manner a gradient of a chemical morphogen could be established or modified. It is also possible that overlapping, multiple gradients of differentially charged molecules could be established by an electrical field. The alteration of the electrical field would then disrupt the chemical gradient and the signals received by the cells of the embryo. It is interesting that one way in which to induce rumplessness in the chick is to provide an excess of or an inappropriately timed exposure to a putative chemical morphogen, retinoic acid, at the same stages of development as those examined in the present study (Griffith and Wiley, 1989; 1991). Furthermore, the limb bud defects produced in the present study with shunting the endogenous electrical field are also suggestive of this indirect role for the field in light of the suspected function of retinoic acid as a signaling molecule in vertebrate limb development (Brickell and Tickle, 1989; Summerbell and Maden, 1990). In any case, it appears that in some manner cells sense their position in an electrical field and respond appropriately. The disruption of this field alters their response.

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