Short Review

Pattern formation in early developmental stages of amphibian embryos

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This article is dedicated to Professor Etienne Wolff on the occasion of his retirement

SUMMARY

An internal factor (or factors), which determine the endoderm and the mesoderm anlage, seem to exist already in certain regions of the oocyte and very early developmental stages. The factor(s), which is (are) protein in nature, has (have) similar properties to the one isolated from chicken embryos. The induction of the neural plate seems to be mediated by a membrane mechanism. The segregation of the primarily formed anlagen occurs by secondary tissue interactions.

The causal morphological analysis of the determination and differentiation processes in early developmental stages has been successfully carried out, especially in insects, sea urchins, amphibians and mammals. In all these investigations the final outcome of isolation and transplantation experiments has been evaluated by histological analysis of the differentiated tissues. In such experiments the term determination, which has been used in several ways, can be defined as follows: A certain region of a cleaving egg or an embryo is said to be determined if after isolation it forms the same tissues as in the intact embryo.

Differentiation depends on the differential synthesis of RNA's and proteins. Ribosomes are very actively synthesized during amphibian oogenesis and stored in the oocyte (Wallace & Birnstiel, 1966; Brown & Dawid, 1968). In amphibian embryos the synthesis of messenger-RNA is low during cleavage. It increases from the blastula stage on. Shortly afterwards a considerable synthesis of 4S-RNA was observed. The synthesis of embryonic ribosomal-RNA becomes accelerated in the gastrula stage (Brown & Littna, 1966; Gurdon & Woodland, 1968; Woodland & Gurdon, 1968). Proteins which are newly formed during the early developmental stages are in part translated on so-called ‘maternal’

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messengers which are synthesized and stored in the oocytes, and in part on
messengers, which are newly synthesized in the cleaving egg after fertilization.
When early embryos or tissues of them are treated with Actinomycin D to
inhibit the synthesis of messenger-RNA, these latter proteins cannot be pro-
duced and a series of differentiation processes cannot take place (Nakamura,
1969). The translational products of 'maternal' messengers include histones and
microtubule proteins (Gross, Jacobs-Lorena, Baglioni & Gross, 1973; Raff,
Colot, Selvig & Gross, 1972). The proteins synthesized on maternal messengers
as well as the ribosomes synthesized during oogenesis are cell constituents
which are needed during cleavage of the large yolk-rich eggs.

It may be asked whether small quantities of macromolecules, which are
directly involved in the control of determination and differentiation processes,
are already distributed in different regions of the egg. Such substances, which
are not RNA but protein in nature, seem indeed to be present. Their existence
was inferred from experiments which were aimed to determine the inducing
ability of isolated areas of the cleaving egg and embryo. The most interesting
result was the discovery by Spemann and Mangold (1924) that the presumptive
mesodermal area of gastrula stages (the so-called 'marginal zone' of earlier
stages) can induce neural and, to some extent, mesoderm-derived tissues also in
gastrula ectoderm after heteroplastic transplantation. This led to the assumption
that the presumptive mesodermal area forms an organization centre in the
amphibian embryo. Its neural-inducing activity was not lost after heat treatment
(Bautzmann, Holtfreter, Spemann & Mangold, 1932).

More recently the inducing ability as well as the capacity for self-differentia-
tion of isolated regions of very early developmental stages have been investi-
gated. From such experiments the timing of organ determination can be
inferred more clearly. The presumptive mesodermal area forms mesoderm-
derived organs when isolated in the morula stage or even earlier (Nakamura,
1969). A better differentiation of mesoderm-derived organs was observed when
the mesodermal zone was isolated together with the presumptive ectoderm
(unpublished experiments by Hildegard Tiedemann, 1969). The presumptive
endoderm as an anlage is already determined very early in development. The
various endodermal organs, however, differentiate much later under the induc-
tive influence of the neighbouring ectomesenchyme or mesoderm (Holtfreter,
1939a; Okada, 1960). So far, no experiments are known in which the endoderm
is forced to build organs which normally arise from the other two germ layers.

The inducing activity of the isolated presumptive endodermal area has
recently been tested from the uncleaved fertilized egg up to the neurula stage
(Nakamura, Takasaki & Ishihara, 1971; Asashima, 1975). It has been shown
that endoderm taken from the uncleaved egg and 4-cell stages induces blood
cells and coelomic epithelium in 10–30% of the cases, whereas endoderm from
later stages in addition induced muscle, notochord and pronephric tubules.
Blastula endoderm has the highest inducing activity. Within the endoderm
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Regional differences of its mesoderm-inducing capacity have been observed. The dorsal endoderm preferentially induces notochord and muscle, whereas the ventral endoderm preferentially induces blood and coelomic epithelium (Boterenbrood & Nieuwkoop, 1973).

The marginal zone (the presumptive mesodermal area) induces mesoderm-derived organs from the morula stage onwards or even earlier and both the marginal zone and the presumptive endodermal region induce intestine in a small percentage of cases.

It can be concluded from these experiments that inducing factors for mesoderm and endoderm are located in the presumptive mesodermal and endodermal regions very early in development. In untreated isolated ectoderm on the other hand no endoderm, mesoderm or neural-inducing activity has been found up to the gastrula stage, but some masked inactive neural and also to a small extent mesoderm-inducing factors seem to be present in gastrula ectoderm. These factors are activated by treatment of gastrula ectoderm with ethanol or phenol.¹

The experiments indicate that animal–vegetal as well as dorsoventral gradients of one or more factors (see below) exist already very early in development. These gradients could perhaps be formed by cytoplasmic movements after fertilization or the gradients could be prelocated in the surface structures of the unfertilized egg. This may correlate with the positional information as defined by Wolpert (1969).

Ectoderm up to the gastrula stage has been considered as an embryonic region which is not determined to form specialized tissues. However, after isolation from cleavage up to early gastrulae the ectoderm region forms typical epithelial cells with a characteristic peripheral zone free of yolk platelets and embryonic pigment. Approximately one-tenth of the ectoderm cells becomes ciliated (Grunz, 1973, 1976). The typical alignment of the cells in the intact embryo depends however on the mesenchyme underlying the epidermis (Holtfreter, 1939b). Obviously the ectoderm is already determined and there is no ‘undetermined’ region even in early embryos.

However, the direction of differentiation which the ectoderm takes is not yet irreversibly fixed but can still be changed by addition of appropriate inducing factors. It can form mesodermal, endodermal or neural tissues. Therefore ectoderm of early gastrula stages can be used as a test system for inducing factors (Mangold, 1923; Holtfreter, 1933; Becker, Tiedemann & Tiedemann, 1959).

The results which have been described extend the ‘organizer’ experiment to early embryonic stages and different regions. They do not give insight into the mechanisms which lead to the differentiation of certain types of cells. The molecular control mechanisms are still far from being resolved, but some clues have evolved more recently. New results suggest that the mechanisms for the

¹ A proteoglycan which inhibits the biological activity of the factors has been extracted from chicken embryos (Born, Tiedemann & Teidemann, 1972) and amphibian tail-bud stages.
determination of the mesoderm and endoderm anlage and for the determination of the neural system are quite different.

A homogeneous factor which in gastrulae induces ectoderm-, endoderm- and mesoderm-derived tissues has been isolated from 11- to 13-day-old chicken embryos. This factor is proteinaceous in nature. After several preparative steps the final degree of purification was attained by repeated isoelectric focusing (isoelectric point \( \sim \text{pH} 8.1 \)) and SDS-acrylamide electrophoresis (Geithe et al., 1975). A molecular weight of 30000–32000 Daltons was calculated by different methods. The factor is inactivated by pepsin or trypsin, but not by ribonuclease. Heating at pH 8 and reagents which reduce S-S bridges also inactivate the factor (Tiedemann, Tiedemann, Born & Kocher-Becker, 1969).

The isolation of a vegetalizing factor from amphibian embryos is much more difficult. Only small amounts of embryos which contain a large portion of inactive yolk proteins are available. Therefore highly purified factors have not been isolated from amphibian embryos. However, extracts which contain crude factors could be obtained from cleavage or gastrula stages. The molecular weight of the vegetalizing factor in amphibian embryos seems to be somewhat higher. But further experiments are needed because the factor may be associated with high molecular weight carrier proteins (Faulhaber, 1970; Faulhaber & Lyra, 1974). Like the chicken factor the amphibian factor can be extracted with phenol without loss of activity. It is inactivated by trypsin.

Neuralizing activity has been found in a large number of tissues. However, in contrast to the vegetalizing factor, a neuralizing factor could not be obtained in a highly purified form. The neuralizing factor is not inactivated by disulphide-bond reducing agents and is not heat-sensitive. The neuralizing activity is lost after treatment with proteolytic enzymes. In homogenates the neuralizing and the vegetalizing factors in part are bound to cellular membranes and in part are found in the cytosol together with soluble proteins.

When the vegetalizing or the neuralizing factors are bound to an insoluble matrix as BrCN-sepharose or bromoacetylcellulose, which prevents the factors from penetrating into the cells and then tested on ectoderm, they behave quite differently. The vegetalizing factor loses its biological activity almost completely. This indicates that the factor must penetrate into the ectoderm cells to exert its biological activity (Tiedemann, Born & Tiedemann, 1976) and preliminary experiments with the labelled factor indicate that it actually does so (Grunz, 1976). However, in normogenesis a similar factor is already present in the presumptive endodermal–mesodermal region (see p. 2) and it follows that it does not migrate into this region from outside.

When on the other hand a neuralizing factor is bound to BrCN-sepharose its biological activity is preserved, while small amounts of the vegetalizing factor which contaminate the more crude fractions of the neuralizing factor are inactivated.

\( ^1 \) BrCN-sepharose blocked with ethanolamine and BrCN-sepharose coupled globulin has no inducing activity.
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inactivated again. This excludes the possibility that the factors are split from the sepharose matrix by proteolytic enzymes which could be present in the crude neuralizing fractions. The experiment suggests that the neuralizing factor, in contrast to the vegetalizing factor, acts on the outer plasma membrane. Transfilter experiments (Saxén, 1961; Toivonen et al. 1975) and experiments with soluble factors (Becker et al. 1959) have revealed that a cell to cell contact is not an absolute requirement for neural induction. It is not known whether in normal embryogenesis a neural inducer, which is part of the plasma membrane of the inducing cells of the archenteron roof, interacts with the plasma membrane of ectoderm cells, or whether a factor is released from the archenteron roof, diffuses a short distance and then interacts with the plasma membrane of ectoderm cells.

Carefully performed experiments have shown that cyclic-AMP, cyclic-GMP and their mono- and dibutyryl-derivatives in a concentration range from $10^{-3}$ to $10^{-5}$ M do not enhance the rate of neural inductions over that observed in the control experiments. Therefore it can be excluded that the neuralizing factor enhances the activity of a membrane bound AMP- or GMP-cyclase (Grunz, 1976). Whether a protein is released from the plasma membrane needs further investigation. It should be mentioned that a soluble neuralizing factor, which has been extracted from amphibian embryos, is inactivated by proteolytic enzymes (Tiedemann, Becker & Tiedemann, 1961), whereas small molecular weight substances with neuralizing or vegetalizing activity so far could not be extracted from amphibian embryos.

It is well known that isolated ectoderm of some species is easily neutralized by changing the concentration of ions in the medium (Barth & Barth, 1962) or by other unspecific means; cited in Saxén & Toivonen, 1962). This would be compatible with the hypothesis that in these species a membrane-bound neuralizing agent is released easily by a change of membrane conformation.

The biological activity of the vegetalizing factor is inhibited when the factor is combined with chicken or Xenopus DNA, but not when combined with different species of RNA or double stranded homopolymeric or heteropolymeric polynucleotides. The inhibition results from the binding of the factor to DNA as shown by their co-sedimentation in sucrose gradients (Tiedemann, Born & Tiedemann, 1972). This may suggest that the added DNA could compete with the DNA in the target cells. But further experiments are needed to test this hypothesis. The neuralizing factor on the other hand is not inhibited by combination with DNA. In addition RNA isolated from a crude nuclear preparation of Xenopus embryos inhibits the vegetalizing factor to some extent. At higher ionic strength the inhibition by DNA is enhanced (excluding unspecific ionic bonds as the major type of interaction), whereas the inhibition by the RNA fraction is abolished.

1 On ectoderm of Triturus alpestris, the species most frequently used for testing tissue extracts and purified factors 'unspecific' stimuli have very little effect.
Other possible mechanisms have to be considered. Treatment of blastula ectoderm with Li⁺ causes mesodermal as well as neural differentiations (Masui, 1961; Grunz, 1968). This could suggest an action of the vegetalizing factor on the Na⁺ pump. But a distinct change of the intracellular Na⁺ and K⁺ concentrations is observed only 72 h after induction with the vegetalizing factor. Treatment of ectoderm (*Triturus alpestris*) with Quabain for several hours raised the intracellular Na⁺ concentration fourfold, but did not lead to the formation of mesodermal or neural differentiations (Siegel & Grunz, 1976). The change in the Na⁺ and K⁺ concentration seems to be a consequence rather than the cause of cell differentiation. This is also true for the change of mutual cell affinities which is observed about 24 h after induction with the vegetalizing factor.

It could be argued that the mitotic rate of certain classes of ‘predetermined’ ectodermal cells would be stimulated selectively by different inducing factors. However, it is unlikely that such a mosaic of cells exists, because almost the total cell population of ectoderm explants can be induced to notochord and myoblasts or to neural tissue. Furthermore by introducing ‘predetermined’ cells the determination problem would be shifted to another level only.

The segregation of the endoderm–mesoderm anlage is still a matter of speculation. At higher concentrations the purified vegetalizing factor induces endodermal structures like endodermal epithelium and intestine and at lower concentrations or after a shorter induction period it also induces mesodermal structures like notochord, muscle, renal tubules and blood cells (Kocher-Becker & Tiedemann, 1971; Grunz, 1976). It is not completely excluded that two different molecules, which must be very closely related in size and charge, are responsible for mesoderm and endoderm induction. On the other hand, a common anlage could be induced, which later segregates (perhaps under the influence of ectodermal factors) into endoderm and mesoderm anlagen or a single factor could induce different organs at different threshold concentrations. Finally endoderm could be induced primarily and then mesodermal organs could be induced by the endoderm. In some explants, however, only mesodermal organs have been found. But it is possible that a small endoderm anlage may have been formed but either not expressed or lost.

Yamada (1940) has made the interesting observation that the organs which are formed from different presumptive mesodermal regions change to a more ‘dorsal’ type (i.e. blood cells to nephric tubules or nephric tubules to myoblasts) when the notochord anlage is added to the explants. From these experiments the existence of another factor has been suggested. It seems to be likely that several factors are involved, but, so far, no experiments are known which show conclusively whether two or more of such factors or different

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1 Whether Li⁺ ions activate masked internal inducers or whether they may change the conformation of chromatin directly is an open question.

2 The molecular basis could be a graduated differential affinity of the factor to structurally related moderately heterogeneous DNA sequences with a regulatory function.
threshold concentrations of one factor are involved in the induction of endoderm and of mesoderm-derived organs.

The segregation of the induced neural anlage (neural plate) of amphibian embryos into forebrain, hindbrain and spinal cord takes place by interaction with mesodermal cells, which come in close contact with the neuralized cells (Saxén, Toivonen & Vainio, 1964; Toivonen, 1972). The neuralized cells lose the ability to undergo transformation less than 10 h after the early neurula stage. The mesoderm loses its capacity to transform the neuralized cells a few hours later. The transforming action depends on the ratio of mesodermal to neuralized cells. When the amount of mesodermal cells is increased neural tube instead of hindbrain is formed.

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