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Short Contribution

5-Azacytidine changes gene expression and causes developmental arrest of early chick embryo

NIKOLAS ZAGRIS* and TASOS PODIMATAS

Division of Genetics and Cell and Developmental Biology, Department of Biology, University of Patras, Patras, Greece

ABSTRACT Methylation of DNA appears to be an important maintenance mechanism for inhibiting gene expression during development in vertebrates. 5-azacytidine (5-azaC) is used as an experimental tool for demethylation and it induces differentiation in various systems. In the chick embryo, the first cellular migrations signal the onset of primitive streak and gastrula formation and result in neural induction and morphogenesis of the embryonic axis. In the present work with the early chick embryo, 5-azaC perturbs normal cellular migrations and the embryos produce an atypical short, thickened primitive streak. These embryos have the tendency to form neural tissue but the embryonic axis shows sparse identity of patterning along its length. A small percentage of embryos display formation of double embryonic axes. Blastula embryos show reduced expression of some polypeptides and express characteristic polypeptides which are not present in morula embryos normally. Under the influence of 5-azaC, blastula embryos expressed all the polypeptides which are characteristic of embryos at both the morula and the blastula stages. If 5-azaC perturbs DNA methylation as the chick embryo develops from the histologically simple blastula, then the wave of methylation which has been reported to start at the late blastula and continues during postgastrulation in vertebrate embryos does not seem to be important for the induction of mesodermal and of neural tissues, but is important for the patterning of these tissues.

KEY WORDS: *embryonic axis, imprinting, DNA methylation, 5-azacytidine, chick embryo*

The cytidine analog 5-azacytidine (5-azaC) has been shown to induce differentiation in various systems (Taylor and Jones, 1979; review Cedar and Razin, 1990). The studies of the effects of 5-azaC on cell functions and differentiation have demonstrated that 5-azaC incorporation into genomic DNA leads to its hypomethylation (Jones and Taylor, 1980; Creusot *et al.*, 1982; Jones, 1985), and thus possibly allows inactive genes to become expressed (Iguchi-Ariga and Schaffner, 1989).

The molecular structure of 5-azaC, with the 5-carbon atom of the pyrimidine ring replaced by a nitrogen atom, renders it incapable of accepting the methyl group in the enzymatic methylation reaction occurring in newly synthesized DNA molecules. 5-azacytidine, as a free base, is known to interfere with normal methylation by inactivating the DNA methyl transferase (Doerfler, 1983; Santi *et al.*, 1983). It seems that DNA methylation influences gene expression probably by altering DNA-protein interactions, and certain methyl moieties may also interfere with specific protein recognition (review Tate and Bird, 1993).

There seems to be an inverse relationship between DNA methylation and gene expression. Almost all tissue-specific genes are found fully methylated in cell types which do not express the genes, while they are unmodified in cells which show active transcription (reviews Cedar and Razin, 1990; Lewis and Bird, 1991; Bird, 1992). The maintenance of the DNA methylation pattern by DNA methylases may account for the stable control of gene expression in differentiated cells (review Tate and Bird, 1993). Vertebrates utilize methylation to inactivate certain DNA sequences and regulate gene expression during development. Mouse development is characterized by loss of genomic methylation during preimplantation development followed by a *de novo* methylation at the late blastocyst stage (Monk *et al.*, 1987). Methylation continues during postgastrulation and hence could be a mechanism initiating, or confirming, differential programming in the definitive germ layers (Monk *et al.*, 1987; reviews Cedar and Razin, 1990; Bird, 1992; Chen *et al.*, 1993; Tate and Bird, 1993).

The chick blastoderm at stage X is a disc about 2 mm in diameter and is considered homologous to the amphibian late morula. Clusters of cells observed on its lower surface may be regarded as the first signs of hypoblast and as marking the beginning of blastula formation. When the hypoblast is completely formed, the blastoderm consists of the epiblast and the primary hypoblast (stage XIII) and is considered homologous to the amphibian blastula (Vakaet, 1970; review Eyal-Giladi, 1991). The primitive streak (PS) starts developing in the epiblast under the influence of the hypoblast in the stage XIII chick blastoderm, and it is thought to elongate due to

Abbreviations used in this paper: 5-azaC, 5-azacytidine; PS, primitive streak.

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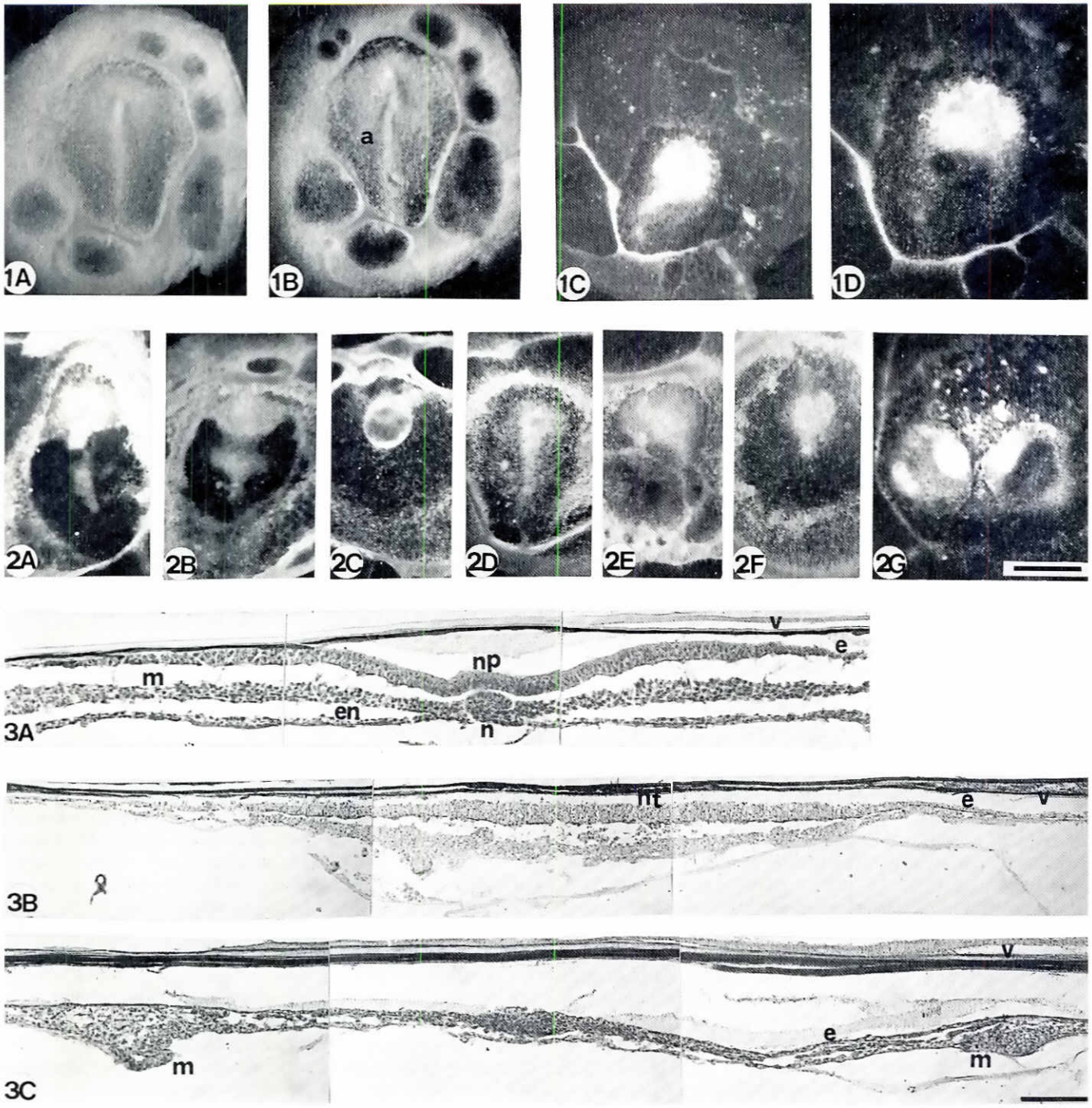
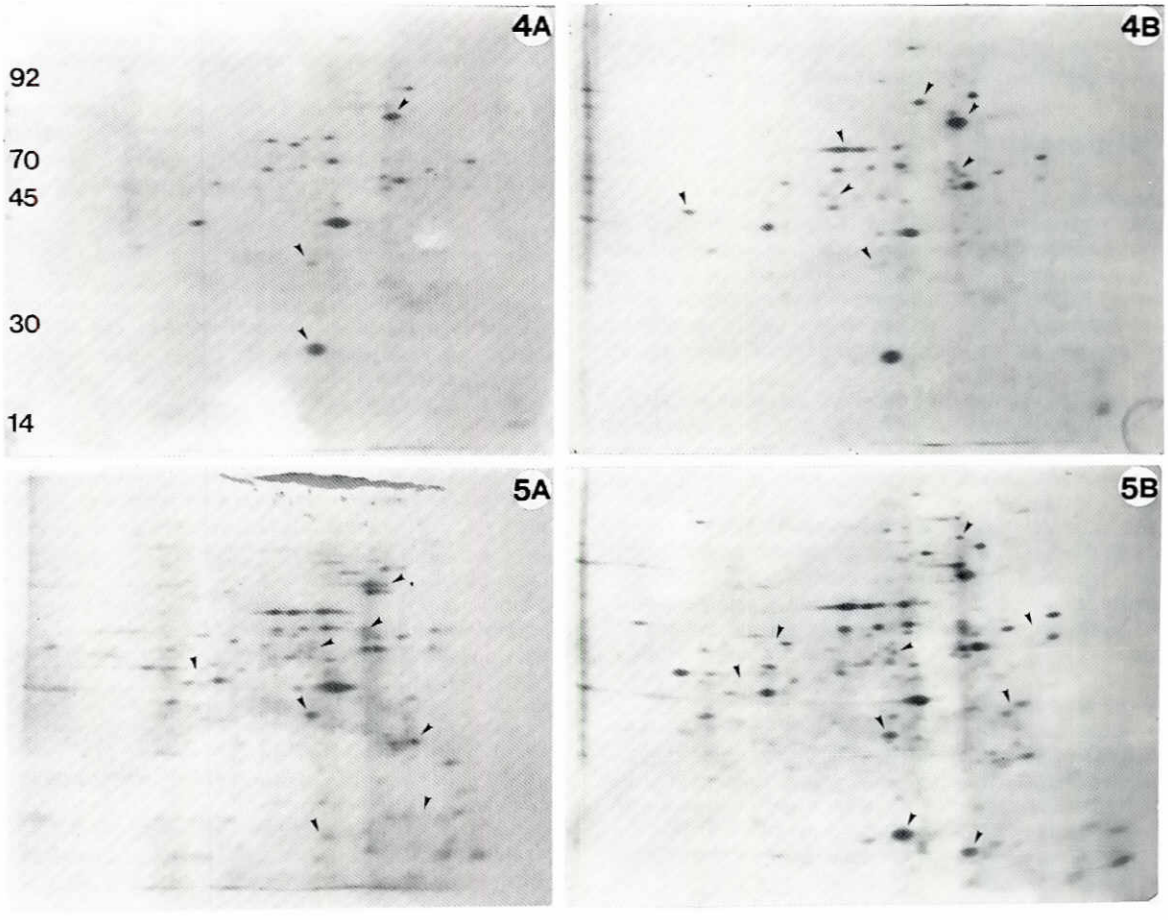


Figs. 1-2. 5-Azacytidine inhibits region identity along the embryonic axis in chick. *Embryos at the morula stage were cultured on plain egg albumen* **(1A,B)** *or in egg albumen containing 5-azaC* **(1C,D, 2A-G)** *for 24 h* **(1A,C)** *and for 30 h* **(1B,D, 2A-G)**. *a, embryonic axis. Bar, 1 mm.*

Fig. 3. 5-Azacytidine does not interfere with mesodermal and neural induction. *Transverse sections through the neural plate region of the blastoderm presented in Fig. 1B* **(A)** *and through the neural plate region* **(B)** *and a more posterior region* **(C)** *of blastoderm in Fig. 1D. Sections (7 μm) stained with hematoxylin-eosin. e, ectoderm; en, endoderm; m, mesoderm; n, notochord; np, neural plate; nt, neural tissue; v, vitelline membrane. Bar, 30 μm.*

an active pushing forward as well as backward (Vakaet, 1962). After it has attained its full length at stage 4 (Hamburger and Hamilton, 1951), the PS starts regressing posteriorly while it induces the embryonic axis which is progressively outlined anteriorly (Nicolet, 1971).We study the capacity of azaC to interfere with morphogenesis of the embryonic axis as the chick embryo develops from the histologically simple morula.

Embryos at the morula stage cultured in the continuous presence of 5-azaC (40 μM) displayed abnormal cellular migrations which produced an atypical, short, thick primitive streak and an



Figs. 4-5. 5-Azacytidine changes gene expression in early chick embryo. *Two dimensional gel pattern of polypeptides of chick embryos at the morula and blastula stages in culture with 5-azaC. Embryos at the morula* **(4A,B)** *and blastula* **(5A,B)** *stages cultured in chick Ringer solution in the absence* **(A)** *or in the presence* **(B)** *of 5-azaC (40 μM) at 37°C for 8 h (control) or 18 h (experimental) and labeled with ^{35}S-methionine the last 5 h in culture. Samples were analyzed by isoelectric focusing gels and subjected to two-dimensional electrophoresis. Isoelectric focusing is from left (pH 7.2) to right (pH 4.4). Molecular mass (daltonsx10^3) of polypeptides identified was determined according to electrophoretic migration of standards (Rainbow markers). Arrowheads indicate changes in major polypeptides.*

atypical neural plate. The embryonic axis showed a rudimentary notochord, an abnormal neural plate and sparse lateral mesoderm. Representative embryos are presented after 24 h (Fig. 1C) and 30h (Figs. 1D, 2A-G) in culture. The development of these embryos was arrested at this stage. A small percentage (5%) of morula stage treated embryos showed formation of double embryonic axes (Fig. 2G). In a parallel culture, the control embryo cultured on plain egg albumen is presented for comparison at the same time points (Fig. 1A,B). Sections through the blastoderm in Fig. 1C show neural induction anteriorly (Fig. 3B) and non-segmented mesoderm more posteriorly (Fig. 3C). Massive blood island formation was produced in embryos in 5-azaC in contrast to control embryos in which blood formation is sparse and is rarely seen even after staining with benzidine peroxide solution. The action of 5-azaC in turning on globin genes in various organisms and cell culture systems is well documented (Creusot *et al.*, 1982; Charache *et al.*, 1983). Concentration of 5-azaC at 40 μM is the lowest that gave us consistent results. Sections through the control embryo presented in Fig. 1B show the notochord and beginning of the neural fold (Fig. 3A).

The patterns of polypeptide synthesis in embryos at the morula and blastula stages under the influence of 5-azaC are presented in Figs. 4 and 5. In embryos that started culture at the morula stage, the majority of polypeptides synthesized in the presence of 5-azaC (Fig. 4B) are identical qualitatively to those of the control (Fig. 4A). However, 5-azaC does stimulate the increased synthesis of several polypeptides found only in trace amounts in control embryos (Fig. 4A,B). Embryos that started culture at the early blastula stage (stage XII) show reduced expression of some polypeptides, and show expression of characteristic polypeptides which are present in trace amounts or are absent in morula embryos normally. The blastula embryos cultured on 5-azaC (Fig. 5B) express all the polypeptides that are expressed in the control embryos both at the morula and blastula stages (Figs. 4A, 5A). Arrowheads point to major changes in polypeptides (Figs. 4,5). The change in gene expression is an interesting result and may reflect changes in methylation patterns caused by 5-azaC. The embryonic axis shows an antero-posterior imprint but there is sparse identity of the specific regions along its length. If 5-azaC perturbs DNA methylation as the chick embryo develops from the histologically simple blastula, then the wave of methylation which has been reported to start at the late blastula and continues during post

gastrulation in vertebrate embryos does not seem to be important for the induction of mesodermal and neural tissues, but is important for the patterning of these tissues.

Experimental procedures

Embryos and culture

Fertilized chick eggs were used. Embryos at stage X (Eyal-Giladi and Kochav, 1976) were removed from the eggs and cultured according to New (1955). Embryos were placed on plain egg albumen (control) or on egg albumen containing 5-azaC to give final concentrations of from 40 μM to 120 μM and were cultured at 37°C for various times. 5-azaC (Sigma) stock solution 4.5 mM was prepared and filter-sterilized before dilution into the culture medium.

Treated and control embryos were observed and photographed at various time intervals. A total of 55 embryos were used in different experiments, usually 7 experimental and 3 control embryos were cultured per experiment. Sterile procedures were employed in all embryo cultures.

Following culture, the embryos were subsequently fixed in Carnoy fixative (formula B) (Humason, 1972), were dehydrated through graded ethanol solutions, embedded in paraffin and sectioned serially. Sections (7 μm) were deparaffinized, rehydrated sequentially in alcohols of decreasing strength down to hydration, stained with Mayer hematoxylin and counterstained with eosin.

Radiolabeling and fluorography

Embryos at stages X and XII were cultured in Ringer solution in the absence (control) or presence of 5-azaC at a final concentration of 40 μM for 12 h at 37°C. For the last 5 h of this culture period, the embryos were placed in fresh Ringer solution in the absence or presence of 5-azaC containing in addition 335 μCi L-[^{35}S] methionine (1,245 Ci/mmol; New England Nuclear 009T) per milliliter. Samples were prepared as described previously (Zagris and Panagopoulou, 1992).

The species of labeled polypeptides were analyzed by two-dimensional gel electrophoresis using isoelectric focusing in the first dimension (O'Farell, 1975) and polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) in the second dimension and fluorography as described previously (Zagris and Matthopoulos, 1985, 1986; Zagris and Panagopoulou, 1992).

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