Postimplantation mouse embryos cultured *in vitro*. Assessment with whole-mount immunostaining and *in situ* hybridization

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ABSTRACT The postimplantation embryos of rodents have been particularly convenient to study in culture using the whole embryo culture (WEC) system developed by New. Two serious limitations of the method will be illustrated in the present paper and proposals will be made to improve the quality of the information. The first limitation is that the developmental period amenable to culture has not been significantly extended in recent years. In the present paper, we show that the culture of mouse presomitic stages for 48 h leads to poorly reproducible results and frequent dysmorphogenic embryos. We also show that early somite stages cultured for 54 h or less have a normal growth and differentiation. In constrast, the culture of these embryos for 72 h results in subtle abnormalities of the head and the first branchial arch. The second limitation is that the gross morphology and histology are often not informative enough to distinguish between overall toxicity and developmental toxicity. We suggest some improvements by the association of WEC with two specific techniques: 1) whole-mount immunostaining of sensory ganglia and nerves and 2) *in situ* hybridization on histological sections using molecular probes for some developmental genes. Embryos reaching about the 30 somite stage at the end of the culture were processed for whole-mount immunostaining of sensory ganglia and nerves. We show that these structures are very sensitive to the noxious effects of $HgCl_2$ and valproate. Both developmental retardations and dysmorphogeneses of the cervical ganglia and nerves were observed. Embryos were also exposed *in vitro* to low concentrations of all-trans-retinoic acid (AT-RA) and processed for *in situ* hybridization with radiolabeled anti-sense RNA probes for the Hoxb-1 and Hoxb-2 developmental genes. Three-dimensional reconstructions of the expression domains were performed. The data show that AT-RA induces ectopic expression domains of Hoxb-1. Our experiments demonstrate that techniques such as immunostaining and *in situ* hybridization can significantly expand the information obtained from whole postimplantation embryo culture.

KEY WORDS: *whole embryo culture, branchial nerves/ganglia, in situ hybridization, three dimensional reconstruction, developmental genes*

Introduction

Whole embryo culture (WEC) of postimplantation rodent embryos is a well established methodology (New, 1971; Cockroft, 1990; Van Maele-Fabry *et al.*, 1993) that is routinely used in many laboratories to study normal and abnormal development. One serious limitation of the method is that the developmental period amenable to culture has not been significantly extended during recent years. When the method is used for *in vitro* developmental toxicity tests, this limitation is important since many developmental toxicants (e.g. retinoic acid) can induce abnormalities in embryos younger than presomite or early somite stages. In addition, many dysmorphogeneses will not become morphologically manifest before stages older than 11 days when the culture has to be completed. The culture period is also a considerable limitation in developmental studies since many interesting developmental processes are not accessible. There is no simple and reproducible method to raise mouse gastrulation stages (day 7 embryos) *in vitro*

Abbreviations used in this paper: AT-RA, *all-trans* retinoic acid; CRL, crown-rump length; EB, early allantoic bud; EHF, early headfold stage; HL, head length; ISH, *in situ* hybridization; LB, late allantoic bud stage; LHF, late headfold stages; MAX, maxillary processes; MD, mandibular processes; OB, no allantoic bud stage; PROS, prosencephalon; r2, 3, 4, rhombomeres 2, 3 and 4; SCORE, morphological score of Brown and Fabro (1981) modified by Van Maele-Fabry *et al.* (1989); SOM, number of pairs of somites; VPA, valproate; WEC, whole embryo culture; YSD, yolk sac diameter.

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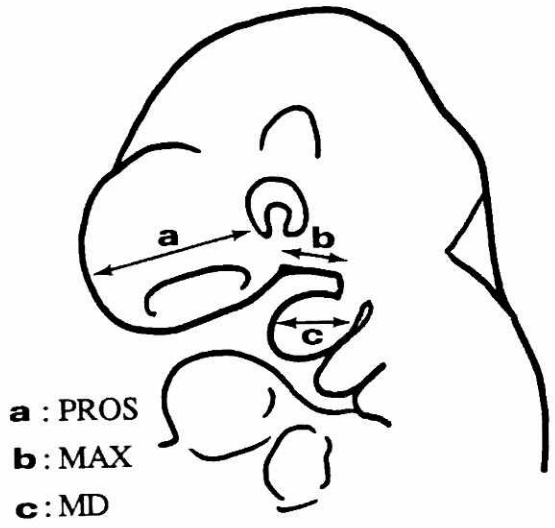

Fig. 1. Drawing of the head of a day 11 mouse embryo *showing three measures performed to assess 3-4 somites embryos cultured during 72 h: the length of the prosencephalon (PROS), the lengths of the maxillary (MAX) and mandibular (MD) processes*

for (Sadler, 1979; Tam and Snow, 1980; Sadler and New, 1981; Cockroft, 1990; Van Maele-Fabry *et al.*, 1995b). It is also difficult to culture early somite mouse embryos for 72 h and more in a completely satisfactory way. In the present paper we will illustrate these difficulties by describing the results of a few experiments aimed at evaluating the development of mouse embryos cultured either at presomite stages or for an extended period of culture.

Another limitation of the method is that at high concentrations the vast majority of chemicals will induce *in vitro* some types of dysmorphogeneses as a result of nonspecific effects on a number of cellular events such as proliferation and migration. Since the culture remains limited to early developmental stages, morphological methods such as observation of live embryos at low magnification, histology or scanning electron microscopy, are not informative enough to distinguish between overall toxicity and developmental toxicity. One way to overcome this problem is to use low concentrations and powerful specific techniques to assess the embryos at the end of the culture. The association of such techniques with WEC should improve the quality of information obtained in developmental toxicity tests and should help us to better understand the genesis of malformations observed at birth.

Some structures such as the cervical sensory ganglia and nerves become well differentiated at the end of the culture though they have long remained difficult to observe at these early stages. The observation of these structures may be of great interest in developmental toxicity studies because craniofacial malformations may often be associated with alterations of specific ganglia/nerves (Couly *et al.*, 1994). A whole-mount immunostaining technique using a monoclonal antineurofilament antibody allows visualization of the early development of branchial nerves/ganglia (Dodd *et al.*, 1988). This technique associated with the WEC system has allowed the present authors to detect unsuspected dysmorphogeneses in mouse embryos exposed *in vitro* to developmental toxicants such as ethanol (Van Maele-Fabry *et al.*, 1995c), valproate (VPA) (Gofflot *et al.*, 1996), mercuric chloride (Van Maele-Fabry *et al.*, 1996) methyl mercuric chloride (Van Maele-Fabry *et al.*, 1995a) and AT-RA (Clotman *et al.*, 1997). In this paper, we will summarize some of the experiments performed with VPA and $HgCl_2$. The results show that the ganglia and nerves are very sensitive to the noxious interference of these chemicals.

Finally, when applied *in situ* on histological sections of embryos, molecular probes for some developmental genes label small subpopulations of cells that cannot be identified by morphological methods. The use of such probes should allow us to detect if some subpopulations are specifically affected (alteration of volume, position, form, etc.) as compared to other populations (Picard *et al.*,

TABLE 1

DEVELOPMENT OF EMBRYOS EXPLANTED AT PRESOMITIC AND EARLY SOMITIC STAGES AND CULTURED FOR 48 h TO 72 h IN STANDARD CULTURE CONDITIONS

Stage at explantation	Cult. period (h)	N. embryos	Final stages (SOM)	N. dead (%)	N. dysm. (%)	YSD (mm)	CRL (mm)	HL (mm)	SCORE
Presomitic stage									
OB-EB	48	25	19.8±1.1 (n=22)	2 (8)	12 (52)	3.7 ± 0.3	2.8±0.2 (n=22)	1.3±0.1 (n=22)	48.9±5.2 (n=22)
LB	48	21	21.2±1.0 (n=17)	0	12 (57)	3.9±0.2	2.9±0.3	1.4±0.1	53.4±3.4 (n=17)
EHF	48	20	22.1±0.9 (n=19)	0	2 (10)	4.0 ± 0.3	3.1±0.2	1.5±0.1 (n=19)	55.5±4 (n=19)
LHF	48	20	23.7±1.2 (n=19)	0	2 (10)	4.2 ± 0.3	3.4±0.2	1.6±0.1 (n=19)	58.7±1.8 (n=19)
EHF - LHF	48	50	24.2±1.2 (n=16)	2 (4)	30 (63)	4.1±0.3 (n= 18)	3.3±0.2 (n=16)	1.6±0.1 (n=16)	57.9±2.7 (n= 16)
Early somitic stage									
3-4 som.	48	17	26.0±0.9	0	1 (6)	4.1±0.3	3.5±0.2 (n=16)	1.8±0.1 (n=16)	59.6 ± 2.9
3-4 som.	54	25	30.0±1.3	2 (8)	2 (9)	4.8±0.4	4.2±0.3	2.2±0.2	ND
3-4 som.	72	21	33.9 ± 1.1	0	3 (14)	ND	5.2 ± 0.3	2.7 ± 0.2	ND

Development of embryos explanted at presomitic and early somitic stages and cultured for 48 h to 72 h in standard culture conditions. (OB), no allantoic bud; (EB), early allantoic bud; (LB) late allantoic bud; (EHF), early headfold and (LHF), late headfold stages (Downs and Davies, 1993). N. embryos, total number of cultured embryos; SOM, number of pairs of somites; N. dead (%), number and percentages of dead embryos; N. dysm. (%), number and percentages of living embryos displaying at least one dysmorphogenic feature; YSD, yolk sac diameter; CRL, crown-rump length; HL, head length; SCORE, morphological score of Brown and Fabro (1981) modified by Van Maele-Fabry *et al.* (1989). Quantitative data are given as mean±SD. When indicated in brackets (n=), the observations were performed on a number of embryos different than the number of living embryos. ND, not done.

1997). In the present paper we will report experiments on mouse embryos exposed *in vitro* for a short time to low concentrations of AT-RA. We will show that probes for two developmental genes allow us to identify in exposed embryos a number of abnormalities in hindbrains that appear histologically normal.

Results

Embryo culture

Presomitic stages

Data on death and dysmorphogeneses of embryos of different presomitic stages after 48h of culture are given in Table 1. The rates of dead embryos were low and never exceeded 5% except in the group constituted by the younger embryos (no allantoic bud, OB; early allantoic bud, EB). In this group, a rate of 8% was observed. Rates of dysmorphogenic embryos after 48 h culture were highly variable: incidences of 10% of dysmorphogenic embryos were observed in two experiments with embryos of early head fold (EHF) and late head fold (LHF) stages whereas 63% of dysmorphogenic embryos were observed in another experiment with embryos of the same stages. Rates of about 55% were observed in earlier presomitic stages (OB-EB and late allantoic bud, LB). The most frequent abnormalities of the dysmorphogenic embryos involved the caudal portion of the embryo (including the caudal neural tube), the turning of the embryos, the heart, the cephalic neural tube, the optic system and the allantois. Other systems were also affected but to a lesser degree. They included the yolk sac circulatory system, the maxillary process, the otic system, the mandibular process and the forelimb. Only few dysmorphogenic embryos showed these abnormalities. The frequency of each abnormality according to the presomitic stage at the beginning of the culture is presented elsewhere (Van Maele-Fabry *et al.*, 1995b). It has to be stressed that 67% of the dysmorphogenic embryos in all groups displayed the same abnormality affecting the caudal portion of the embryo (abnormal shape, tail underdeveloped and/or outside the yolk sac) (Fig. 2).

Data on growth parameters including the yolk sac diameter (YSD), the crown-rump length (CRL) and the head length (HL) as well as the data on differentiation parameters including the number of pairs of somites (SOM) and the morphological score (SCORE) are summarized in Table 1. An increase of all growth and differentiation parameters was observed as a function of increasing initial stage.

Early somitic stage

Data on death and dysmorphogeneses of 3-4 somites embryos cultured during 48, 54 and 72 h are given in Table 1. No or low (8%) percentages of dead embryos were observed. The incidences of dysmorphogenic embryos were low (6 to 14%; Table 1) and

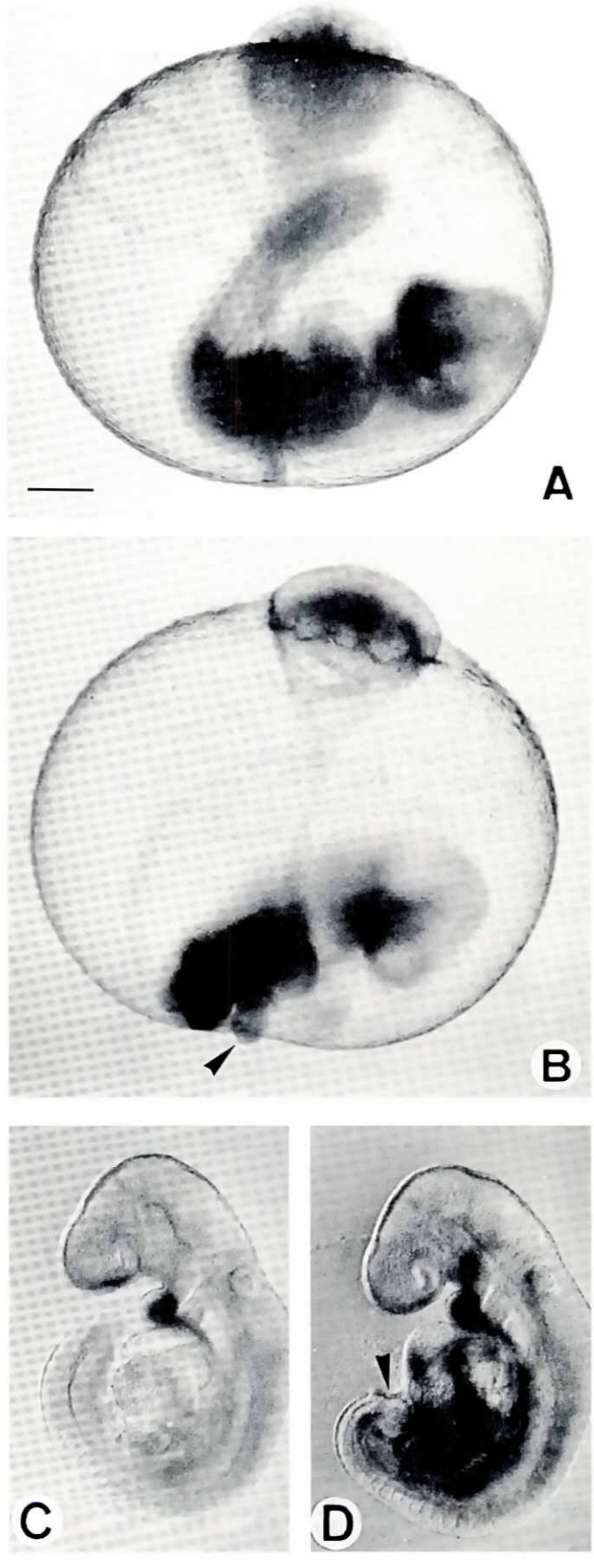

Fig. 2. Mouse embryos at the end of the 48 h culture period beginning at the late allantoic bud (LB) presomitic stage. (A and B) *Embryos surrounded by the yolk sac and by the amnion.* **(C and D)** *Embryos without the membranes.* **(A and C)** *Embryos showing normal morphological features.* **(B and D)** *Dysmorphogenic embryos displaying an abnormal caudal portion: tail partially outside the yolk sac* **(B)** *and underdeveloped* **(D)** *as compared to normal embryos* **(A and C).** *Abnormalities are indicated by arrowheads. Bar, 500 μm.*

TABLE 2

GROWTH AND DIFFERENTIATION OF EMBRYOS WITH 32 TO 36 PAIRS OF SOMITES EXPLANTED *EX VIVO* AND CULTURED FOR 72 h

Parameters	*Ex vivo*	*In vitro*
N. embryos	37	21
SOM	34.2±1.5	33.9±1.1
CRL (mm)	5.08±0.33	5.18±0.27
HL (mm)	2.76±0.23	2.70±0.15
PROS (mm)	0.88±0.10	0.72±0.06*
MAX (mm)	0.46±0.07	0.58±0.13*
MD (mm)	0.55±0.06	0.44±0.04*

Growth and differentiation of embryos with 32 to 36 pairs of somites explanted *ex vivo* or cultured for 72 h. The cultured embryos had 3-4 pairs of somites at the beginning of the culture period. N. embryos, SOM, CRL, HL, as in Table 1; PROS, prosencephalon length; MAX, maxillary length; MD, mandibular length. Quantitative data are given as mean±SD. Asterisks indicate a statistically significant difference (*$p<0.01$) evaluated by using Student's *t* test.

increased slightly with the duration of the culture period. The head and the branchial arches were normal after 48 and 54 h of culture. However, after 72 h of culture, an unusual appearence of the head and of the maxillary and mandibular processes was observed. In order to better define the abnormality, three additional parameters were used for the assessment of embryos cultured for 72 h: the lengths of the prosencephalon (PROS), of the maxillary (MAX) and of the mandibular processes (MD) (Fig. 1). The data of cultured embryos were compared with the data of *ex vivo* embryos displaying the same number of pairs of somites and are summarized in Table 2. As compared to the *ex vivo* embryos, a statistically significant decrease of the PROS and MD as well as an increase of the MAX was observed.

Branchial nerves and ganglia

Branchial nerves and ganglia of embryos of 6-7 or 8-9 pairs of somites cultured for 48 or 44 h, respectively, and exposed during 24 h to $HgCl_2$ and to VPA, respectively, showed three types of anomalies: developmental retardations, dysmorphogeneses and left-right asymmetries.

Developmental retardations were detected by using the reference table of Van Maele-Fabry *et al.* (1996) presented in Table 3. In this Table, for each branchial nerve/ganglion, the chronological appearance of developmental patterns was summarized and scores were attributed to indicate successive developmental stages. The patterns of development of nerves/ganglia were recorded and compared between *in vitro* control and treated embryos. $HgCl_2$ induced a pronounced retardation in the differentiation of ganglion/nerve V and a slight retardation in the differentiation of ganglia/nerves VII and IX. The ganglia/nerves VIII and IX were not retarded (Van Maele-Fabry *et al.*, 1996). VPA induced a delay in the development of nerves/ganglia V, IX and X. The development of nerve VII was delayed to a lesser extend (Gofflot *et al.*, 1996).

Dysmorphogeneses of ganglia/nerves were also recorded in embryos exposed to the two chemicals. The variations and/or abnormalities were described and quantified separately for the embryos treated with $HgCl_2$ (Van Maele-Fabry *et al.*, 1996) and with VPA (Gofflot *et al.*, 1996). These results are summarized in Table 4 and illustrated in Figure 3. $HgCl_2$ induced hight percentages of abnormalities of ganglion/nerve V (e.g. abnormal shape of the ganglion, abnormal ophthalmic branch) and fusions between ganglia/nerves IX and X. Disorganized fibers between ganglia/nerves VII-VIII and IX and between ganglia/nerves IX and X were also more frequently observed than in the control embryos. VPA induced defects in the four ganglia. The main abnormalities were a reduced dorsal component of ganglion V, the absence of dorsal root of ganglion IX, a disorganized dorsal part of ganglion X and diffuse ventral fibers in nerves VII-VIII. In addition, scattered fibers were observed around and between ganglia.

At the highest concentration of $HgCl_2$, the variations and/or abnormalities recorded were different according to the sides with a more pronounced effect on the right side of the embryos.

In situ hybridization and three dimensional reconstruction

Exposure to AT-RA induced modifications in the pattern of expression of developmental genes. Figure 4 illustrates one type

TABLE 3

REFERENCE TABLE FOR EMBRYONIC DEVELOPMENT OF BRANCHIAL NERVES AND GANGLIA OBSERVED MACROSCOPICALLY AFTER IMMUNOSTAINING OF DAY-10,-10.5 EMBRYOS

Nerve[a]	Score[b]	Description of the pattern[c]
V	1	- ganglion without ventral branches
	2	- ophthalmic branch
	3	- ophthalmic branch; additional fibers towards the periphery
	4	- ophthalmic and mandibular branches
	5	- slightly developed ophthalmic, mandibular and maxillary branches
	6	- three well developed branches
VII	1	- ganglion with few fibers towards the periphery
	2	- one fascicle of fibers reaches the root of the 2nd branchia arch
	3	- most fibers in the 2nd branchial arch
	4	- additional fibers towards the 1st branchial arch
VIII	1	- ganglion without lateral fibers
	2	- few fibers to the otic vesicle
	3	- thick bundle of fibers to the otic vesicle
IX	1	- no dorsal root; no or few ventral fibers
	2	- thin dorsal root; no or few ventral fibers
	3	- thin dorsal root; ventral fibers reach the 3rd branchial arch
	4	- dorsal root well developed; ventral fibers reach the 3rd branchial arch
	5	- additional fibers branch perpendicularly towards the root of the 2nd branchial arch
X	1	- ganglion with unorganized dorsal rootlets
	2	- few organized rootlets converging ventrally in one narrow bundle
	3	- many rootlets converging into one thick bundle

Reference table for embryonic development of branchial nerves and ganglia observed macroscopically after immunostaining of day-10,-10.5 embryos. For each ganglion, the table presents the chronological appearance of developmental patterns. a) Branchial ganglion and/or nerve: (V) trigeminal, (VII) facial, (VIII) vestibulocochlear, (IX) glossopharyngeal and (X) vagus; b) developmental scores corresponding to specific patterns described in c).

of modification detected in the expression of Hoxb-1. In the control embryos, Hoxb-1 transcripts were restricted to the rhombomere 4 (r4) and to the neural crest cells migrating facing r4. In the neural tube of the 3-4 somite stage treated embryos, additional sites of expression were detected more cranially than the normal site of expression in r4. A gap without Hoxb-1 transcripts was observed between the normal site and the ectopic sites. The ectopical expression of Hoxb-1 was often asymmetrical.

The superposition of the expression patterns of Hoxb-1 and Hoxb-2 allowed to precisely compare their boundaries and to assess the position of the ectopic expression sites of Hoxb-1 (Fig. 5). In the control embryos, Hoxb-2 was expressed from the caudal part of the neural tube to a sharp cranial border corresponding to the r2/r3 boundary. A co-expression of Hoxb-1 and Hoxb-2 was therefore observed in r4, as well as in the neural crest cells facing r4. In the treated embryo presented in Figure 5, no modification was detected in the expression of Hoxb-2. The ectopic sites of expression of Hoxb-1 were located in r3 since they were included in the region where Hoxb-2 was expressed.

The three-dimensional reconstruction revealed the complexity of the ectopic expression of Hoxb-1 (Fig. 6). Control embryos showed the restricted expression of Hoxb-1 in r4 and the cranial border of expression of Hoxb-2 at the r2/r3 boundary. In the treated embryos, Hoxb-1 was additionally detected in patches located more cranially than r4. The ectopic sites were randomly distributed. The majority of these sites were located in r3 where Hoxb-2 was expressed. Some sites were detected more cranially than the cranial border of expression of Hoxb-2. These sites were therefore located in r2.

Discussion

Embryo culture

In vitro development of presomitic mouse embryos has been reported to be less reliable than the development of rat embryos of the same stage (Sadler and New, 1981; New, 1990). We have shown in the present report that presomitic mouse embryos of 4 different stages have a poorly reproducible development *in vitro* and often display dysmorphogeneses (10 to 63%) after 48 h of culture. By far the most frequent anomaly was the abnormal development of the tail and a protrusion of the tail outside the vitelline membrane. This anomaly suggests that the culture conditions do not allow an appropriate process of gastrulation. We conclude that, in contrast to rat embryos, presomitic stage mouse embryos cannot be satisfactorily cultured for 48 h using the described conditions.

Mouse embryos of early somite stage can be cultured with success for 48 h and 54 h. The percentages of dysmorphogenic embryos were 6% and 9%, respectively. In contrast, culture of these embryos for 72 h often results in discrete anomalies in the head and in the first branchial arch (Table 2). Inadequate supply of nutriments and oxygen is probably the cause of these anomalies since the blood circulation in the vitelline membrane is declining and is not adequately replaced by the allantoic circulation at the end of the culture. The data presented in Tables 1 and 2 suggest strongly that whole embryo culture of early somitic embryos (3-4 SOM) during more than 54 h should not be used to examine the effects of teratogens. For this reason, older embryos (6-7 and 8-9 SOM) were used in the next section and were cultured during 44 h or 48 h. Much work is still to be done to improve the culture

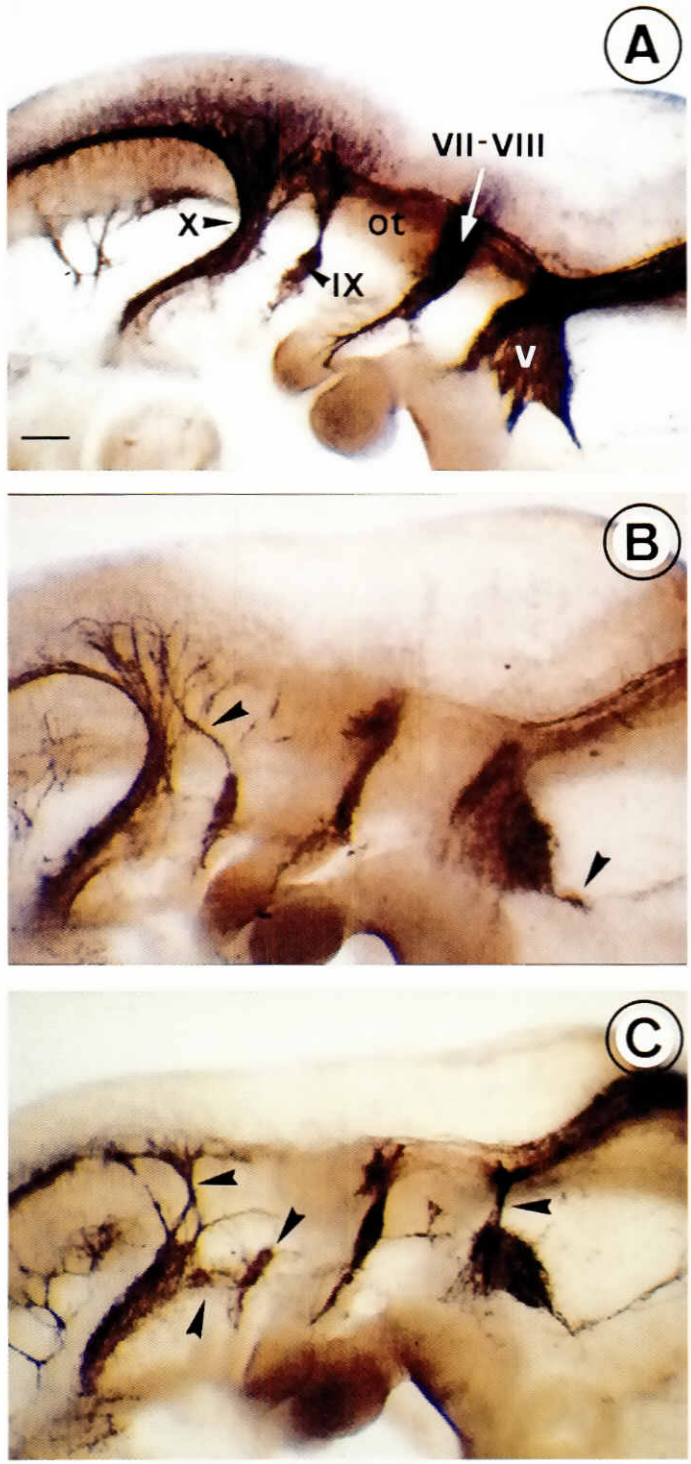

Fig. 3. Pattern of development of branchial nerves/ganglia of cultured mouse embryos immunolabeled with a monoclonal antibody (2H3) against the 155x10³ Mr neurofilament protein. (A) *Control embryo,* **(B and C)** *embryos exposed to 25 μM $HgCl_2$ and 750 μM VPA, respectively. The right sides of the embryos are presented, cranial is to the right and dorsal is to the top. Branchial nerves/ ganglia are designated in* **(A)** *with Roman numerals as follows: (V) trigeminal, (VII) facial, (VIII) vestibulocochlear, (IX) glossopharyngeal and (X) vagus. ot, otic vesicle. Arrowheads in* **(B and C)** *indicate the main differences observed in the treated embryos as compared to the control embryos. Bar, 100 μm.*

conditions for the earliest and latest developmental stage of mouse embryos.

Branchial nerves and ganglia

For each nerve/ganglion, the reference Table 3 (Van Maele-Fabry *et al.*, 1996) provides the normal pattern of chronological development in *ex vivo* embryos. Some control or treated embryos may display in some ganglia/nerves additional features (e.g. Table 4, ganglion IX, fibers between ganglia IX and X) not reported in Table 3 or some differences in the patterns described in Table 3 (e.g. Table 4, ganglion V, reduction of the dorsal part of ganglion V). When these additional features and differences in patterns are observed frequently in control embryos, they are defined as variations. We define as abnormalities those features and differences observed frequently in treated embryos and not or rarely observed in control embryos. When high percentages of variations were observed in the controls and slightly higher percentages were observed in the treated embryos, we did not conclude to an effect due to the treatment. Only marked effects were taken into consideration.

$HgCl_2$ induces pronounced developmental retardation of nerve/ganglion V and a slight retardation in the differentiation of nerves/ganglia VII and IX. Important alterations of the ventral part of nerve V were also reported suggesting a preferential interference with the placodal neuroblasts. In addition, asymmetric defects were reported at the highest concentration (Van Maele-Fabry *et al.*, 1996).

On the other hand, VPA induces a delay in the development of all branchial nerves/ganglia as well as alterations in the shape and organization of these nerves/ganglia. The alterations affected mainly the dorsal part of nerves/ganglia V, IX and X derived from rhombencephalic neural crest cells. This suggests that VPA interferes preferentially with the neural crest derived components (Gofflot *et al.*, 1996). The diversity of the effects observed with the two compounds suggests that the observed abnormalities are due to specific effects on the branchial nerves/ganglia and demonstrate the usefulness of this approach in developmental toxicology studies.

Caracteristic craniofacial and/or brain abnormalities have been described at birth both in human and animals after exposure to VPA (DiLiberti *et al.*, 1984; Nau and Hendrickx, 1987) or $HgCl_2$ (Gale and Ferm, 1971; Gale, 1981; for review, see Schardein, 1985) but abnormalities of the sensory cervical ganglia have not been described to our knowledge. Our present observations should stimulate new studies in search of a possible association between craniofacial syndromes induced by teratogens and malformations of sensory cervical ganglia. In addition, they suggest that great care should be paid in the search for abnormal behavioral, sensory and psychomotor development when facial syndromes and minor facial abnormalities are observed in children that have been exposed *in utero* to potential teratogens.

In situ hybridization

Retinoic acid has been shown to induce the ectopic expression of Hoxb-1 in r2 (Marshall *et al.*, 1992; Wood *et al.*, 1994). Here, we

TABLE 4

VARIATIONS AND/OR ABNORMALITIES IN THE DEVELOPMENT OF BRANCHIAL NERVES AND GANGLIA OBSERVED AFTER *IN VITRO* EXPOSURE TO $HgCl_2$ AND VPA

GGL/nerve[a]	Anomaly/Variation[b]	Treatment[c]			
		HgCl2		VPA	
		Control (n=18)[d] Left/Right	(25 µM) (n=15)[d] Right[e]	Control (n=19)[d] Left/Right	(750 µM) (n=15)[d] Left/Right
V	- reduction of ganglion: • overall	0 (0)	0 (0)	1 (5)	4 (29)
	• dorso-ventral	0 (0)	1 (7)	0 (0)	0 (0)
	• dorsal part	1 (6)	2 (13)	3 (16)	7 (50)
	- abnormal shape of ganglion	0 (0)	9 (60)	0 (0)	0 (0)
	- abnormal ophthalmic branch	0 (0)	8 (53)	0 (0)	0 (0)
	- long thin ventral fibers	0 (0)	7 (47)	0 (0)	0 (0)
VII-VIII	- diffuse ventral fibers	3 (17)	1 (7)	0 (0)	5 (36)
	- fibers between ganglia VII-VIII and IX	0 (0)	6 (40)	0 (0)	4 (29)
IX	- no dorsal root, but a well developed ventral branch into 3rd branchial arch	1 (6)	2 (13)	1 (5)	4 (29)
	- dorsal root/ganglion fused with nerve X	2 (11)	7 (47)	6 (32)	1 (7)
	- fibers emerging from periphery of ganglion	1 (6)	4 (27)	4 (21)	6 (43)
	- fibers between ganglia IX and X	4 (22)	8 (53)	7 (37)	6 (43)
X	- disorganized dorsal root	0 (0)	1 (7)	2 (10)	5 (36)
	- very short dorsal rootlets	0 (0)	1 (7)	0 (0)	0 (0)
	- thin fascicles of few fibers	0 (0)	0 (0)	1 (5)	6 (43)
	- one very thin bundle of fibers	0 (0)	0 (0)	0 (0)	5 (36)

Variations and/or abnormalities in the development of branchial nerves and ganglia observed after *in vitro* exposure to $HgCl_2$ and VPA. a) Branchial ganglion and/or nerve: (V) trigeminal, (VII) facial, (VIII) vestibulocochlear, (IX) glossopharyngeal and (X) vagus; b) abnormalities and/or variations observed for each ganglion. We define variations as the patterns observed frequently in the control embryos. We define abnormalities as the patterns observed frequently in treated embryos and rarely in control embryos; c) treatment: for each group, the number and percentages (in brackets) of embryos displaying a given variation/abnormality are presented; d) number of examined embryos; e) the right side of the embryos was considered due to asymmetries in variations/abnormalities at this concentration.

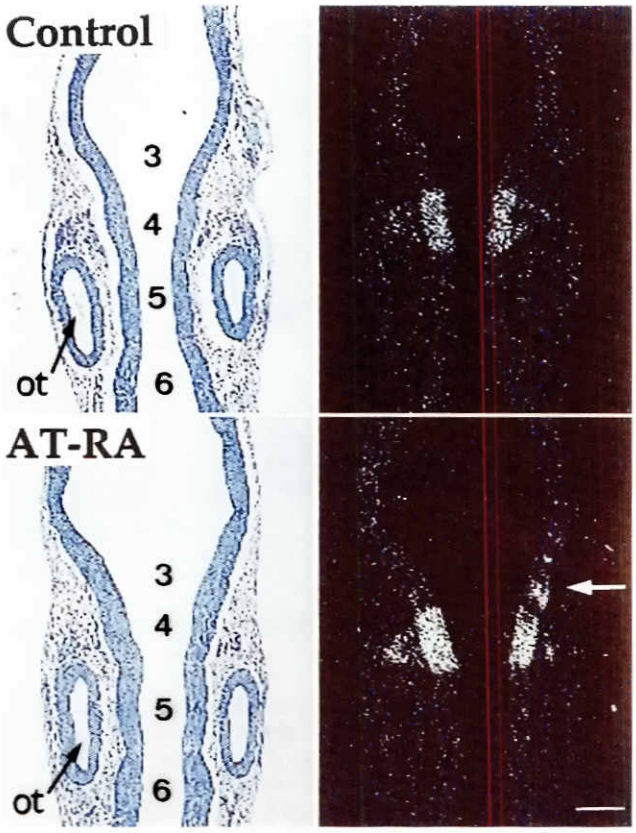

Fig. 4. Expression of Hoxb-1 on coronal sections of a control and an AT-RA (1 μM) treated embryo. *A stained section (left) and an adjacent section of the same embryo hybridized with the Hoxb-1 probe (right) are presented. Cranial is to the top, caudal is to the bottom.* **(A)** *In the control embryos, the expression of Hoxb-1 was observed in the rhombomere 4 (r4) and in the neural crest cells facing r4.* **(B)** *In the neural tube of the treated embryos, additional sites were detected more cranial than the normal expression in r4 (arrow). Bar, 100 μm.*

report the abnormal expression of Hoxb-1 in r3. The comparison of the boundaries of expression of Hoxb-1 and Hoxb-2 makes the location of this ectopic site unambiguous. This modification can be interpreted as a lower induction of the expression of Hoxb-1 by retinoic acid as compared to the previous reports (Marshall *et al.*, 1992; Wood *et al.*, 1994). It can be explained by exposure to a lower effective concentration of retinoic acid, as the level of induction depends on the concentration (Papalopulu *et al.*, 1991). The left-right asymmetry of the alteration is difficult to explain. It may be similar to asymmetries in malformations that have been reported repeatedly previously after exposure to a variety of teratogens (Greenaway *et al.*, 1986; Brown *et al.*, 1989; Fantel *et al.*, 1991). A full description and quantification of the modifications induced by retinoic acid in the expression pattern of Hoxb-1, Hoxb-2 and Krox-20 is presented and discussed elsewhere (Clotman *et al.*, 1997).

The results presented here illustrate the complementarity of the direct observation of sections and of the image analysis performed after *in situ* hybridization. The observation of the sections is necessary to evaluate the intensity of the signal, to identify possible artifacts and to compare signals on standardized sections. However, the lack of histological clues makes sometimes the precise location of the boundaries of expression difficult. The superposition of patterns allows the location of the boundaries of gene expression relative to each other. Furthermore, it can reveal co-expression or mutually exclusive expression domains that are difficult to assess from the individual sections. The three-dimensional reconstruction allows us to visualize the whole patterns of expression, to compare the borders of expression and to assess the symmetry or asymmetry of the expression between the left and the right side or according to the dorso-ventral axis. It also allows us to compare embryos that are cut in different planes. These three types of analyses are therefore complementary for a precise description of the pattern of gene expression.

We conclude that the use of very specific techniques such as immunostaining and *in situ* hybridization should significantly expand the usefulness of the whole embryo culture system and considerably improve the quality of the information it can provide. These improvements should in the near future increase the attractiveness of whole embryo culture in the fields of embryology and developmental toxicology.

Materials and Methods

Embryo culture

Animals

NMRI mice were used. Presomitic and early somitic stage embryos were obtained as previously described (Van Maele-Fabry *et al.*, 1995b). Presomitic embryos were classified according to their stage of develop-

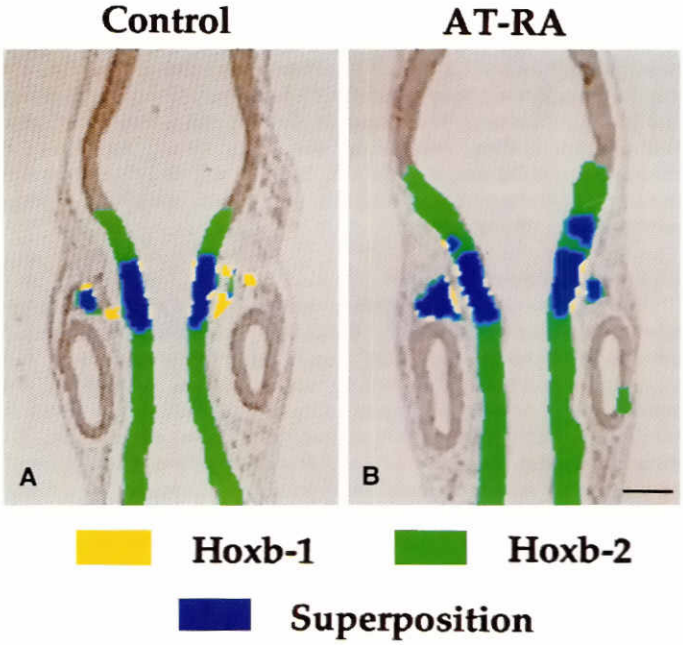

Fig. 5. Superposition of the expression patterns of Hoxb-1 (yellow) and Hoxb-2 (green). *The regions co-expressing Hoxb-1 and Hoxb-2 are presented in blue.* **(A)** *In the control embryos, Hoxb-1 and Hoxb-2 were co-expressed in r4 and in the neural crest cells facing r4. The cranial border of Hoxb-2 corresponds to the r2/r3 boundary.* **(B)** *In the treated embryos, additional sites of expression of Hoxb-1 were detected. These ectopic sites were located in the region cranial to r4 where Hoxb-2 was expressed. The expression of Hoxb-2 was not modified in this embryo. Bar, 100 μm.*

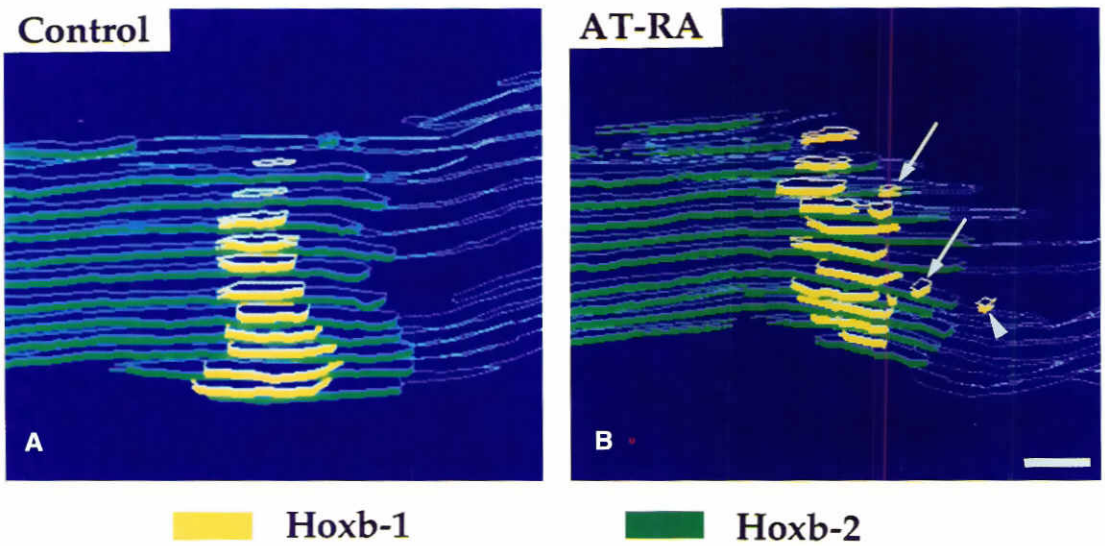

Fig. 6. Three-dimensional reconstruction of the expression pattern of Hoxb-1 and Hoxb-2 in the hindbrain of a control and a treated embryo. *To avoid a transparency effect, the hindbrain has been cut sagitally and the right side of the neural tube is displayed. Cranial is to the right and dorsal is to the top.* **(A)** *In control embryos, Hoxb-1 is expressed in r4 and Hoxb-2 is expressed up to the r2/r3 boundary.* **(B)** *Additional sites of Hoxb-1 expression are detected in treated embryos. Patches of ectopic sites are cranial to r4, located either in r3 (arrows) where Hoxb-2 is also expressed or in r2 (arrowhead). These sites are randomly distributed. Bar, 100 μm.*

ment based on the morphology of the embryos (Downs and Davies, 1993). The following stages were selected in the present study: the three substages of the neural plate stage including the no allantoic bud (OB), the early allantoic bud (EB) and the late allantoic bud (LB) stages and the two substages of the headfold stages including the early headfold (EHF) and the late headfold (LHF) stages.

Culture method

Whole embryos were cultured as previously described (Van Maele-Fabry *et al.*, 1995b). The culture was carried out at 38°C. In the experiments on presomitic stages, the culture medium consisted of a mixture of mouse, rat and human serum (1:2:5 by vol.). In all the other experiments on somitic stages, the culture medium consisted of a mixture of rat and human serum (1:4 by vol.). After each 24 hour period, the embryos were transferred to fresh medium. Embryos were gassed with 5% O_2, 5% CO_2 and 90% N_2 at the beginning of the culture. A 20% O_2, 5% CO_2 and 75% N_2 mixture was used after 24 h of incubation and a 40% O_2, 5% CO_2 and 55% N_2 mixture was used after 48 h of culture.

Assessment of the response

The assessment of the response was performed as previously described (Van Maele-Fabry *et al.*, 1995b). At the end of the culture period, the embryos were examined at 38°C under a dissecting microscope. Yolk sac circulation is established at 9-11 pairs of somites. At the end of the culture, all embryos of the present study had about 20 or more pairs of somites. Therefore, embryos without heartbeat and/or yolk sac circulation were considered dead and were not further analyzed. The remaining embryos were morphologically examined for the presence of external dysmorphogeneses. Embryos were considered dysmorphogenic when they displayed at least one structural malformation or when they displayed a severe retardation limited to one or to some developmental features (e.g. when embryos of more than 18 somites had open neural tube or had neither limb buds nor limb crests). The embryos were judged to be dysmorphogenic on the basis of data on the normal development of *in vivo* mouse embryos of 0 to 30 pairs of somites (Van Maele-Fabry *et al.*, 1992). The degree of differentiation was evaluated by counting the number of pairs of somites (SOM) and by using the morphological score table of Brown and Fabro (1981) modified by Van Maele-Fabry *et al.* (1990) (SCORE). Yolk sac diameter (YSD), crown-rump length (CRL) and head length (HL) were taken as growth indicators. Three additional parameters were used for the assessment of embryos cultured during 72 h: the length of the prosencephalon (PROS) as measured from the rostral end to the optic vesicle, the lengths of the maxillary (MAX) and mandibular (MD) processes (Fig. 1).

Whole-mount immunostaining of sensory nerves and ganglia

The immunostaining technique was performed as described by Mark *et al.* (1993) and by Van Maele-Fabry *et al.* (1995c). In brief, embryos of 6-7 or 8-9 pairs of somites were cultured for 48 or 44 h, respectively and were exposed during 24 h to 25 μM $HgCl_2$ or to 750 μM VPA, respectively. At the end of the culture, embryos were fixed in paraformaldehyde, treated with the mouse monoclonal antibody 2H3 against the $155x10^3$ Mr neurofilament protein (Developmental Studies Hybridoma Bank, Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, USA), washed and treated with the peroxidase-conjugated sheep antimouse Ig (Fab fragments) (Boerhinger Mannheim, Germany). The peroxidase deposits were visualized using solutions of the substrate 4-chloro-1-naphtol (Merck, Darmstadt, Germany). Embryos were then washed in 30% ethanol and photographed. The development of branchial nerves and ganglia was evaluated using the reference table established by Van Maele-Fabry *et al.* (1996) and presented in Table 3.

In situ hybridization and three-dimensional reconstruction

In situ hybridization

Embryos displaying 3-4 pairs of somites were exposed for 8 h to 1 μM AT-RA (Sigma Aldrich Chemie, Bornem, Belgium) dissolved in dimethylsulfoxide and cultured for additionnal 40 h without chemical. The *in situ* hybridization (ISH) was performed mainly according to the procedure described by Ruberte *et al.* (1990) and recently detailed elsewhere by Picard *et al.* (1997). In brief, embryos were washed in phosphate-buffered saline (PBS), fixed overnight at 4°C in 4% paraformaldehyde dissolved in PBS, dehydrated in a graded series of ethanol, cleared in benzene and embedded in paraffin wax at 65°C. Coronal sections of 6 μm were collected on 0.5% gelatin/0.5% alun chrome coated slides. Serial sections were equally distributed onto six slides. The first slide was stained with toluidine blue and the remaining slides were hybridized each with a different probe. The procedure provides one series of sections with excellent histological quality and 5 series of sections for direct comparison of expression domains of 5 developmental genes on neighboring sections of the same embryos. The antisense RNA probes were synthesized in a standard T7 polymerase reaction (Stratagene enzymes and reagents) using (^{35}S)CTP,

followed by a partial alkaline hydrolysis to reduce the average probe length to about 200 nucleotides. The probes for Hoxb-1 (Wilkinson *et al.*, 1989) and Hoxb-2 (Rubock *et al.*, 1990) have already been described. These were gifts of R. Krumlauf. All slides were exposed to Kodak NTB-2 autoradiography emulsion for 3 weeks.

Three-dimensional (3D) reconstructions and pattern superpositions

The 3D reconstructions were performed on the 6 μm sections. Sections were displayed using a microscope (Laborlux S, Leitz) through a CCD camera (TK-890E, JVC) on the RGB monitor of a PC/ compatible computer (Kontron Elektronik GMBH). The software used was the Vidas Rel. 2.1 (Kontron Elektronik GMBH). The sections were digitized as 256 grey levels images. Each bright-field and dark-field section was aligned on the nearest bright-field section. The digitized images were treated to automatically extract the structures of interest (the histological features or the ISH labeling). The outlines of these structures were manually rectified to correct for artefacts, with the digitized image corresponding to the nearest bright-field section displayed as background. For the three-dimensional reconstruction, the structures of interest were manually selected and introduced in the 3D-software, the ISH labeling being put in different channels to be displayed in different colours, and the reconstruction was performed. For the comparison of the patterns of expression, the structures corresponding to the labeling were displayed in colors and superposed on the digitized image of the nearest bright-field section.

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