Evolution of the developmental scores of sixteen morphological features in mouse embryos displaying 0 to 30 somites

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ABSTRACT A precise framework of morphological developmental events observed macroscopically in early postimplantation mouse embryos aged 8-10 days (0-30 somites) is established. The quantitative evolution of the developmental score of 16 features as a function of the developmental stage of the embryos (expressed in number of somites) is presented. Thirty-one groups of ten embryos, each with 0 to 30 somites, were scored for each feature according to the previous description of the authors. In addition, the variation of individual structures as a function of embryonic developmental stages is evaluated. It is suggested that the framework of differentiating individual structures at given developmental stages will help to plan experiments in developmental biology of rodents and will facilitate the interpretation of results in developmental toxicity.

KEY WORDS: *postimplantation, mouse, embryo, development*

Introduction

Early postimplantation embryos are characterized by the rapid production of new tissues and dramatic morphological changes. They are extensively used in order to progress in the understanding of normal and abnormal developmental processes. During the last few years, the study of normal development of rodent embryos has gained new interest due to the considerable progress in the understanding of genes that control early mammalian embryogenesis (reviews in Gehring, 1987; Kessel and Gruss, 1990; Wilkinson and Krumlauf, 1990; Ruiz i Altaba, 1991). Study of the expression of these genes during early organogenesis would be facilitated if it were known exactly which morphological stages these studies should be referred to. In addition, knowledge of the differentiation of individual structures at given developmental stages is critical for the interpretation of studies in developmental toxicity performed both *in vivo* or *in vitro*. This knowledge would be facilitated by tables providing precise data on the evolution of individual macroscopical structures according to developmental stages. Various scoring systems (Brown and Fabro, 1981; Sadler and Warner, 1984; Klug *et al.*, 1985; Van Maele-Fabry *et al.*, 1990) have been devised to estimate the differentiation of postimplantation embryos. They are composed of lists of embryonic features or primordia. The development of each feature is divided into defined morphological stages and a score is attributed to each stage. A chart of schematic illustrations of the morphological stages of sixteen developmental features observed macroscopically in mouse embryos aged 8-10 days (0-30 somites) has been published by the present authors (Van Maele-Fabry *et al.*, 1990). However, the correlation of the morphological stages of all individual structures with embryonic developmental stage (number of somites and/or age) has not been described.

The first aim of the present work is to establish a framework of morphological developmental events in early postimplantation mouse embryos. This framework will allow easy selection of a developmental stage during which a given structure undergoes a given morphological change. It will also afford a rapid overview of all major changes underway at a given developmental stage.

It is well known that early embryos of a given age may reach different developmental stages as defined by the number of somites. The variation in developmental stages may be observed even within a single litter, particularly in the mouse (Herken and Anschütz, 1981; Tam, 1981; Kapron-Bras and Trasler, 1988; Van Maele-Fabry *et al.*, 1988). Variations in the morphological stages of a given structure in embryos of a given number of somites have often been mentioned. For example, it has been recognized that the closure of the cranial neural tube does not coincide with the attainment of a definite somite number (Macdonald *et al.*, 1989; Theiler, 1989). However, the extent of the observed variations has not been the object of thorough studies.

The second aim of the present paper is to evaluate the variation of the morphological stages of individual structures as a function of embryonic developmental stage as defined by the number of somites. An accurate knowledge of these variations would make it possible to prevent misinterpretations of data obtained in embryotoxicity studies and to define for a given structure the limits of a retardation in differentiation. Retardation in the differentiation of an individual feature may be defined as an abnormally low score

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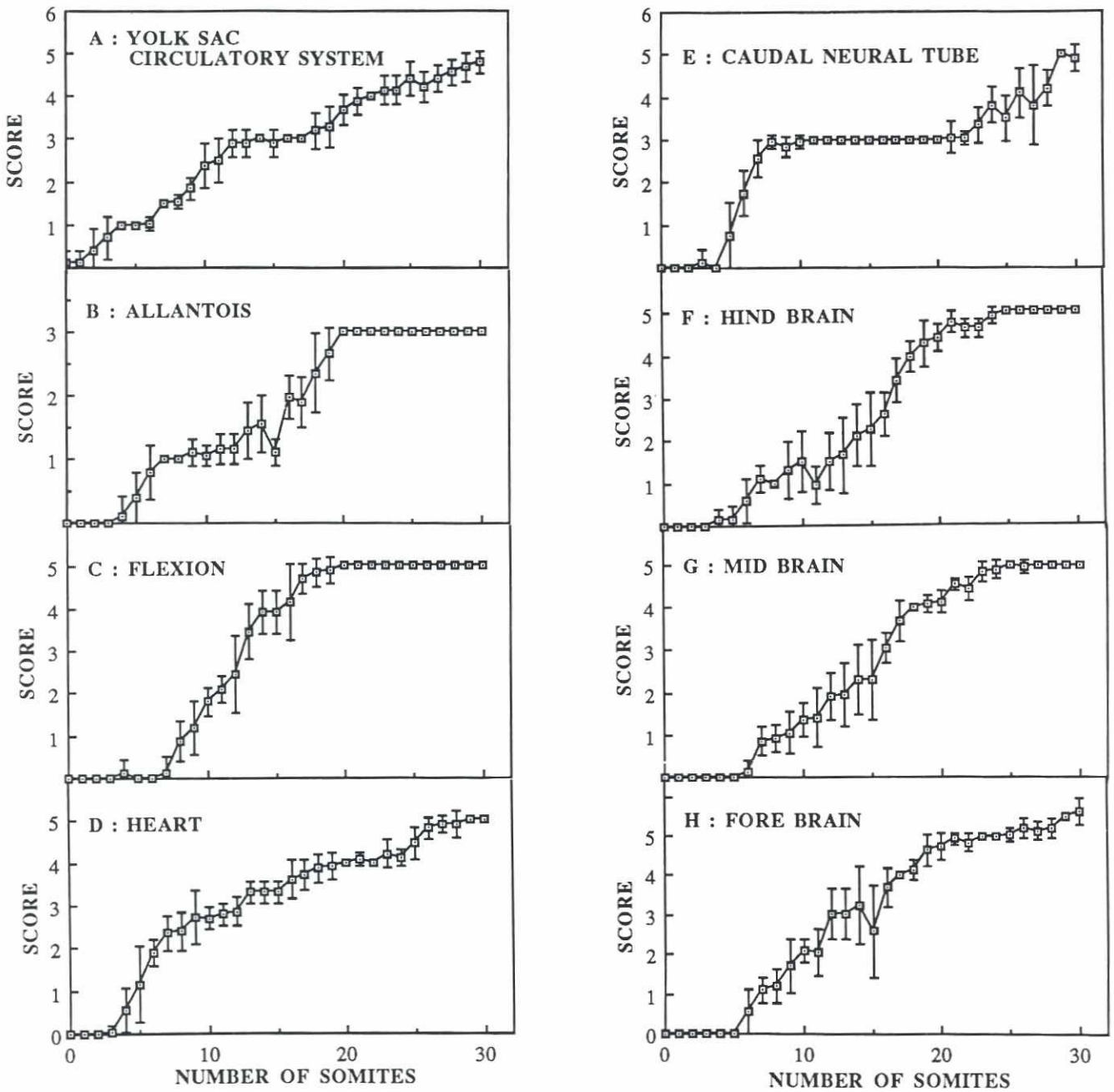
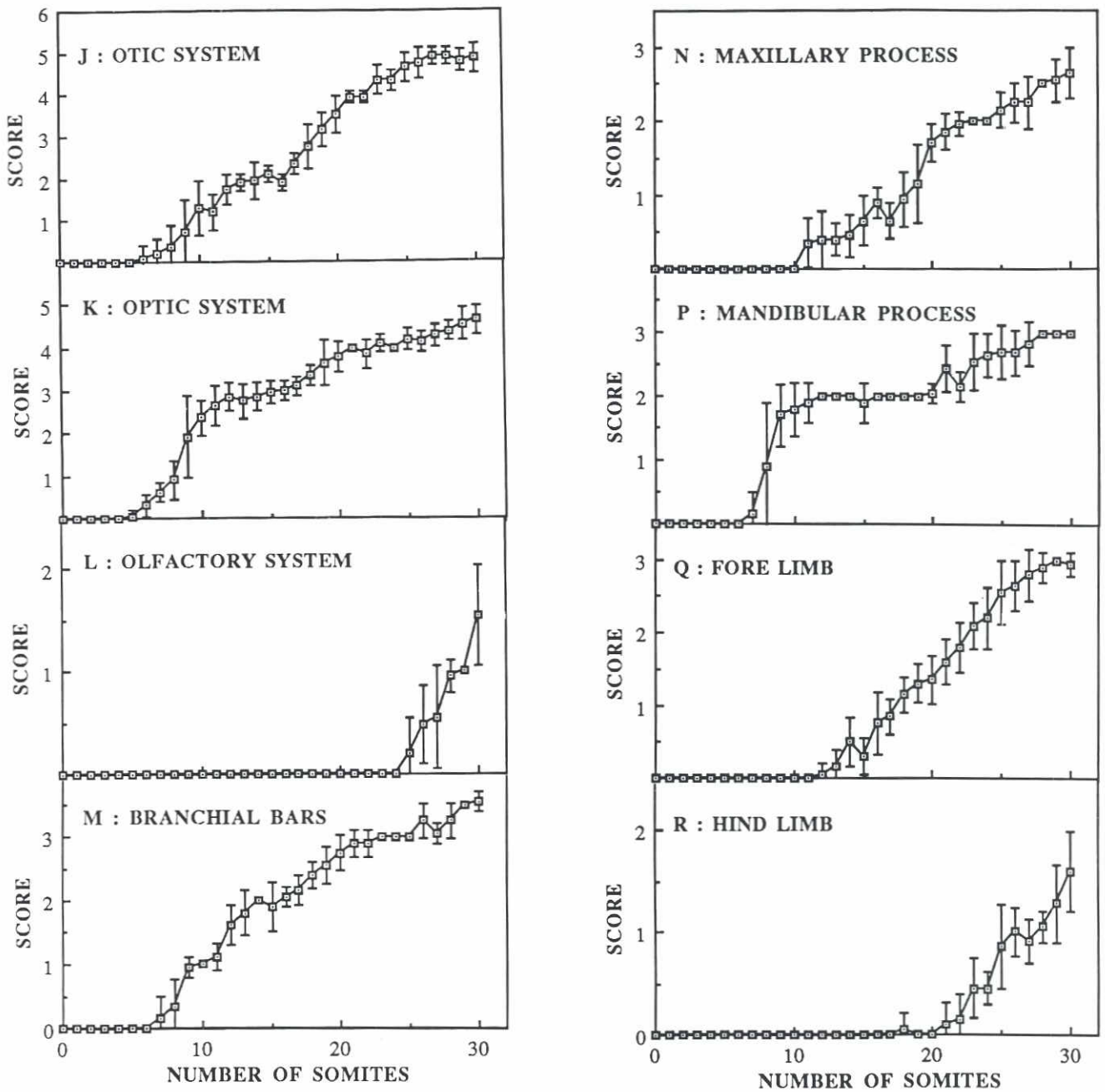


Fig. 1. Correlation between developmental scores of sixteen morphological features and number of somites. *Whole or half scores were attributed to each of the 16 features of individual embryos, according to Van Maele-Fabry* et al. *(1990). Mean and standard deviation were calculated for each group of ten embryos and expressed as a function of the number of somites.*

of the structure as compared to the score of the same structure in normal embryos of the same developmental stage. The detection of retardation in differentiation of one or several features induced by a chemical would make it possible to better specify the adverse effects of a xenobiotic on the embryonic development.

Results

The present data are based on the detailed description and schematic illustrations of the morphological evolution of each of 16 features published previously (Van Maele-Fabry *et al.*, 1990). For



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each of these morphological features, the quantitative evolution of the mean scores (± standard deviation) of ten embryos is plotted as a function of the number of somites (Fig. 1). The development of individual features is characterized by different quantitative patterns.

A. Yolk sac circulatory system

The evolution of the score may be described as proceeding in two phases of steady increase (from 0 to 12 somites and from 17 to 30 somites) separated by a possible plateau between 12 and 17 somites. Score 1 is attributed when a corona of blood islands, with

or without anastomoses, are visible. During the first phase, a major event is the establishment of the vitelline circulation (score 2). This event is observed for embryos displaying approximately 9 to 11 somites. The plateau is observed for score 3. Score 3 is attributed when a full plexus of yolk sac vessels can be observed and when the origins of the vitelline artery and of the vitelline vein are still widely separated. During the second phase of steady increase, the origins of the two vitelline vessels migrate first close to each other (score 4) and then progressively separate from each other distally with the yolk stalk becoming narrow and obliterated (score 5).

B. Allantois

During the first phase, extending to 3 somites, no score is attributed. The allantois is free in the exocoelom. During the second phase, from 4 to 7 somites, the score increases linearly to reach score 1, when the allantois fuses with the chorion without showing any visible organisation. The third phase is a plateau at score 1 between 7 and 12 somites. The fourth phase, between 12 and 20 somites, shows an increase in the score from 1 to 3. This phase is characterized by the development of the allantoic vessels (score 2) leading to the establishment of the umbilical circulation (score 3). No further increase in the score is observed during the fifth phase (plateau from 20 to 30 somites).

C. Flexion

No score is attributed before about 7 somites. During this phase, the embryo remains curved dorsally with the characteristic inversion of the embryonic sheets. The increase of the score is linear between 6 and 18 somites. During this second phase, the embryo turns progressively (rotation 1/4, 1/2 and 3/4 corresponding to scores 1, 2 and 3, respectively) to become dorsally convex (score 4) with a spiral torsion at score 5. No further increase of the score is observed after 20 somites.

D. Heart

No score is attributed before 3 somites. The second and the third phases are two successive exponential phases. The second phase extends from 3 to 24 somites. During this phase, the heart rudiment initially visible as a horseshoe-like thickening of the mesoderm surrounding the front end of the embryo (score 1) develops rapidly. It appears successively as a beating s-shaped cardiac tube (score 2) and as a convoluted cardiac tube (score 3) to reach a three-chambered appearance (bulbus cordis, ventriculus communis and atrium commune) (score 4). The third phase extends from 24 to 30 somites to reach score 5. Score 5 is attributed when a four-chambered appearance is observed (dividing atrium commune).

E. Caudal neural tube

No score is attributed before about 5 somites. The second phase occurs between 4 and 8 somites as a rapid increase of the score. Scores 1, 2 and 3 are attributed, respectively, when the neural folds are closing but not fused, when the neural folds are fused at level of somites 4-5 and when the posterior neuropore is formed but open. During this phase, one observes the first major event of the feature as the posterior neuropore is forming and remains open (score 3). The third phase is a plateau at score 3 between 8 and 22 somites. Finally, during the fourth phase, extending from 22 to 30 somites, the score increases to 4 when the posterior neuropore is closing with only a small opening remaining present, and to 5 when the posterior neuropore is closed.

Cranial neural tube: hind brain, mid-brain, forebrain

The sequence of morphological events in cranial neural tube closure has been clearly established by Macdonald *et al.*, 1989. The points of contact and of fusion of the neural folds have been described by these authors. Their «closure stage» nomenclature is used in the present paper. Numbers indicate the order of appearance of sites of initiation of contact and fusion.

The first contact between the neural folds occurs in the cervical region (spinal cord) (closure 1) and the fusion proceeds bidirectionally (caudally and rostrally). Rostrally, the fusion meets closure 4 (rhombencephalon). The cephalic contact (closure 2) is initially made in the posterior prosencephalic region and the fusion proceeds also bidirectionally. During approximately the same period, contact and fusion begin at the most rostral part of the prosencephalon (closure 3). Closure 3 proceeds caudally until it meets closure 2. Closure 4 appears in the caudal part of the rhombencephalic region. Fusion proceeds rostrally until it covers the rhombencephalic region and until it meets closure 2.

Authors differ over the definition and location of the anterior neuropore. For Macdonald *et al.* (1989) the anterior neuropore is the most anterior opening of the neural tube and is therefore located in the prosencephalic region. In the present paper, the anterior neuropore is defined as the last open region of the anterior neural tube and is therefore located in the rhombencephalic region. The latter definition is in accordance with descriptions of other authors (Theiler, 1989; Brown, 1990).

F. Hind brain

No score is attributed before 4 somites. During the second phase, the score increases steadily from 3 to 21 somites with a possible plateau between 7 and 11 somites. This plateau corresponds to score 1, when the neural folds elevate to display a V-shape in tangential view. Score 2 is attributed when the edges of the rhombencephalic folds are closer to each other and have a U-shape in tangential view. The contact of the neural folds of the rhombencephalic region (closure 4) is in process at score 3 and the fusion proceeds caudally to rostrally. Score 4 is attributed when the anterior neuropore is closed. Embryos of more than 18 somites display a closed anterior neuropore. Score 5 is attributed when a pronounced pontine flexure with transparent roof of 4th ventricle is observed. During the last phase, after 24 somites, no increase of the score is observed.

G. Mid-brain

No score is attributed before 6 somites. The second phase is a linear increase in the score between 5 and 23 somites. During this phase, the neural folds have a V-shape (score 1) and then a U-shape (score 2) in tangential view. Subsequently, the neural folds of the mesencephalic region make contact (closure 2) and the fusion proceeds caudally to the anterior rhombencephalic region (score 3). Score 4 is attributed when the mesencephalon is closed. This event occurs in embryos displaying more than 17 somites. Score 5 is attributed when a visible division between mesencephalon and diencephalon is observed. No further increase of the score is observed after 23 somites.

H. Forebrain

No score is attributed before 6 somites. From 5 to 21 somites, the evolution of the score may be described as linear. During this phase, the neural folds have a V-shape (score 1) and then a U-shape

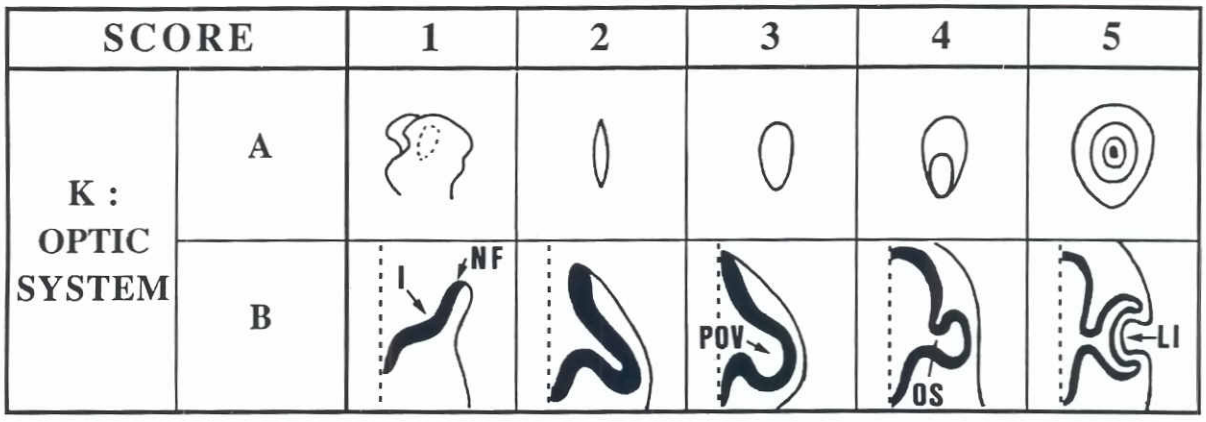

Fig. 2. Illustrations of the development of the optic system in embryos from 0 to 30-somite stages. (A) *Macroscopic appearance of the optic system in lateral view according to Van Maele-Fabry* et al. *(1990). Score 1: sulcus opticus; score 2: elongated optic primordium; score 3: ovoid optic primordium; score 4: primary optic vesicle with open optic stalk (darkened region within the optic vesicle); score 5: optic vesicle becoming circular and displaying an additional central region.* **(B)** *Schematic illustration of the corresponding histologic events. The illustrations represent transverse sections through the left half of the cephalic region. The dotted line indicates the sagittal plane. Score 1: indentations in the optic primordium; score 2: narrowing of the optic sulcus and lateral extension of the optic evagination; score 3: formation of the primary optic vesicle; score 4: formation of the open optic stalk; score 5: invaginating optic vesicle and initiation of the lens invagination. Abbreviations: I, indentation in the optic primordia; NF, neural fold; POV, primary optic vesicle; OS, open optic stalk; LI, lens invagination.*

(score 2) in tangential view. The initiation of contact of the prosencephalic neural folds occurs at closure point 2 (score 3). The closure of the prosencephalon proceeds from two directions: rostrally from closure 2 and caudally from closure 3. Score 4 is attributed when the prosencephalon is completely closed and score 5 is attributed when the fissura telodiencephalica is formed. The first cephalic contact of the neural folds is observed in embryos displaying about 12 to 15 somites. The prosencephalon is completely fused when the embryos display more than 16 somites. A short plateau may be described between 21 to 25 somites. This plateau corresponds to score 5. From 25 to 30 somites one observes a second increase in the score corresponding to the elevation of the telencephalic hemispheres (score 6).

J. Otic system

No score is attributed before 6 somites. The second phase is linear, extends from 5 to 12 somites and is characterized by the formation of the indented otic primordium (score 1). A plateau is observed between 12 and 16 somites. This plateau corresponds to the formation of the otic pit (score 2). Another linear increase occurs between 16 and 25 somites to reach score 5. Score 3 is attributed when the otic vesicle is closed but not yet separated from the epidermis. The stalk is not easily observed on a lateral view of fresh specimens but results in an apparent small indentation in the cavity. Score 4 is attributed when the otic vesicles are completely separated from the epidermis. Score 5 is attributed when the otic vesicle develops a dorsal recess. Scores 3, 4 and 5 appear in embryos displaying about 19, 22 and 27 somites, respectively. No further increase in the score is observed after 25 somites.

K. Optic system

No score is attributed before 5 somites. The score increases rapidly from 6 to 11 somites, to reach score 3. Indentations in the two optic primordia form the optic sulci (Fig. 2, score 1). Score 2 is attributed when, macroscopically, these evaginations appear elongated in lateral view. This view corresponds to the narrowing of the optic sulcus and to the lateral extension of the optic evagination (Fig. 2, score 2). Score 3 is attributed when the elongated primordium becomes ovoid in lateral view. This corresponds to the formation of the primary optic vesicle (Fig. 2, score 3). Score 4 is attributed when a darkened region appears within the optic vesicle in lateral view. This appearance corresponds to the formation of the open optic stalk (Fig. 2, score 4). Score 5 is attributed when the primordium becomes circular and displays an additional central region in lateral view. This stage corresponds to the invaginating optic vesicle and to the initiation of the lens invagination (Fig. 2, score 5). Scores 2, 3, and 4 are observed in embryos displaying about 9, 16, and 21 somites, respectively. At 30 somites, most embryos have not yet reached score 5.

L. Olfactory system

No score is attributed before 25 somites. Subsequently, the score increases linearly from 25 to 30 somites. The olfactory plate (score 1) is observed in embryos displaying 28 somites. Score 2 is attributed when the olfactory plate displays a rim. At 30 somites, most embryos have not yet reached score 2.

M. Branchial bars

No score is attributed before 7 somites. A steady increase in the score is observed from 7 to 21 somites. This phase is characterized by the formation of branchial bars I (mandibular processes), II and III corresponding to scores 1, 2 and 3, respectively. A plateau is observed from 21 to 27 somites (score 3). Finally, a short increase from 27 to 30 somites leads to the progressive formation of the fourth branchial bar.

N. Maxillary process

The maxillary processes are fully developed after the 30-somite stage. However, the region between the head and the first branchial bars undergoes clear morphological changes during the period

considered in this paper. No score is attributed before 11 somites. The score increases slowly from 11 to 30 somites. Score 1 is attributed when no maxillary anlage is visible but when the first branchial bars (mandibular processes) are separated from the forehead by a deep cleft. The first anlage of the maxillary process (score 2) appears clearly in embryos displaying 22 somites. Score 3 is attributed when the development of the maxillary anlage has modified the appearance of the cleft between the mandibular process and the forebrain. This cleft is strongly elongated. Due to the development of the maxillary anlage, the elongated cleft becomes located between the maxillary and mandibular processes.

P. Mandibular process

No score is attributed before 7 somites. The score increases steadily from 7 to 12 somites. This phase is characterized by the appearance of the processes (score 1) and leads to score 2 when the ventral edges of the mandibular processes are just touching each other. A plateau is subsequently observed between 12 and 20 somites. A second steady increase is observed between 20 and 28 somites. During this phase, the ventral edges of the processes join though they are not yet fused (score 3). No further increase in the score occurs at 28 to 30 somites.

Q. Forelimb

No score is attributed before 12 somites. The increase in the score is linear from 11 to 28 somites. The first sign of forelimb development begins to appear at 12 somites (score 1) and the forelimb buds (score 2) are clearly present in embryos displaying 22-23 somites. Score 3 is attributed at about 29 somites when the forelimb buds become paddle-shaped.

R. Hindlimb

No score is attributed before about 21 somites. The increase in the score is linear from 20 to 30 somites. The first sign of hindlimb development begins to appear approximately at 21 somites and a distinct evagination of the Wolffian crest is visible for embryos displaying at least 26 somites (score 1). At 30 somites, most embryos have not yet reached score 2 (hindlimb buds clearly present).

Discussion

In the past, most mammalian embryologists have used age as a measure of developmental stage. This has proven to be an inadequate and often misleading criterion. Embryos only a few hours apart in age may significantly differ in their developmental state (Beddington, 1987). Expressing the development of morphological features as a function of the number of somites has the advantage of taking into account the developmental variation of embryos of the same gestational age. The present study has been restricted to embryos of 0 to 30 somites since the most rapid changes in macroscopical features occur during this period. However, in spite of this more accurate measure of the developmental stage, important variations in the score values of one feature may be observed for embryos having a same number of somites. The extent of these variations differs considerably according to the morphological feature and somite stage. As an example, for the midbrain, important variations are observed among the groups of ten embryos displaying 6 to 17 somites (e.g., a standard deviation of 41% of the mean is observed in the embryos displaying 15 somites). For embryos displaying more then 17 somites, the standard deviation never exceeds 6% of the mean (e.g., a standard deviation of 6% of the mean is observed in embryos displaying 20 somites). These variations may be explained by genetic factors that differ among individual embryo and by the delay needed for the production of a new somite pair (about 2 h; Brown, 1990). Interpretation of the score chart and evaluation of the developmental state of the embryo (somite number, scores of individual features) are additional sources of variation.

The evolution of the score of each developmental feature displays a strikingly individual pattern within the limits of our study (Fig. 1). In most cases the evolution of the development of the features is not continuous from 0 to 30 somites. The yolk sac circulatory system is the only structure undergoing a steady increase from 0 to 30 somites (with a possible short plateau). All other structures display an initial lag before the initiation of macroscopical development. This lag is short (3 to 7 initial somites) for most features. The olfactory system, the maxillary process and the fore- and hindlimbs initiate their macroscopic development at much later somite stages. Some of the structures, such as the flexion, the mid-brain, the olfactory system, the maxillary processes and the limbs develop in a single phase with a steady increase of the score. Other structures, such as the allantois, the heart, the caudal neural tube, the hindbrain, the forebrain, the otic and optic system, the branchial bars and the mandibular processes develop according complex developmental curves that may be divided into two or more phases.

The accurate developmental stages observed in the present paper for the major events of the different features are in fair agreement with descriptions reported in the literature (Macdonald *et al.*, 1989; Theiler, 1989; Kaufman, 1990).

Precise quantitative evolution of the development of individual features may make it possible to detect differences in the developmental pattern of a single feature among strains. Such differences could partially explain variations among strains in liability to dysmorphogenesis induced by teratogens. As an example, some authors (Macdonald *et al.*, 1989; Juriloff *et al.*, 1991) have performed a precise staging of the anterior neural tube closure in different mouse strains. This staging documents differences among normal mouse strains in morphology of cranial neural tube closure. These authors hypothesize that the observed genetic polymorphism in the location of the first site of fusion between the cranial neural folds in normal mouse embryos may be one basis for differences among strains in liability to exencephaly induced by teratogens. The description of mouse development provided by the present paper may help to uncover subtle differences in relative developmental pace of individual structures with other species often used in developmental toxicity studies such as the rat and the rabbit.

In addition, the establishment of the quantitative evolution of the development of each primordium as a function of the developmental stage of the embryo may make it possible to detect specific retardation of a primordium induced by embryotoxic xenobiotics. Retardation of one primordium may be considered as a prelude to dysmorphogenesis (Brown, 1990) or alternatively, as a specific adverse effect on the embryonic differentiation which has to be distinguished from structural malformations. In the whole embryo culture system, specific retardation of one primordium currently escape in routine assessment of postimplantation embryos cultured *in vitro* and exposed to xenobiotics or to adverse culture conditions. Indeed, in most of these *in vitro* studies, the total nu-

merical score (Brown and Fabro, 1981; Van Maele-Fabry *et al.*, 1990) for all features is used to estimate overall differentiation of the embryos. Xenobiotics or adverse culture conditions are usually considered as affecting the embryonic differentiation when the mean of the total scores of embryos of a treated group is statistically significantly lower than the mean of the total scores of the control embryos (for example Repetto *et al.*, 1990; Stein *et al.*, 1990; Hansen and Grafton, 1991; Kapron-Bras and Hales, 1991). Consequently, a specific retardation of a primordium will be masked in the overall score. The present authors propose to specify adverse effects of xenobiotics on embryonic differentiation and to distinguish this effect from structural malformations by reporting the number of embryos displaying a retardation in differentiation of a specific primordium as an additional criterion for the assessment of embryonic development.

Materials and Methods

Animals

NMRI mice were used. Each male was caged overnight with two nulliparous females, which were checked for a vaginal plug the following morning. Embryonic age 0 was defined as the midpoint of the dark cycle during which copulation took place. Animals were killed by cervical dislocation at various times on days 8 to 10 of gestation.

Dissection of embryos

Embryos were explanted according to the procedure described by New (1971) and adapted for the mouse by Van Maele-Fabry *et al.* (1988). Embryos with 0 to 30 somites were selected in order to obtain ten embryos with each number of somites. Consequently, a total of 310 conceptuses were examined, fresh and without fixation, under the dissecting microscope.

Evaluation of the embryonic differentiation for each individual feature

The 310 embryos were examined for yolk sac circulation; allantois development; embryonic flexion; heart; closure of the neural tube; telen-, mesen-, and rhombencephalon; otic, optic and olfactory systems; branchial bars; mandibular and maxillary processes; formation of limb buds. The embryos were scored according to the morphological scoring system of Brown and Fabro (1981) modified by Van Maele-Fabry *et al.* (1990). The modified score has more intermediate stages for several features. The mean scores ± standard deviation of each individual feature were expressed as a function of the number of somites.

Acknowledgments

The authors are indebted to Ms F. Gofflot for help in the composition of the figures. They thank Mr. J. Lepage for animal husbandry. G. Van Maele-Fabry is supported by a FIRST fellowship of the Région Wallonne (Belgium).

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