Development 109, 775–786 (1990)
Printed in Great Britain © The Company of Biologists Limited 1990

Hox-2.3 upstream sequences mediate lacZ expression in intermediate mesoderm derivatives of transgenic mice

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Summary

The mouse *Hox-2.3* gene contains an *Antp*-like homeobox sequence and is expressed in a spatially restricted anteroposterior domain during development. To study the molecular basis of this differential gene regulation, we set out to characterize the *cis*-regulatory elements mediating *Hox-2.3* expression during embryogenesis. We show that a fragment extending 1316 base pairs (bp) upstream of the transcription start site, thus corresponding to the *Hox-2.4/Hox-2.3* intergenic sequences is capable of mediating luciferase gene transcription in transfected cells *in vitro* and *lacZ* expression in transgenic mice. The β-galactosidase-staining pattern in embryos was found to be strikingly similar to the *Hox-2.3 in situ* hybridization pattern in intermediate mesoderm derivatives: high levels of both *Hox-2.3* transcripts and β-galactosidase activity were found in the mesonephric duct-derived epithelium of the meso- and metanephric kidney and associated ducts, from the time these structures first appeared on throughout development. The transgene apparently lacks sequences needed for correct *Hox-2.3* expression in somitic and lateral plate mesoderm and in neurectoderm. These results document the involvement of distinct regulatory elements in *Hox* gene expression in subsets of cells with distinct developmental fate, situated at similar positions along the anteroposterior axis of the embryo.

Key words: homeobox gene, *Hox-2.3*, *in situ* hybridization, transcription regulation, luciferase reporter gene, *lacZ* reporter gene, transgenic mice.

Introduction

In *Drosophila*, homeobox-containing genes have been identified among the genes controlling pattern formation (reviewed in Gehring, 1987). The discovery of homeobox-containing genes in vertebrate species (Levine *et al.* 1984; McGinnis *et al.* 1984) soon stimulated many investigators to set out searching for the function of these genes during mouse development (Colberg-Poley *et al.* 1985*a*, 1985*b*; Hart *et al.* 1985; Jackson *et al.* 1985; Joyner *et al.* 1985; Awgulewitsch *et al.* 1986; Duboule *et al.* 1986; Rubin *et al.* 1986; Wolgemuth *et al.* 1986; and references in Holland and Hogan, 1988*b*). Many of the mouse homeobox genes encode proteins that are *Antennapedia (Antp)*-like. As in *Drosophila* these genes are clustered. Four clusters of *Antp*-like homeobox genes have been described in the mouse, all genes within a cluster being transcribed in the same orientation. As soon as sequences could be compared, it became clear that the genes from the four complexes could be aligned, suggesting that these complexes arose by duplication of an ancestral cluster (Hart *et al.* 1987). Recently, work on sequence comparison, structural organization and differential expression suggested that the vertebrate *Hox* clusters are true homologs of the insect *Antp* and *Bithorax* homeotic gene complexes: in the mouse as well as in *Drosophila*, a correlation was found between the order of the genes in their cluster and the anteroposterior (AP) position of their rostral expression boundary in the central nervous system (CNS) and in the mesoderm (Gaunt *et al.* 1988; Duboule and Dollé; 1989; Graham *et al.* 1989; Dressler and Gruss, 1989); the more 3′ a gene lies in a cluster, the more anterior the rostral border of its expression domain in the CNS and in the mesoderm. In mid-gestation embryos, the rostral expression boundaries in the CNS are being found at more anterior positions than those in the mesoderm (reviewed in Holland and Hogan, 1988*b*).

A large amount of work on the expression of mouse *Antp*-like genes suggests that these genes play a crucial role during pattern formation, like *Antp* and *Bithorax* genes do during specification of segment identity in the fly (reviewed in Holland and Hogan, 1988*b*, and in Dressler and Gruss, 1988). Examination of *in situ* hybridization results led to the conclusion that the time

when *Hox* gene expression is first detected lies within the interval when position of tissues becomes assigned along the rostrocaudal axis of the embryo (Gaunt, 1987), providing circumstantial evidence that *Hox* genes specify positional cues for AP axis determination (discussed in Gaunt, 1987). Moreover, experiments by Wilkinson *et al.* (1989) provided direct evidence for segment-related domains of expression of *Hox-2* genes in the developing hindbrain, strengthening the hypothesis that mouse *Antp*-type *Hox* genes may have a role in the establishment of positional information during embryogenesis.

Studying the expression pattern of genes throughout development thus can provide clues as to the function of these genes during pattern formation. On the other hand, to study the regulation of the *Hox* genes themselves both individually and in the context of their cluster is likely as well to lead us towards a better understanding of the gene function(s) and possibly of the mechanisms providing positional signalling along the developing body axis.

We have characterized by *in situ* hybridization the *Hox-2.3* expression pattern in embryos, previously determined by Northern blot analysis (Deschamps *et al.* 1987*b*). We also started to characterize the *cis*-regulatory elements mediating differential *Hox-2.3* expression during embryogenesis. Here we describe a *Hox-2.3* promoter element capable of driving the luciferase reporter gene in transfected cells and the *lacZ* gene in transgenic mice. Furthermore, we show that this *Hox-2.3* promoter region is able to generate a *lacZ* staining pattern in transgenic embryos which is very similar to the *Hox-2.3* expression pattern in only a subset of *Hox-2.3* positive tissues: a regulatory element responsible for expressing the gene in the epithelium of the urogenital system derived from the mesonephric duct is present in the *Hox-2.3* 5′ flanking region and is thus capable of functioning outside the context of the *Hox-2* cluster. The expression pattern of the transgene diverged from that of the endogenous gene in the central and peripheral nervous system, and in somitic and lateral plate mesoderm derivatives, implying that sequences responsible for *Hox-2.3* expression in these tissues are located outside the *Hox-2.4*/*Hox-2.3* intergenic region. Further implications for the existence of distinct regulatory elements that mediate *Hox-2.3* expression in different embryonic derivatives originating at a similar level along the rostrocaudal axis, and for the genetic organization of individual loci in the clusters, are discussed.

Materials and methods

Recovery of embryos

For all embryos used, mid-day after the copulation plug was noticed was taken as day 0.5. Embryos used for *in situ* hybridization experiments were obtained from a closed outbred colony of Swiss mice. The postimplantation development of this strain lags up to half a day behind that described in Theiler (1972). Embryos used in the *lacZ* work were produced by mating transgenic animals with C57Bl6×DBA2 F1 mice. The rate of development of these embryos corresponds to the description in Theiler.

In situ *hybridization*

In situ hybridization experiments were carried out on paraffin-embedded tissue sections according to Wilkinson *et al.* (1987), with the following modifications i) butanol was used instead of toluene before embedding in paraffin wax; ii) 6 μm sections were cut, loaded on a drop of water and dried onto slides which had been pretreated with a 2% solution of the binding silan TESPA (Sigma) in acetone for 10 s, then washed twice in acetone, once in water and dry-baked at 42°C; iii) the hybridization mixture contained 60 mM dithiothreitol (DTT) instead of 10 mM DTT; iiii) after high-stringency wash and autoradiography, staining was with 0.5% toluidine blue in water.

The probes used for *in situ* hybridization were as follows: ^{35}S-labelled antisense RNA probes were synthesized in a direction opposite to that of normal transcription using either T7 or SP6 RNA polymerase. Control (sense) probes gave no specific labelling. One of the three *Hox-2.3* antisense probes that have been used in *in situ* hybridizations experiments was transcribed from a 521 bp sequence extending from the *Bgl*II site 43 bp before the end of the homeobox to the BamH1 site in the 3′ untranslated region, 98 bp upstream from the polyadenylation site (see Fig. 1); a second probe was from the same *Bam*HI site in the 3′ untranslated region to the polyadenylation site 88 bp farther; the third probe was from a 296 bp subclone representing unique *Hox-2.3* coding sequences just upstream of the conserved hexapeptide coding sequence (Meijlink *et al.* 1987), in the first exon. Probes except the shortest one were partially hydrolyzed as described in Wilkinson *et al.* (1987). No difference in the specificity of the three probes was observed.

Construction of hybrid genes

Standard recombinant DNA procedures were followed, according to Maniatis *et al.* (1982). The *Hox-2.3* upstream region used in the present work was completely sequenced (Verrijzer *et al.* 1988, and unpublished but see accession number X06762 from the EMBL Nucleotide Sequence Data Base). *Hox-2.3* coordinates are relative to a localized transcription start site (Verrijzer *et al.* 1988). To generate the *Hox-2.3*/luciferase fusion constructs a *Sma*I (−1316) to *Sma*I (+207) *Hox-2.3* DNA fragment cloned into the pGEM-blue vector was subjected to Bal31 treatment in order to remove the *Hox-2.3* ATG and following coding sequences. Sequencing of the resulting clone mapped the 3′ end of the deletion to nt +81. *Hox-2.3* sequences (from −1316 to +81) were cut out of the vector using polylinker restriction sites, made blunt by the Klenow DNA polymerase and cloned into the blunt ended *Hin*dIII site of the promoterless pSV0ALΔ5′ luciferase containing vector obtained from De Wet *et al.* (1987). This generated the Sm1.3L construct. Sc2L was made by adding *Hox-2.3* 5′ sequences to the Sm1.3L plasmid using the *Sac*I (−2104) to *Sac*I (−169) *Hox-2.3* subclone. Deletion from the *Sac*I site (−169) to +81 generated clone Sm1.3 DL. Sl0.2L only contains *Hox-2.3* sequences encompassing −207 to +81. pSV2ALΔ5′ was a construct in which the luciferase gene is driven by the SV40 early promoter and enhancer; this plasmid was generously provided to us by Dr Subramani (De Wet *et al.* 1987). Control construct LK1.5L was made by cloning the 1.5Kb *Kpn*I fragment from the cIind1ts857 *lambda* phage DNA (Gibco-BRL) in front of the luciferase gene from the pSV0LΔ5′ vector.

The *Hox-2.3*/*lacZ* construct was made the following way: *Hox-2.3* sequences between the *Sma*I site (−1316) and the

*Sma*I site (+207) were inserted at the blunt-ended *Sal*I site of pMoMuLVnlslacZLTRΔEnh. This vector is the same as described in Bonnerot *et al.* (1987), except for the 3'LTR from which the enhancer was deleted as in Linney *et al.* (1984).

Tissue culture cells and DNA transfection

C1003 EC cells (McBurney, 1976) and the differentiated Fib9 cell line derived from it (A. Piersma and C. Mummery, unpublished) were grown in DMEM/F12 medium supplemented with 7.5% FCS. Retinoic acid (RA;10^{-6} M) treatment was as described in Deschamps *et al.* (1987*a*, 1987*b*) and was for up to one day starting one day after the DNA had been applied to the cells. The transfection protocol was a modification of the standard calcium phosphate coprecipitation method (Graham and Van der Eb, 1972): per 6 cm diameter tissue culture dish, 10 μg supercoiled DNA was added to 250 μl Hepes-buffered saline solution (42 mM Hepes, 275 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4 and 10 mM dextrose, pH 7.05). While mixing, 250 μl of 250 mM $CaCl_2$ was added and the precipitate was allowed to form at room temperature for 25 min. Cells were washed with the Hepes-buffered saline solution diluted twice and directly covered with the DNA coprecipitate. After 30 min at room temperature, 4 ml of medium at 37°C was added, and incubation was continued overnight in a CO_2 incubator. The next morning, the DNA containing medium was replaced by fresh medium and incubation was continued for one day before harvesting the cells.

In most experiments, DNA of a *lacZ* gene under the control of a house keeping gene promoter (hydroxy-3-methylglutaryl-coenzyme A reductase promoter, characterized by M. Methali and R. Lathe, personal communication), was used as an internal control: 5 μg of *HMG/lacZ* DNA (a generous gift from Dr R. Kothary) was coprecipitated with the 5 μg luciferase construct DNA, and β-galactosidase and luciferase activities were measured in the same extracts.

Luciferase and β-galactosidase transient assays

Luciferase activity was measured in transfected cell extracts according to De Wet *et al.* (1987) with the following modifications: cells were washed with PBS, and directly harvested in 0.5 ml extraction buffer (100 mM potassium phosphate [pH 7.8], 10 mM dithiothreitol) by scraping. Cells were lysed by three cycles of freezing in liquid nitrogen and thawing at 37°C. Membranes and cell debris were pelleted out by centrifugation at 4°C. Protein concentration of each extract was determined using the Biorad assay reagent. Up to 75 μl sample of each extract was added to 265 μl of a reaction mixture containing 90 μl of 100 mM glycylglycine, 90 μl of 20 mM ATP and 54 μl of 100 mM $MgSO_4$ in a small test tube and the tube was placed in a Lumac 3M luminometer. The reaction was started with the injection of 100 μl 1 mM luciferin (Boehringer, Mannheim). Light emission was displayed and recorded every second for 10 s. This way of following the peak light emission and the time course of the reaction proved to be equally accurate as using a chart recorder.

In the cotransfection experiments, β-galactosidase activity was measured in the extracts prepared for the luciferase assay. To 180 μl extract, 20 μl of a 1 M sodium phosphate buffer (pH 6.8) containing 0.1 M KCl, 10 mM $MgSO_4$ and 0.5 M β-mercapto-ethanol was added; the reaction was initiated by adding 40 μl of the β-galactosidase substrate analog orthonitrophenyl-β-D-galactopyranoside (ONPG, Sigma) at 4 mg ml^{-1}. Incubation was for 1 to 3 h at 37°C. The reaction was stopped by increasing the pH with 100 μl of 1 M Na_2CO_3. After a short centrifugation to get rid of any possible precipitated materials, the colorimetric assay was read in a spectrophotometer at a 420 nm wavelength.

Production of transgenic mice

Linear *Hox-2.3/lacZ* DNA insert was excised from vector sequences by *Pst*I restriction enzyme digestion, separated on an agarose gel and purified by the glass powder method (Vogelstein and Gillespie, 1979). A 6 μg ml^{-1} DNA solution was injected into the male pronucleus of zygotes from C57Bl6×DBA2 F1 females mated to 129/Sv males (about 800 copies of linearized molecules per zygote). *In vitro* manipulations and oviduct transfers were according to standard methods. Transgenic mice carrying the *Hox-2.3/lacZ* DNA were identified by tail DNA analysis and bred further to establish transgenic lines. The number of integrated copies of the transgene was determined by Southern analysis using a *lacZ* probe. As a control, DNA from cells in culture harboring respectively one and three *lacZ* proviruses was treated in parallel.

β-galactosidase histochemical analysis

Embryos and adult kidneys were stained as whole mounts as follows: fixation was with 4% paraformaldehyde in PBS at 4°C during 20 min (9.5 and 10.5 day embryos) or 60 to 80 min (12.5 and 14.5 day embryos); embryos at 14.5 days and later were first intracardially perfused in order to improve penetration of the fixative. Fixed tissues were washed with PBS during 30 min at 4°C, two to three times depending on their size. Staining was carried out at 30°C usually overnight in a solution of X-gal (Sigma) at a final concentration of 0.4 mg ml^{-1} made from a 40 mg ml^{-1} stock in DMSO, with 4 mM $K_3Fe(CN)_6$, 4 mM $K_4Fe(CN)_6.6H_2O$, 2 mM $MgCl_2$ in PBS.

After staining, tissues and embryos were rinsed with PBS and kept at 4°C in a 30% sucrose PBS solution until being photographed as whole mounts and subsequently frozen in OCT compound (Miles laboratory) for cryostat sectioning at 30 μm. Sections were viewed after further staining by incubating them overnight in X-gal solution at 30°C. No or a very light counterstaining of the sections with phloxine was performed. Staining of non-transgenic embryos and adult kidneys either as whole mounts or in sections never showed any detectable β-galactosidase activity in the organs we were concerned with.

Results

Definition of a Hox-2.3 promoter using luciferase as a reporter gene

To get insight into the molecular mechanism accounting for *Hox-2.3* expression, we set out to test *Hox-2.3*/ reporter gene constructs and deletions thereof in *in vitro* cell systems. As the firefly luciferase system was reported to be remarkably sensitive (De Wet *et al.* 1987), we constructed chimeric transcription units containing the luciferase gene and presumptive *Hox-2.3* regulatory elements (Fig. 1B). We introduced these constructs by transfection into cells in culture where *Hox-2.3* is expressed or inducible by retinoic acid (RA). In undifferentiated EC cells *Hox-2.3* mRNA does not accumulate; high levels of *Hox-2.3* transcripts are found, however, when these cells are treated with RA (Deschamps *et al.* 1987*b*; Meijlink *et al.* 1987). Induction of homeobox gene mRNA accumulation in EC

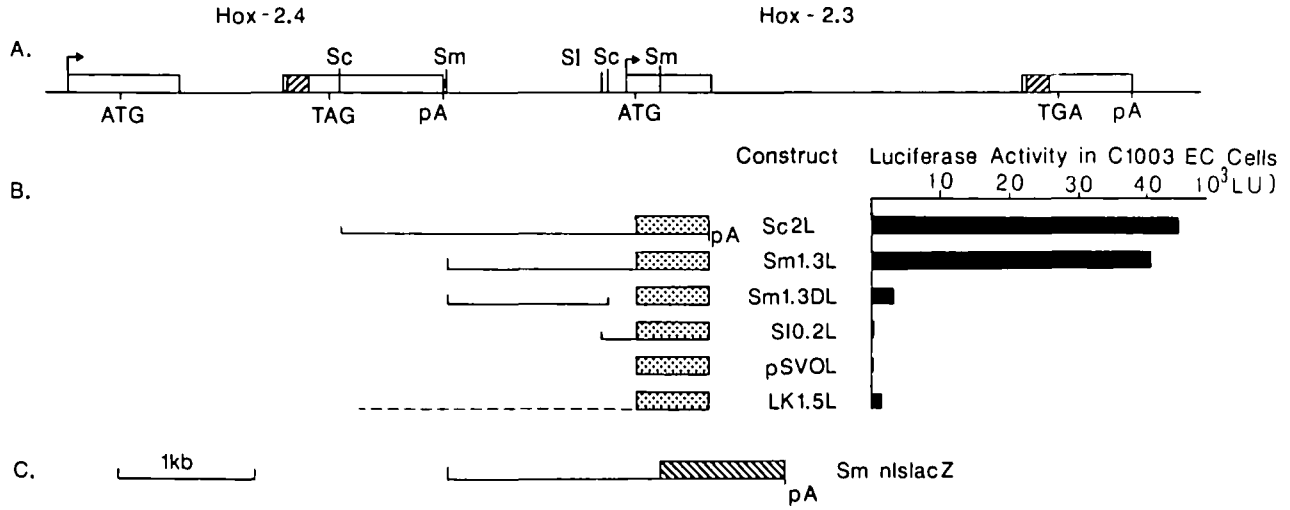


Fig. 1. (A) Genomic map of the *Hox-2.4* and *Hox-2.3* region. Arrows indicate the origin and orientation of transcription; open boxes represent exons; hatched boxes indicate the homeoboxes. pA stands for polyadenylation site. Beginning and end of coding regions are also indicated. Regarding *Hox-2.4*, polyadenylation site and beginning and end of the coding region are from Kongsuwan *et al.* (1989). Only the restriction sites are indicated that are relevant to the construction of the fusion genes in Fig. 1B and 1C (B) *Hox-2.3*/luciferase recombinant plasmids. Different lengths of *Hox-2.3* upstream sequences (shown in A) were inserted into the promoterless luciferase carrying plasmid pSV0ALΔ5′ (De Wet *et al.* 1987) (see Materials and methods). Control constructs were pSV0ALΔ5′, pLK1.5L where the 1.5 *Kpn*I fragment from the *lambda* phage genome is inserted upstream of the luciferase gene, and pSV2ALΔ5′ where the SV40 early promoter and enhancer drive the luciferase gene. Stippled boxes represent the luciferase coding region. pA is the SV40 transcription termination and polyadenylation site. The right part of the figure represents the luciferase activity measured in lysates from C1003 EC cells transiently transfected with these constructs. Enzymatic activity is expressed in Arbitrary Light Units and is the average of measurements from 5 experiments. (C) *Hox-2.3*/*lacZ* hybrid transcription unit used to generate transgenic mice. *nlslacZ* is a β-galactosidase-encoding gene modified so that the enzyme is targeted to the cell nucleus (Kalderon *et al.* 1984; Bonnerot *et al.* 1987). pA is the transcription termination and polyadenylation site from a Moloney Murine leukemia Virus LTR from which the enhancer has been deleted (see Materials and methods).

cells is RA-dependent and is not required for cell differentiation (Deschamps *et al.* 1987*a*), in keeping with the hypothesis that RA used *in vitro* may be an effector that mimics, if not is, a physiological inducer of *Hox* genes *in vivo*. We used C1003 EC cells, C1003 cells treated with RA, and a differentiated fibroblast-like derivative of C1003, Fib9 (A. Piersma and C. Mummery, unpublished) as we found that the latter constitutively expresses *Hox-2.3* at a high level. Luciferase activity was measured two days after transfection (Fig. 1B and Table 1). In C1003 EC cells, construct Sm1.3L that contains almost the entire intergenic region between *Hox-2.4* (Kongsuwan *et al.* 1989) and *Hox-2.3* (Meijlink *et al.* 1987, 1989) 5′ of the luciferase gene (Fig. 1A and B) gave rise to about 100 times more luciferase activity than a promoterless construct (pSV0ALΔ5′) and to about 30 times more activity than a control construct containing a randomly chosen 1.5 kbp of λ phage DNA 5′ of the luciferase gene, all these constructs being derived from the same basal plasmid.

The Sm1.3L construct contains the transcription start site previously mapped by S1 protection experiments (Verrijzer *et al.* 1988; Meijlink *et al.* 1989). A 3′ deletion of the *Hox-2.3* fragment driving the luciferase gene in the Sm1.3L construct – generating construct Sm1.3DL – gave rise to a more than 10-fold drop in activity (Fig. 1B). A construct containing only the proximal

Table 1. luciferase *transient assays*

	cells		
construct	C1003EC	C1003+RA	Fib9
Sc2L	44000	86000	69000
SM1.3L	41000	80000	54000
Sm1.3DL	3300	8000	3000
S10.2L	200	700	700
pSV0ALΔ5′	320	600	100
LK1.5L	1200	5000	1700
pSV2ALΔ5′	7400000	20000000	5700000

DNA from the different constructs (see Fig. 1B) was transfected in C1003 EC cells, in C1003 cells treated with RA for one day, and in Fib9 cells. Luciferase activity is expressed in Arbitrary Light Units. Each value is the average of the values obtained in 2 (for Sc2L in C1003+RA) to 5 experiments (in all other cases). See Results section for interpretation of the differences between the luciferase values obtained in the presence or in the absence of RA.

Hox-2.3 portion of Sm1.3L (S10.2L) gave virtually no activity (Fig. 1B), suggesting that both DNA fragments, −1316 to −169, and −207 to +80, contain sequences needed for transcription activity. To investigate whether sequences upstream from the *Sma*I site and thus inside the *Hox-2.4* transcription unit were involved in *Hox-2.3* transcriptional regulation 772 more nucleotides upstream of the *Sma*I site were included in a new construct (Sc2L, Fig. 1B). No important increase

in transcription was noticed in Sc2L *versus* Sm1.3L (Fig. 1B). Similar results were obtained for all constructs in C1003 EC cells and in Fib9 cells (Table 1).

We wanted to relate the *Hox-2.3* promoter activity to that of a standard promoter in the cell systems used, and therefore we tested the SV40 early promoter/luciferase construct pSV2ALΔ5' (De Wet *et al.* 1987) in parallel with the above described *Hox-2.3*/luciferase constructs (Table 1). This made clear that the *Hox-2.3* promoter is weak compared to the SV40 early promoter in these experiments.

When luciferase was assayed in extracts of C1003 transfected cells treated for up to one day with RA, enzyme activity was increased by approximatively the same factor two to three for the *Hox-2.3*/luciferase constructs as for the controls (Table 1); cotransfection with a *lacZ* construct (kindly provided by R. Kothary) in which *lacZ* is under the control of the promoter of the 'house keeping' gene, hydroxy-3-methylglutaryl-coenzymeA reductase, (*HMG;* M. Mehtali and R. Lathe, personal communication), and staining of duplicate culture dishes with X-gal enabled us to establish that RA increased the number of *lacZ* expressing cells at least threefold. Moreover in similar cotransfection experiments where the luciferase values were normalized using the β-galactosidase catalytic activity measured in the same extracts, no significant difference between *Hox-2.3*/luciferase activity in the presence or in the absence of RA could be observed (not shown). This leads us to the conclusion that the RA effect observed in the luciferase transfection experiments (Table 1) is due to a facilitation either of DNA transfection or of expression of transfected DNA in general, or both. Given the fact that the RA treatment used induces *Hox-2.3* transcripts to accumulate at least 50 to 100 times in C1003 cells (Deschamps *et al.* 1987*b*), we conclude that this regulation by RA is not mediated by sequences present on the *Hox-2.3*/luciferase hybrid genes in this *in vitro* system.

Hox-2.3 expression detected by in situ *hybridization*

While a relevant *in vitro* system can be expected to allow studies to be made on the basal transcription regulatory elements of *Hox* genes, the hypothetical involvement of these genes in cell position signalling during embryogenesis is to be approached by *in vivo* experiments such as testing *Hox*/reporter gene constructs in transgenic mice. Before studying the expression pattern of *Hox-2.3/lacZ* transgenes in embryos *in vivo*, we performed *in situ* hybridization experiments to establish how the *Hox-2.3* endogenous pattern is set up and evolves during embryogenesis. We knew from RNA analysis in dissected 13.5-day embryos that gene expression at this stage is the strongest in the spinal cord, starting rostrally at a level posterior to that of *Hox-2.1* taken as a control (Deschamps *et al.* 1987*b*). Establishment and evolution of the boundaries of *Hox-2.3* expression along the AP axis within the central and peripheral nervous system and within the mesoderm, as well as a detailed description of the expression pattern in these tissues throughout embryogenesis will be submitted separately (RV and JD, in preparation). Attention in the present communication will particularly focus on *Hox-2.3* expression in intermediate mesoderm derivatives where faithful expression of our *Hox-2.3/lacZ* construct was detected.

The first sign of *Hox-2.3* expression in the nephrogenic area was detected by *in situ* hybridization experiments in 9.5-day embryos (about 20 somites, Theiler stage 14). At this stage *Hox-2.3* expression is restricted along the AP axis: analysis of transverse and sagittal sections revealed that the posterior part of the embryo was labelled, while the cephalic and heart region did not express the gene (not shown). This was true as well in day 10.5 embryos (Theiler stage 16; Fig. 2A,B showing a 32-somite embryo). Expression was clearly detected in the spinal cord and in somitic, lateral plate (stomach primordium) and intermediate (mesonephric) mesoderm. Mesonephric duct and tubule epithelium specifically displayed a high density of silver grains (Fig. 2C,D). At Theiler stage 17 (35–39 somites), the ureteric bud emerges as an evagination from the mesonephric duct and will then start branching to form the collecting tubules of the metanephric or definitive kidney. In 12.5-day embryos (Theiler stage 20), the mesonephric duct and metanephric kidneys gave a very strong *Hox-2.3* hybridization signal (Fig. 3A,B). The degenerating mesonephric tubules were less intensely labelled (not shown). Transcripts were localized in the epithelium of the mesonephric ducts and tubules as was the case in 10.5 day embryos, and in the epithelium of the branching ureteric tree (collecting tubules of the metanephros) and ureters (Fig. 3A,B). Fig. 3 also shows that the spinal cord expresses *Hox-2.3* at a very high level, the spinal ganglia being labelled as well although at a lower level. One day later (Theiler stage 21), the ureteric bud induces the surrounding mesenchyme to condense and form the comma-shaped and s-shaped bodies (reviewed in Rugh, 1968 and Saxen, 1987). High amounts of *Hox-2.3* transcripts were observed in the collecting tubules in 14.5 day embryos (Theiler stage 22) (Fig. 4A–D). We cannot exclude that weak *Hox-2.3* expression also occurs in the comma-shaped and s-shaped tubuli. The epithelium of the mesonephric (Wolffian) ducts and of the ureters gave a high *Hox-2.3* signal, both in 12.5- and 14.5-day embryos. Evidence for *Hox-2.3* expression in the Müllerian – paramesonephric – duct (future oviduct and uterus in the female) was obtained with 14.5-day sections containing both Wolffian and Müllerian ducts (as shown in Fig. 4A,B). High *Hox-2.3* expression was observed all along the spinal cord, as was the case in 12.5-day embryos, while the 14.5-day spinal ganglia gave a lower signal (not shown). High expression of *Hox-2.3* persists in adult kidneys and spinal cord as has been reported earlier (Meijlink *et al.* 1987).

Hox-2.3/lacZ *expression in transgenic mice*

In order to test whether *Hox-2.3* upstream sequences containing a S1-mapped transcription start site and shown to possess promoter activity *in vitro* are able to mediate gene transcription *in vivo*, and are involved in

differential gene regulation during embryogenesis, the sequences between *Hox-2.4* and *Hox-2.3* (Fig. 1A) were coupled to the *E. coli lacZ* gene and used to generate transgenic mice. The *lacZ* gene used was a modified version encoding nuclear-targeted β-galactosidase (Kalderon *et al.* 1984; Bonnerot *et al.* 1987) (Fig. 1C and see Materials and methods). Six transgenic founder animals were identified by Southern blotting

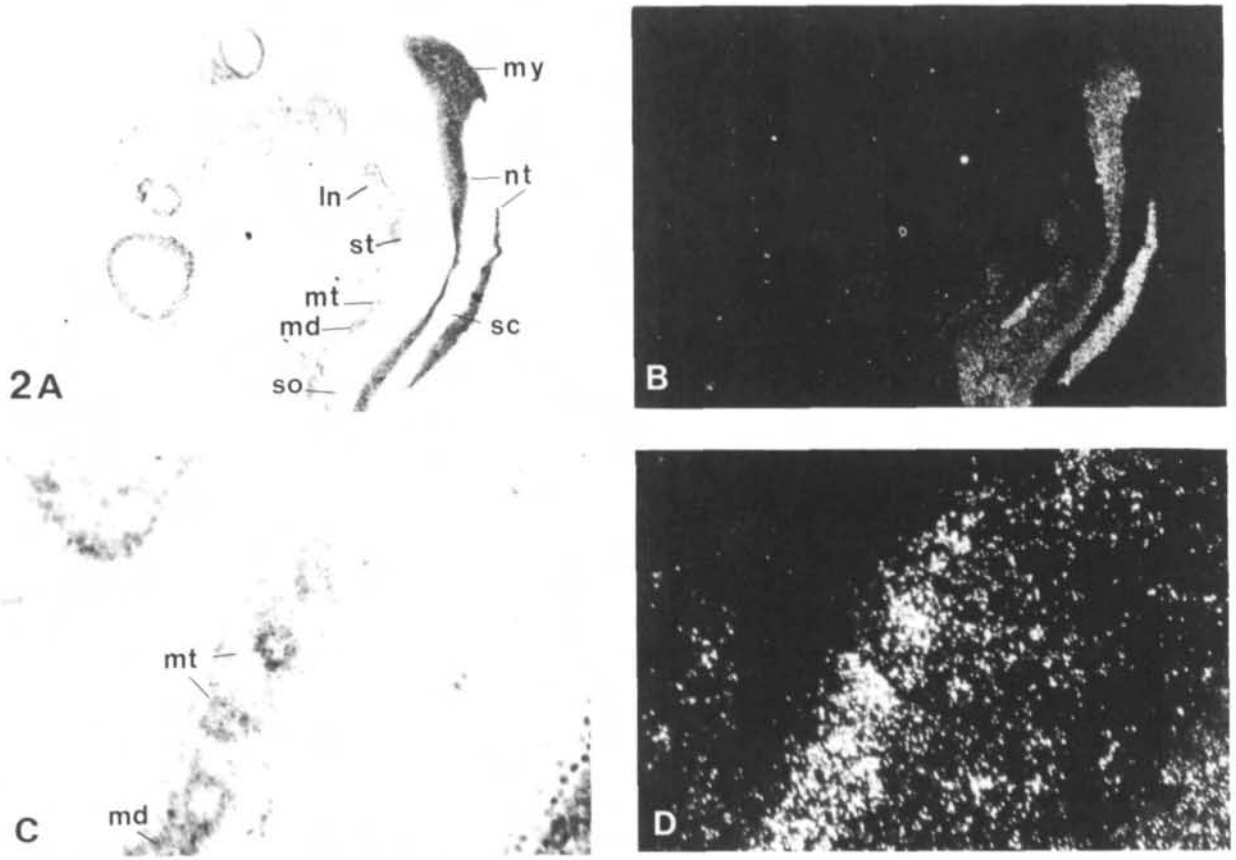


Fig. 2. *Hox-2.3* transcripts detected by *in situ* hybridization on longitudinal sections of a 10.5-day embryo (Theiler stage 15, 35 somites). (A) Bright-field photograph of the whole embryo section; (B) Dark-field illumination of section in (A). Magnification 22 fold. (C,D) Higher magnification (160×) of the mesonephric region, showing *Hox-2.3* expression in the mesonephric tubules and duct and photographed under bright-field (C) and dark-field (D) illumination. Hybridization was with a *Hox-2.3* antisense probe (see Materials and methods) and exposure was for 20 days. nt, neural tube; sc, spinal canal; ln, lung; ms, mesonephric tubules, md, mesonephric duct; st, stomach primordium; so, somite.

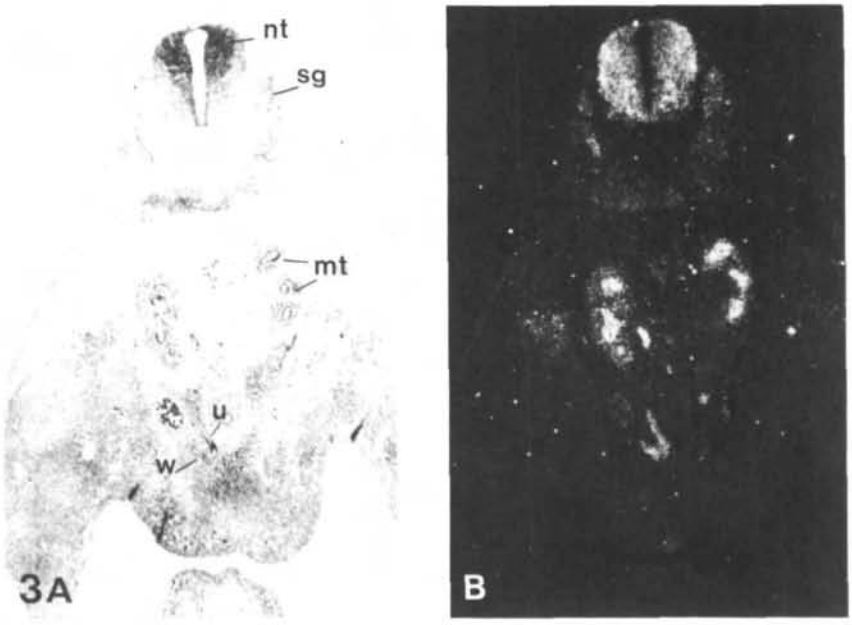


Fig. 3. *Hox-2.3* expression in the lumbar area of a 12.5-day embryo (Theiler stage 20). Transverse section through the metanephric region, hybridized with a *Hox-2.3* antisense probe and photographed under bright-field (A) and dark-field (B) conditions. Exposure was for 17 days. nt, neural tube; sp, spinal ganglia; mt, metanephric collecting tubules; u, ureter; w, Wolffian (mesonephric) duct. Magnification 22 fold.

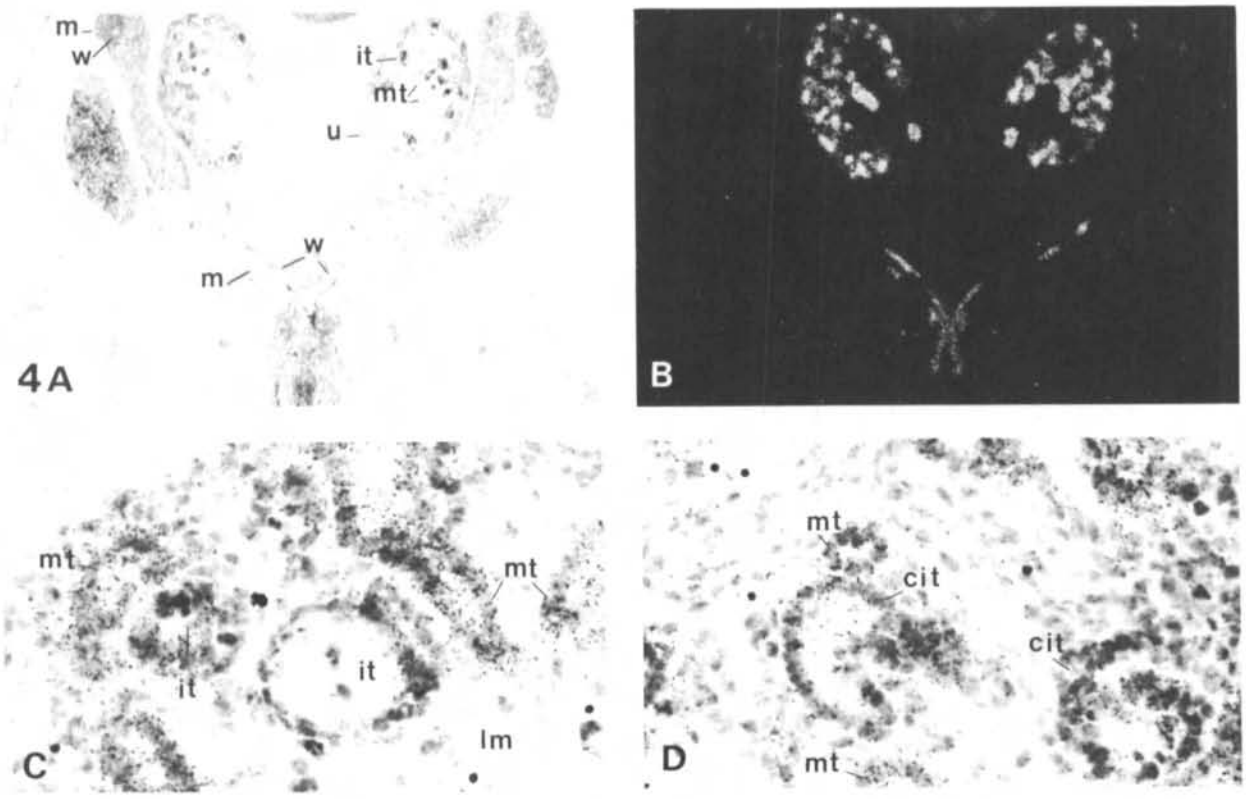


Fig. 4. *Hox-2.3* expression in the urogenital region of a 14.5-day embryo (Theiler stage 22). *In situ* hybridization on a transverse section with a *Hox-2.3* antisense probe. (A) Bright-field and (B) dark-field photograph. Signal is clearly detected in the metanephric collecting tubules, in the ureters and Wolffian and Müllerian ducts. Magnification 22 fold. (C,D) Higher magnifications (255×) of parts of a nearby parallel section to that shown in (A) and (B), photographed under bright-field conditions. Exposure was for 18 days. mt, metanephric collecting tubules; it, induced tubule; cit, comma-shaped induced tubule; lm, loose mesenchyme; u, ureter; w, Wolffian duct; m, Müllerian duct.

experiments using tail biopsy DNA (not shown) and were bred further. Four of these transgenic lines did not express the transgene. Transgenic offspring from line 23 (harboring two copies of the transgene) and from line 21 (carrying about ten copies of the transgene) were analyzed extensively for β-galactosidase staining at various stages of embryogenesis. From whole-mount staining of mid-gestation embryos, it was obvious that *Hox-2.3/lacZ* expression was very strong in the developing kidneys (Fig. 5A showing a 14.5-day embryo from line 23).

β-galactosidase-positive tissues were detected around day 9.5 (Theiler stage 15) along the nephrogenic cord (Fig. 5B showing a 25-somite embryo, and 5C showing a 30-somite embryo from line 23). Labelled cells were found ventro-lateral to the somites, from the level of the anterior limb bud on to more caudal regions. In day-10.5 (Fig. 5D) and day-11.5 (Fig. 5E) embryos, the mesonephric (Wolffian) duct, the ureteric bud and developing metanephros, and the ureters were clearly stained. At day 12.5 and 14.5 *p.c.*, (Theiler stage 21 and 23, respectively) embryos possessed stained cells in the epithelium of the mesonephric ducts, in that of the branching ureteric tree (metanephric collecting tubules) and in that of the ureters (Fig. 5F and G). Ureters and mesonephric ducts were labeled all the way down to their junction with the urogenital sinus (future bladder). Examination of histochemically stained tissue sections from 14.5 day old transgenic embryos (Fig. 5K,L) led us to the conclusion that the epithelium of the collecting tubules, mesonephric ducts and ureters was selectively labelled, whereas the epithelium induced from the surrounding mesenchyme by the ureteric buds (comma- and s-shaped bodies which will develop into renal tubules, and glomerular (Bowman's) capsules [reviewed in Saxen, 1987]) did not show any β-galactosidase activity (Fig. 5K,L). No *lacZ* expression was observed in the Müllerian ducts. Examination of this and other sections allowed us to conclude that expression of the transgene takes place exclusively in the epithelium derived from the mesonephric duct. From embryonic day 15 on and in postnatal male mice the epididymis, vas deferens and seminal vesicle (all derived from the mesonephric duct) were positive for *lacZ* expression (Fig. 5H,I). Adult kidneys were stained as whole mounts, revealing a strong signal along the collecting tubules running from the pelvis radially throughout the medulla and cortex (Fig. 5J). *Hox-2.3/lacZ* expression in the urogenital system was thus maintained throughout embryogenesis and in transgenic adults. A pattern identical in time and space was observed in the urogenital system for the second

transgenic line analyzed (not shown, and Fig. 5I as an example). The level of *Hox-2.3/lacZ* expression in line 21 was lower than in line 23 in all positive tissues. Variation of the expression level of a transgene in independent lines, irrespective of the number of integrated copies is a well-known phenomenon.

Aside from the β-galactosidase staining pattern that reflected endogeneous *Hox-2.3* expression in mesonephric duct-derived structures of the urogenital system, β-galactosidase-positive cells were also visible in the nervous system, although according to a pattern different from that of *Hox-2.3*. In embryos from line 23 at 11.5 days, lightly stained cells were visible in the mesencephalon, in the anterior and posterior myelencephalon and along, as well as in the neural tube. At day 12.5, embryos from this line showed weak transgene expression in the nervous system as described for day 11.5. X-gal-stained sagittal and transverse sections enabled us to analyze the distribution of the stained cells in the spinal cord: two longitudinal columns of labelled cells were observed to run parallel to the spinal canal all along the spinal cord. The dorso-ventral position of these columns of cells was medio-lateral to the spinal canal (not shown). In day 14.5 embryos, label was still observed in the spinal cord, apparently decreasing posteriorly to the cervical region. All spinal ganglia were lightly stained at this stage (see Fig. 5A). In embryos from line 21, lacZ expression at 11.5 day is detected in a few widespread cells in the mesencephalon while rare labelled cells were also detected in the spinal cord.

Transgene expression that is consistent neither with *Hox-2.3* expression nor between lines 23 and 21 probably reflects an effect of the integration sites of the transgene: in line 23, label was also detected at the basis of the third visceral arch and later in the epithelium of the olfactory pits. In line 21, a few β-galactosidase-positive cells were present on the surface of the muzzle in 14.5 day embryos. As position effects are frequently observed on the expression of transgenes (Allen *et al.* 1988; Khotary *et al.* 1988; Gossler *et al.* 1989), it was not surprising to observe some integration site-dependent *Hox-2.3/lacZ* expression in our transgenic mouse lines.

Fig. 5. Spatial distribution of β-galactosidase-positive cells in *Hox-2.3/lacZ* transgenic embryos and adult urogenital tract. (A) Whole-mount X-gal staining of a day 14.5 embryo (Theiler stage 23) from line 23 showing most intense label in the kidneys, and revealing other sites of transgene expression: the myelencephalon and spinal cord region. (B and C) *In situ* detection of β-galactosidase activity in a 9.5-day embryo (25 and 30 somites, respectively, Theiler stage 15) from line 23, stained with X-gal. The two labelled nephrogenic cords (nc) are seen, ventro-lateral to the somites. (D) X-gal staining of a whole-mount 10.5-day embryo from line 23 showing the labelled primary ureteric buds (ub) evaginating from the mesonephric duct (nc). (E) β-galactosidase positive cells in the mesonephric ducts (md), branching ureteric buds of the metanephros (M) and ureters (ur) of a 11.5 day embryo. (F and G) X-gal staining of a whole mount 12.5- and 14.5-day embryo (Theiler stages 21 and 23, respectively) from line 23, showing the more differentiated metanephric kidneys with numerous blue collecting tubules (mt); mesonephric ducts (md) and ureters (ur) are stained as well. (H and I) *LacZ* positive derivatives of the mesonephric duct – vas deferens (v), epididymis (ep), seminal vesicle (sv), ureters (ur) and collecting tubules of the kidney (K) – in 3-day old male mice from line 23 and 21, respectively. (Continued overleaf.)

Discussion

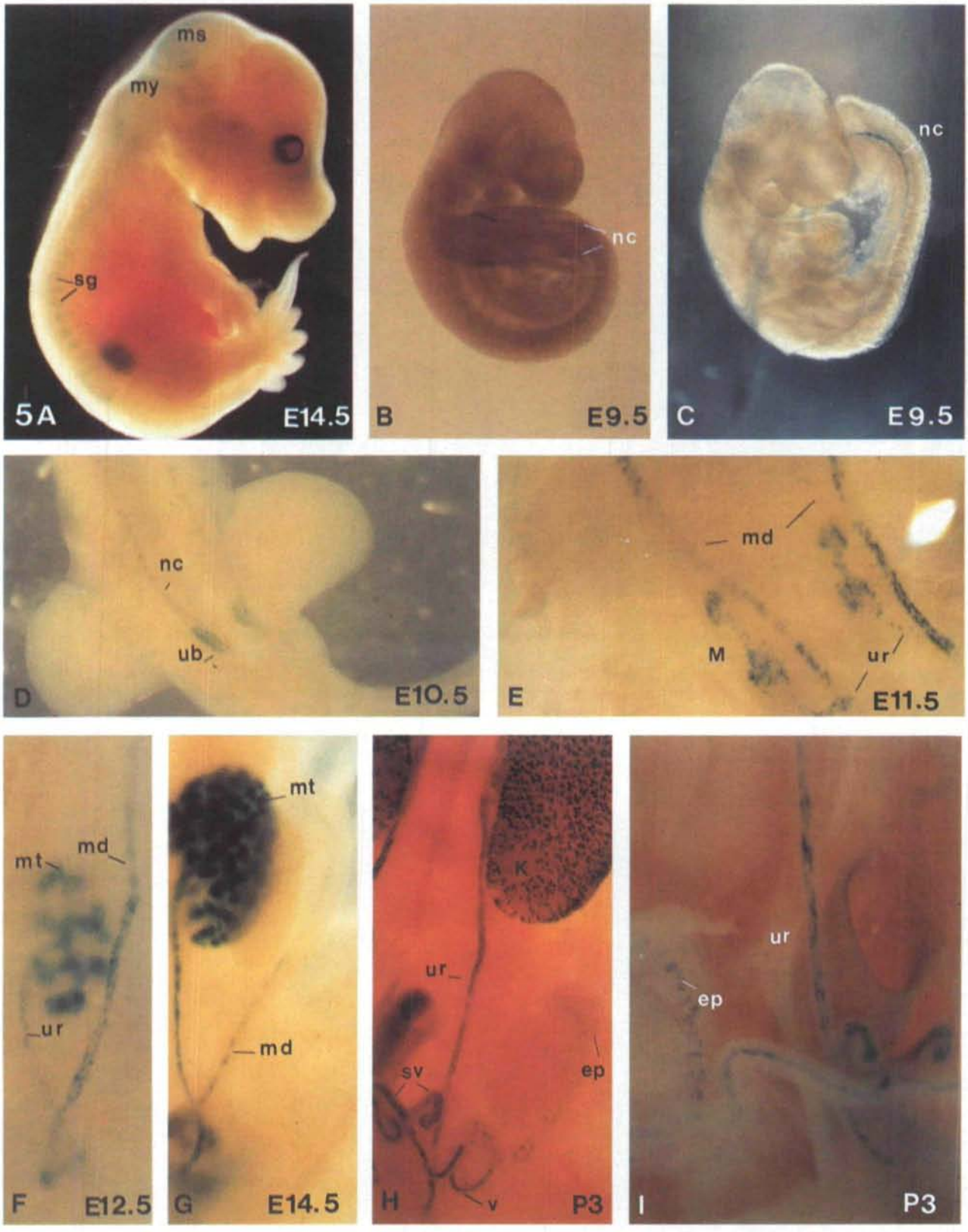
Like all the mouse homeobox genes of the *Antp* type studied so far, *Hox-2.3* is expressed according to a temporally and spatially restricted pattern in the embryonic central and peripheral nervous system and in derivatives of the axial, lateral plate and intermediate mesoderm (Deschamps *et al.* 1987*b*; Graham *et al.* 1989; this paper; RV and JD, unpublished results).

We addressed the question of the molecular basis of this differential genetic control, since identification of regulatory elements influencing individual *Hox* genes within a cluster might shed some light on their interrelationship and on their function. We demonstrate in this communication that *Hox-2.3* upstream sequences containing a S1-mapped transcription start site and encompassing 1.3 kbp 5′ to this site are active in driving transcription of a reporter gene in transfected cells and in transgenic mice.

These *Hox-2.4/Hox-2.3* intergenic sequences contain a promoter mediating transcription of the luciferase gene in transfected cells *in vitro*. A similar level of transcription was observed in cells expressing (Fib9) or not expressing (C1003 EC) *Hox-2.3* at a high level. As no difference could be detected either upon measuring luciferase in EC cells with or without RA, we conclude that the *Hox-2.3* upstream sequences present on the luciferase constructs might define a promoter that is constitutively active at a low level in a transient assay in the *in vitro* cell systems used thus far.

The use of a chimeric transcription unit composed of *Hox-2.3* upstream sequences encompassing this promoter, and the bacterial *lacZ* gene to generate transgenic mouse lines, has enabled us to demonstrate that this *Hox-2.3* region is responsible for differential gene expression *in vivo*. The *Hox-2.3* sequences used contain *cis*-acting control sequences mediating selective *lacZ* expression in the epithelium of the urogenital system derived from the mesonephric duct, a site of high *Hox-2.3* expression. These sequences are likely to be responsible for the regulation of *Hox-2.3* in the corresponding mesodermal tissue. The fact that the expression pattern of the transgene differs from that of the endogenous gene in somitic and lateral plate mesoderm and in neurectoderm suggests that the transgene is lacking sequences that account for *Hox-2.3* expression in these tissues. A conclusion from these observations is that distinct regulatory elements are used in different subsets of cells expressing *Hox-2.3*.

The vertebrate urinary apparatus arises from the intermediate mesoderm, as a nephrogenic column that shows a markedly metameric character. The most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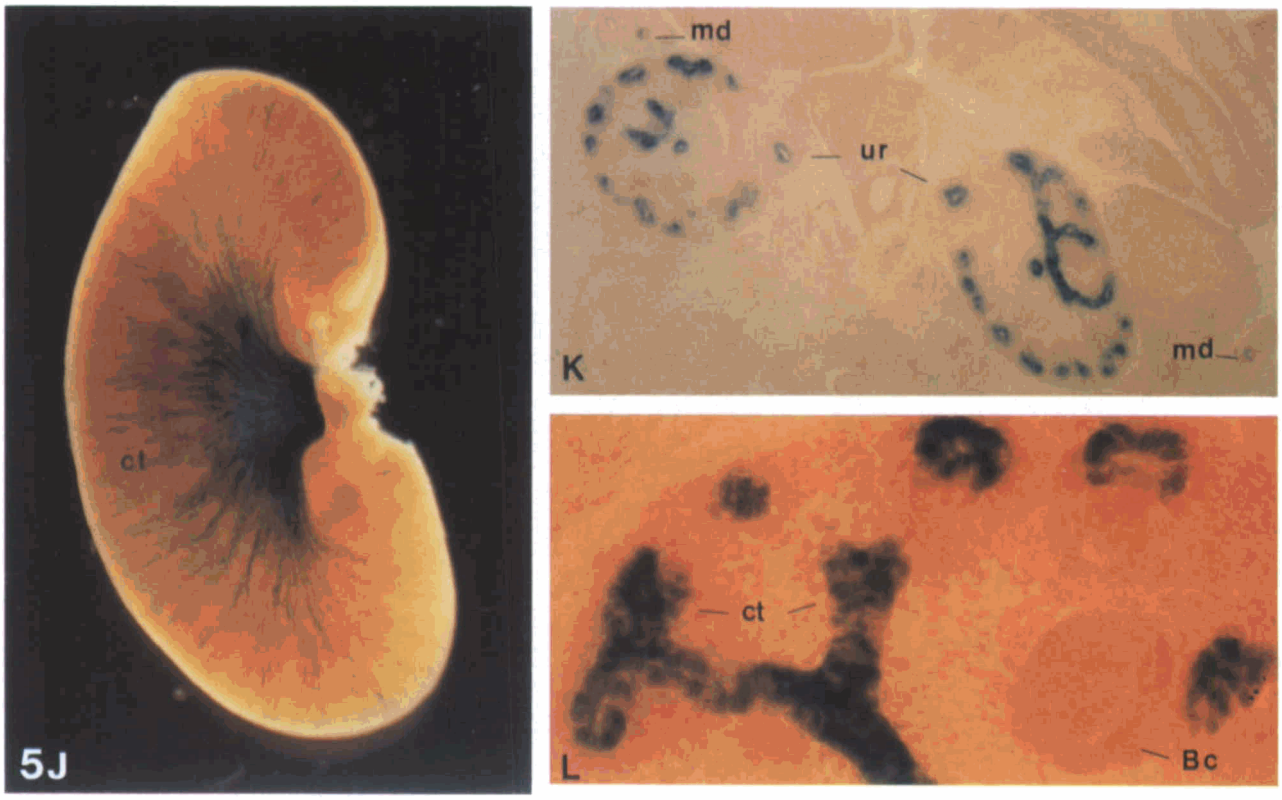

Fig. 5. continued. (J) X-gal staining of a whole-mount kidney from a four month old F_1 progeny of line 23 transgenic mice showing labelled collecting tubules (ct) gathering in the pelvis. (K) Histological identification of β-galactosidase-expressing cells on a transverse tissue section in the urogenital region of a 14.5 day embryo. *LacZ*-positive cells are found in the epithelium of the collecting tubules, ureters (ur) and mesonephric ducts (md). The differentiating glomeruli (induced tubules and future Bowman's capsule) do not show any blue cells. (L) Higher magnification of part of the section shown in (K). ct, collecting tubules; Bc, forming Bowman's capsule.

anterior part, the pronephros remains very rudimentary and without functional significance. The mesonephros, lying caudally to the pronephros, is composed of a number of renal vesicles, the nephrons, connected to the mesonephric or Wolffian duct. The metanephros, which will replace the mesonephros as functional excretory organ, arises partly from the mesenchyme of the posterior part of the nephrogenic cord, and partly from the ureteric bud growing out from the mesonephric duct and subsequently branching extensively to form the collecting tubules. The metanephric excretory units, the nephrons, develop from the metanephric mesenchyme after an inductive signal has been emitted by the tips of the branching ureteric tree (reviewed in Hamilton and Mossman, 1972, and Saxen, 1987). In the male embryo, the mesonephric duct, originally concerned with urinary excretion, partially degenerates and eventually becomes part of the genital tract – epididymis, vas deferens, seminal vesicle. In the female embryo, the primitive excretory duct undergoes degeneration and does not contribute to the reproductive tract.

In the meso- and metanephros, *Hox-2.3* and *Hox-2.3/lacZ* appear to be expressed at a high level in the ureter cell lineage: the epithelium of the mesonephric ducts and their derivatives, ureters and collecting tubules of the metanephros. Although some expression of *Hox-2.3* in the epithelium induced from the metanephric mesenchyme cannot be excluded, the level would be weak compared to that of the N-myc protooncogene, the transcription of which has been specifically associated with early differentiation stages of the induced tubules (Mugrauer *et al.* 1988).

One can only speculate about the physiological meaning of persistent *Hox-2.3* expression in mesodermal cells derived from the mesonephric duct from 9.5 day on throughout development and in adults. In agreement with the proposition that homeobox gene products may serve as positional cues during vertebrate development (for recent reviews, see Holland and Hogan, 1988*b*, Dressler and Gruss, 1988, and Akam, 1989), *Hox-2.3*-expressing cells belong to structures, the origin of which lies in the same rostrocaudal domain along the anteroposterior axis. The nephrogenic cord arises at a level within the *Hox-2.3* expression domain defined in the spinal cord and somitic mesoderm, as the mesonephric tubules appear at a level posterior to somite 11 in a 24 somite embryo, and the metanephros at the level of somite 26 in 34 somite embryos (Torrey, 1943), and as the most rostral somite to express *Hox-2.3* is somite 11 or 12 at similar stages of development (RV and JD, unpublished results). *Hox-2.3/lacZ* positive cells might have received a positional signal early during embryogenesis, with which *Hox-2.3*-expression would correlate; gene expression would be maintained through the subsequent cell generations either as a nonerased memory of the original 'label', or because *Hox-2.3* plays an additional and independent role in the urogenital epithelium in embryos and in adults.

Hox gene expression in the kidney is a recurrent observation: in all cases where *Hox* transcript level and localization have been analyzed by *in situ* hybridization in meso- and metanephros, cells in both these structures have been shown to be positive for mRNA accumulation (Holland and Hogan, 1988*b* as a review; Dressler and Gruss, 1989; Dollé and Duboule, 1989; Galliot *et al.* 1989; Bogarad *et al.* 1989). This situation is not unexpected since the rostral boundaries of *Hox* gene expression in the mesoderm occupy an AP position anterior to, or within the meso-and metanephric region (Duboule and Dollé, 1989; Dressler and Gruss, 1989). Interestingly, comparing the anterior expression boundaries of *Hox-2.3* (this paper), *Hox-5.2* and *Hox-5.3* (Dollé and Duboule, 1989) in the 12.5-day mesonephric tissue makes clear that these boundaries are displaced relatively to each other along the AP axis, as are the boundaries found in the somitic mesoderm. This observation suggests an involvement of *Hox* gene expression in positional signalling within the mesonephric duct derivatives. Interesting as well is the fact that the cell type where *Hox* transcripts accumulate in the meso- and metanephros also differs depending on the gene: for instance, genes like *Hox-1.4* (Galliot *et al.* 1989), *Hox-1.5* (Gaunt, 1988), *Hox-5.3* (Dollé and Duboule, 1989) are expressed homogeneously in the meso- and metanephric mesenchyme with no enhancement in tubules, whereas *Hox-2.1* (Jackson *et al.* 1985; Krumlauf *et al.* 1987; Holland and Hogan, 1988*a*), *Hox-5.2* (Dollé and Duboule, 1989) and *Hox-2.3* (this paper) are expressed particularly strongly in tubular epithelium.

Another major site of *Hox-2.3* expression from 8.5 day on throughout embryogenesis is the rostrocaudally defined domain in the nervous system, the posterior myelencephalon, the spinal cord and the dorsal root ganglia being positive for transcript accumulation in mid-gestation embryos (Deschamps *et al.* 1987*b*; Graham *et al.* 1989; this paper; RV and JD, in preparation). The transgenic lines examined did not give a β-galactosidase-staining picture similar to the *Hox-2.3* expression pattern in these structures. The conclusion is that *Hox-2.3* gene expression in the nervous system operates *via* a mechanism involving (additional) sequences located outside the transgene. One possibility is that a neurospecific element is required for proper high expression in the nervous system and is not present on the transgene. Alternatively, sequences responsible for the establishment of the AP-restricted *Hox-2.3* expression domain in both neurectoderm and mesoderm might not be present on the transgene, and expression of *Hox-2.3* and *Hox-2.3/lacZ* in the mesonephric duct-derived epithelium would correlate with an independent tissue-specific function of *Hox-2.3* in these structures.

An interesting observation results from the comparison of the β-galactosidase-staining pattern of the two mouse lines examined in detail: localized expression of *Hox-2.3/lacZ* in a subset of cells widespread in the mesencephalon of both families of embryos suggests that this feature is inherent to the transgene. This was not due to the reporter gene, as a number of *lacZ* transgenic lines with other promoters do not show β-galactosidase-positive cells in these structures (CB and JFN, unpublished results). This property might result

from the insensitivity of the transgene to a negative regulation normally modulating *Hox-2.3* expression in mesencephalon cells, as no *Hox-2.3* transcripts accumulate in the mesencephalon that is located outside the *Hox-2.3* expression boundaries. The fact that primary cultures of embryonic mesencephalon cells respond to RA treatment by an abundant accumulation of *Hox-2.3* transcripts whereas, for example, limb bud mesenchymal cells do not (Deschamps *et al.* 1987*b*) is in keeping with *Hox-2.3* being turned on in the CNS by a mechanism different from that in certain other tissues. This hypothesis of the absence of a negative regulation in mesencephalon cells may also apply to the weak expression of the transgene in the spinal cord and spinal ganglia that also differs from the endogenous pattern.

Altogether, we conclude from our *Hox-2.3/lacZ* experiments that regulatory sequences selectively mediating *Hox-2.3* expression in derivatives of the intermediate mesoderm are present on the transgene, and that sequences involved in *Hox-2.3* expression in cells lying at a similar rostrocaudal level in the spinal cord, in spinal ganglia and in somitic and lateral plate mesoderm derivatives are located elsewhere. Therefore, the regulatory mechanism leading to *Hox-2.3* expression in cells derived from the intermediate mesoderm would not be identical to that employed by cells from somitic and lateral plate mesoderm and from the neurectoderm. A similar conclusion as to the existence of distinct regulatory elements upstream of an *Antp*-type *Hox* gene was drawn by Zakany *et al.* (1988), who showed that *Hox-1.3* most proximal 912 bp mediate expression of a *Hox-1.3/lacZ* transgene in a specific region of the embryonic spinal cord, while expression in the *Hox-1.3*-positive mesodermal tissues (Dony and Gruss, 1987; Dressler and Gruss, 1989) does not take place. An important difference between the situations concerning *Hox-1.3* and *Hox-2.3*, is that *Hox-2.3/lacZ* expression is observed in the urogenital system of the transgenic embryos/mice whereas the *Hox-1.3/lacZ* transgene is transcribed in the spinal cord. This difference reveals that the regulatory element(s) directing gene expression in the spinal cord, positive for all *Hox* genes, is (are) not always located directly upstream of the gene to be controlled; in the case of *Hox-2.4* and *Hox-2.3*, such an element might be located upstream of, or within *Hox-2.4* and influence both genes. It is also possible that the putative 'kidney element' present upstream of *Hox-2.3* controls several genes of the cluster. Future experiments will test these speculations.

An analysis of the spatial and temporal activity of the promoter of *Hox-1.1*, the homolog of *Hox-2.3* in cluster 1, has recently been performed using *Hox-1.1/lacZ* transgenic mice (Püschel *et al.* 1990). 3.6 kbp sequences upstream and 1.7 kbp sequences downstream from *Hox-1.1* were fused to *lacZ* and shown to direct gene expression according to a pattern identical to that of *Hox-1.1* at early stages of development (7.5 to 8.5 days). At later stages, the patterns diverged in their caudal expression boundary and in the tissue distribution of the transcripts. Clearly, the *Hox-1.1* sequences present on the construct are endowed with a regulatory potential different from that of the 1.3 kbp *Hox-2.3* upstream region. For a direct comparison between *Hox-1.1/lacZ* and *Hox-2.3/lacZ*, differences in the constructs should be born in mind which might be causally related to the difference in expression pattern of the transgenes: *Hox* sequences present on *Hox-1.1/lacZ* extend farther upstream than those on *Hox-2.3/lacZ* do, and they contain intragenic and downstream sequences. In addition, the fact that there is no counterpart to *Hox-2.4* in the *Hox-1* cluster (Duboule and Dollé, 1989; Graham *et al.* 1989) makes it very likely that the 5′ flanking regions of the two members of the *Hox-1.1* subfamily extensively differ from each other. Duplication of the putative ancestral gene cluster (reviewed in Akam, 1989), as it gave rise to alterations within the clusters, thus might have provided the gene family with an additional variety of regulatory mechanisms, possibly enriching the repertoire of cellular instructions during embryogenesis.

We wish to thank Drs A. Graham and R. Krumlauf for introducing us to the *in situ* hybridization technique. C. K. is thankful to Dr M. Rassoulzadegan for teaching her the art and skill of generating transgenic mice. We also acknowledge Dr S. Subramani for sending us the luciferase clones, Drs M. Mehtali and R. Lathe for allowing us to use their cloned *HMG* promoter and Dr R. Kothary for providing us with the *HMGlacZ* plasmid. Many thanks are due to M. Hameleers for constructing the Sm1.3L plasmid, and to Dr K. Lawson for stimulating discussions and for reading and criticizing the manuscript. We also would like to thank Dr P. Ekblom and Dr G. Mugrauer for their help in interpreting the gene expression patterns in developing kidney. This work was supported by grants from the 'Association Française contre les Myopathies', the 'Association pour la Recherche sur le Cancer', and the 'Institut National de la Recherche Médicale' to J.-F.N.

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(*Accepted 10 April 1990*)