Gene Expression, Vol. 11, pp. 199–210
Printed in the USA. All rights reserved.

1052-2166/03 $20.00 + .00
Copyright © 2003 Cognizant Comm. Corp.
www.cognizantcommunication.com

Comprehensive Expression Profiling of Highly Homologous 39 Hox Genes in 26 Different Human Adult Tissues by the Modified Systematic Multiplex RT-PCR Method Reveals Tissue-Specific Expression Pattern That Suggests an Important Role of Chromosomal Structure in the Regulation of Hox Gene Expression in Adult Tissues

MIYAKO YAMAMOTO, DAISAKU TAKAI,[1] FUMIYA YAMAMOTO, AND FUMIICHIRO YAMAMOTO[2]

Cancer Genetics and Epigenetics Program, Burnham Institute, 10901 N. Torrey Pines Rd., La Jolla, CA 92037

Homeobox genes play a crucial role as molecular address labels in early embryogenesis by conferring cell fate and establishing regional identity in tissues. Homeobox gene expression is not restricted to the early development, but it is also observed in the differentiated cells in adult tissues. To have a better understanding of the functionality of homeobox gene expression in adult tissues in physiological and pathological phenomena, it is important to determine the expression profiles of Hox genes. We established a system to study the expression of 39 human Hox genes by the modified Systematic Multiplex RT-PCR method. Using this system, we have systematically examined their expression in 26 different adult tissues. The results showed tissue-specific differential expression. They also revealed that the posterior tissues generally express more Hox genes than the anterior tissues and that the genes located centrally in the Hox Gene Complexes are expressed in more tissues than the genes located at the 5′ or 3′ end of the complexes. Instead of similar expression patterns among paralogous genes, we found that several neighboring Hox genes on the same chromosomes exhibited similar tissue-specific expression pattern, which may suggest that the regulation of Hox gene expression may be more dependent on chromosomal structure in adult tissues.

Hox genes Homeobox genes Systematic Multiplex RT-PCR Hierarchical clustering algorithm Tissue-specific expression Adult tissues

Accepted September 22, 2003.
[1]Present address: National Institute of Radiological Sciences, Chiba-shi, Chiba 263-8555, Japan.
[2]Address correspondence to Fumiichiro Yamamoto, Ph.D., The Burnham Institute, 10901 N. Torrey Pines Rd., La Jolla, CA, 92037. Tel: (858) 646-3116; Fax: (858) 646-3173; E-mail: fyamamoto@burnham.org

HOMEOTIC mutations, originally discovered in *Drosophila*, were found to transform parts of the body into structures normally appropriate to other positions (23,27). A whole set of homeotic selector genes encodes a system that determines the head-to-tail patterns of the fly segments in *Drosophila*. Many mutations in these genes exhibit a recessive lethal phenotype and the affected embryos usually fail to survive beyond hatching, which led to the conclusion that these genes are critical in embryonic development. They possess a highly conserved 183-bp homeobox in the coding regions, and encode proteins that specifically bind to DNA and act as transcriptional modulators. The helix–turn–helix secondary structure of the homeobox-encoded homeodomain seems to be responsible for this binding action (6,18). Homologous genes have also been identified in mammalian genomes. Many of them are highly related to the *Antennapedia* gene, and are called Hox genes. In humans, 39 functional Hox genes are clustered in four complexes (HOX A–D Gene Complexes) (1,2,17,22). Besides laying down the anteroposterior character of embryos, the Hox gene family has also been shown to play an important role in the creation of multiple germ layers that give rise to all of a creature's tissues and organs (12). These include genitourinary organs (13,26, 36), skin (28), gut (5), hindbrain (29), and sphincter (34). Hox genes were suggested to be a link between patterning and the segmentation clock (16,30,35).

Novel isolated and divergent homeobox genes have continuously been identified. The human genome sequence published in February 2001 revealed that there are at least 178 homeobox sequences in the genome, 160 of which may be translated into homeodomains in functional proteins (31). In spite of this large number, most studies have focused on only one to several homeobox genes, possibly due to the absence of a simple and reliable method to examine the expression of multiple homeobox genes. Detection of homeobox gene expression by the hybridization of DNA microarrays with probes prepared from total RNA may not be an easy task because of the relatively low abundance of homeobox gene transcripts and significant sequence homology among genes. By modifying the Systematic Multiplex RT-PCR (SM RT-PCR) (33), we established a system to systemically analyze the expression of 39 human Hox genes. Using this system, we have examined their expression in 26 differential adult tissues. The moderately high-throughput data obtained have then been subjected to statistical analysis.

MATERIALS AND METHODS

Individual PCR, Confirmation of Band Identities, and Optimization of Multiplex PCR Conditions

Individual PCR reactions were performed using respective common region sequence primer (CRP) and unique region sequence primer (URP) pairs and genomic DNA. AccuPrime SuperMix II (Invitrogen) was used with 5 pmol each of primers and 100 ng of genomic DNA in 10-μl reactions. The following PCR reaction protocol was used: 94°C 2 min followed by 32 cycles of 94°C 30 s, 60°C 30 s, and 68°C 1 min 30 s, followed by 68°C 10-min incubation. Small aliquots of the PCR reaction products were added with loading dye and electrophoresed through an 8% polyacrylamide gel. The gel was then stained with ethidium bromide and gel pictures were taken. Once the amplification of unique bands of expected sizes was confirmed, we separated amplified DNA fragments in the remaining aliquots from unincorporated primers and substrates through affinity chromatography using QIAquick PCR Purification Kit (QIAGEN). Eluted DNA was then used to directly determine the nucleotide sequences of the amplified DNA fragments by cycle sequencing dideoxy termination method using BigDye Terminator Sequencing Kit (PE Applied Biosystem). The CRP primers used for respective PCR amplification was used as sequencing primer. Multiplex PCR was performed using genomic DNA as a template. Initially 5 pmol each of primers was mixed and aliquots were used for PCR. The same PCR cycle protocol employed for the individual PCR was first examined for multiplex PCR. The concentrations of primers were later adjusted to unify the band intensities. Those primers that did not produce unique bands in multiplex PCR were replaced with new primers until a set of primers that amplify all the bands of expected sizes was obtained. The nucleotide sequences and concentrations of the respective primers determined from optimization are listed in Table 1. The annealing temperature for the amplification of Hox C gene fragments was raised to 62°C. Multiplex PCR reactions was also performed, using primers that lacked one pair each to examine the authenticity of amplified bands. To determine the yields of individual DNA fragments during PCR, multiplex PCR was performed with variable numbers of amplification cycles and variable amounts of genomic DNA templates, and the yields were measured.

Systematic Multiplex RT-PCR

Total RNA from human adult tissues was purchased from Clontech, Stratagene, and Invitrogen.

We examined the expression of 39 Hox genes by the modified SM RT-PCR method. Total RNA was reverse transcribed into cDNA using oligo dT as primer and Advantage RT-for-PCR Kit (Clontech). The quality and quantity of cDNA were examined by PCR using control primers for G3PDH. All the cDNAs prepared gave satisfactory amplifications and were therefore used for the successive SM RT-PCR experiments. The same PCR protocol optimized for genomic DNA was used except the amplification cycles were reduced to 28 cycles. Twenty nanograms of cDNA was used in the experiments.

Hierarchical Clustering Algorithm

After electrophoresis, the polyacrylamide gels were stained with ethidium bromide, and the banding patterns were visualized. The intensities of the bands generated from 100 ng of genomic DNA templates were rated as 7 in the 1–10 scale, allowing more subdivision of weak bands. The intensities of the corresponding bands from the SM RT-PCR using cDNA templates were rated from 1 (minimum) to 10 (maximum). An Excel file was created that contained the information on gene names, tissue names, and band intensities. The dataset was then imported into the GeneSpring software to examine the relationships among the tissues and the genes, using a hierarchical clustering algorithm. An experiment tree was constructed by measuring the distance in similarity with the standard settings having a separation ratio of 0.5 and minimum distance of 0.001.

RESULTS

Modified SM RT-PCR Strategy to Analyze Hox Gene Expression

To systematically analyze Hox gene expression, we modified the SM RT-PCR method (33). We planned a strategy to simultaneously amplify DNA fragments from Hox genes in the individual Hox Gene Complexes. We designed two primers per gene, which would amplify DNA fragments of different sizes in single exons; one conserved region sequence primer (CRP) and the other unique region sequence primer (URP), in place of two unique region primers used in the original SM RT-PCR method. The strategy is schematically shown in Figure 1 with the genes in Hox B Gene Complex as examples. SM RT-PCR is performed under the conditions where control genomic DNA template produces a ladder of Hox gene DNA fragments of similar intensity. The PCR reactions are then subjected to polyacrylamide gel electrophoresis and the banding patterns are visualized. Relative, rather than absolute, quantity is semiquantitatively determined by comparing the band intensities of corresponding bands among different cDNA reactions and also with control genomic DNA reactions. The SM RT-PCR of 39 Hox genes consists of four reactions representing four Hox Gene Complexes.

Establishment of SM RT-PCR System of 39 Human Hox Genes

We first designed the respective CRP and URP combinations that would produce specific amplification in the individual PCR reactions. Genomic DNA was used as template. When PCR resulted in the amplification of additional band(s), the URP was replaced with a new URP to a different unique sequence while maintaining distinct fragment size. The nucleotide sequences of the CRPs and URPs that gave specific amplifications and were used in the SM RT-PCR studies are listed in Table 1, together with the expected sizes of DNA fragments amplified with the respective primer pairs. Results of the individual PCR reactions using those pairs of primers are shown in Figure 2a. We determined the nucleotide sequences of the amplified DNA fragments and confirmed their identity. We then optimized the conditions of the SM RT-PCR by elaborating primer concentrations until genomic DNA, which contained equal numbers of those genes, produced bands of similar intensities. The optimal concentrations of CRPs and URPs determined are also listed in Table 1. The PCR reaction products were electrophoresed in parallel with the individual PCR reactions, and the results are also shown in Figure 2a. These results clearly demonstrated that simultaneous amplification of 9–11 Hox gene fragments in a single tube multiplex PCR reaction was feasible. The same results also showed the reproducibility of the technique because the two independent multiplex PCR reactions with two different genomic DNA (lanes G1 and G2) gave almost identical banding patterns. To confirm the identities of bands produced from multiplex PCR, we also performed multiplex PCR experiments using a mixture of CRPs and URPs that lacked a single pair of primers in all the combinations. PCR products were electrophoresed and the results are shown in Figure 2b. The multiplex PCR reaction containing all the primers was used as control (lane ALL). In the fragment size range shown in the figure, three weak unwanted background bands were observed in the Hox C reaction: one close to the Hox C13 band and one between the C4 and 8 bands and the other between the C5 and C10 bands.

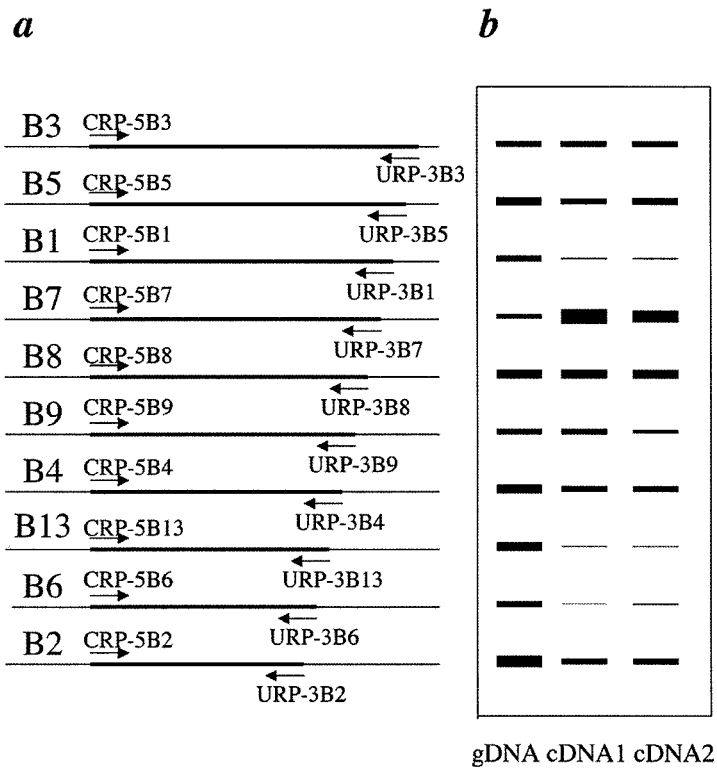

Figure 1. Modified Systematic Multiplex RT-PCR strategy. (a) Designing of primers. The modified SM RT-PCR scheme is presented with Hox B genes as examples. Two primers are designed for the individual members; one conserved region primer (CRP) and the other unique region primer (URP). The locations of the URPs are designed to produce DNA fragments of different sizes. The intron sequences are avoided so that the same size fragments are amplified from genomic DNA and cDNA. (b) Polyacrylamide gel electrophoresis. After PCR, the cDNA reaction products are analyzed by parallel polyacrylamide gel electrophoresis together with the genomic DNA reaction product(s).

Therefore, care should be taken when interpreting the results of C13 gene expression when using the present system. All the other bands produced from genomic DNA template were demonstrated to be unique and have derived from the respective Hox genes. We then determined the yields of DNA fragments after different numbers of amplification cycles in combination with different amounts of genomic DNA template as shown in Figure 2c. Based on the results, the cycle number for SM RT-PCR was set to 28 cycles so that the amplification would not reach the point of saturation for individual fragments amplified from control reactions using 100 ng of template genomic DNA.

SM RT-PCR Expression Profiling of Hox Genes in Adult Tissues

We examined the expression of 39 Hox genes, using RNA from 26 different human adult tissues. Genomic DNA was used as control. Human tissue RNA was reverse transcribed and the resultant cDNA was subjected to PCR under the conditions optimized using genomic DNA. Results are shown in Figure 3. Two independent control PCR reactions of genomic DNA once again gave almost identical banding patterns. The banding patterns of the SM RT-PCR reactions of different tissue RNA were not the same. Several tissues showed similar expression patterns, whereas several others showed unique patterns. For example, Hox B13 was found abundantly transcribed in the prostate tissue. We investigated the correlation between tissues and gene expression using our modestly high-throughput gene expression data of 39 Hox genes in 26 human tissues. We first assigned a number between 1 (minimum) and 10 (maximum) to the intensities of bands produced from cDNA templates. Number 7 was used to represent the intensities of the corresponding bands from universal control genomic DNA templates, and the number was either increased or decreased based on band intensity. We next sorted the tissues based on the total sum of the numbers. The 39 Hox genes were categorized into paralogous gene groups. Results are shown in Figure 4. The examination of the data panel showed the tendency of the tissues located posteriorly to express wider variety of Hox genes in higher quantity. The panel also

TABLE 1
PRIMERS USED FOR THE SM RT-PCR OF HOX GENES

Gene Name	Fragment Size (bp)	5′ CRP Primer Sequence	3′ URP Primer Sequence	Final Concentration (nM)
Hox A				
A1	112	GAGACCCAAGTGAAGATCTGGTT	CCTTCTCGTCGTTTCCTGGCG	45
A2	182	GAGAGACAAGTGAAAGTGTGGTT	AGAAGGGCCCCAGAGACGCTAAG	91
A3	101	GAGCGCCAGATCAAGATCTGGTT	CTTGGAGACTGGCCCCCCGAT	45
A4	124	GAGCGCCAGGTCAAGATCTGGTT	CTTTCCCTGGTGGGCCGGCAGAGGC	45
A5	173	TTGCCTCTCCGAGAGACAAATTAAAATCTGGTT	ACTGACACTACGCGGGATCCGCTAATA	159
A6	240	GAGCGCCAGATCAAGATCTGGTT	CTCCCCTGAAGCTGCGGAAGCCCC	45
A7	200	GAGCGCCAGATTAAGATCTGGTT	GGCCCCGGATCGGCCCCTCATTC	227
A9	217	GAGAGGCAGGTCAAGATCTGGTT	GAGGTTTAGAGCCGCTTTGTGCG	45
A10	141	ACGGACAGACAAGTGAAAATCTGGTT	GGAAGTGAAAAAACCGCGTCGCCTGG	45
A11	90	GATCGTCAAGTCAAAATCTGGTT	TGGATTTGCTGAGTAGTACTGTAAACG	159
A13	128	GAGCGGCAGGTCACAATCTGGTT	TTCTGAAGCGTTTCTTCAAGTTGCCTTCTTGC	45
Hox B				
B1	205	GAAACACAGGTCAAGATTTGGTT	GGAAGCCCCATTGGTGGCTAGGT	50
B2	121	GAAAGGCAGGTCAAAGTCTGGTT	GGTCGCAGATGTCCTCCAGGG	250
B3	241	GAGCGGCAGATCAAGATCTGGTT	GGTAGTTGGAGGGCAGCGCGTAG	75
B4	142	GAGCGCCAGATCAAGATCTGGTT	CGCGGGGGCCTCCATTGGGC	100
B5	220	GAGCGCCAGATCAAGATCTGGTT	GCAGGCTTGTGGGAACCGGTC	50
B6	125	GAGAGGCAGATCAAGATATGGTT	CCAGCACCTTACTCGGCCTGTTTTTCTTCC	50
B7	180	GAAAGACAGATCAAGATTTGGTT	CTGGGCTTCTCTTCGTCTCCCTTTCTCATG	175
B8	168	GAGAGACAGGTCAAAATCTGGTT	CTTGTCGCCCTTCTGCGCGTC	250
B9	156	GAGAGACAAGTCAAAATCTGGTT	GCACTGGCTTTGCAGTCGTCACAT	50
B13	134	GAGCGCCAGATTACCATCTGGTT	CCAGGACACCCCCACTTTCGC	50
Hox C				
C4	150	GAGAGGCAGATCAAAATCTGGTT	GTGGTCTTCAGAAGTACCCGGGGTA	83
C5	95	GAGAGACAGATCAAGATCTGGTT	CCCCGCTGCCTCTAAAGAGCCT	194
C6	195	GAGCGACAGATCAAAATCTGGTT	GGGAGAGAGGGGTGGCAGGGAC	111
C8	180	GAGAGACAAGTGAAGATCTGGTT	GGGGTCTTTCTTTTTTGCTTAGTCCTTGTT	194
C9	126	GAGCGGCAGGTCAAAATCTGGTT	TTTCCCTTGGCTGTGCTGAGG	56
C10	110	GACAGACAAGTCAAAATCTGGTT	CGCTCTCAGGTGAAATTAAAATTGGAGGT	278
C11	118	GACCGACAAGTGAAAATTTGGTT	AAAGGGCCCGGTCTGCAG	556
C12	142	GACCAGCAGGTCAAGATCTGGTT	TTTGCTCTCTGCCAGGGACAGG	111
C13	215	GAGCGCCAGGTAACCATCTGGTT	GCGCGGTTCTTCTCTTTCCTTAACA	56
Hox D				
D1	106	GACACGCAAGTCAAAATCTGGTT	GAAGTTGGAGGGGAGCCACAG	111
D3	94	GAACGCCAGATCAAGATCTGGTT	ACTGGCTAGCCGGCGAGTGCAGGATG	278
D4	201	GAGCGCCAGATCAAGATCTGGTT	CCAGGGTCCCCACTTCTATAAGGTCG	83
D8	140	GAGAGACAGGTAAAAATCTGGTT	CTGTCTTCCTCCAGCTCTTGGGC	278
D9	185	GAGAGACAGGTCAAAATCTGGTT	CTGGAGAGTTTCTGGAAATCAAGCACCAAACA	278
D10	122	GACAGGCAGGTCAAGATTTGGTT	TCAGACCGGCCTCAGACCTAAGA	111
D11	162	GACCGGCAAGTCAAAATCTGGTT	GAGCAGGCTGGTGGGAAGG	194
D12	98	GACCAGCAAGTCAAAATCTGGTT	CACGCGCGCGGCTAGTAG	194
D13	130	GAGAGACAAGTGACCATTTGGTT	GACAACCGAATGGCTTCTAAGCTGTC	111

showed that the Hox genes centrally located in the Hox Gene Complexes are more expressed than those located at the 5′ or 3′ end. We further analyzed the dataset, by using a hierarchical clustering algorithm. Results are shown in Figure 5. The dendrogram at the top lists the 26 human tissues studied and indicates the degrees of relatedness among tissues in terms of Hox gene expression. Functionally and anatomically related tissues demonstrated weak degrees of relationship in their expression patterns. Figure 5 also shows the degrees of relatedness among Hox genes in regard to gene expression. We anticipated that the paralogous Hox genes would be clustered by this statistical analysis. However, only weak correlation was observed. In contrast to this, several genes in the same Gene Complexes, such as Hox A3 and A4 genes, Hox B3 and B4 genes, and separately Hox D4, D8, and D9 genes, showed similar expression patterns.

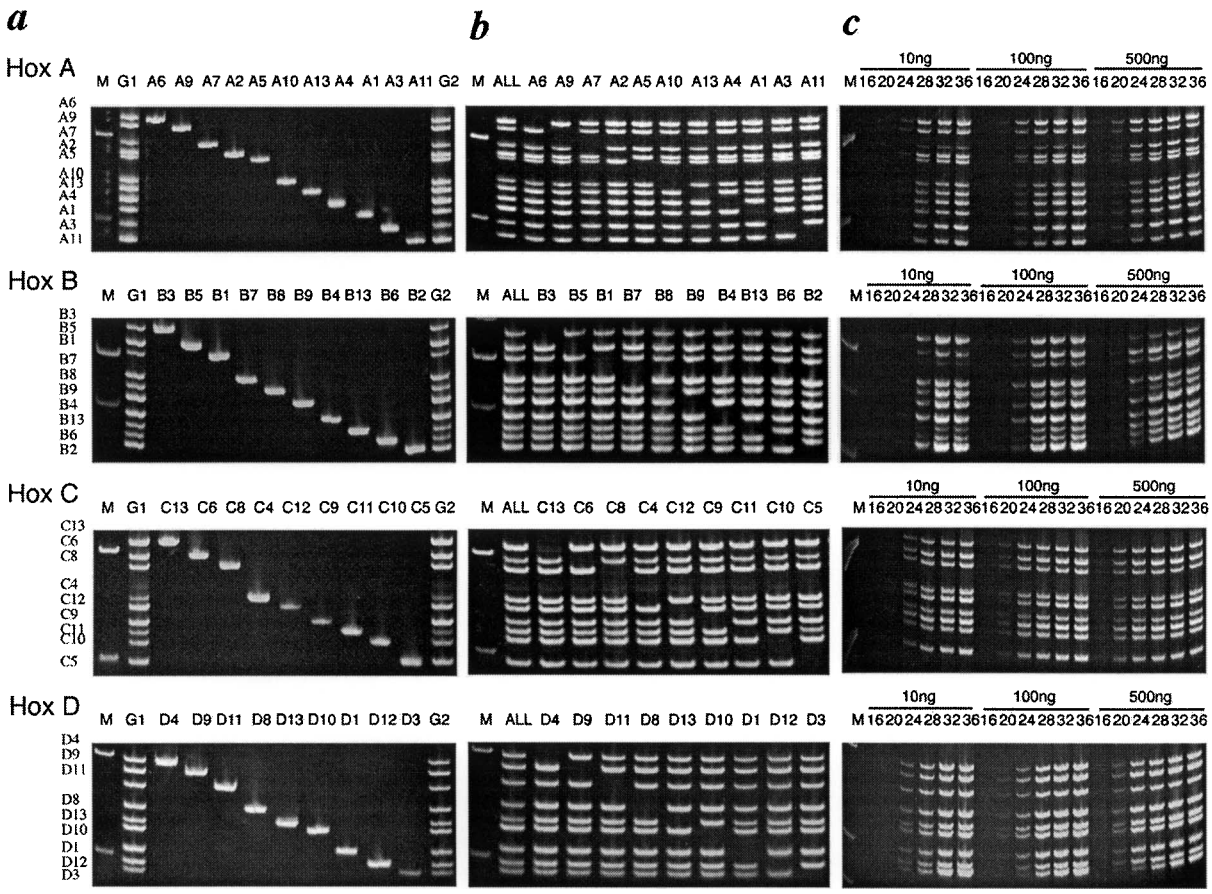

Figure 2. Three steps of establishment of the SM RT-PCR system for the 39 Hox genes. (a) Individual and multiplex PCR reaction products. Genomic DNA was used as template, and the PCR reaction products were analyzed by polyacrylamide gel electrophoresis. The names of the Hox genes whose fragments were amplified are listed on the top and the left sides of the gels. The lanes G1 and G2 show the results of multiplex PCR using two different preparations of genomic DNA. M denotes the 100-bp-ladder molecular size marker. (b) PCR of genomic DNA using primers lacking one pair. PCR was performed using primers that lacked one pair in all the combinations. The names of the genes whose primer pairs were absent in the reactions are listed on the top of the gel. The reaction with complete set of primers is shown in lane ALL. (c) Relationship between the cycle numbers of PCR and the yields of individual DNA fragments. PCR was performed using 10, 100, and 500 ng of genomic DNA as template. The reactions were stopped after 16, 20, 24, 28, 32, and 36 cycles of amplification followed by incubation for 10 min at 68°C.

DISCUSSION

Hundreds of papers have been published reporting expression analyses of homeobox genes. Most of those studies, however, analyzed only one to several genes. In the remaining limited number of studies that analyzed more genes, two methods were primarily used: the Northern hybridization method (8–10) and the RT-PCR Survey method (7,11,24,32). By modifying the SM RT-PCR method (33), we have developed a system to examine the expression of 39 Hox genes in four SM RT-PCR reactions. As opposed to the Northern hybridization method, which requires microgram quantity of RNA, the SM RT-PCR needs much less RNA. Therefore, it can be applied to the expression analyses of experimental and clinical specimens where RNA amount is limited. Another advantage of SM RT-PCR over the Northern hybridization method is that it does not require homeobox gene-specific probes. Compared with the RT-PCR Survey method that consists of PCR using two degenerate primers, and cloning and sequencing of the resultant amplified DNA fragments (7), the SM RT-PCR method is time- and cost-efficient and suitable for the simultaneous analysis of multiple samples as demonstrated in this analysis of 26 human tissues.

We initially attempted to modify the RT-PCR Survey method to quantify the Hox gene messages using

the DNA microarray technology. We amplified Hox gene transcript cDNA using two degenerate primers and then fluorescently labeled the RT-PCR products with CY5 or CY3, using Klenow enzyme and CY5-dCTP or CY3-dCTP. After the labeling reactions are stopped, both probes were mixed, and unincorporated dNTP were removed. The probes were then hybridized with DNA microarray of 76-mer oligodeoxynucleotides corresponding to the internal sequences of the amplified fragments from respective Hox genes that were spotted on glass slide by the comparative genomic hybridization (CGH) method (3,20,25). Fluorescence signal hybridized to each target spot was measured using a fluorescence scanner. We tried avoiding homologous sequences as much as possible while designing the 76-mer oligo panel, but were not successful. The pilot experiment, using predefined mixtures of Hox gene fragments as PCR templates, still resulted in the cross-hybridization of several other Hox gene sequences in the microarray (data not shown). Therefore, we switched to a gel electrophoresis-based size separation approach of SM RT-PCR, which may be advantageous in discriminating homologous sequences as it provides information on not only band intensities but also on the sizes of DNA fragments. Different from the original SM RT-PCR method where two URPs were used for PCR, we utilized conserved region sequences close to the 3′ end of homeobox to design the CRPs for the SM RT-PCR method. The other URPs were chosen for the

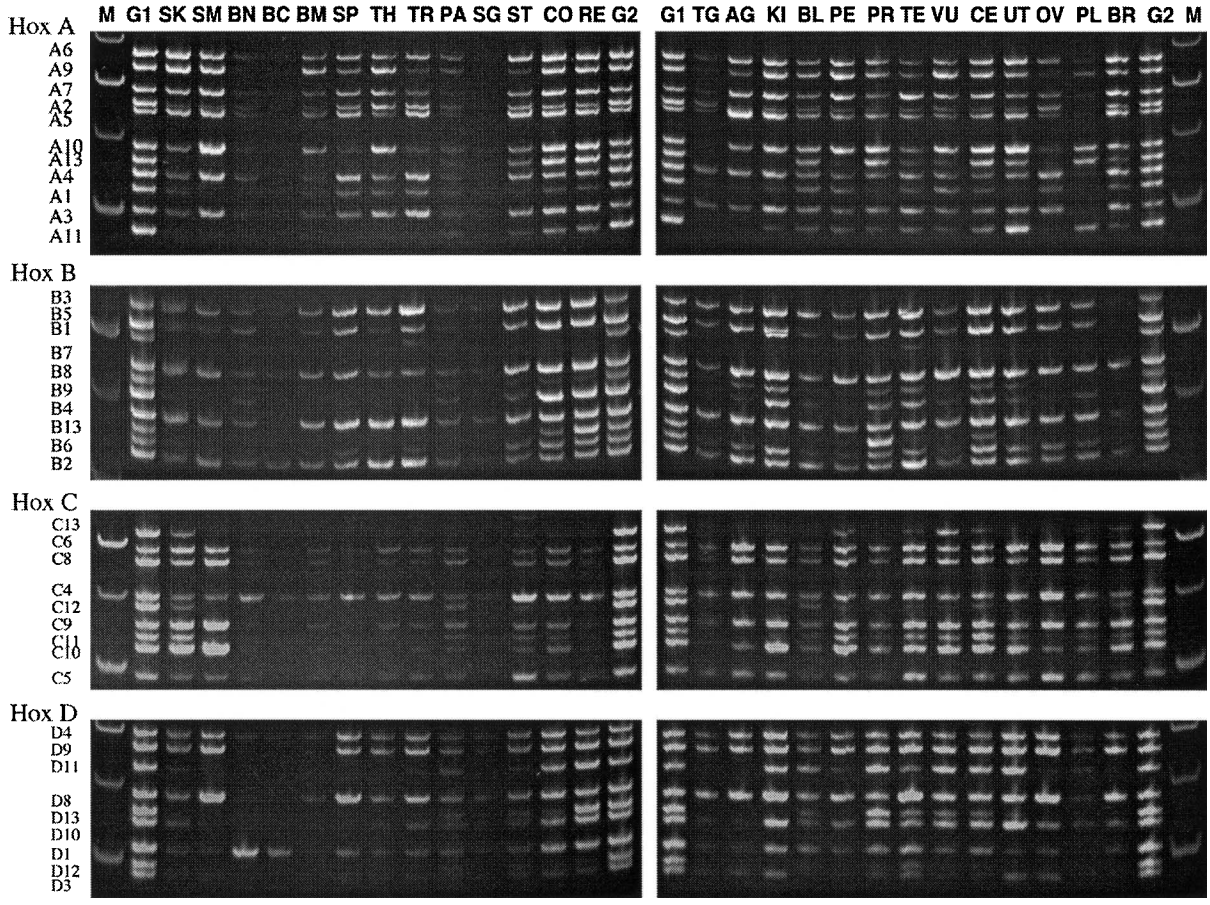

Figure 3. Differential Hox gene expression in human adult tissues. The SM RT-PCR was performed using RNA from 26 different human tissues. Reaction products were analyzed by polyacrylamide gel electrophoresis. Genomic DNA from two individuals was used as control templates and the results are shown in lanes G1 and G2. The symbols used are AG (adrenal gland), BC (brain cerebellum), BL (bladder), BM (bone marrow), BN (brain cerebrum), BR (breast), CE (cervix), CO (colon), KI (kidney), OV (ovary), PA (parotid gland), PE (penis), PL (placenta), PR (prostate), RE (rectum), SG (salivary gland), SK (skin), SM (skeletal muscle), SP (spleen), ST (stomach), TE (testis), TG (thyroid gland), TH (thymus), TR (trachea), UT (uterus), and VU (vulva). M denotes the size marker. The expected locations of the DNA fragments from the Hox genes are also shown.

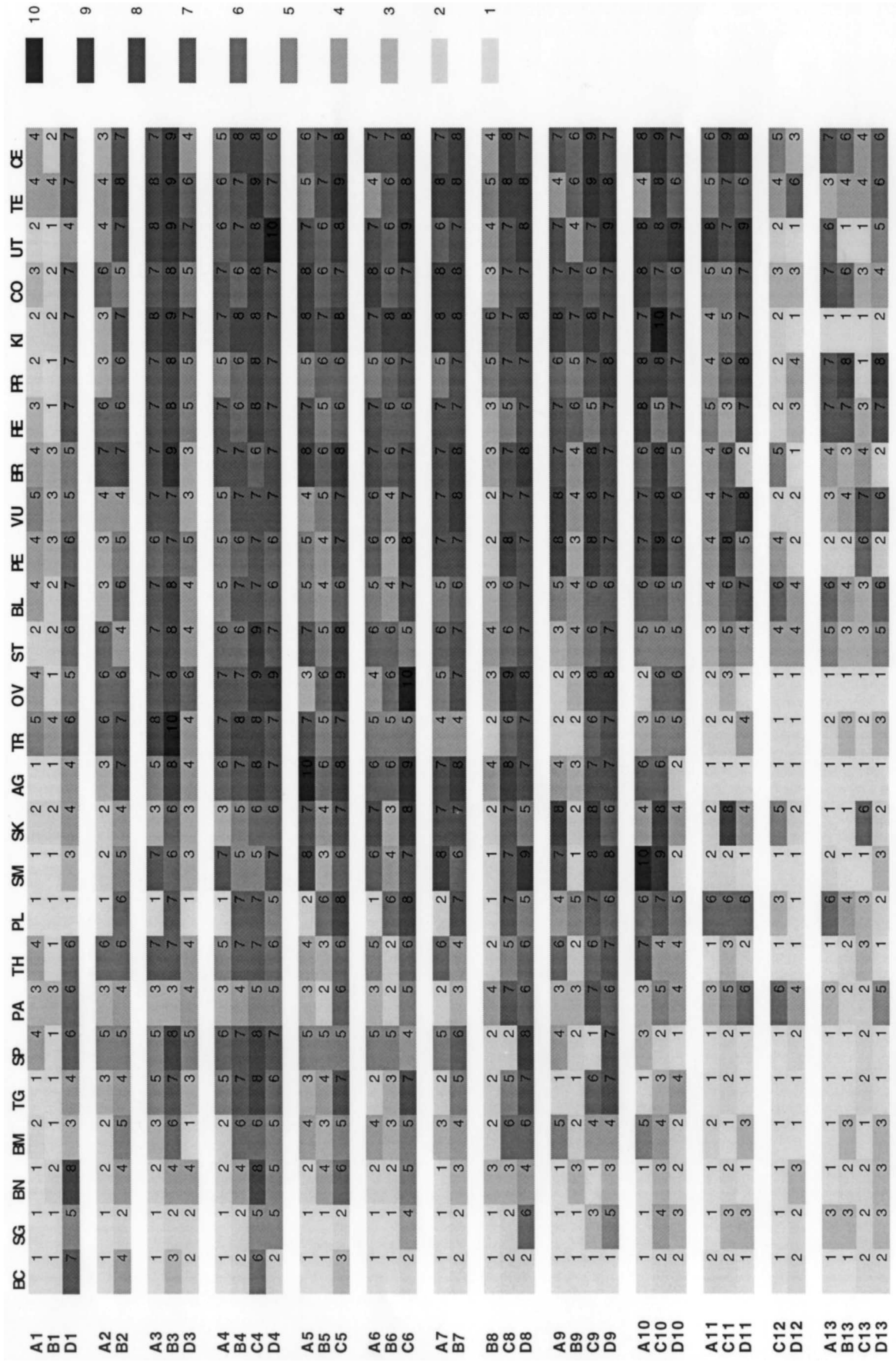

Figure 4. Hox gene expression pattern of human adult tissues. The paralogous Hox genes are gathered into a group. The expression levels are indicated by the number (10 highest and 1 lowest) as well as the brightness of boxes.

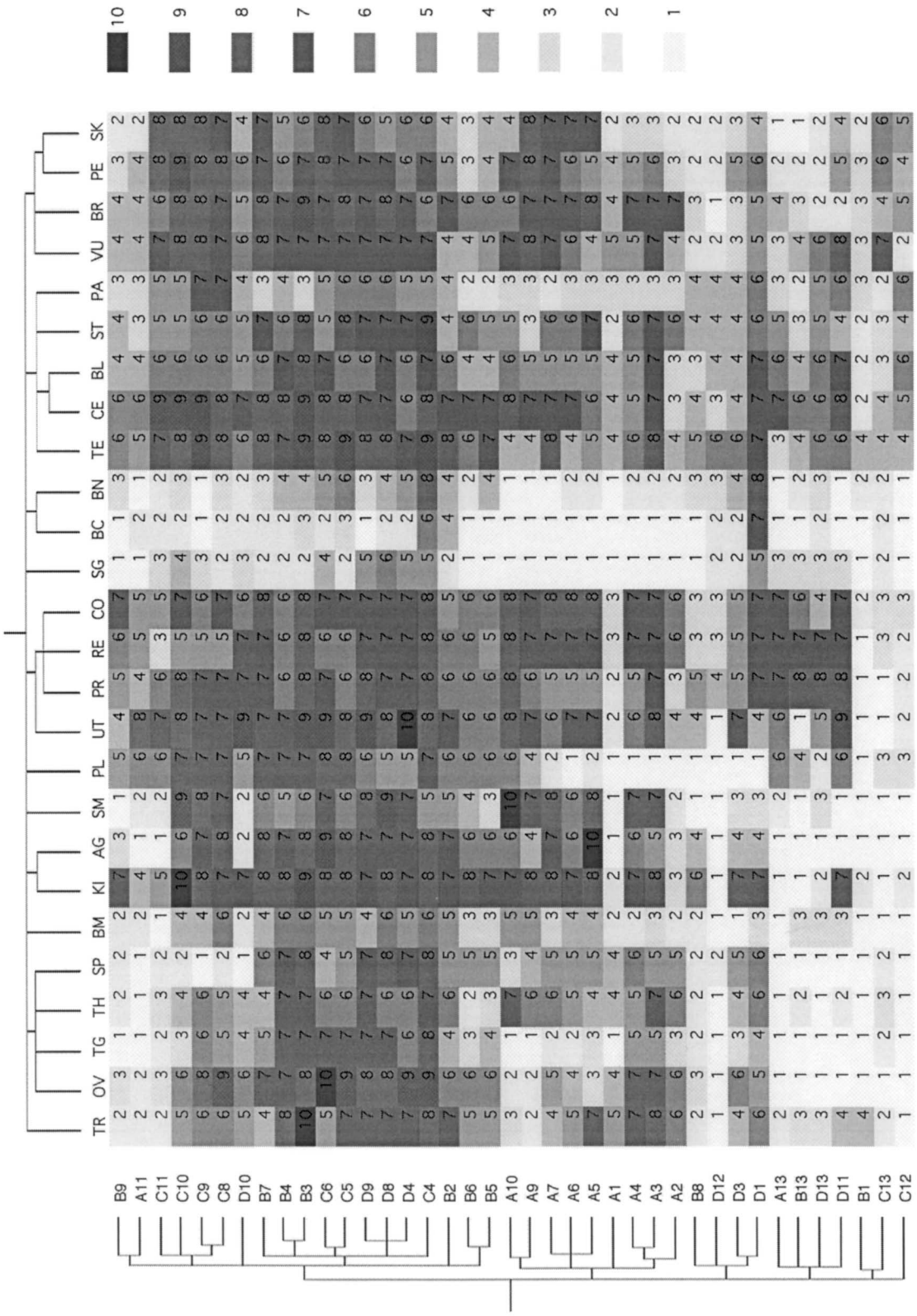

Figure 5. Hierarchical clustering algorithm analysis of Hox gene expression data. The Hox gene expression dataset obtained by the modified SM RT-PCR was converted into numbers based on the band intensity. The GeneSpring software was used to analyze the relationships among tissues and Hox gene expression. The dendrogram at the top of the panel indicates the degrees of relationship among different tissues, and the dendrogram at the left indicates the degrees of relationship among different Hox genes regarding gene expression.

3′ downstream unique sequences around termination codons. With this strategy, the 5′ ends of the amplified DNA fragments are fixed, which may greatly decrease the chance of producing artificial recombinants during PCR.

In the regular RT-PCR method, PCR is designed to amplify DNA fragments containing intron sequence(s) to differentiate the amplification from cDNA and the amplification from contaminating genomic DNA. In the SM RT-PCR method, we intentionally use primers to amplify DNA fragments of the same sizes from genomic DNA and cDNA. Accordingly, genomic DNA that contains equal number of genes can be used as a universal control template to standardize PCR efficiency, and the relative abundance of messages from the genes in different complexes may be evaluated by normalizing the band intensities using individual bands from the control genomic DNA reactions. The contaminating genomic DNA produces bands of similar intensity as shown in Figure 2c. Therefore, unless the genes are amplified or deleted due to some pathological conditions, a small degree of genomic DNA contamination in RNA preparation may not be a major problem. Even if contamination does occur, it will be easily diagnosed from the banding patterns and solved by treating RNA with RNAse-free DNAse I before cDNA synthesis. The SM RT-PCR of DNA fragments from the genes belonging to different complexes may also be achieved by designing appropriate primer combinations to amplify those genes of interest. In spite of these advantages, the SM RT-PCR method is not free from being defective. The results obtainable are not exactly quantitative because of the competition among different genes for limited amounts of DNA polymerase and substrates during PCR. Nonetheless, semiquantitative data can be obtainable. Once the gene(s) that exhibit differences or alterations in expression are identified by the SM RT-PCR method, other methods, such as real-time RT-PCR (19), may then be used to quantify the expression more accurately. In situ hybridization and immunohistochemistry may be useful in identifying the exact locations of cells that express those specific homeobox genes.

We examined the expression of Hox genes in RNA from a variety of human adult tissues and observed differences in Hox gene expression among different tissues. The results of the SM RT-PCR experiments shown in Figure 3 present the information on the expression of Hox genes in tissues at a total of 1014 data points (39×26). Among them, some coincided well with previously published data (8,10). Many of the other data are novel and have never been reported. By this moderately high-throughput study, we observed that the tissues posteriorly located tend to express a wider variety of Hox genes in higher quantity. We also found that the Hox genes located at the 5′ or 3′ end are expressed in a rather limited number of tissues. Although we observed a correlation in tissue expression among paralogous genes as shown in Figure 4, the degree of relation was not as strong as we anticipated. The hierarchical clustering algorithm analysis failed to verify this correlation among paralogous genes. This may be due to the small number (one) of RNA samples per tissue analyzed in the present study as some variations may exist in profiles among different individuals and different tissue specimens due to polymorphism and varied constituency of cell populations. However, we think it unlikely because several tissues, including kidney and colon, showed the identical banding patterns using multiple numbers of different RNA preparations (data not shown). Instead of similar expression patterns among paralogous genes, we found that several neighboring Hox genes on the same chromosomes exhibited similar tissue-specific expression pattern. The meaning of this observation is unclear. However, considering that early phase of establishment and later phase of maintenance of gene expression are regulated differently (21), these results may suggest that the regulation of Hox gene expression may be more dependent on chromosomal structure in adult tissues.

In addition to the comprehensive expression profiling of human adult tissues, the SM RT-PCR system of Hox genes may also be applied for other studies of both physiological and pathological phenomena. Those may include studies of early development, stem cell differentiation, aging, and aging-associated diseases. The research on Hox gene expression in the aging process may be especially beneficial because aging may be a continuation of development and this process may also be controlled by Hox gene function. We have developed a system to efficiently study the expression of 39 human Hox genes. In addition to the Hox gene family, other homeobox gene families also exist (4,14,15). These genes are also deeply involved in organogenesis during embryo development and, therefore, may be incorporated into the present system. Similar systems may also be produced for mice and other organisms.

ACKNOWLEDGMENTS

We thank T. T. Luong for technical and secretarial assistance. This work was supported by NIH, Department of Defense Breast Cancer Research Program, and California Breast Cancer Research Program grants to F.Y.

REFERENCES

1. Acampora, D.; D'Esposito, M.; Faiella, A.; Pannese, M.; Migliaccio, E.; Morelli, F.; Stornaiuolo, A.; Nigro, V.; Simeone, A.; Boncinelli, E. The human HOX gene family. Nucleic Acids Res. 17:10385–10402; 1989.
2. Akam, M. Hox and HOM: Homologous gene clusters in insects and vertebrates. Cell 57:347–349; 1989.
3. Albertson, D. G.; Ylstra, B.; Segraves, R.; Collins, C.; Dairkee, S. H.; Kowbel, D.; Kuo, W. L.; Gray, J. W.; Pinkel, D. Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. Nat. Genet. 25:144–146; 2000.
4. Bachiller, D.; Macias, A.; Duboule, D.; Morata, G. Conservation of a functional hierarchy between mammalian and insect Hox/HOM genes. EMBO J. 13: 1930–1941; 1994.
5. Beck, F.; Tata, F.; Chawengsaksophak, K. Homeobox genes and gut development. Bioessays 22:431–441; 2000.
6. Biggin, M. D.; Tjian, R. A purified *Drosophila* homeodomain protein represses transcription *in vitro*. Cell 58: 433–440; 1989.
7. Calvo, R.; West, J.; Franklin, W.; Erickson, P.; Bemis, L.; Li, E.; Helfrich, B.; Bunn, P.; Roche, J.; Brambilla, E.; Rosell, R.; Gemmill, R. M.; Drabkin, H. A. Altered HOX and WNT7A expression in human lung cancer. Proc. Natl. Acad. Sci. USA 97:12776–12781; 2000.
8. Cillo, C.; Barba, P.; Freschi, G.; Bucciarelli, G.; Magli, M. C.; Boncinelli, E. HOX gene expression in normal and neoplastic human kidney. Int. J. Cancer 51:892–897; 1992.
9. Cillo, C.; Cantile, M.; Mortarini, R.; Barba, P.; Parmiani, G.; Anichini, A. Differential patterns of HOX gene expression are associated with specific integrin and ICAM profiles in clonal populations isolated from a single human melanoma metastasis. Int. J. Cancer 66: 692–697; 1996.
10. De Vita, G.; Barba, P.; Odartchenko, N.; Givel, J. C.; Freschi, G.; Bucciarelli, G.; Magli, M. C.; Boncinelli, E.; Cillo, C. Expression of homeobox-containing genes in primary and metastatic colorectal cancer. Eur. J. Cancer 6:887–893; 1993.
11. Dick, M. H.; Buss, L. W. A PCR-based survey of homeobox genes in *Ctenodrilus serratus* (Annelida: Polychaeta). Mol. Phylogenet. Evol. 3:146–158; 1994.
12. Dickman, S. Possible new roles for HOX genes. Science 278:1882–1883; 1997.
13. Dolle, P.; Izpisua-Belmonte, J. C.; Brown, J. M.; Tickle, C.; Duboule, D. HOX-4 genes and the morphogenesis of mammalian genitalia. Genes Dev. 5:1767–1767; 1991.
14. Duboule, D. How to make a limb? Science 266:575–576; 1994.
15. Duboule, D.; Morata, G. Colinearity and functional hierarchy among genes of the homeotic complexes. Trends Genet. 10:358–364; 1994.
16. Dubrulle, J.; McGrew, M. J.; Pourquie, O. FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. Cell 106:219–232; 2001.
17. Graham, A.; Papalopulu, N.; and Krumlauf, R. The murine and *Drosophila* homeobox gene complexes have common features of organization and expression. Cell 57:367–378; 1989.
18. Han, K.; Levine, M. S.; Manley, J. L. Synergistic activation and repression of transcription by *Drosophila* homeobox proteins. Cell 56:573–583; 1989.
19. Higuchi, R.; Dollinger, G.; Walsh, P. S.; Griffith, R. Simultaneous amplification and detection of specific DNA sequences. Biotechnology 10:413–417; 1992.
20. Kallioniemi, O. P.; Kallioniemi, A.; Sudar, D.; Rutovitz, D.; Gray, J. W.; Waldman, F.; Pinkel, D. Comparative genomic hybridization: A rapid new method for detecting and mapping DNA amplification in tumors. Semin. Cancer Biol. 4:41–46; 1993.
21. Karch, F.; Weiffenbach, B.; Peifer, M.; Bender, W.; Duncan, I.; Celniker, S.; Crosby, M.; Lewis, E. B. The abdominal region of the bithorax complex. Cell 43:81–96; 1985.
22. Kessel, M.; Gruss, P. Murine developmental control genes. Science 249:374–379; 1990.
23. McGinnis, W.; Levine, M. S.; Hafen, E.; Kuroiwa, A.; Gehring, W. J. A conserved DNA sequence in homoeotic genes of the *Drosophila* Antennapedia and bithorax complexes. Nature 308:428–433; 1984.
24. Moretti, P.; Simmons, P.; Thomas, P.; Haylock, D.; Rathjen, P.; Vadas, M.; D'Andrea, R. Identification of homeobox genes expressed in human haemopoietic progenitor cells. Gene 144:213–219; 1994.
25. Pinkel, D.; Segraves, R.; Sudar, D.; Clark, S.; Poole, I.; Kowbel, D.; Collins, C.; Kuo, W. L.; Chen, C.; Zhai, Y.; Dairkee, S. H.; Ljung, B. M.; Gray, J. W.; Albertson, D. G. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. Nat. Genet. 20:207–211; 1998.
26. Renucci, A.; Zappavigna, V.; Zakany, J.; Izpisua-Belmonte, J. C.; Burki, K.; Duboule, D. Comparison of mouse and human HOX-4 complexes defines conserved sequences involved in the regulation of Hox-4.4. EMBO J. 11:1459–1468; 1992.
27. Scott, M. P.; Weiner, A. J. Structural relationships among genes that control development: Sequence homology between the Antennapedia, Ultrabithorax, and fushi tarazu loci of *Drosophila*. Proc. Natl. Acad. Sci. USA 81:4115–4119; 1984.
28. Stelnicki, E. J.; Komuves, L. G.; Kwong, A. O.; Holmes, D.; Klein, P.; Rozenfeld, S.; Lawrence, H. J.; Adzick, N. S.; Harrison, M.; Largman, C. HOX homeobox genes exhibit spatial and temporal changes in expression during human skin development. J. Invest. Dermatol. 110:110–115; 1998.
29. Trainor, P. A.; Krumlauf, R. Patterning the cranial neural crest: Hindbrain segmentation and Hox gene plasticity. Nat. Rev. Neurosci. 1:116–124; 2000.
30. Vasiliauskas, D.; Stern, C. D. Patterning the embryonic axis: FGF signaling and how vertebrate embryos measure time. Cell 106:133–136; 2001.

31. Venter, J.; Adams, M. D.; Myers, E. W.; Li, P. W.; Mural, R. J.; Sutton, G. G.; et al. The sequence of the human genome. Science 291:1304–1351; 2001.
32. Wang, Y.; Hung, C.; Koh, D.; Cheong, D.; Hooi, S. C. Differential expression of Hox A5 in human colon cancer cell differentiation: A quantitative study using real-time RT-PCR. Int. J. Oncol. 18:617–622; 2001.
33. Yamamoto, M.; Yamamoto, F.; Luong, T. T.; Williams, T.; Kominato, Y.; Yamamoto, F. Expression profiling of 68 glycosyltransferase genes in 27 different human tissues by the systematic multiplex reverse transcription-polymerase chain reaction method revealed clustering of sexually related tissues in hierarchical clustering algorithm analysis. Electrophoresis 24:2295–2307; 2003.
34. Zakany, J.; Duboule, D. Hox genes and the making of sphincters. Nature 401:761–762; 1999.
35. Zakany, J.; Kmita, M.; Alarcon, P.; de la Pompa, J. L.; Duboule, D. Localized and transient transcription of Hox genes suggests a link between patterning and the segmentation clock. Cell 106:207–217; 2001.
36. Zappavigna, V.; Renucci, A.; Izpisua-Belmonte, J. C.; Urier, G.; Peschle, C.; Duboule, D. HOX4 genes encode transcription factors with potential auto- and cross-regulatory capacities. EMBO J. 10:4177–4187; 1991.