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Expression of Homeobox-containing Genes in Primary and Metastatic Colorectal Cancer

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Homeobox genes are a network of genes encoding nuclear proteins functioning as transcriptional regulators. Human and murine homeobox genes of the HOX family are organised in four clusters on different chromosomes. Gene order within each cluster is highly conserved, perhaps in direct relation to their expression. Homeobox genes have recently been involved in normal development and oncogenesis. We have analysed HOX gene expression in normal human colon and in primary and metastatic colorectal carcinomas. The majority of HOX genes are active in normal adult colon and their overall expression pattern is characteristic of this organ. Furthermore, the expression of some HOX genes is identical in normal and neoplastic colon indicating that these genes may exert an organ-specific function. In contrast, other HOX genes exhibit altered expression in primary colon cancers and their hepatic metastases which may suggest an association with colon cancer progression.
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INTRODUCTION

COLORECTAL CARCINOMAS rank high among the most frequent human malignancies. Such tumours may arise from benign adenomatous polyps, which later progress to adenocarcinomas through several mutational steps [1]. Some of these events have been better understood through the identification of the genes 'Familial Adenomatous Polyposis' (FAP) and 'Deleted in Colorectal Cancer' (DCC) involved in colon tumorigenesis [2–3]. The overall biological characteristics of colorectal cancers, and of neoplastic tissues in general, result from accumulated genetic alterations rather than from the order in which these events occur with respect to one another [1]. Even though several important genes have been identified, other events, which remain to be elucidated, may well take place during the progression of colon cancer.

Homeobox (HOX) genes are a family of genes containing a common 183-nucleotide sequence. The HOX encodes a 61 amino acid domain, the homeodomain (HD), which includes a helix-turn-helix motif responsible for the DNA binding ability of HOX-containing proteins [4]. On the basis of structural similarities and direct evidence that *Drosophila* homeodomain proteins are capable of binding DNA sequences and modulating transcriptional activity, it is generally accepted that HD proteins are transcriptional regulators [5]. The HOX was originally discovered in genes controlling *Drosophila* development [6] and has subsequently been isolated in other, evolutionarily distant species, such as nematodes and vertebrates [7]. Different HOX gene families have evolved which encode HD of different types or classes. Among these HD the *Drosophila antennapaedia* (Antp) HD defines one consensus sequence referred to as class I HD [4]. Mammalian class I HOX genes are clustered in restricted regions of the genome (HOX loci) on four distinct chromosomes that presumably evolved by duplication of a primordial gene cluster [8]. A striking finding is that the order of genes within each cluster is also highly conserved throughout evolution, suggesting that the physical organisation of HOX genes may be essential for their expression [10]. HOX genes are expressed during embryogenesis in a tissue-specific and frequently stage-related fashion [11]. Expression of individual HOX genes has been detected in normal adult tissues [8–12].

A possible association between genes that control transcription and those involved in the oncogenic process has been postulated on the basis of several independent observations. Constitutive expression of the HOX-2.4 gene may entail oncogenic consequences in mice [13]. Mice homozygous for a null mutation in the HOX-1.5 and HOX-1.6 genes show major morphological abnormalities [14–15]. The growth factor activin activates HOX gene expression in developing Xenopus embryos [16]. The coordinate regulation of HOX genes may play an important role in human haemopoietic differentiation [17]. HOX gene

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expression appears to be altered in renal cancer compared to normal human kidney tissues [12].

In line with the above association between HOX genes, development and oncogenesis, our aim has been to determine whether the physical organisation of HOX genes might be a part of a regulatory network involved in the control of such processes. We have thus analysed the expression of a panel of 38 HOX genes in adult human tissues originating from normal intestinal mucosa or liver parenchyma from colorectal carcinoma biopsy samples and liver metastases from colorectal cancers. We have identified HOX genes (HOX1J, HOX2F) whose expression remains unaltered during progression of colorectal tumours. We interpret this result as an intestinal-specific expression which may suggest the involvement of the corresponding homeoproteins in organ-specific functions. Expression of other HOX genes (HOX2C, HOX4F), however, is altered in primary and metastatic colorectal cancer suggesting the possible implication of these transcriptional regulators in colon tumorigenesis.

MATERIALS AND METHODS

Normal and neoplastic colon, liver metastases from colorectal carcinoma patients and normal liver adjacent to metastatic foci were obtained from untreated, non-selected patients at the Istituto di Patologia Chirurgica, University of Florence (Italy) and at the Centre Hospitalier Universitaire Vaudois (CHUV, Lausanne, Switzerland). During colectomy, the tumour mass was dissected and a sample from non-necrotic colorectal cancer tissue as well as another of intact normal colonic mucosa, from an area within a few centimeters of the tumour, were taken. In the case of liver metastasis of primary colorectal cancer, a biopsy of the metastatic mass and another from a zone of nearby intact normal liver were taken. The surgically removed specimens were snap-frozen in liquid nitrogen and stored at −80°C until RNA was extracted.

RNA extraction and analysis

Frozen tissues were pulverised in a blender. Total RNA was extracted by the guanidinium thiocyanate technique [18] and poly (A)+ selected by one passage on oligo (dT) cellulose columns. Poly (A)+ RNA was run on 1.25% agarose–formaldehyde gels, transferred to nylon (Schleicher and Schuell, NY13N) membranes by northern capillary blotting. Five micrograms of poly (A)+ RNA were hybridised to 10^7 cpm of DNA probe, labelled by nick-translation to a specific activity of $3–8 \times 10^8$ dpm/μg. The probes contained the 3′ untranslated regions specific for each of the 38 HOX genes as previously reported [19–21]. Prehybridisation and hybridisation were carried out as described [22]. After washing under stringent conditions (30 mmol/l NaCl/3 mmol/l sodium citrate/0.2% sodium dodecyl sulphate at 65° C), the blots were exposed for 1–7 days at −70°C to Kodak XR-5 films in an X-omatic intensifying screen cassette.

RESULTS

We analysed the expression of the four HOX gene clusters in normal human intestinal mucosa and in tumour samples derived from patients with primary colorectal cancer. Poly (A)+ RNA from normal and neoplastic tissues were hybridised by northern blotting with probes containing the 3′ untranslated region specific for each of the 38 HOX genes, organised in four large clusters, HOX1 to HOX4, located on chromosomes 7, 17, 12, and 2, respectively, as shown in Fig. 1.

The pattern of HOX gene expression as observed in normal intestinal mucosa is illustrated in Fig. 1. Of the 38 HOX genes tested, 29 appear to be actively expressed in normal intestinal mucosa. Overall, the normal colon shows very strong expression of genes in HOX1 and HOX2 loci, whereas genes within the HOX3 and HOX4 clusters function at much lower levels. The entire HOX1 locus, containing 11 genes (HOX1J through HOX1F), is expressed, although at different levels for individual genes. The genes located at both 5′ and 3′ ends of the locus are strongly expressed, while two contiguous genes (HOX1B and HOX1C) in the middle part of the locus appear to be barely detectable. Virtually all the genes of the HOX2 cluster are expressed with the exception of HOX2I, located at the 3′ far end of the locus. In contrast, five contiguous genes at the 5′ end of the HOX3 locus (HOX3G through HOX3B) remain silent, while a group of four genes (HOX3A through HOX3E) are intensively expressed. Genes of the HOX1, HOX2 and HOX3 clusters are thus switched on or off in normal intestinal mucosa in blocks containing a variable number of contiguous genes within each locus. In contrast, in the HOX4 locus, only a small group of three contiguous genes is expressed, whereas the remaining genes are alternatively switched on or off. The complex pattern of the whole HOX gene clusters, illustrated in Fig. 1, is characteristic of the normal colonic mucosa. The same analysis performed in normal kidney [12], lung (Tiberio *et al.*, in preparation) or liver (Fig. 2) exhibits different patterns of expression with different HOX genes switched on or off in these organs.

Figure 2 illustrates HOX gene expression in normal colonic mucosa as compared to colorectal cancer tissues in terms of positive and negative expression. Only qualitative differences in the patterns of HOX gene expression have been taken into account. No differences in expression can be detected between normal and neoplastic colon with regard to genes at the 5′ end of the HOX1 locus, HOX1J through HOX1G, which are constantly highly expressed. In contrast, marked heterogeneity is observed in biopsy samples of primary tumours as compared to normal and neoplastic tissues in the expression of the 3′ HOX1 genes (HOX1D through HOX1F). Virtually all the HOX2 genes are active and their expression does not vary when normal tissues are compared to colorectal cancer specimens. Similarly, a group of five genes at the 5′ end of the HOX3 locus (HOX3G through HOX3B) remain silent in colon cancer as well as in normal tissues. In contrast, the 3′ end HOX3 genes (HOX3A through HOX3E) appear to be heterogeneously expressed in colorectal cancers. Finally, the HOX4 locus is characterised by a marked variable expression of HOX genes.

Several differences in expression of individual HOX genes have indeed been detected when comparing normal intestinal mucosa to primary colon carcinomas. The HOX2C gene (Fig. 3) is expressed in normal colonic mucosa with two transcripts of 1.4 and 1.6 kb. The short mRNA is more abundantly expressed than the long one. However, in primary colon cancer biopsies, the two mRNA are equally abundant. Furthermore, in some tumour biopsies two longer additional transcripts (2.3 and 2.7 kb), previously described in other organs, are transcribed.

The gene HOX4B is expressed in normal intestinal mucosa, two transcripts of 5.4 and 2.8 kb being barely detectable (Fig. 3). The molecular mechanisms regulating the expression of this gene have been well elucidated [23]. Two alternative promoters underlie the transcription of two classes of HOX4B-specific mRNA: the 5.4 and 2.8 kb transcripts are driven from a distal promoter while the 4.2 and 1.4 kb transcripts originate from a proximal promoter. The two promoters are differentially

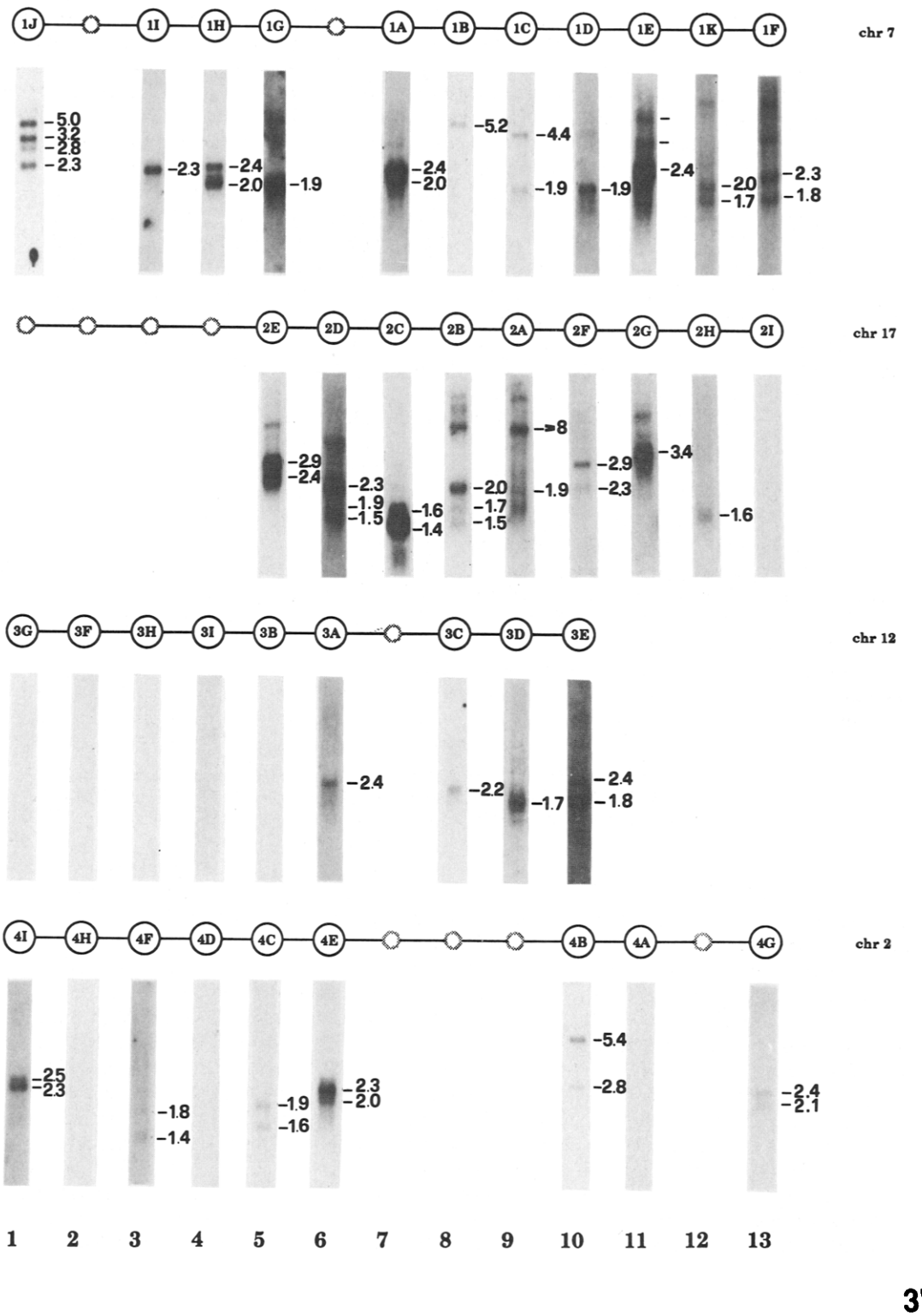

Fig. 1. HOX gene expression in normal human colon. HOX genes are aligned horizontally according to their physical position on the chromosomes and vertically on the basis of maximal sequence homology of the homeodomain. The homeodomains are indicated in the circles above each lane. Small stippled circles indicate homeodomains predicted in the scheme but not yet found. The 13 homeodomain groups are indicated at the bottom. Transcript sizes are given in kb.

regulated in a tissue- and stage-specific manner and respond differentially to retinoic acid induction [24]. The expression of HOX4B in colorectal cancer is shown in Fig. 3. In eight out of 12 biopsies tested this gene is silent (Fig. 3, T1). In three out of 12 cases it shows a level of expression comparable to that seen in normal intestinal mucosa with two transcripts of 5.4 and 2.8 kb (Fig. 3, T2). In one case out of 12 it shows an intense expression due to the activation of the proximal promoter with two transcripts of 4.2 and 1.4 kb (Fig. 3, T3).

The HOX gene expression pattern in normal adult human

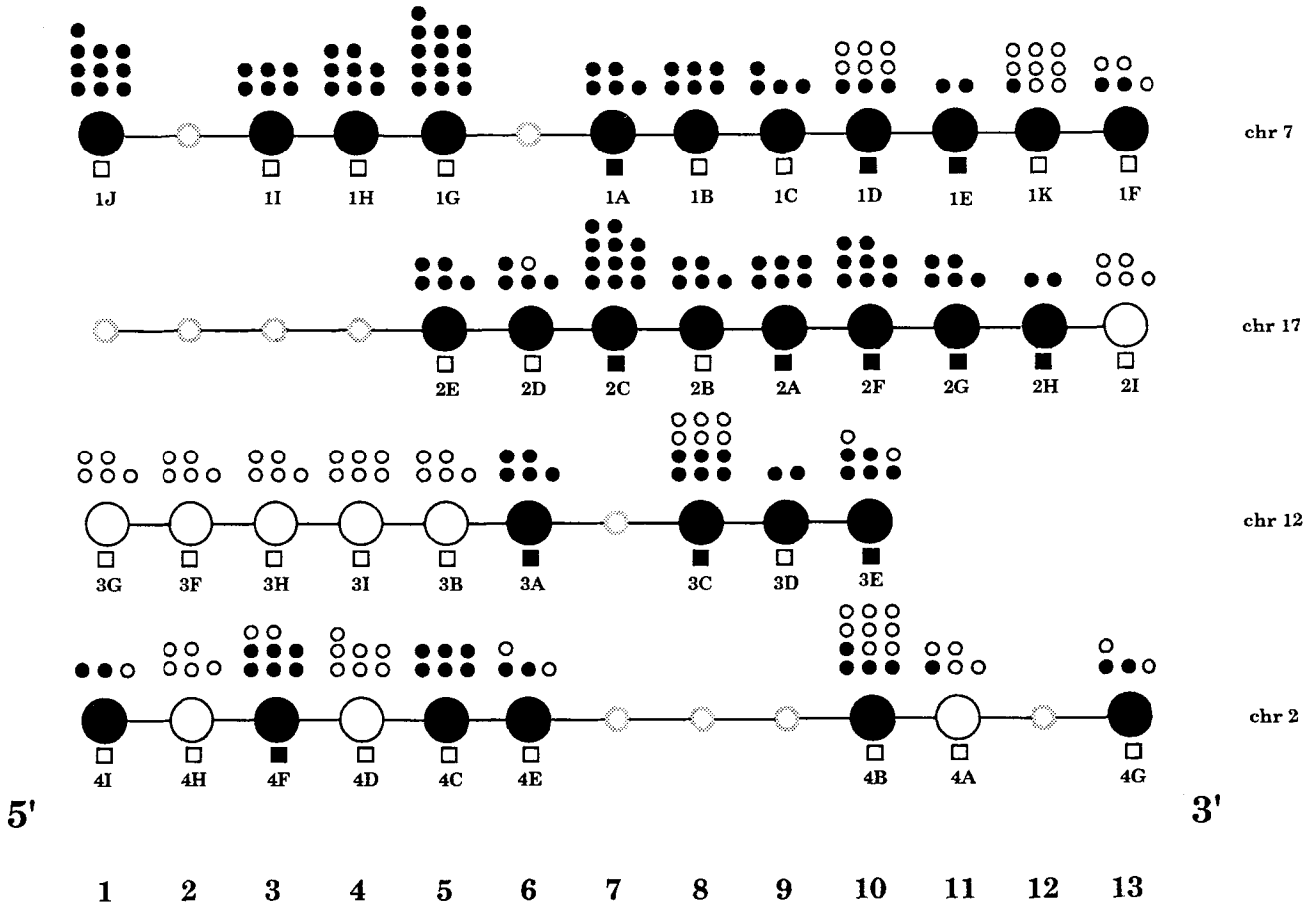

Fig. 2. Diagram of HOX gene expression in normal and neoplastic colon, and in normal liver. Different symbols indicate different tissues. Closed or open symbols indicate active or silent HOX genes, respectively. (●○) represent normal colon; (●○) represent each individual cancer biopsy analysed; (■□) represent normal liver.

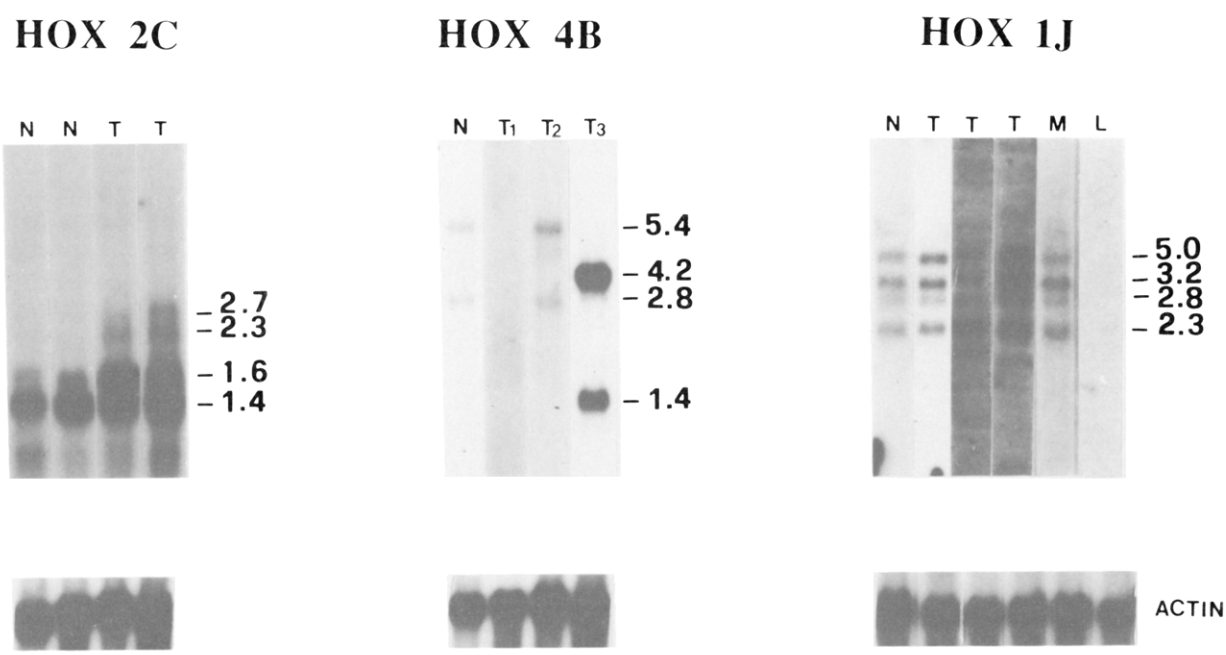

Fig. 3. Expression of HOX2C, HOX4B, HOX1J in normal and neoplastic colon. Expression of HOX2C, HOX4B and HOX1J genes in normal colonic mucosa (N) in primary colorectal cancer (T) in normal liver (L) and in liver metastases originating from colorectal cancer (M). Transcript size is indicated in kb. Control hybridisation to a β-actin probe is shown.

liver is reported in Fig. 2. We found only 12 out of 38 HOX genes actively expressed, their expression being predominant at the 3′ end of the loci HOX1, HOX2 and HOX3. The HOX2 locus indeed shows the highest number of HOX genes expressed (five out of nine). A single gene of the HOX4 locus, HOX4F, is expressed, and furthermore, its position is the most 5′ relative to the whole panel of genes expressed in the liver.

We have compared HOX gene expression in liver metastases from colorectal cancer, in the surrounding normal liver, in primary colon cancers and in the normal colonic mucosa coming from the same patient. The HOX1J gene is expressed in normal intestinal mucosa and in colorectal cancer with four transcripts (5.0, 3.2, 2.8 and 2.3 kb) (Fig. 3). This gene belongs to the block of genes at the 5′ of the locus HOX1 whose expression appears to be unaltered in all the primary and metastatic colorectal cancers we tested. This gene is completely silent in normal liver. Similar results are observed with other genes: Fig. 4 shows expression of the HOX2E gene, in the HOX2 locus, which expresses two mRNA of 2.4 and 2.9 kb in normal intestinal mucosa, in primary colon cancer and in liver metastases from the same patient. No expression of this gene has been detected in the normal liver.

The HOX1A gene expresses two transcripts (2.4 and 2.0 kb) in normal colon and in primary colon cancer. In normal liver the HOX1A gene is barely detectable with two mRNA of 2.0 and 4.3 kb. In addition to the two mRNA expressed in the normal colon, individual liver metastasis show two additional transcripts (4.3 and 1.8 kb) which are detectable together (Fig. 4, M3) or separately (Fig. 4, M2-M4), suggestive of post-transcriptional alterations.

The HOX4F gene in normal colonic mucosa expresses two transcripts (1.8 and 1.4 kb), whereas in normal liver it displays one 2.0 kb transcript only. This gene is expressed in 80% of all primary tumours analysed so far and shows an extra mRNA of 2.0 kb in 20% of the biopsies. In liver metastases the expression of HOX4F appears to be highly variable (Fig. 4). Some metastases very intensively express three transcripts (2.0, 1.8 and 1.4 kb) (Fig. 4, M6), others do not show any expression at all (Fig. 4, M7) or express a unique band of 2.0 kb (Fig. 4, M9); finally, others express the same transcripts as those detected in primary colon cancer samples (Fig. 4, M8).

DISCUSSION

The experiments presented here were aimed at determining whether the physical organisation of HOX genes reflects a regulatory network involved in normal intestinal organogenesis and/or in colon cancer progression. Therefore, we have analysed the expression of the four HOX gene clusters in normal human colon as well as in primary and metastatic colorectal cancer. Our results show that the majority of HOX genes are active in normal adult colon and that their overall expression pattern is characteristic of this organ. Furthermore, our observations highlight that the expression patterns of some HOX genes are identical in normal and neoplastic colon indicating that these genes may be involved in organ-specific functions. In contrast, other HOX genes exhibit altered expression in primary colon cancers and their hepatic metastases which may suggest an association with colon cancer progression.

In the normal colon HOX genes are highly expressed with respect to both the number of HOX genes turned on and the abundance of individual HOX transcripts. Interestingly, the genes of the HOX1, HOX2 and HOX3 clusters are turned on or off in blocks, containing a variable number of contiguous genes within each locus. In contrast, contiguous genes of the HOX4 cluster are switched on or off in an alternate fashion. The expression pattern of the HOX4 cluster is peculiar to the colon since in the haematopoietic system [17] and in the normal kidney [12] HOX4 genes are coordinately expressed. The coordinate regulation of HOX genes is consistent with the idea that one or more upstream promoter elements account for the concerted expression of HOX genes in differentiating systems. Experimen-

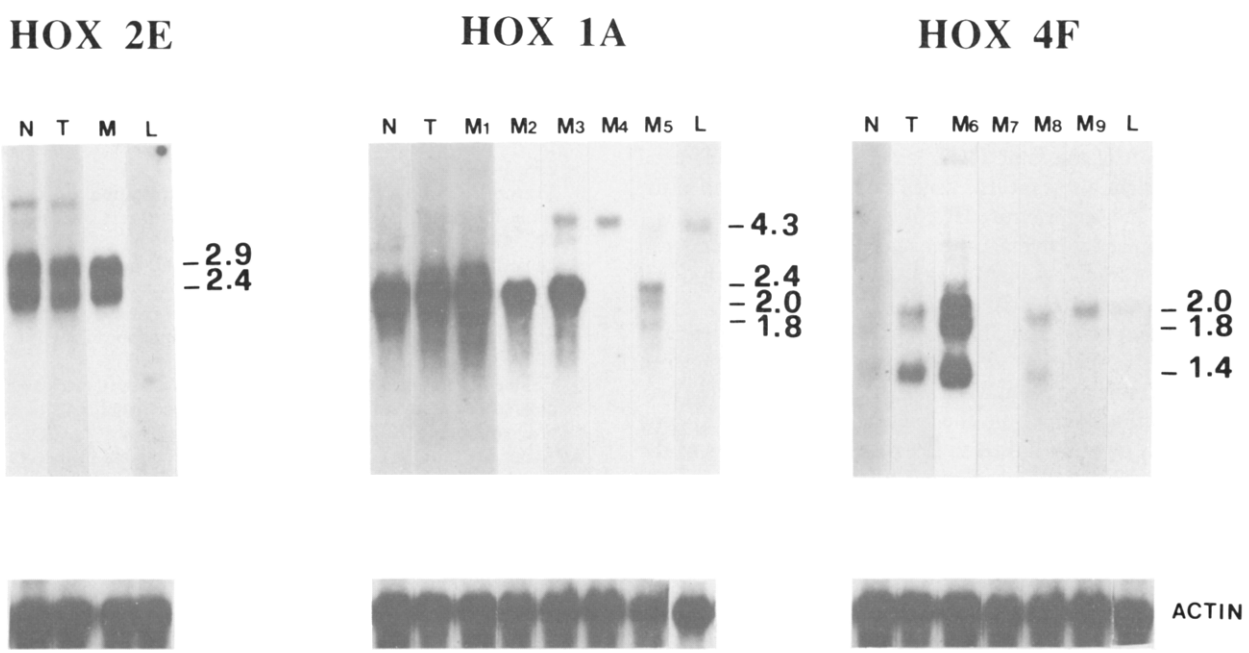

Fig. 4. Expression of HOX2E, HOX1A, HOX4F in normal and neoplastic colon. Expression of HOX2E, HOX1A and HOX4F genes in normal colonic mucosa (N) in primary colorectal cancer (T) in normal liver (L) and in liver metastases originated from colorectal cancer (M). Transcript size is indicated in kb. Control hybridisation to a β-actin probe is shown.

tal evidence for a major promoter upstream of several HD-containing exons of the HOX3 locus has been reported [25].

Comparing different normal human organs such as liver, colon, kidney and lung suggests that HOX genes may display overall patterns of expression that are characteristic for each organ. Furthermore, the expression of several specific HOX genes appears to vary among individual organs. For example, we have observed identical expression patterns for a block of four genes located at the 5′ end of the HOX1 locus (HOX1J through HOX1G) in the normal colon as well as in all our biopsy samples, whether these originated from the primary or from the metastatic tumours so far tested. In contrast, the expression of the same group of genes is different in normal kidney [12], liver (Fig. 2) and lung (Tiberio, personal communication). In addition to their probable organ-specific function, the HOX genes and their expression may become a means to molecularly fingerprinting different normal adult tissues.

In colorectal cancer the expression of individual HOX genes is sometimes altered as compared to that seen in normal colonic mucosa. For instance, the HOX2C and HOX4B genes, although both expressed in normal and neoplastic colon, display different classes of transcripts according to their size. For the HOX2C gene, two transcripts with high molecular weight, already described in teratocarcinoma cells activated by retinoic acid [19], are detected in primary tumours vs. normal colon. In normal colon and in 25% of the primary colon cancer biopsies, the HOX4B gene exhibits the two transcripts driven from the distal promoter. The majority of colorectal cancer specimens do not express this gene. However, in only one out of the 12 cancer biopsies tested, we did observe a switching off of the distal promoter and a switching on of the proximal one driving two alternative transcripts.

Biopsy samples of primary tumours are markedly heterogeneous according to the expression patterns of HOX genes located at the 3′ end of the HOX1 locus. For example, HOX1 genes from 1D through 1F, are consistently expressed in normal tissue, whereas they are silent in most colorectal cancer specimens of our series (Fig. 2). Indeed, transgenic mice overexpressing the HOX gene Hox 1.4 (the murine analog of the human gene HOX1D) exhibit abnormal gut development, resulting in an inherited abnormal megacolonic phenotype [26] reminiscent of human congenital Hirschsprung's disease. Such discontinuous turning off of the same HOX genes in different colorectal cancer specimens also concerns genes located at the 3′ end of the locus HOX3 and on the entire HOX4 locus. Taken together, our results on primary colorectal cancer suggest that HOX genes can be regulated at transcriptional as well as at a post-transcriptional level, possibly by differential splicing.

Two explanations may be proposed for the differences observed in the patterns of HOX gene expression between normal and malignant colon tissues. First, these differences may reflect intrinsic changes in the mechanisms of HOX gene transcription, possibly linked to the acquired properties of the transformed cell phenotype. Second, they may predominantly reflect differences between the various cell populations present in the normal colon, and the dominant clonal population produced during colorectal cancer cell proliferation.

Some HOX genes (HOX1A, HOX4F) exhibit altered expression in metastatic lesions compared to primary colorectal tumours and to normal intestinal mucosa. Novel transcripts can indeed be expressed in some metastases compared to primary tumours or different amounts of mRNA may be produced in metastatic vs. primary colon cancer. However, it has not been possible to identify any specific alterations of HOX gene expression that would systematically characterise metastatic vs. primary colorectal cancer tissues. In contrast, other HOX genes (HOX1J, HOX2E) display identical patterns of expression in normal colon, primary colorectal cancer and corresponding liver metastases when these are derived from one individual patient. If this holds true with large series, it may perhaps become possible to use HOX genes as molecular probes to determine the site of a primary cancer when dealing with probable metastases of unknown primary origin.

We could not establish any correlation between HOX gene expression and the clinical outcome of our patients due to (a) the low number of cases analysed; (b) the small amount of RNA Poly (A)+ obtained from several biopsies; (c) the high number (38) of HOX genes we attempted to screen for each specimen and (d) the choice of using northern blotting for the purpose of obtaining quantitative and qualitative data on HOX gene expression. Consequently, our results must be taken as only indicative of general trends in HOX gene expression in normal and neoplastic colon. More clinically oriented analyses involving a much larger number of cases are needed to identify any specific relation between patterns of expression of HOX genes and to confirm their possible role in colon cancer progression.

In conclusion, our observations suggest that homeoproteins may play a role in the evolution of human solid tumours. Future experiments and DNA analysis are required in order to understand the involvement of HOX genes in human cancer. A wide variety of sequence-specific DNA-binding proteins, including *myb*, *jun*, *fos*, Rb, Sp1 and GATA-1 have indeed been implicated in the mechanisms of oncogenesis [27–29]. Futhermore, there is evidence that interactions between different DNA-binding proteins and other proteins that form part of transcriptional complexes are essential in normal differentiation and may be altered in oncogenesis [30]. The alterations of HOX gene expression we detected in colorectal cancer would stress once again the importance of the role that transcription factors play in the evolution of neoplasia.

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Tissue Concentrations of Prothymosin Alpha: A Novel Proliferation Index of Primary Breast Cancer

Fernando Dominguez, Carlos Magdalena, Esperanza Cancio, Elena Roson, Jesus Paredes, Lourdes Loidi, Juan Zalvide, Maximo Fraga, Jeronimo Forteza, Benito J. Regueiro and Jose L. Puente

In 71 patents with classic invasive ductal carcinomas, levels of prothymosin alpha (PTα), as assayed by a radioimmunoassay that detects thymosin alpha 1 (the NH_2-terminal fragment of PTα), were significantly greater in tumour samples than in normal breast tissue. PTα levels were correlated with (a) the number of positive axillary lymph nodes ($r_s = 0.5384$, $P < 0.01$), and (b) the percentage of tumour cells in the S or G2/M phase as assessed by flow cytometry ($r_s = 0.5027$, $P < 0.01$). Since the beginning of this study in 1989, 21 patients have presented distant metastases, all of whom were previously shown to have tumour PTα levels greater than 124 ng of thymosin alpha 1/mg protein. The present report indicates that PTα might be used to identify breast cancer patients at high risk for distant metastases.
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INTRODUCTION

A MAJOR EFFORT in cancer research is aimed at finding new parameters that enable more precise means of identifying patients at high risk for local recurrences and distant metastases [1]. There are numerous indications that prolonged, increased cell proliferation is necessary for the development of tumours, particularly for hormonally related tumours, tumours secondary to various chemical exposures or virally related tumours (for a review see [2]). In breast cancer, cell proliferation indices have been shown to be prognosis-related [3].