**INTRODUCTION**

Homeobox-containing genes (Hox) code for nuclear proteins termed homeoproteins (Levine and Hoey, 1988; McGinnis and Krumlauf, 1992). The homeobox is a highly conserved stretch of 183 bp that codes for a homeodomain (HD) that confers on the products of these genes the ability to recognise and bind specific DNA sequences (Gehring et al., 1990).

Hox genes were first identified in *Drosophila* where they cooperate in controlling the development of the body plan, providing positional information along the anteroposterior axis (Levine and Hoey, 1988; Kessel and Gruss, 1990). Clustering of homeotic genes was also first recognised in *Drosophila* where class I Hox genes (those containing a homeobox most closely related to the archetypal Antennapedia homeobox) are located in two genomic regions, the Antennapedia and the Bithorax complexes (Gehring and Hiromi, 1986).

In vertebrates class I Hox genes are present in at least four clusters located in four different chromosomes (Boncinelli et al., 1988; Duboule and Dollé, 1989; Graham et al., 1989). Their role in controlling vertebrate morphogenesis was demonstrated by several experiments in which their normal regulation was disrupted (Wright et al., 1989; Ruiz i Altaba and Melton, 1989; Cho et al., 1991) or they were inactivated in the germline (Lufkin et al., 1991; Chisaka and Capecchi, 1991).

The pattern of expression of Hox genes has been extensively studied during embryogenesis in man, mouse and *Xenopus*, where they are expressed in overlapping domains along the anteroposterior body axis (Gaunt et al., 1988; Akam, 1989) following the 5′-posterior/3′-anterior rule, first observed in *Drosophila* and termed colinearity (Lewis, 1978). Several Hox genes have also been found expressed in normal adult tissues, most notably during haematopoiesis (Lawrence and Largman, 1992), in tumour cells and in cultured cell lines of myeloid, lymphoid, erythroid, epithelial and neural origin (Kongsuwan et al., 1988; Peverali et al., 1990; Magli et al., 1991; Corte et al., 1993).

Platform - nuclease localisation associated with the nucleolus. We used monoclonal antibodies to study the distribution of three homeoproteins, namely HOXB7, HOXC6 and HOXD4. The immunoreactivity to antibodies against HOXC6 protein in *Xenopus laevis* embryonic tissues is restricted to one or two spots within the nucleus; this distribution partially overlaps that of fibrillarin, a protein of the fibrillar zone of the nucleoli. Indirect immunofluorescence analysis of the distribution of HOXB7 protein in 3T3 cells, and of HOXD4 protein in human neuroblastoma and Raji lymphoma cell lines and activated lymphocytes, results invariably in a nucleolar localisation. Purified nucleoli from stimulated T lymphocytes, and Raji cells contain an activity capable of binding, in a gel retardation assay, to an oligonucleotide specifically recognised by the HOXD4 homeoprotein. This activity is specifically removed by anti-HOXD4 antibodies and is found associated in southwestern blots with a single band with an apparent Mr, of 30,000, corresponding to that of recombinant HOXD4.

The functional significance of the nucleolar localisation of Hox proteins remains to be determined.

**SUMMARY**

Homeoproteins encoded by genes of the Hox family are nuclear proteins believed to act as transcription factors and to participate in the determination of the body plan. Here we show that in several vertebrate cells, they exhibit a sub-nuclear localisation associated with the nucleolus.

We used monoclonal antibodies to study the distribution of three homeoproteins, namely HOXB7, HOXC6 and HOXD4. The immunoreactivity to antibodies against HOXC6 protein in *Xenopus laevis* embryonic tissues is restricted to one or two spots within the nucleus; this distribution partially overlaps that of fibrillarin, a protein of the fibrillar zone of the nucleoli. Indirect immunofluorescence analysis of the distribution of HOXB7 protein in 3T3 cells, and of HOXD4 protein in human neuroblastoma and Raji lymphoma cell lines and activated lymphocytes, results invariably in a nucleolar localisation. Purified nucleoli from stimulated T lymphocytes, and Raji cells contain an activity capable of binding, in a gel retardation assay, to an oligonucleotide specifically recognised by the HOXD4 homeoprotein. This activity is specifically removed by anti-HOXD4 antibodies and is found associated in southwestern blots with a single band with an apparent Mr, of 30,000, corresponding to that of recombinant HOXD4. The functional significance of the nucleolar localisation of Hox proteins remains to be determined.

**Key words:** nucleolus, homeogene, differentiation
proteins encoded by human genes HOXB7, HOXC6 and HOXD4 produced in insect cells using the baculovirus expression system (Corsetti et al., 1992).

MATERIALS AND METHODS

Monoclonal antibodies

The monoclonal antibodies 3B2 (anti-HOXC6), 5BL (anti-HOXB7) and 4CS (anti-HOXD4) were prepared as previously described (Corte et al., 1981). Briefly, Balb/c mice were injected intraperitoneally at 7-day intervals with 10 mg of oligonucleotide affinity-purified recombinant HOXC6, HOXB7 or HOXD4. Mice were killed 4 days after the fourth injection and spleen cells were fused with P3X63Ag8U1 (P3U1) myeloma cells according to the method of Gefter et al. (1977). The hybridoma supernatant was tested in ELISA plates coated with nuclear lysates of infected S9 cells expressing the HOXB7, C6 or D4 homeoprotein. Cultures positive for the relevant protein only were cloned in soft agar.

Cell culture and staining

Paraffin sections were prepared for staining according to published procedures (Levi et al., 1987). For immunofluorescence, sections were deparaffinised in xylene (3x 2 minutes), rehydrated and incubated sequentially with the primary monoclonal antibody (10 µg ml⁻¹ in PBS, 5% foetal calf serum (FCS); overnight), biotinylated goat anti-mouse IgG secondary antibodies (10 µg ml⁻¹ in PBS, 5% FCS; 2 hours) and FITC-conjugated streptavidin (5 µg ml⁻¹ in PBS, 5% FCS; 30 minutes). Laser scanning confocal microscopy was performed using a Zeiss Axioskop microscope equipped of a Sarastro 2000 (Molecular Dynamics) laser and image-processing system. Mouse 3T3 cells were cultured on glass coverslips in DME containing 10% FCS, fixed in PBS/3.7% paraformaldehyde for 5 minutes and permeabilised with 0.1% Triton X-100. After 3 hours of incubation with the primary antibody (10 µg ml⁻¹) the slides were incubated for 1 hour with FITC-conjugated goat anti-mouse Ig antibodies (10 µg ml⁻¹). SK-N-SH human neuroblastoma cells cultured on glass coverslips in RPMI 1640 containing 15% FCS were treated with 10 mM all-trans retinoic acid for 10 days (Peverali et al., 1990). The cells were fixed in 50:50 (v/v) methanol/acetic acid and stained with 4CS as above except that the second antibody was TRITC-conjugated. Raji cells attached to a polylysine-coated glass slide were fixed and stained as above. Peripheral blood lymphocytes, containing approximately 70% T cells, were obtained by fractionation over a Ficoll gradient and either used immediately or stimulated with the T cell mitogen PHA in RPMI 1640 containing 10% FCS. HeLa cells were transfected by the standard calcium phosphate method using the pRC/CMV vector (Invitrogen) containing the relevant cDNA under the cytomegalovirus promoter. Cells were harvested after two days and stained as above.

Electrophoretic mobility shift assay

Mobility shift experiments were performed with the blunt-ended double-stranded oligonucleotide 4B-26 (5'-GCCAAGGCCCATAG-GCTACCGAATT-3') as described (Corsetti et al., 1992). Nuclei were obtained from Raji cells by centrifugation at 100 g, of cells lysed in PBS containing 0.5% NP40 and 10 mM MgCl₂, through a cushion of 0.25 M sucrose containing 10 mM MgCl₂. After two washings in 0.25 M sucrose, the nuclear extract was obtained by resuspending the nuclear pellet in two volumes of 25 mM HEPES, pH 7.9, 620 mM NaCl, 1 mM DTT, 1 mM MgCl₂ (Buffer HS). Purified nucleoli were prepared from the nuclei by successive cycles of sonication and centrifugation through a cushion of 0.88 M sucrose as described (Busch, 1967). ‘Core’ nucleoli were prepared by digestion with 25 µg/ml of DNase I for 30 minutes at RT as described (Bolla et al., 1985). Nucleolar extracts were obtained resuspending the nucleolar pellet in two volumes of Buffer HS. For immunodepletion 0.3 ml of nucleolar extract were rotated for 1 hour at 4°C with 50 µl of Sepharose 4B beads coupled either to 4CS or to normal human Igs, and the supernatant was recovered by low speed centrifugation. The procedure was repeated once more before incubation of the supernatant with the labelled oligonucleotide.

RESULTS

After the first selection, the specificity of the three monoclonal antibodies was tested by indirect immunofluorescence on S9 monoclonal antibody (green) and anti-fibrillarin antibody (Lapeyere et al., 1990) (red). Nucleoli of neural cells are stained by both antibodies, whereas nucleoli from liver are only stained by anti-fibrillarin antibodies. A,B, ×400; C,D, ×5000; E, ×2000.

Fig. 1. Distribution of 3B2 (anti-HOXC6) immunoreactivity in developing Xenopus tissues. Strong staining is always localised within the nucleolus. (A) Confocal microscope analysis of a section through a rostral region of the spinal cord of a stage 45 embryo. Double staining with 3B2 monoclonal antibody (green) and a polyclonal anti-N-CAM antibody (red). Most neuronal cell bodies are positive for both molecules. While N-CAM stains all neuronal plasma membranes, anti-HOXC6 immunoreactivity is localised in one or two spots within the nucleus. ep, ependyma. (B) Section through the visceral part of a stage 41 embryo, double stained with propidium iodide and 3B2 monoclonal antibody. All nuclei are stained in orange. Nuclei of the intestinal epithelium contain a bright yellow spot corresponding to the stain of 3B2 monoclonal antibody. Nuclei of the overlying epidermids (e) are not stained by 3B2 antibody. (C-E) High magnification confocal analysis of sections through the nervous system (C,D) and through the liver (E). Double staining with 3B2
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cells expressing different homeoproteins. As shown in Table 1 each antibody stained Sf9 cells that expressed the protein used to produce it and did not stain Sf9 cells transfected with the other homeoproteins.

The monoclonal antibody 3B2, raised against the human HOXC6 protein, recognises the homologous protein (known as XlHbox-1) in Xenopus embryos (Levi et al., 1993) and stains all tissues known to express XlHbox-1 mRNA. The same

Fig. 2. Staining of human and mouse cells with 5BL (anti-HOXB7), 3B2 (anti-HOXC6) and 4C5 (anti-HOXD4) monoclonal antibodies. (A) Mouse 3T3-J2 cells stained with 5BL. (B) Mouse 3T3-J2 cells stained with 3B2. (C) Mouse 3T3-J2 cells stained with 4C5. (D) Raji cells stained with 5BL. (E) Raji cells stained with 3B2. (F) Raji cells stained with 4C5.
A typical pattern of staining was observed in all positive cells including, for example, spinal cord neurons (Fig. 1A) and epithelial cells of the intestine (Fig. 1B). A closer inspection of the tissue sections revealed that the immunoreactivity was always confined to one or two spots within the nucleus of the positive cells. As only one pair of chromosomes contributes to the nucleolus in *X. laevis*, these data suggest a nucleolar localisation for the HOXC6 protein. To confirm the nucleolar localisation we double stained the sections with 3B2 antibody and polyclonal antibodies directed against fibrillarin, a major protein of the fibrillar zone of the nucleolus. In all cases, high magnification analysis (Fig. 1C, D) showed colocalization of the immunoreactivity with a largely, albeit not entirely, overlapping pattern. No 3B2 immunoreactivity was found in nucleoli of the embryonic liver, which were, however, positive with anti-fibrillarin antibodies (Fig. 1E). The 4C5 and 5BL antibodies did not recognise any epitope in *Xenopus* embryos.

In view of the results obtained in tissue section we used the 3B2 antibody and two other antibodies, 5BL and 4C5 directed against HOXB7 and D4, respectively, to investigate the localisation of Hox proteins in cultured cells where one can determine much more closely the possible relations with the cell cycle and where biochemical analysis is much easier. The reactivity of the 4C5 antibody (anti-HOXD4) in human cells was tested using a neuroblastoma cell line, SK-N-SH, which was previously shown to express HOXD4 mRNA after stimulation with retinoic acid (Peverali et al., 1990). In accordance with the mRNA expression, the 4C5 antibody stained SK-N-SH cells only after treatment with retinoic acid (Fig. 2A, B), while 5BL and 3B2 were always negative. In the absence of cell lines in which expression of HOXB7 or C6 could be induced, we performed transfection experiments in HeLa cells, which constitutively express HOXD4 but not HOXB7 and C6 mRNA. Only monoclonal antibody 4C5 (anti-HOXD4) stained untransfected cells, while 5BL (anti-HOXB7) and 3B2 (anti-

### Table 1. Reactivity of the three monoclonal antibodies to Sf9 cells expressing different homeoproteins

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<th>Homeoproteins:</th>
<th>HOXA10</th>
<th>HOXB7</th>
<th>HOXC6</th>
<th>HOXD4</th>
<th>EVX1</th>
<th>EMX1</th>
<th>EMX2</th>
<th>OTX2</th>
<th>Uninfected</th>
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<tr>
<td>3B2</td>
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Sf9 cells infected with each recombinant baculovirus with a low multiplicity were harvested 48 hours post-infection and stained with each antibody.

![Fig. 3. Staining of human cells with 5BL (anti-HOXB7) and 4C5 (anti-HOXD4) monoclonal antibodies. (A) SK-N-SH human neuroblastoma cells before induction with retinoic acid, stained with 4C5. (B) SK-N-SH human neuroblastoma cells induced with retinoic acid, stained with 4C5. (C) Human resting peripheral blood lymphocytes stained with 4C5. (D) Human peripheral blood T cells 24 hours after stimulation with phytohemagglutinin (PHA), stained with 4C5. (E) Human peripheral blood T cells 72 hours after stimulation with PHA, stained with 4C5. (F) Human peripheral blood T cells 72 hours after stimulation with PHA, stained with 5BL. ×630.](image-url)
HOXC6) were positive only in cells transfected with HOXB7 and C6, respectively (data not shown).

We detected immunoreactivity only to the 5BL antibody in mouse 3T3-J2 cells, which express the HoxB7 mRNA (data not shown) (Fig. 3A, B, C), while the 4C5 antibody stained human Raji Burkitt lymphoma cell line (Fig. 3D, E, F), in agreement with the mRNA expression data (Celetti et al., 1993). In all instances one to several spots were stained per nucleus, which is consistent with the fact that several mammalian chromosomes contribute to the nucleolus, namely five pairs in human and six in mouse cells. Again, in agreement with the mRNA expression data, the anti-HOXC6 antibody was consistently negative on all cell lines.

Very few resting T lymphocytes from peripheral blood are stained by the 4C5 antibody (Fig. 2C), while most of PHA-stimulated T cells (Fig. 2D, E) are positive. Thus, a homeoprotein recognised by the 4C5 antibody is expressed in T lymphocytes upon stimulation. The pattern of staining is consistent with the stages of nucleolar fusion in different phases of the cell cycle (Anastassova-Kristeva, 1977). After stimulation, T cells are activated but do not start to divide until after 48-72 hours. Accordingly, 24 hours after stimulation, all cells show a single large spot, corresponding to the single large nucleolus typical of the late G1 and S phases (Fig. 2D), while two days later, when division has started, most cells show several spots (Fig. 2E), a pattern consistent with the variable degree of nucleolar fragmentation typical of the other phases of the cell cycle.

Confirmation of these observations was derived from an independent set of experiments. We purified nucleoli from both Raji cells and activated T lymphocytes, which could be grown in sufficient numbers, and tested the presence of HOX product by a DNA band-shift assay. Using a specific oligonucleotide, a band-shift activity was observed in nucleolar extracts from both Raji cells (Fig. 4A, lane 2) and stimulated T lymphocytes (Fig. 4B, lane 1) but not in extracts from whole nuclei (Fig. 4A, lane 1). This activity is absent from the nucleoli of resting T lymphocytes (Fig. 4B, lane 2). In addition, the band-shift activity present in the nucleoli of Raji cells and stimulated T lymphocytes showed the same gradient of affinity for three different oligonucleotides as the recombinant HOXD4 produced with the baculovirus (Corsetti et al., 1992). The highest affinity is for the oligonucleotide 4B-26 present in the promotor region of the HOXD4 gene (Fig. 3A, lane 2 and 3B, lane 1); weaker binding is observed to cp11-25 and even weaker to HCR-19 (not shown). Furthermore, the binding is specifically competed for by increasing amounts of the same unlabelled oligonucleotide.

In southwestern blots of nucleolar extracts from Raji cells, the oligonucleotide 4B-26 revealed a single band with the expected Mr of 30,000 (Fig. 5B, lane 4), in good agreement with the apparent Mr of the recombinant HOXD4 (Corsetti et al., 1992) and different from that of HOXA10, B7, C6, EVX1, EMX1, EMX2 and OTX2. Although highly homologous, the polypeptide chains of homeoproteins do vary widely in length. No such band was visible in nucleolar extracts from resting T cells, which are not stained by the 4C5 antibody (Fig. 5B, lane 5).

The band-shift activity can be specifically removed from nucleolar extracts of Raji cells by repeatedly incubating them with Sepharose-bound 4C5 antibody (Fig. 4C, lane 2) but not with Sepharose-bound non-specific antibodies (Fig. 4C, lane 1).

The protein is not loosely associated with the nucleoli as it can be extracted only with high salt concentrations, > 0.5 M NaCl, and cannot be released from the nucleoli by treatment with 25 µg/ml DNase I for 30 minutes.

### DISCUSSION

Using three functional complete recombinant homeoproteins, HOXB7, HOXC6 and HOXB4, produced by the baculovirus system (Corsetti et al., 1992), we have prepared monoclonal antibodies specific to each one of them. In the course of a study aimed at defining the expression of these factors at the single-cell level in *Xenopus* embryo (Levi et al., 1993) in human lymphocytes and cell lines and in mouse 3T3 (unpublished data), the high definition afforded by these antibodies revealed an unexpected nucleolar localisation of the homeoproteins. This localisation was confirmed in *Xenopus* by double staining with an anti-fibrillarin antibody. The conclusion is independently confirmed by showing the presence, in highly purified nucleoli from human activated T lymphocytes and Raji cells, of a protein with the expected Mr and DNA-binding specificity. That this protein was the one recognised by the antibody was confirmed by the disappearance of the specific DNA-binding activity after immunodepletion of nucleolar extracts. The homeoprotein cannot be removed from the purified nucleoli by treatment with DNase I and can be eluted only with an NaCl concentration >0.5 M, indicating a strong association with the core of the nucleoli and ruling out the possibility that the protein is uniformly distributed within the nucleus and trapped specifically by the nucleolar structures. It is worth noting that the concentration of the homeoprotein in whole nuclear extracts (including nucleoli) was below the limit of detection of the gel retardation assay, while the ~50-fold purification...
to define their pattern of expression within a body or a tissue and not within the cell itself.

The observation that three Hox proteins exhibit a nucleolar localisation in three different species and in different cell lineages, seems to indicate that the phenomenon is general and cannot be explained by a role played by a particular homeo-protein controlling a precise differentiation step.

At least two hypotheses can be considered to account for our observation. According to the first hypothesis, none of the nucleolar functions is regulated by the Hox proteins. The proteins are active in different nuclear compartments and become transiently associated with the nucleolus, which would function like a storing device, i.e. a sort of reservoir used to keep their nuclear concentration constant. Alternatively, as the subcellular location usually indicates the potential function of a protein, we have to consider the possibility of a specific function of these proteins in the nucleolus. Even if the various roles played by the nucleolus in the cell are still poorly understood, it is well known (Hadjolov, 1985) that morphological and biochemical alterations in the nucleoli precede and accompany major events in the life of cells; for instance, the surge of ribosome biogenesis in cells undergoing the G₀-G₁ transition. Other regulatory proteins with a nucleolar localisation (Bouche, 1987; Su et al., 1993) have been shown to be involved in the regulation of cell growth by controlling rRNA synthesis. Homeoproteins are believed to control differentiation through the regulation of specific batteries of genes. It could well be that they also control the proliferation rate by modulating nucleolar function.

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obtained by isolating the nucleoli increased its concentration not only above this limit but also above the limit of south-western blot, a much less sensitive technique. It must be stressed that these results do not imply that Hox proteins are localised exclusively to the nucleolus. They could well be present in the nucleus at a concentration too low to be detected by these techniques and yet high enough to function.

The homeoproteins are regulatory factors that cooperate to control cell differentiation and proliferation (Levine and Hoey, 1988; McGinnis and Krumlauf, 1992). Although it is now generally accepted that they exert this control by binding to specific DNA sequences present in promoters and enhancers of target genes, little is known about the cellular processes regulated by these proteins and about the mechanism by which this control is exerted. To our knowledge, their precise intracellular localisation has not been investigated, either because of lack of suitable reagents or because the study was intended...

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