Expression of HOXC4 Homeoprotein in the Nucleus of Activated Human Lymphocytes

By Raffaella Meazza, Antonio Faiella, Maria Teresa Corsetti, Irma Airoldi, Silvano Ferrini, Edoardo Boncinelli, and Giorgio Corte

We have analyzed the expression of homeoproteins of the HOX family in resting and activated lymphoid cells and in neoplastic lymphoid cell lines by the use of monoclonal antibodies (MoAbs) already shown to react with the homeoproteins HOXA10, HOXC6, and HOXD4, respectively. Anti-HOXA10 and C6 MoAbs did not show any reactivity with the lymphoid cells tested, whereas anti-HOXD4 MoAb stained few resting peripheral blood lymphocytes (PBLs) and most phytohemagglutinin (PHA)-stimulated PBLs as early as 6 hours after stimulation. The pattern of staining of PHA-activated PBLs is reminiscent of the stages of nuclear fragmentation in different phases of the cell cycle. The MoAb reacted also with activated or Epstein-Barr virus-transformed B cells, with clonal or polyclonal T and natural killer (NK) cells, with leukemic T-cell lines, and with a Burkitt’s lymphoma cell line. RNase protection experiments, performed with probes specific for HOXD4 or for the highly homologous HOXA4, HOXB4, and HOXC4, belonging to the same paralogy group, indicated that only HOXC4 mRNA is present in resting or activated PBLs. Northern blot analysis on polyA+ RNA from activated PBLs or Raji cells showed the presence of two different HOXC4 transcripts of 2.8 and 1.9 kb. Gel retardation and Southwestern blot assays showed the presence of a 32-kD homeoprotein with DNA-binding properties typical of a HOX4 homeoprotein in nucleolar extracts of PHA-activated, but not of resting, lymphocytes. Taken together, these data indicate that the HOXC4 homeoprotein is expressed in activated and/or proliferating lymphocytes of the T-, B-, or NK-cell lineage, whereas it is weakly expressed in a minority of resting cells. The early expression and the nucleolar localization suggest an involvement of HOXC4 in the regulation of genes controlling lymphocyte activation and/or proliferation.

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MATERIALS AND METHODS

Isolation, Culture, and Staining of Lymphocytes and Cell Lines

Peripheral blood mononuclear cells (PBMCs) from normal volunteers were isolated by Ficoll-Hypaque density gradients. PBMCs were used either immediately or after stimulation in culture with 1% (vol/vol) phytohemagglutinin (PHA; Gibco, Grand Island, NY) or with 1 mg/mL OKT3 MoAb for different time intervals. Culture medium was RPMI 1640 supplemented with 10% fetal calf serum (FCS). B-cell–enriched populations were obtained from tonsillar mononuclear cells depleted from T cells by rosetting with neuraminidase-treated sheep red blood cells (SRBCs). These B-cell–enriched populations were activated in culture with an optimal dilution of Staphylococcus aureus Cowan strain 1 (SAC) and with 100 U/mL of human recombinant interleukin-2 (IL-2) for 72 hours. T- and natural killer (NK)-cell clones and polyclonal lines used in this study were obtained from purified T- or NK-cell populations by the use of a PHA-dependent microculture system, as previously described. They were expanded in IL-2–containing medium and characterized for their surface markers expression by indirect immunofluorescence and cytofluorometric analysis, using commercially available MoAbs, as previously described.

MoAbs to HOXA10 and HOXD4 were produced and characterized as previously described. For the assessment of reactivity with anti-HOX MoAbs, cells were washed in medium without FCS, attached to poly-L-lysine–coated multistep slides (ICN Biomedicals, San Francisco, CA), and fixed in cold methanol/acetic acid (50/50 vol/vol). After drying, slides were saturated with phosphate-buffered saline (PBS) containing 10% FCS and then stained overnight at 4°C with the appropriate dilution of 4CS, 3B2, or D7.87 anti-HOX MoAbs or with UCHT-1 (anti-CDS MoAb, kindly provided by Dr Peter Beverley, ICRF, London, UK) or with an irrelevant MoAb as controls. After five washings, fluorescein isothiocyanate (FITC)-

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conjugate goat antimouse Ig was added for 2 hours. The slides were washed extensively and scored for positivity.

For immunoprecipitation, Sf9 cells were labelled for 1 hour with 100 μCi of [35S]-methionine 2 days after infection with the relevant recombinant Baculovirus. The nuclear extract, obtained as previously described, was incubated for 1 hour with the 4C5 antibody and rotated for 3 hours with 50 μL of Sepharose-bound protein A. After washing three times with PBS, the Sepharose beads were boiled in gel loading buffer and loaded onto a 12% polyacrylamide gel.

**RNA Analysis**

**RNase protection.** Cytoplasmic RNA was extracted and hybridized overnight in 40-μg aliquots to radiolabeled RNA probes corresponding to the analyzed HOX genes or actin RNA probe at 55°C. Mixtures were then digested with RNase A and T1 (1 hour at 32°C) and proteinase K, extracted with phenol-chloroform, and precipitated in ethanol. Electrophoresis was performed on a 6% polyacrylamide sequencing gel, which was then dried and exposed for 24 to 48 hours at −70°C to Kodak XAR5 film. Quantitation of the relative amounts of protected mRNA was performed by standard densitometry.

**Northern blot.** Total RNA was extracted by the guanidine thiocyanate technique. Poly(A)+ was selected by one passage on oligo (dT) cellulose column, run in 25 μg on 1.0% agarose-formaldehyde gel, and transferred to nylon membrane by Northern capillary blotting. Filters were hybridized to 107 cpm of HOXC4 probe labeled by nick translation to a specific activity of 3.6 × 108 dpm/mg, washed at high stringency (final wash, 0.1% sodium dodecyl sulfate [SDS], 0.1× SSC at 60°C), and exposed for 72 hours to Kodak XAR5 film. HOXC4 probe was a 250-bp EcoRI/HindIII clone.

**Gel Retardation and Southwestern Blot Analysis**

Mobility shift experiments were performed with the blunt-ended double-stranded oligonucleotide 4B-26 (5′GCCAAGCCCAATTAGGCTACCTGAACT3′) as described.99 Nuclei were obtained from resting and from 24-hour PHA-activated PBLs by centrifugation at 700 g of cells lysed in PBS containing 0.5% NP40 and 10 mmol/L MgCl2, through a cushion of 0.25 mol/L sucrose, 10 mmol/L MgCl2. After two washings in 0.25 mol/L sucrose, the nuclear extract was obtained resuspending the nuclear pellet in 2 vol of 25 mmol/L HEPES, pH 7.9, 620 mmol/L NaCl, 1 mmol/L DTT, and 1 mmol/L MgCl2 (buffer HS). Purified nucleoli were prepared from the nuclei by successive cycles of sonication and centrifugation through a cushion of 0.88 mol/L sucrose. “Core” nucleoli were prepared by digestion with 25 μg/mL of DNase I for 30 minutes at room temperature. Nucleolar extracts were obtained by resuspending the nucleolar pellet in 2 vol of buffer HS.

For Southwestern blot analysis, a nucleolar extract from resting or PHA-activated lymphocytes was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% polyacrylamide gel and transferred to nitrocellulose according to standard proce-
Table 1. Reactivity of Normal and Neoplastic Lymphoid Cells With Anti-HOX MoAbs

<table>
<thead>
<tr>
<th>Cells</th>
<th>Lineage (phenotype)*</th>
<th>Reactivity With MoAb Anti-HOX1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal line</td>
<td>T CD3^+4^+8^+ TCRß^+^</td>
<td>A10</td>
</tr>
<tr>
<td>Polyclonal line</td>
<td>T CD3^+4^+8^+ TCRy^+^</td>
<td>-</td>
</tr>
<tr>
<td>Clone G1.MV</td>
<td>T CD3^+4^+8^+ TCRy^+^</td>
<td>NT</td>
</tr>
<tr>
<td>Clone DM 2.6</td>
<td>T CD3^+4^+8^+ TCRß^+^</td>
<td>-</td>
</tr>
<tr>
<td>Clone DM4.8</td>
<td>T CD3^+4^+8^+ TCRß^+^</td>
<td>-</td>
</tr>
<tr>
<td>Polyclonal line</td>
<td>NK CD3^+16^+</td>
<td>-</td>
</tr>
<tr>
<td>Clone</td>
<td>NK CD3^+16^+</td>
<td>-</td>
</tr>
<tr>
<td>Line 82 EBV</td>
<td>EBV-transformed B cell</td>
<td>NT</td>
</tr>
<tr>
<td>Line 51 EBV</td>
<td>EBV-transformed B cell</td>
<td>NT</td>
</tr>
<tr>
<td>Jurkat</td>
<td>T-ALL</td>
<td>-</td>
</tr>
<tr>
<td>MOLT 13</td>
<td>T-ALL</td>
<td>-</td>
</tr>
<tr>
<td>MOLT 4</td>
<td>T-ALL</td>
<td>-</td>
</tr>
<tr>
<td>H9</td>
<td>T-ALL</td>
<td>-</td>
</tr>
<tr>
<td>CEM</td>
<td>T-ALL</td>
<td>-</td>
</tr>
<tr>
<td>RAJI</td>
<td>Burkitt’s lymphoma</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviation: NT, not tested.

* Surface antigens of clones and polyclonal T or NK cell lines were studied by indirect immunofluorescence and cytofluorimetric analysis.

† Reactivity of anti-HOX MoAbs was evaluated by indirect immunofluorescence on fixed cells and scored by fluorescence microscope examination.

RESULTS

Reactivity of Resting and Activated Lymphocytes With Antithomeoprotein MoAbs

We have analyzed the expression of three different human HOX proteins in resting and activated lymphocytes by means of the MoAbs D7.87, 3B2, and 4C5, which react with the homeoproteins HOXA10, HOXC6 and HOXD4, respectively. These MoAbs were raised against recombinant proteins produced in insect cells using the baculovirus expression system and specifically reacted with nuclear extracts of infected cells expressing the respective immunizing homeoprotein. Indirect immunofluorescence on fixed cells showed that a small proportion of resting PBLs (ranging from 3% to 15% in 5 different donors) was weakly stained by 4C5 (anti-HOXD4) MoAb (Fig 1A). Conversely, most of the PHA-stimulated PBLs displayed a bright nuclear staining with this MoAb (Fig 1B through F). Similar data were obtained when anti-CD3 MoAb was used as mitogenic stimulus (data not shown). In time/course experiments, the staining pattern changed significantly after PHA stimulation. A significant nuclear staining was observed as soon as 3 hours after stimulation, was distinctly visible after 6 hours, and reached maximal intensity at 24 hours. The staining was concentrated in a single bright spot in each nucleus from 6 to 24 hours after stimulation, whereas several (up to 5) nuclear spots were observed at 72 hours. This pattern of nuclear staining may reflect a localization of immunoreactivity in the nucleolus, which displays characteristic morphologic changes along the replicative cell cycle. Thus, a single bright spot was evident at 6 to 24 hours after PHA stimulation, corresponding to the single large nucleolus typical of the G1 phase of the cell cycle, whereas, at 48 to 72 hours, several spots were observed, in accordance with the nucleo-
Table 2. Expression of HOXA4, B4, C4, and D4 Genes in Lymphoid Cells by RNAse Protection Assay

<table>
<thead>
<tr>
<th>HOX Gene</th>
<th>PBLs Resting</th>
<th>PHA-Activated PBLs</th>
<th>Raji</th>
<th>Jurkat</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4</td>
<td>–</td>
<td>+/–</td>
<td>++++</td>
<td>NT</td>
</tr>
<tr>
<td>B4</td>
<td>–</td>
<td>–</td>
<td>++++</td>
<td>–</td>
</tr>
<tr>
<td>C4</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
<td>+++++</td>
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<tr>
<td>D4</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Abbreviation: NT, not tested.

lar fragmentation occurring in the progression of the cell cycle. Parallel ³H-thymidine uptake experiments confirmed that DNA synthesis started after 48 hours of stimulation (data not shown). No immunoreactivity was observed using 3B2 and D7.87 MoAbs in both resting (Fig 1G and H) and activated PBLs (data not shown). We further investigated the reactivity of these MoAbs in cultured human T-cell clones and polyclonal lines maintained in culture with recombinant IL-2 (rIL-2) for different time intervals (1 to 4 weeks). As shown in Table 1, this analysis showed that 4C5 MoAb reacted with all the T cells analyzed, independently of the CD4/CD8 surface phenotype and of the T-cell receptor (TCR) molecules (either α/β or γ/δ) expressed. Also, in these cells, 3B2 and D7.87 MoAbs displayed no immunoreactivity. In addition, the human T-cell leukemia lines Jurkat, Mol4, Molt13, CEM, and H9 were stained by 4C5 MoAb (Table 1). We next investigated the reactivity of the antibody in activated B- and NK-lymphoid cells. As shown in Table 1, resting tonsilar B-cell populations displayed little or no reactivity with 4C5 MoAb, whereas B-cell blasts induced by SAC + rIL-2, Epstein-Barr virus (EBV)-transformed B-cell lines, the Raji Burkitt’s lymphoma cell line, and IL-2–cultured polyclonal CD3-16+ NK-cell lines were brightly stained by 4C5 MoAb. All these cells displayed a similar pattern of immunoreactivity consisting of single or multiple nuclear spots. Therefore, a nuclear immunoreactivity with 4C5 MoAb is characteristic of activated and/or proliferating lymphocytes, independently of their B-, T-, or NK-cell lineage.

Resting and PHA-Activated Normal T Lymphocytes and Jurkat and Raji Cell Lines Express HOXC4 But Not HOXD4 mRNA

It is known that HOX genes are organized in four clusters on different chromosomes. These clusters can be aligned so that the genes are grouped into 13 vertical groups on the basis of their position in the chromosomes. The vertically aligned genes are referred to as paralogs and display a very high degree of homology. Therefore, there is a high probability that a MoAb raised against a particular homeoprotein encoded by an HOX gene displays cross-reactivity with the homeoproteins encoded by the paralog genes. To test whether the reactivity to 4C5 MoAb in lymphocytes was related to HOXD4 gene expression or to the expression of the paralog genes HOXA4, HOXB4, or HOXC4, we performed RNase protection experiments using probes specific for the four different paralogs. As shown in Fig 2 and Table 2, resting PBLs, PHA-activated PBLs, and Raji and Jurkat cells expressed mRNA specific for the HOXC4 gene, in accordance to previous reports, whereas HOXD4 mRNA was
Fig 5. Effect of cycloheximide and actinomycin D treatment on the expression of HOXC4 by human PHA-activated PBLs. (A) PBLs 8 hours after stimulation; (B) PBLs 8 hours after stimulation treated with cycloheximide; (D) PBLs 8 hours after stimulation treated with actinomycin D; and (C) unstimulated untreated control PBLs. Cells were fixed and stained with 4C5 MoAb as in Fig 1.

The presence of HOXC4 mRNA also in resting lymphocytes, in which only a weak staining with 4C5 MoAb was observed in a small proportion of cells, may be explained in different ways. The expression of HOXC4 protein may be regulated at the posttranscriptional level so that very low amounts or no HOXC4 protein is present in resting lymphocytes. Alternatively, the HOXC4 protein may be diffusely present in the cytoplasm, in which it may fail to react with 4C5 MoAb. Cell activation would result in the entrance of the HOXC4 protein in the nucleus and the subsequent concentration of this protein in the nucleolus would allow its detection by MoAb 4C5. To test these hypotheses, we studied the immunoreactivity with 4C5 MoAb in lymphocytes that had been stimulated with PHA for 8 hours in the presence of either actinomycin D (0.5 mg/mL) or cycloheximide (50 mmol/L). Actinomycin D, which blocks transcription, only partially inhibited immunoreactivity, whereas a complete inhibition was observed in the presence of cycloheximide, which blocks mRNA translation into proteins (Fig 5). These data indicate that HOXC4 protein is indeed rapidly synthesised after PHA activation and, together with the finding that HOXC4 mRNA is present also in resting lymphocytes, suggest that the expression of HOXC4 in activated lymphocytes is regulated at the posttranscriptional level.

Evidence for a 32-kD DNA-Binding Homeoprotein in the Nucleoli of Activated Lymphocytes

Because further characterization of the HOX protein identified in activated lymphocytes could not be performed by Western blotting because the 4C5 MoAb does not recognize the denatured protein or by immunoprecipitation because of the very small amount of protein, we performed gel retardation assays on nucleolar extracts from resting or PHA-activated lymphocytes. These assays were performed using the synthetic oligonucleotide 4B-26, known to bind with
high affinity to HOXD4 and to other homeoproteins belonging to the same paralogy group, which share the same homeodomain. As shown in Fig 6A, nucleolar extracts of activated lymphocytes contained a DNA-binding protein capable of binding to 4B-26, whereas no DNA-binding activity was observed in the nucleoli of resting PBLs.

The molecular characteristics of the DNA-binding protein present in the nucleoli of activated PBLs were analyzed by Southwestern blot. Nucleolar extracts were size-fractionated by SDS-PAGE, blotted, and probed with radiolabeled 4B-26 in the presence of an excess of unlabeled poly(dI-dC)(dI-dC). As shown in Fig 6B, a single band of approximately 32 kD was detected only in the nucleolar extracts of activated lymphocytes; this molecular weight is comparable to that of the recombinant HOXC4 protein produced in Sf9 cells.

**DISCUSSION**

Taken together, these data indicate that the HOXC4 homeoprotein is expressed in the nucleus of proliferating lymphocytes of the T, B, and NK lineages. The synthesis of HOXC4 in T cells starts immediately after mitogen stimulation and the homeoprotein accumulates in the nucleus several hours before the onset of DNA replication and cell proliferation. The localization of the immunoreactivity, together with the changes in the number of nuclear spots along the proliferative cell cycle, is strongly suggestive of a predominant accumulation of the homeoprotein in the nucleus. Indeed, nucleolar extracts from activated T cells contained a 32-kD homeoprotein with DNA-binding properties typical of a HOX4 homeodomain. In a more detailed study of the nucleolar localization of homeoproteins, it was shown that the homeoprotein present in the nucleoli of Raji cells could be removed by the same 4C5 antibody used in this study. The homeoprotein present in activated T cells was identified as HOXC4, because the mRNAs specific for the other members of the paralogy group were not detectable in PBLs. The 4C5 MoAb, raised against HOXD4, does indeed react with the highly homologous HOXC4, but not with several other recombinant homeoproteins.

Because homeoproteins represent a family of DNA-binding proteins that regulate gene expression during the embryonic morphogenesis and control the differentiation process, it is conceivable that one or more of them could regulate the expression of genes involved in the regulation of the proliferation and differentiation of lymphoid cells. In this context, previous reports indicated that the human non-class I homeobox gene HB2 and HOXB genes are switched on in activated T lymphocytes, in which they have been implicated in the control of cell activation and growth. On the other hand, other DNA-binding proteins are activated by mitogenic signals and subsequently migrate into the nucleus, where they act as transcription factors for genes specifically involved in lymphocyte activation, such as those encoding for lymphokines or their receptors. However, the finding that the expression of HOXC4 gene product is found in all lymphoid cell lineages suggests that the regulatory activity of this homeoprotein is not restricted to T-cell lineage-specific genes. The expression of HOXC4 in proliferating but not in resting lymphocytes and its nucleolar localization may indicate a regulatory role on genes present in the nucleolus, such as those encoding for ribosomal RNA and other unknown genes. In this context it is well known that resting lymphocytes display a relatively low number of ribosomes and a low level of protein synthesis that sharply increase during the blast transformation. On the other hand, these results do not imply that the HOXC4 homeoprotein localizes exclusively in the nucleolus. The homeoprotein could be present elsewhere in the nucleus at a concentration below the sensitivity of immunofluorescence and yet high enough to exert its regulatory activity on genes involved in the activation and/or proliferation of lymphoid cells. Resting T lymphocytes have an amount of HOXC4 mRNA comparable to that of activated T lymphocytes, but the homeoprotein is synthesized only after stimulation. This finding indicates that its synthesis is regulated at the translational level. The existence of this type of regulation not only implies the need for an immediate synthesis of HOXC4 upon activation, suggesting a regulatory role of the early stages of lymphocyte stimulation, but also that the presence of a Hox protein should not be inferred on the basis of the expression of its mRNA alone and should be confirmed either by immunologic or functional evidence.

**REFERENCES**


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