The EMBO Journal Vol.16 No.10 pp.2892–2906, 1997

Rox, a novel bHLHZip protein expressed in quiescent cells that heterodimerizes with Max, binds a non-canonical E box and acts as a transcriptional repressor

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Proteins of the Myc and Mad family are involved in transcriptional regulation and mediate cell differentiation and proliferation. These molecules share a basic-helix–loop–helix leucine zipper domain (bHLHZip) and bind DNA at the E box (CANNTG) consensus by forming heterodimers with Max. We report the isolation, characterization and mapping of a human gene and its mouse homolog encoding a new member of this family of proteins, named Rox. Through interaction mating and immunoprecipitation techniques, we demonstrate that Rox heterodimerizes with Max and weakly homodimerizes. Interestingly, bandshift assays demonstrate that the Rox–Max heterodimer shows a novel DNA binding specificity, having a higher affinity for the CACGCG site compared with the canonical E box CACGTG site. Transcriptional studies indicate that Rox represses transcription in both human HEK293 cells and yeast. We demonstrate that repression in yeast is through interaction between the N-terminus of the protein and the Sin3 co-repressor, as previously shown for the other Mad family members. *ROX* is highly expressed in quiescent fibroblasts and expression markedly decreases when cells enter the cell cycle. Moreover, *ROX* expression appears to be induced in U937 myeloid leukemia cells stimulated to differentiate with 12-*O*-tetradecanoylphorbol-13-acetate. The identification of a novel Max-interacting protein adds an important piece to the puzzle of Myc/Max/Mad coordinated action and function in normal and pathological situations. Furthermore, mapping of the human gene to chromosome 17p13.3 in a region that frequently undergoes loss of heterozygosity in a number of malignancies, together with the biochemical and expression features, suggest involvement of *ROX* in human neoplasia.

Keywords: bHLHZip proteins/Max-interacting proteins/Myc/sin3/transcriptional repressor

Introduction

Myc is a nuclear protein that acts as a key regulator of vertebrate cell proliferation. Its N-terminus includes a proline and glutamine-rich domain, which acts as a transcriptional activator in both mammalian cells and yeast (Kato *et al.*, 1990; Amati *et al.*, 1992, 1993; Kretzner *et al.*, 1992). Its C-terminus contains a helix–loop–helix and a leucine zipper domain (Landschulz *et al.*, 1988; Murre *et al.*, 1989), preceded by a basic region. The combination of these motifs (referred to as bHLHZip) is the hallmark of a class of transcription factors, including USF (Gregor *et al.*, 1990), AP-4 (Hu *et al.*, 1990), CBF1 (Cai and Davis, 1990), TFE-3 (Beckmann *et al.*, 1990) and TFEB (Carr and Sharp, 1990), which bind DNA as homo- and/or heterodimers. Myc cellular functions are carried out through heterodimerization with another bHLHZip protein, called Max (Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991; Amati *et al.*, 1992, 1993). Max is transcriptionally inert and the Myc–Max heterodimer binds the sequences CACGTG and CATGTG, sharing specificity for the E box consensus (CANNTG) with other HLH proteins (for a review see Blackwood *et al.*, 1992a).

Two additional bHLHZip proteins, Mad (Ayer *et al.*, 1993) and Mxi1 (Zervos *et al.*, 1993), have been isolated while searching for Max partners. They share high sequence homology, particularly in the bHLHZip domain and within an amphipathic α-helical region at their N-terminus, which interacts with two mammalian homologs of the yeast transcriptional repressor Sin3 (mSin3A and B) (Ayer *et al.*, 1995; Schreiber-Agus *et al.*, 1995). Given the requirement for both Mad and the N-terminus sequence of Mxi1 for full repression activity, it is likely that the Mad–Max and Mxi1–Max regulation of transcription is not merely achieved by competitive inhibition at the Myc binding site, but requires the recruitment of specific proteins that together mediate transcriptional repression of Myc-responsive target genes. Recently, two additional Max-interacting repressors (Mad3 and Mad4) were identified (Hurlin *et al.*, 1995b). Similarly to the previously reported Mad and Mxi1 proteins, Mad3 and Mad4 contain a bHLHZip domain which interacts with Max and binds to the CACGTG site and an N-terminus region that interacts with the mSin3 proteins.

Max is a stable protein and its expression is not significantly affected by cell growth, as both *MAX* mRNA and Max protein are detected in quiescent cells at levels that are not altered upon mitogenic stimulation (Berberich *et al.*, 1992; Blackwood *et al.*, 1992b). In contrast, Myc

and Mad are known to be unstable proteins (Ayer and Eisenman, 1993) and their expression is tightly regulated throughout cell cycle progression and differentiation. c-*MYC* expression is rapidly induced upon stimulation of quiescent cells with growth factors or mitogens (reviewed in Marcu *et al.*, 1992). In contrast, expression of *MAD* and *MXI1* mRNAs and Mad protein appears to be induced by various differentiation inducing agents in different cell lines (Ayer and Eisenman, 1993; Zervos *et al.*, 1993; Larsson *et al.*, 1994; Hurlin *et al.*, 1995a). Therefore, Myc, Mad, Mxi1 and possibly Mad3 and Mad4 represent the rate limiting components in the Myc/Max/Mad network. Myc levels are high in proliferating cells, Mad levels are low and only Myc–Max complexes can be detected. However, Mad protein synthesis occurs upon differentiation, resulting in a shift from Myc–Max to Mad–Max complexes (Ayer and Eisenman, 1993). Expression studies in the mouse central nervous system and epidermis have consistently revealed that Myc expression is generally associated with the proliferating compartments of tissues, while expression of Mad, Mad3 and Mad4 occurs in growth arrested cells undergoing differentiation. Expression of *MXI1* in both proliferating and differentiating cells suggests a crucial role for the switch from Myc–Max to Mxi1–Max during differentiation (Zervos *et al.*, 1993; Larsson *et al.*, 1994; Hurlin *et al.*, 1995a).

The interactions among the various members of the Myc/Mad/Max family to either activate or repress transcription of target genes is not only the result of the relative intracellular level of Mad proteins (Mad, Mad3, and Mad4) and Mxi1 in comparison with that of Myc, but is also the effect of an active process. Max DNA binding kinetics have been reported to be influenced by casein kinase II phosphorylation status of the protein (Berberich and Cole, 1992; Bousset *et al.*, 1994) and, more recently, cloning of a new spliced form of the p38 kinase (Mxi2) which is able to phosphorylate Max has linked the stress-induced signaling pathway to the Myc superfamily transcription machinery (Zervos *et al.*, 1995). Three physiologically relevant targets of the Myc–Max heterodimer have been described so far, ornithine decarboxylase (Packham and Cleveland, 1995), prothymosin α (Eilers *et al.*, 1991; Gaubatz *et al.*, 1994; Desbarats *et al.*, 1996) and the cell cycle activator CDC25A (Galaktionov *et al.*, 1996).

As predicted from their biological role, both clinical and biological observations substantiate the implication of these transcription factors in a wide range of neoplasias. The *MYC* family genes are activated in Burkitt lymphoma (c-*MYC*) (Battey *et al.*, 1983), neuroblastoma (N-*MYC*) (Brodeur *et al.*, 1984) and small cell lung carcinoma (L-*MYC*) (Nau *et al.*, 1985). A direct role for *MYC* genes in transformation is indicated by the ability of c-*MYC* to transform primary rat embryo fibroblasts in association with the c-Ha-*ras* oncogene (Land *et al.*, 1983; Schwab *et al.*, 1985). Expression of the Mad family proteins suppresses this transformation, which suggests opposite roles for the Myc and Mad proteins with respect to cell transformation (Lahoz *et al.*, 1994; Hurlin *et al.*, 1995b; Schreiber-Agus *et al.*, 1995). Recently, adenovirus constructs encoding Mad have been found to inhibit the proliferation and tumorigenicity of human astrocytomas (Chen *et al.*, 1995). Moreover, *MXI1* allelic loss and mutations have been associated with prostate cancer (Eagle *et al.*, 1995).

Here we report the cloning, mapping, expression and functional analysis of a human gene and its mouse homolog encoding a novel member of the Myc/Mad/Max family.

Results

Isolation of the human ROX and murine rox genes

During a search for transcribed sequences from human chromosome 17p13.3, a *Not*I linking clone (LL132) was identified, indicating the presence of a nearby gene (Ledbetter *et al.*, 1990). We used this clone as a probe for screening two human fetal brain cDNA libraries. Several overlapping clones were obtained and assembled in a 4812 bp cDNA contig. The consensus cDNA sequence of this novel gene, referred to as *ROX*, was generated (DDBJ/EMBL/GenBank accession No. X96401) and found to contain a polyadenylation signal and a poly(A) tail. A partial sequence of the cDNA located in the 3′-untranslated region and containing a polymorphic dinucleotide repeat has been reported elsewhere (Carrozzo and Ledbetter, 1993). An open reading frame (ORF) of 1746 bp was identified, encoding a predicted protein of 582 amino acids (Figure 1A; DDBJ/EMBL/GenBank accession No. X96401). The putative initiation codon was identified at position 213 of the consensus sequence and properly fulfilled Kozak's criteria (Kozak, 1984). All three possible reading frames preceding the initiation codon showed stop codons, suggesting that an alternative initiation codon was unlikely to reside outside the cDNA contig. The sequence of the most 5′ cDNA clone was confirmed on genomic DNA clones. The putative protein sequence contains a bHLHZip domain located at positions 222–299, which shows significant homology to the bHLHZip motif found in other proteins belonging to the Myc/Max family (Figure 1B). Overall, the amino acid sequence shows a high proline content (17.2%).

Cloning of the murine cDNA homolog revealed an ORF of 1773 bp (DDBJ/EMBL/GenBank accession No. Y07609), with 88.5% identity to the human gene, encoding a putative protein of 591 amino acids (92.9% identity, Figure 1A). The bHLHZip domain in the mouse protein was identical to its human counterpart and is located at positions 224–301. The putative human protein lacks nine amino acids which are present in the mouse at positions 38, 39, 406 and 522–527. This latter six amino acid sequence is repeated in tandem in the mouse protein. The mouse cDNA was mapped by *in situ* hybridization to band B on chromosome 11, in the predicted mouse syntenic region. Cross-species conservation of the gene was tested using the ORF as the probe on a Southern blot containing DNA from several species (including human, cow, cat, dog, chicken, rabbit, mouse, hamster and *Drosophila*). Hybridization bands were observed in the lanes corresponding to all species tested, indicating a high degree of conservation (data not shown).

Rox is a nuclear protein

In vitro translation of the mRNA generated from a full-length human *ROX* cDNA produced a protein with an apparent molecular weight of 70 kDa (Figure 2A). This protein is specifically recognized by an antiserum raised

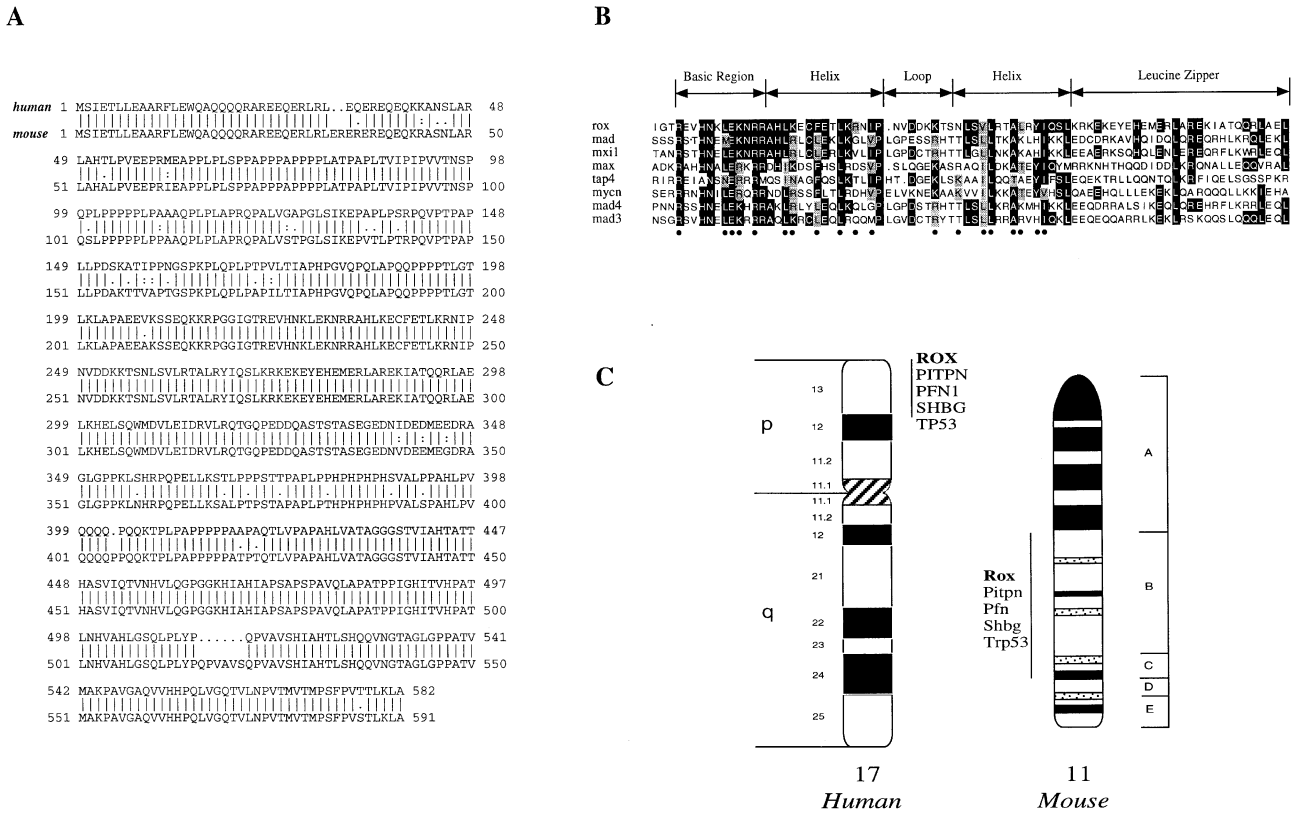

Fig. 1. (**A**) Comparison of the predicted protein products encoded by the human *ROX* (top) and mouse *rox* (bottom) genes. (**B**) Alignment of the bHLHZip of Rox and other bHLHLZip family members. Dots indicate the position of the amino acids of the bHLH consensus (Benezra *et al.*, 1990; Cai and Davis, 1990). Conservative amino acid differences in the consensus are written in black on a gray background. Identical amino acids shared by Rox with other proteins are written in white on a black background. (**C**) Diagramatic representation of human chromosome 17 and mouse chromosome 11. Human genes mapping to 17p13 and their murine homologs mapping to 11 band B–C are indicated.

against a GST protein fused to Rox amino acids 96–219 (Figure 2B). In immunoblot and immunoprecipitation experiments using total lysate of cells transiently transfected with an HA-tagged (HA-*ROX*) expression vector, both the anti-hemagglutinin monoclonal antibody (mAb 12CA5) and the anti-Rox antiserum detect a doublet of ~70 kDa and a series of bands ranging from ~46 to 55 kDa that are not present in non-transfected cells (Figure 3D). The 70 kDa doublet is likely to represent differently modified forms of the entire Rox protein, while the 46–55 kDa bands appear to be the result of proteolytic cleavage of the full-length forms. It remains to be established whether or not this cleavage represents a physiological step during the lifetime of Rox.

The use of anti-Rox antibodies allowed us to determine the subcellular localization of the protein. Indirect immunofluorescence was performed on HeLa cells transiently transfected with an HA-*ROX* expression vector. Figure 2C (left panel) shows a nuclear spotted staining typical of several transcription factors (Spector, 1993). The specificity of the signal was confirmed by co-staining with anti-HA (data not shown) and by staining with preimmune serum (Figure 2C, right panel). The finding that Rox localizes to the nucleus like other members of the Myc superfamily and mSin3A (Abrams *et al.*, 1982; Persson and Leder, 1984; Stone *et al.*, 1987; Blackwood *et al.*, 1992b; Kato *et al.*, 1992; Ayer and Eisenman, 1993; Chin *et al.*, 1995) is consistent with its possible role as a transcription factor.

Rox homodimerizes and heterodimerizes with Max

To test Rox binding specificity, we took advantage of the interaction mating technique, an extension of the two-hybrid system (Finley and Brent, 1994). Rox and a panel of informative bHLHZip and bHLH proteins were expressed as fusion proteins with either the LexA DNA binding domain (bait) or the B42 acidic moiety (prey). The medium high sensitivity pSH18-34 reporter gene was used. Consistent with previous work, we confirmed the interaction between Max and the other members of the Myc family (Blackwood and Eisenman, 1991; Dang *et al.*, 1991; Ayer *et al.*, 1993; Zervos *et al.*, 1993; Hurlin *et al.*, 1995b). Using this assay we also demonstrated that Max strongly interacts with Rox. Furthermore, Rox was also shown to form homodimers (Figure 3A).

To provide independent evidence for Rox dimerization and interaction between Rox and Max, we examined the ability of different bHLHZip members fused to GST and expressed in bacteria to bind *in vitro* translated and labeled Rox. The GST fusion proteins that were used are schematically presented in Figure 3C. *In vitro* translated (IVT) Rox (Figure 3B, top panel), HA-tagged Max (central panel) or LexA-tagged c-Myc (bottom panel) were mixed with purified GST–bHLHZip family member fusions and a low stringency co-immunoprecipitation was performed using an anti-GST antibody and protein A–Sepharose. The bound and recovered proteins were analyzed by 12% SDS–PAGE. The ability of GST–c-Myc, GST–Max, GST–Mxi1 and GST–Rox to bind IVT Max and the ability of

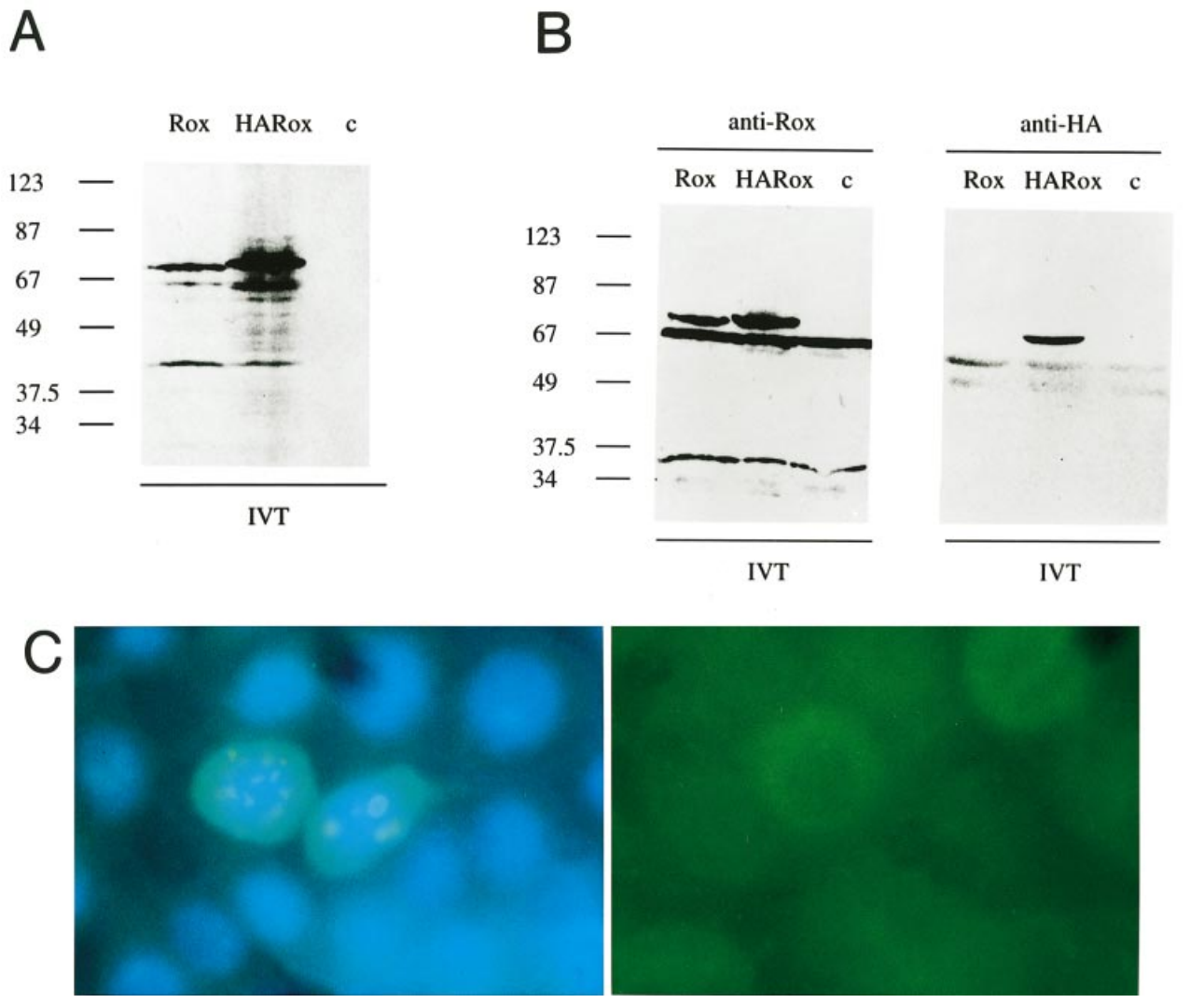

Fig. 2. Rox is a nuclear protein with a molecular weight of ~70 kDa. (**A**) SDS–PAGE of *in vitro* transcribed and translated *ROX* and HA-*ROX* cDNAs. (**B**) Immunoblot of IVT Rox and HA-Rox with anti-GST–Rox (96–219) antiserum and anti-HA monoclonal antibody. (**C**) Immunofluorescence of HeLa cells transfected with an HA-*ROX* expression vector. Cells expressing Rox were stained with anti-GST–Rox antiserum, followed by FITC-conjugated anti-rabbit antibodies and with DNA-specific Hoechst staining (left panel). As a control, HeLa cells transfected with the HA-*ROX* expression vector were also incubated with preimmune serum and stained with FITC-conjugated anti-rabbit antibodies (right panel).

IVT c-Myc to bind GST–Max and, to a lesser extent, GST–c-Myc, are consistent with previous works and confirm that the bacterially expressed fusions are functional. The results shown in the top panel indicate that GST–Max and, to a lesser extent, GST–Rox retain IVT Rox on the matrix. Both the two-hybrid and co-immunoprecipitation data suggest that the only members of the bHLHZip family interacting with Rox are Max and, to a lesser extent, Rox itself.

We confirmed Rox–Max interaction *in vivo*. HA-*ROX*-transfected HEK293 cells were lysed and immunoprecipitated using anti-Max-specific antibodies (anti-Max C-17) under low stringency conditions. The immunoprecipitate was run on 12% SDS–PAGE and subjected to immunoblot analysis using anti-HA antibody. Rox was recovered in the anti-Max immunoprecipitate as an ~70 kDa doublet and a series of bands of ~50 kDa. These bands were also observed in the total extract and in the anti-HA antibody immunoprecipitate from transfected cells, but not in the lysates and immunoprecipitate from non-transfected cells. The same bands were absent in immunoprecipitations with an unrelated antibody, proving the specificity of the interaction (Figure 3D). The ~50 kDa bands were more clearly visible in the immunoprecipitates at a shorter film exposure, when the signal due to the immunoglobulin was fainter (data not shown).

The Max–Rox heteromeric complex preferentially binds CACGYG sequences

Myc–Max, Max–Max, Mxi1–Max, Mad1–Max, Mad3–Max and Mad4–Max complexes strongly bind the canonical CACGTG and CATGTG DNA sites (Blackwood and Eisenman, 1991; Ayer *et al.*, 1993; Zervos *et al.*, 1993; Hurlin *et al.*, 1995b). We performed electrophoretic mobility shift assays (EMSAs) to determine if Rox–Max and Rox–Rox complexes also bind these sites.

Figure 4A shows that a reticulocyte lysate containing IVT Rox was able to bind a labeled oligonucleotide containing the CACGTG sequence (lanes 5–8) and that the observed Rox-containing complex is not present in

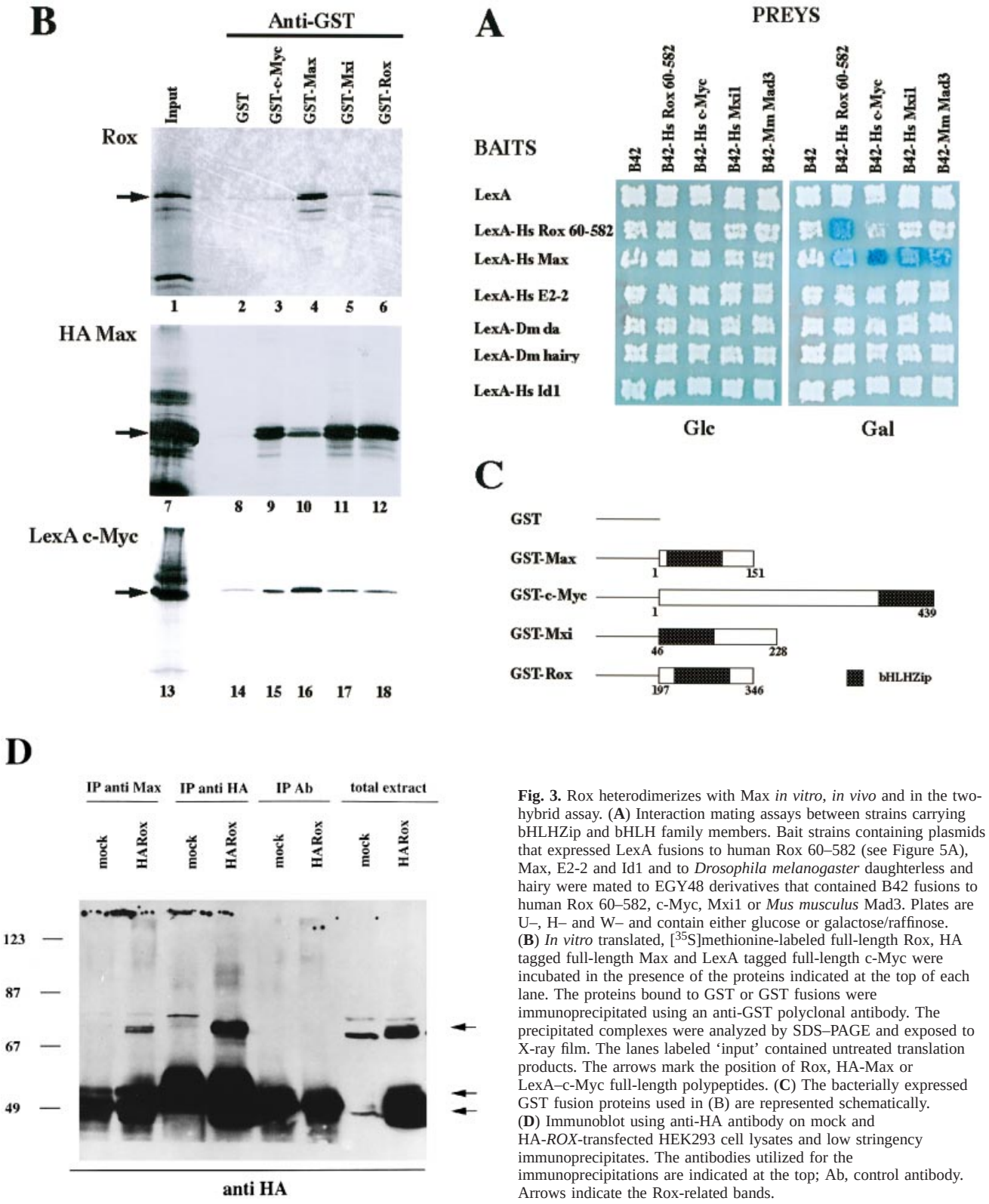

Fig. 3. Rox heterodimerizes with Max *in vitro*, *in vivo* and in the two-hybrid assay. (**A**) Interaction mating assays between strains carrying bHLHZip and bHLH family members. Bait strains containing plasmids that expressed LexA fusions to human Rox 60–582 (see Figure 5A), Max, E2-2 and Id1 and to *Drosophila melanogaster* daughterless and hairy were mated to EGY48 derivatives that contained B42 fusions to human Rox 60–582, c-Myc, Mxi1 or *Mus musculus* Mad3. Plates are U–, H– and W– and contain either glucose or galactose/raffinose. (**B**) *In vitro* translated, [^{35}S]methionine-labeled full-length Rox, HA tagged full-length Max and LexA tagged full-length c-Myc were incubated in the presence of the proteins indicated at the top of each lane. The proteins bound to GST or GST fusions were immunoprecipitated using an anti-GST polyclonal antibody. The precipitated complexes were analyzed by SDS–PAGE and exposed to X-ray film. The lanes labeled 'input' contained untreated translation products. The arrows mark the position of Rox, HA-Max or LexA–c-Myc full-length polypeptides. (**C**) The bacterially expressed GST fusion proteins used in (B) are represented schematically. (**D**) Immunoblot using anti-HA antibody on mock and HA-*ROX*-transfected HEK293 cell lysates and low stringency immunoprecipitates. The antibodies utilized for the immunoprecipitations are indicated at the top; Ab, control antibody. Arrows indicate the Rox-related bands.

the reticulocyte lysate (2–4). Moreover, competition experiments showed that Rox-containing lysates were unable to bind the Myc–Max canonical CATGTG, the Myc–Max non-canonical CACGAG, the CATGCG (Blackwell *et al.*, 1993), the MyoD CAGCTG (Lassar *et al.*, 1989) or the mutated CACGGA sites (lanes 9 and 11–14). Interestingly, the retarded DNA–protein complex could be competed for by the non-canonical Myc–Max CACGCG binding site (lane 10).

To assess more accurately the affinity of interaction between Rox-containing lysates and the non-canonical CACGCG site, the four different CRCGYG sites were assayed for binding in competition with the E box CACGTG site (Figure 4B and C). The CACGTG binding

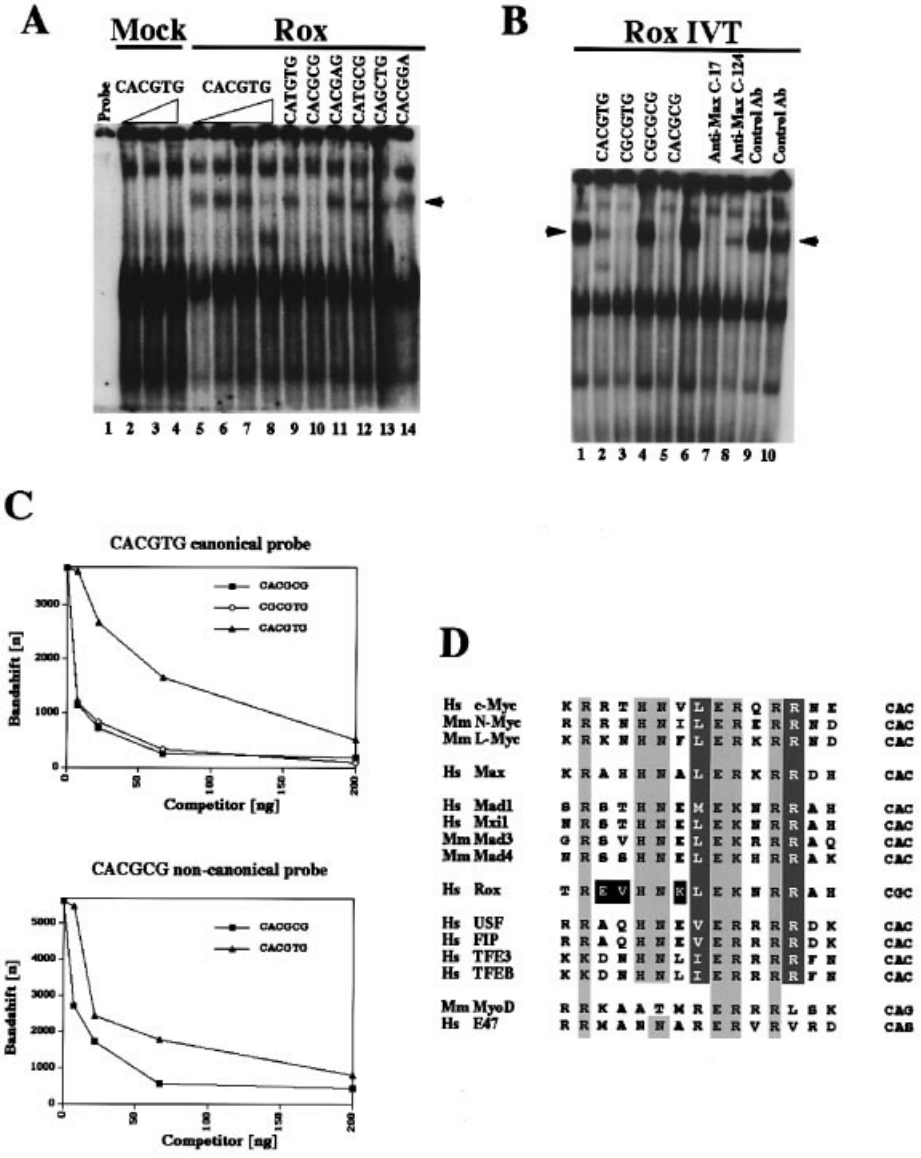

Fig. 4. Rox and Max heterodimers bind DNA and preferentially recognize the non-canonical CACGCG binding site. (**A**) The ability of Rox to bind DNA was examined by EMSA. Either a mock translation or translated Rox were tested for binding to the Myc–Max canonical CACGTG motif oligonucleotide as described in Materials and methods. The following additions were made to the reaction mixtures: lanes 2 and 5, buffer; lane 6, 10 ng unlabeled probe; lanes 3 and 7, 40 ng unlabeled probe; lanes 4 and 8, 160 ng unlabeled probe; lane 9, 200 ng CATGTG AR146/AR147 competitor; lane 10, 200 ng CACGCG AR150/AR151 competitor; lane 11, 200 ng CACGAG AR154/AR155 competitor; lane 12, 200 ng CATGCG AR148/AR149 competitor; lane 13, 200 ng CAGCTG AR152/AR153 competitor; lane 14, 200 ng CACGGA AR138/AR139 competitor. The arrow indicates the Rox-containing DNA–protein complex. (**B**) The Rox–DNA complex contains Max and binds specifically to CACGYG sites. The ability of Rox to bind CRCGYG motifs and the presence of Max in the DNA–protein complex was tested as described in Materials and methods. The following additions were made to the reaction mixtures: lanes 1 and 6, buffer; lane 2, 200 ng unlabeled probe; lane 3, 200 ng CGCGTG AR160/AR161 competitor; lane 4, 200 ng CGCGCG AR159/AR159 competitor; lane 5, 200 ng CACGCG AR150/AR151 competitor; lane 7, 5 μg purified anti-Max C-17 antibody; lane 8, 5 μg purified anti-Max C-124 antibody; lane 9, 5 μg purified unrelated antibody A; lane 10, 5 μg purified unrelated antibody B. (**C**) The Rox–Max complex preferentially recognizes the CACGCG sequence. The ability of the CACGTG (AR100/AR101), CGCGTG (AR160/AR161) and CACGCG (AR150/AR151) sites to compete for binding to the Rox–Max complex was tested. In this EMSA, binding of Rox–Max to labeled CACGTG (top panel) and to labeled CACGCG (bottom panel) probes was competed for with increasing amounts of the indicated cold competitors. Rox–Max–DNA labeled probe complexes were quantitated with a PhosphorImager (Molecular Dynamics) and plotted against the amount of competitor DNA added to the reaction. (**D**) Rox is unique among bHLHZips in its basic region. Alignment of the basic region of bHLHZip and bHLH proteins. Amino acids in one letter code are numbered according to the full-length Max protein. Conserved residues are shown in light gray boxes, residues important for recognition of the central nucleotides of the E box are in dark gray boxes. They were shown to direct contacts between the α-helical basic region and the DNA major groove of the CA–TG recognition sequence (Ferre-D'Amare *et al.*, 1993, 1994; Ellenberger *et al.*, 1994; Ma *et al.*, 1994). Unique sequence features of Rox are highlighted in black. The preferred binding half-sites are shown on the right.

site competed at ~12-fold molar excess, as did the non-canonical CACGCG and CGCGTG sites (Figure 4B, lanes 1–5, and C). The CGCGCG sequence was not able to compete. All the bHLHZip proteins examined so far bind DNA when associated with Max. As Rox was able to homodimerize and heterodimerize with Max and as it was shown that IVT Myc bound DNA together with Max present in the reticulocyte lysate (Littlewood *et al.*, 1992),

we reasoned that Max could be part of the retarded DNA–protein complex observed in Rox-containing lysates. To determine whether Max was itself a component of the DNA–protein complex, supershift experiments using anti-Max purified antibodies were performed (Figure 4B, lanes 6–10). The addition of antibodies directed against the C- (lane 7) or N-termini (lane 8) of Max caused the disappearance of most of the retarded complex, an effect not seen after addition of unrelated purified antibodies. This is consistent with the idea of Rox and Max both being components of the DNA binding complex in these reticulocyte lysates containing endogenous Max.

These data suggest that the Rox–Max heterodimer binds DNA with a new specificity as compared with the other bHLHZip complexes and that Rox–Max binds asymmetrically, with Rox binding the GYG half-site and Max the CAC half-site (Blackwell *et al.*, 1993). Rox has a higher affinity for the GCG half-site than for the GTG half-site. Site-directed mutagenesis of bHLHZip proteins has shown that the basic region is involved in binding to the E box (Amati *et al.*, 1992; Anthony-Cahill *et al.*, 1992; Blackwell *et al.*, 1993; Fisher *et al.*, 1993). Recently, the crystal structures of four bHLH proteins with their cognate DNA were determined and it was shown that there were direct contacts between the α-helical basic region and the DNA major groove of the CA–TG recognition sequence (Ferre-D'Amare *et al.*, 1993, 1994; Ellenberger *et al.*, 1994; Ma *et al.*, 1994). All conserved amino acids in the basic region make either base or phosphate contacts with the E box. The presence of a positively charged residue at position 227 in the Rox protein, which is unique among known bHLHZip and bHLH proteins, is consistent with the observed difference in specificity (Figure 4D). Other Rox-specific features in this region are the negatively-charged residue 223 followed by a hydrophobic residue, which are only found in yeast bHLH PHO4p and CBF1p and human bHLH AP-4 among bHLH and bHLHZip proteins.

Deletion analyses of Rox (Figure 5B) were carried out to determine the interaction site with Max and itself and the capacity of the heteromeric Rox–Max complex to bind DNA. The Rox protein consists of a 221 residue region which contains three Pro/Gln-rich regions, the central bHLHZip region and a 282 amino acid tail with a Pro/Gln/His-rich region. As expected, deletion of either the first 205 residues or the last 262 which retain the bHLHZip region did not abolish the formation of a Rox–Max heterodimer or a Rox–Rox homodimer *in vitro* (Figure 5A and D). Our finding that Rox mutants form heterodimers with Max *in vitro* prompted us to determine whether or not these complexes also retain DNA binding ability (Figure 5C). Reticulocyte lysates containing either Rox N- or C-terminal peptides bind the E box sequence (lanes 3, 6–8 and 11–12). The band shifts produced by expression of the last (523, 467, 387 and 377) or the first (437 and 320) Rox residues are smaller than those produced by expression of full-length Rox, consistent with the formation of a complex incorporating the shorter Rox proteins. The presence of Rox in the retarded DNA–protein complexes was further confirmed by the addition of anti-HA mAb (lanes 4–5) or polyclonal anti-Rox (lanes 13–14, see Figure 2B) to binding reactions. The anti-HA antibody caused the formation of two supershifts (complexes i and j), probably due to the presence of two epitope tags. Addition of anti-Rox antibody to the binding reaction resulted in the disappearance of the DNA–protein complex. These observations indicate that the Rox bHLHZip domain is sufficient for binding to Max, for homodimerization and for binding to the E box. The same domain in c-Myc has previously been found to be sufficient and necessary to bind Max and DNA (Kato *et al.*, 1992).

Rox transcriptional activity

Previous work showed that Myc–Max complexes activate the transcription of a reporter gene driven by multiple E box binding sites, while Mad–Max or Mxi1–Max complexes repress transcription from the same construct by recruiting mSin3A or mSin3B to the DNA (Amati *et al.*, 1992; Kretzner *et al.*, 1992; Ayer *et al.*, 1993; Hurlin *et al.*, 1995b; Schreiber-Agus *et al.*, 1995). To examine the transcriptional properties of Rox, we used a luciferase reporter construct containing four CACGTG binding sites cloned upstream of a thymidine kinase minimal promoter (TKMP). This construct was co-transfected with the HA-*ROX* expression vector in HEK293 cells and the luciferase activity was measured 48 h after transfection. Overexpression was also carried out in the presence of a plasmid containing only the TKMP as control. The $(CACGTG)_4$-driven promoter is characterized by very high activity in the cell, presumably due to the presence of endogenous E box-specific transcription factors. Under these conditions, Rox was able to repress approximately eight times the luciferase activity as the empty vector (Figure 6A). This result was confirmed using a different *ROX* expression vector (pcDL Rox+) compared with a co-transfection using the same plasmid with *ROX* cDNA cloned in the antisense orientation (pcDL Rox–). These data suggest that the Rox protein product is responsible for the observed repression and shows that the degree of repression depends on the concentration of *ROX* expression vector (Figure 6B). The difference observed between the two expression vectors is likely to be due to different strengths of the promoters and to their different 'squelching' effects. This transcriptional property of Rox has been also observed in U2OS and SK-N-BE cell lines (data not shown). Although we assume that this repression is due to Rox–Max heterodimers, we cannot exclude the possibility that it could be due either to Rox–Rox homodimers or heterodimers that contain Rox and a still unidentified partner.

An exception among the cell lines we tested for Rox transcriptional activity is represented by HeLa cells. In this cell line, Rox is able to strongly activate transcription from a $(CACGTG)_4$-driven promoter (data not shown). This discrepancy is likely to be due to the presence of human papilloma virus 18 (HPV-18) in HeLa cells, which might affect Rox transcriptional properties. Further experiments are needed to assess whether the activation observed in HeLa cells reflects a potential step in the progression towards transformation and malignancy.

During the preparation of this manuscript, we learned that the mouse *rox* gene had been isolated independently by another group (P.Hurlin and R.Eisenman, personal communication), who demonstrated transcriptional repression determined by Rox (Mnt in their designation) in HEK293 cells and activation in HeLa cells.

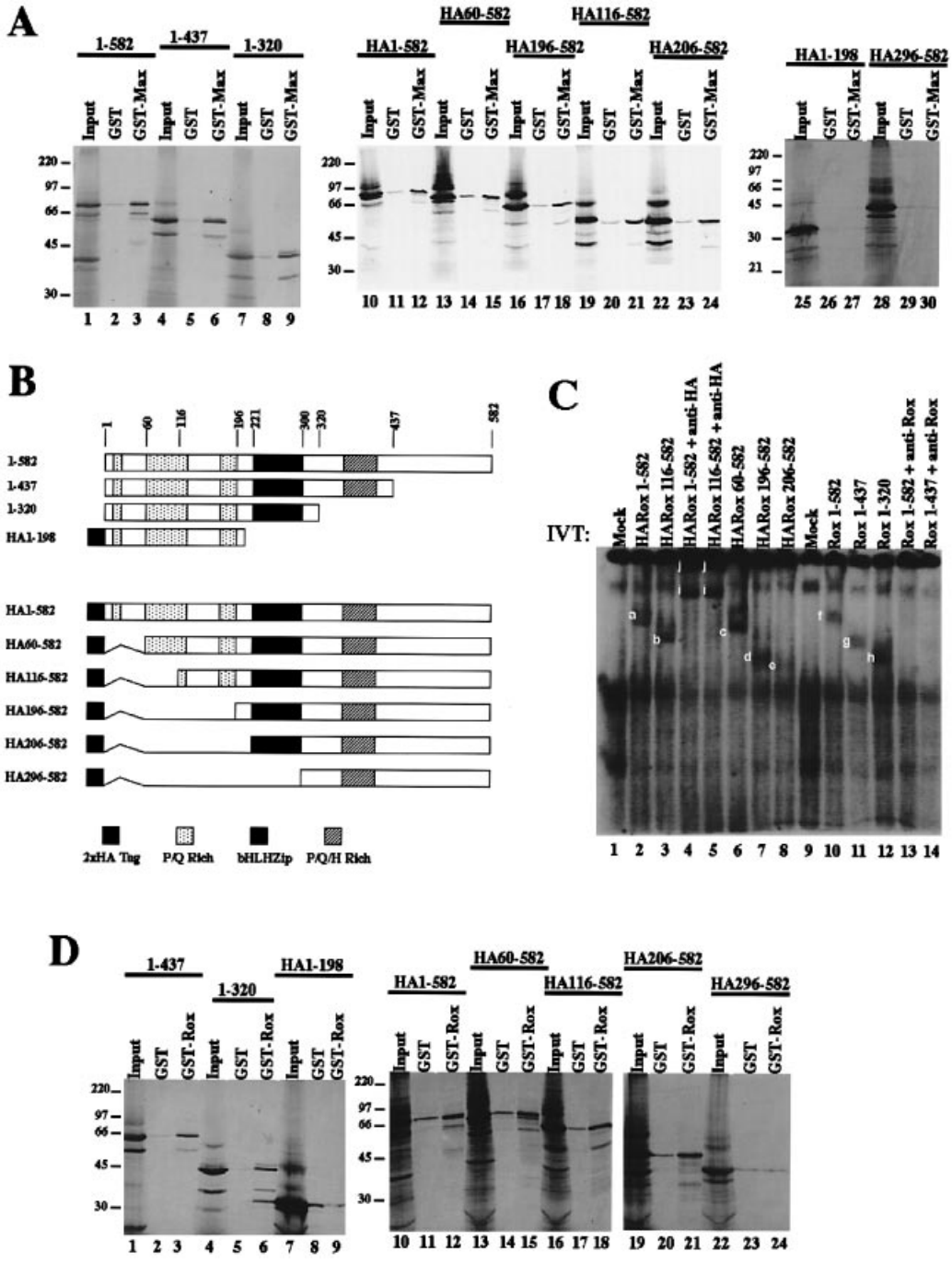

Fig. 5. Structure–function analysis of various Rox constructs as assessed by ability to bind Max, Rox and CACGTG DNA sequences. (**A**) [^{35}S]methionine-labeled, *in vitro* translated Rox and Rox mutants were analyzed by low stringency immunoprecipitation using anti-GST antiserum for binding to GST and GST–Max (see Figure 3C). The immunoprecipitates were then analyzed by 10 or 12% SDS–PAGE. Untreated Rox forms are shown in lanes 1, 4, 7, 10, 13, 16, 19, 22, 25 and 28. (**B**) The various Rox expression constructs utilized in (A), (C) and (D) are shown schematically. The P/Q-rich region is stippled, the bHLHZip domain is darkly shaded and the P/Q/H-rich region is lightly shaded. The C-terminal mutants of Rox are tagged at their N-terminus with a double HA tag sequence (MKGYPYDVPDYASYPYDVPDYAEF), represented here in black. (**C**) *In vitro* translated Rox mutants were tested for binding to the Myc–Max canonical CACGTG motif oligonucleotide. The following additions were made to the reaction mixtures: lanes 1–3 and 6–12, buffer; lanes 4 and 5, 5 μg anti-HA mAb; lanes 13 and 14, 6 μg purified anti-Rox R1 antibody. a, the Max–Rox full-length-containing DNA complex; b–e, the Max–Rox N-terminal truncation-containing DNA complexes; f–h, the Max–Rox C-truncation-containing DNA–protein complexes; i and j, the Max–Rox–12CA5 mAb-containing DNA–protein complexes. The variation in mobility of the protein–DNA complexes and the results of the supershift experiments are consistent with the view that Rox is a member of these complexes. (**D**) Low stringency immunoprecipitation using anti-GST antiserum for binding to GST and GST–Rox (see A and also Figure 3C). Untreated Rox forms are shown in lanes 1, 4, 7, 10, 13, 16, 19 and 22.

The Rox N-terminus interacts with the PAH2 domain of yeast and mammalian Sin3

The ability of Rox to repress transcription in HEK293, U2OS and SK-N-BE cells raised the possibility that Rox, like Mad, Mad3, Mad4 and Mxi1, was bringing a co-repressor to the DNA (Ayer *et al.*, 1995; Hurlin *et al.*, 1995b; Schreiber-Agus *et al.*, 1995). We used a yeast assay to identify the domain(s) of Rox required for

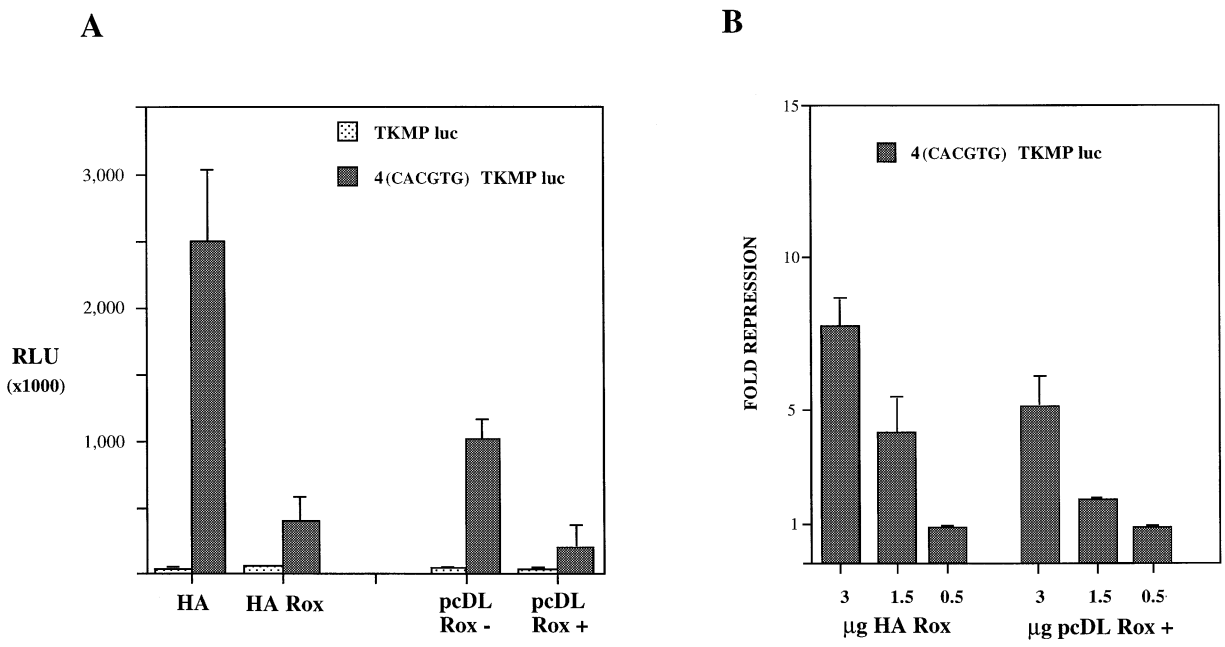

Fig. 6. Rox represses transcription at promoters containing CACGTG binding sites. (**A**) Bars indicate luciferase units (RLU) in cell extracts after transfection with 3 μg of two different *ROX* expression vectors (HA-*ROX* and pcDL Rox+) on $(CACGTG)_4$ TKMP and TKMP luciferase reporter plasmids, with respect to activity in the absence of exogenous Rox and in the presence of an equal amount of empty (HA) or antisense vector (pcDL Rox–). (**B**) Luciferase activity, expressed in fold repression with respect to empty or antisense vectors, in the presence of decreasing amounts (3, 1.5 and 0.5 μg) of the two Rox expression vectors. The data represent an average of at least five independent experiments.

repression, since preliminary observations showed that a LexA–Rox fusion was able to repress transcription in this organism. Plasmids carrying single or multiple *lexA* operators upstream of a *CYC1–lacZ* test gene were transformed in yeast cells, together with expression plasmids encoding either intact LexA, LexA–Rox 1–582 (full-length) or LexA–Rox deletions. The results shown in Figure 7A demonstrate that LexA–Rox 1–582 and LexA–Rox 1–300 fusions repress expression of the β-galactosidase reporter gene at least 3-fold from either one or four *lexA* operators positioned upstream of the UAS and TATA elements. In contrast, expression of the LexA–Rox 60–582 fusion was unable to repress transcription. These observations suggest that the first 59 residues of Rox are critical for repression.

Database searches revealed a striking homology between the N-terminus of Rox and the Sin3-interacting domain (SID) of Mad, Mxi1, Mad3 and Mad4 (Figure 7D) (Ayer *et al.*, 1995; Hurlin *et al.*, 1995b; Schreiber-Agus *et al.*, 1995). These results suggest that Rox, like the Mad family proteins, could repress transcription by interacting with the co-repressor Sin3. In order to verify this hypothesis, we generated a yeast strain deleted for *SIN3* to test the transcriptional activities of the LexA–Rox fusion proteins. The data in Figure 7A demonstrate that repression by Rox requires the presence of the *SIN3* gene. Interaction mating and *in vitro* interaction assays were performed to determine if the Rox N-terminal domain interacts with yeast and mammalian Sin3. The second paired amphipathic helix domain of Sin3 (PAH2) has previously been shown to create the interface required for interaction with the Mad family proteins (Ayer *et al.*, 1995; Hurlin *et al.*, 1995b; Schreiber-Agus *et al.*, 1995). Two-hybrid assays were employed to test the ability of Rox deletions to bind the PAH2 domain of *Saccharomyces cerevisiae* Sin3 and *Mus musculus* Sin3A. Besides achieving the expected interaction between Sin3 and Mad3 (Hurlin *et al.*, 1995b), this experiment demonstrated that Rox interacts with the PAH2 domain of both yeast and mammalian Sin3 via its N-terminal 59 amino acids (Figure 7B).

Independent evidence for Rox and Sin3 interaction was provided by *in vitro* interaction assays. *In vitro* translated and labeled HA-Rox 1–582, HA-Rox 60–582 and mSin3A PAH2 domain were mixed and immunoprecipitated with either an anti-HA antibody or an anti-Sin3 antibody under low stringency conditions (Figure 7C). Whereas interaction was observed between full-length Rox and Sin3, deletion of the first 59 residues of Rox resulted in a loss of interaction.

Overall, these results suggest that Rox represses transcription by recruiting Sin3 to the DNA. As with the Mad family members, this association requires the second PAH domain of Sin3 and an intact SID-like N-terminus of Rox.

Rox expression

The expression pattern of Rox was determined by hybridizing a human cDNA clone to Northern blots containing human and mouse poly(A)$^+$ RNAs from various tissues. A 4.8 kb mRNA species was ubiquitously detected both in mouse and human tissues (Figure 8A). This is in agreement with our preliminary *in situ* hybridization results which show that *ROX* expression is not restricted to specific cell lineages (data not shown), similar to what has been observed for other members of the Myc superfamily (Mugrauer *et al.*, 1988; Zervos *et al.*, 1993; Hurlin *et al.*, 1995b).

As expression of almost all members of the Myc family is tightly regulated during differentiation and cell proliferation (Ayer and Eisenman, 1993; Zervos *et al.*,

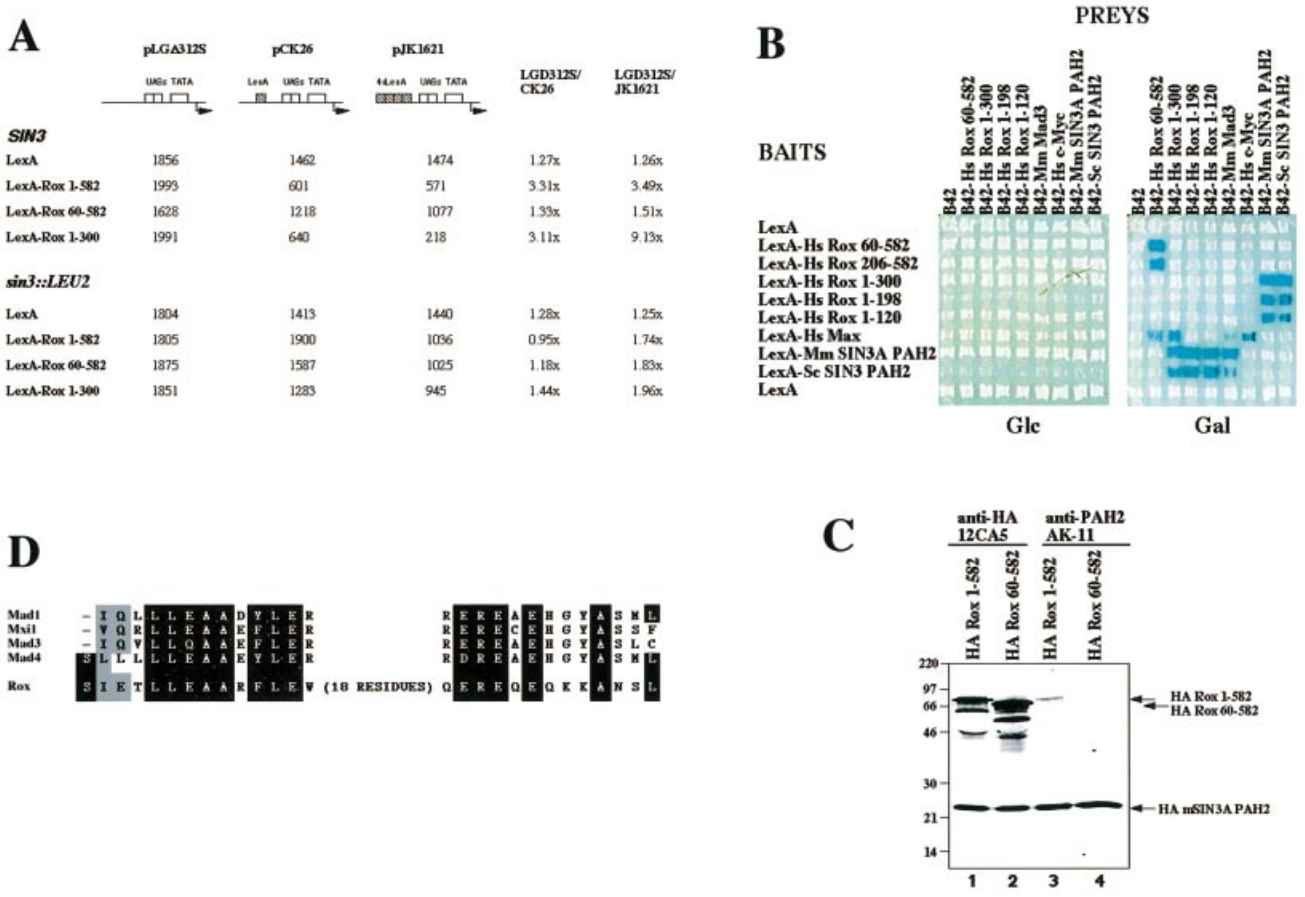

Fig. 7. The Rox N-terminus interacts with mSin3 and ySin3 and represses transcription in yeast cells. (**A**) Rox N-terminus represses transcription in yeast in a Sin3-dependent manner. EGY48 (*SIN3*) and ARY01 (*SIN3*::*LEU2*) cells were transformed with the reporter constructs shown schematically together with an expression plasmid that produced LexA, LexA–Rox 1–582 (full-length), LexA–Rox 60–582 or LexA–Rox 1–300 fusions. The level of repression is expressed as the ratio of β-galactosidase activity from the reporters lacking an operator to those containing one or four *lexA* operators. (**B**) Interaction mating assays between strains carrying Rox deletions or the PAH2 domain of ySin3 and mSin3A. Bait strains containing plasmids that expressed LexA fusions to human Rox 60–582, Rox 206–582, Rox 1–300, Rox 1–198, Rox 1–120 and Max and to the PAH2 domains of *S.cerevisiae* and *M.musculus* Sin3 were mated to EGY48 derivatives that contained B42 fusions to human Rox 60–582, Rox 1–300, Rox 1–198, Rox 1–120 and c-Myc, *Mus musculus* Mad3 and the Sin3 PAH2 domain and *S.cerevisiae* Sin3 PAH2. Plates are U–, H– and W– and contain either glucose or galactose/raffinose. (**C**) *In vitro* translated and [^{35}S]methionine-labeled HA-mSin3A PAH2 was incubated with HA-Rox 1–582 or HA-Rox 60–582 similarly expressed. Immunoprecipitates were performed with the 12CA5 anti-HA tag mAb (lanes 1–2) or the AK-11 anti-mSin3 PAH2 domain polyclonal antibody (lanes 3–4; Santa Cruz). Immunoprecipitates were separated by 12% SDS–PAGE and exposed to X-ray film. (**D**) Amino acid comparison of Rox residues 2–46 with the SID domain of Mad1 (residues 9–34), Mxi1 (9–34), Mad3 (8–33) and Mad4 (5–31). Amino acids conserved between Mad proteins and Rox are shown in black boxes and amino acids conservatively substituted are shown in gray boxes.

1993; Hurlin *et al.*, 1994, 1995a; Larsson *et al.*, 1994), we decided to investigate the expression of *ROX* mRNA in differentiating cells. We used the system of U937 cells induced with phorbol esters. Treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces this human leukemia cell line to differentiate along the monocyte/macrophage pathway (Harris and Ralph, 1985). We measured the expression of *ROX* by an RNase protection assay on total RNA extracted from cells induced for different times with TPA and observed a peak of *ROX* induction 2 h after treatment (Figure 8B). Under our conditions, *MAD* was induced as quickly as 1 h after TPA treatment and was maintained throughout the induction, starting to decrease 8 h after TPA treatment. In the same system, *MYC* was highly expressed in non-treated cells and the message progressively decreased with differentiation (Figure 8B). Consistent with previous reports, the *MAX* mRNA level was stable throughout the experiment (data not shown). A similar pattern of *ROX* and *MAD* induction was observed with vitamin D_3 (data not shown).

This early induction of *ROX* suggested a link between expression and cells ceasing proliferation rather than differentiating. This prompted us to analyze the expression of *ROX* during the cell cycle. Normal quiescent human lung fibroblasts (WI38) were obtained by serum starvation for 3 days. The cells were then stimulated to enter the cell cycle by the addition of 20% serum. RNA samples were collected at several times after serum addition and *ROX* and *MYC* expression were monitored by the RNase protection assay. Under these conditions, the cells start to synthesize DNA ~12 h after serum addition and by 24 h ~80% of the cells had entered S phase (Baldin *et al.*, 1993). As shown in Figure 8C, *ROX* was highly expressed in quiescent cells (t_0) and the message was markedly decreased when the cells entered the cell cycle. *ROX* became highly expressed again ~24–30 h after serum addition, possibly reflecting the quiescent status of confluent cells. As expected, *MYC* RNA was barely detectable in quiescent cells and was transiently induced after mitogenic stimulation.

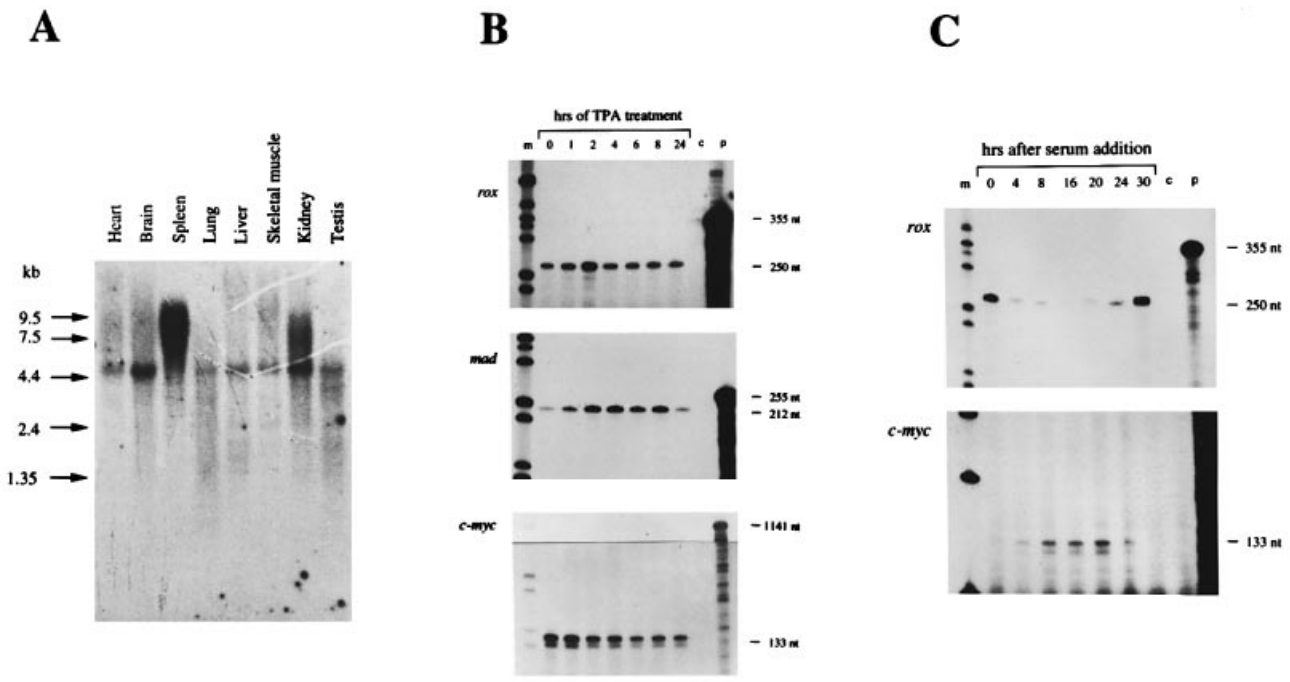

Fig. 8. ROX is ubiquitously expressed in adult tissues, but regulated during differentiation and proliferation. (**A**) Northern Blot analysis of adult human tissues probed with a *ROX* cDNA fragment. *ROX* mRNA is detected in all tissues as a 4.8 kb band. (**B**) RNase protection on total RNA isolated from U937 cells induced for different times with 5 nM TPA. *ROX*, *MAD* and *MYC* messages were monitored using the probes described in Materials and methods. m, molecular weight marker; 0–24, hours of TPA treatment; c, control with yeast RNA; p, untreated probe. (**C**) RNase protection analysis of *ROX* and c-*MYC* on total RNA from WI38 normal quiescent fibroblast induced to enter the cell cycle. 0, serum starved cells; 4, 8, 16, 20, 24 and 30, hours after serum addition; m, molecular weight marker; c, control with yeast RNA; p, untreated probe.

These results suggest that signals that modulate cell proliferation, growth factors and cell contact exert a strong and rapid control on *ROX* expression.

Discussion

We describe the isolation of a human gene and its murine homolog encoding a novel Max-interacting protein, named Rox. Similar to other Max-interacting proteins, Rox expression is ubiquitous in various tissues and cell lines but is tightly regulated during cell growth. In differentiating U937 myeloid leukemia cells, *ROX* mRNA is up-regulated within 2 h, returning to basal levels as early as 4 h after induction. This early activation of *ROX* appears to be mostly related to the cell stopping proliferating rather than starting differentiating. Consistent with this observation, we found that the presence of *ROX* mRNA is associated with the quiescent state of normal fibroblasts. Mitogenic stimulation induces a rapid and consistent down-regulation of *ROX* mRNA, concomitant with up-regulation of c-*MYC*. Our results show that extracellular conditions which modulate cell proliferation exert strong and rapid control on *ROX* mRNA regulation in the reverse direction to c-*MYC*, suggesting that Rox may act as a Myc antagonist, like Mad, Mxi1, Mad3 and Mad4.

Like the Mad family proteins, Rox represses transcription of an E box-driven reporter gene in HEK293, U2OS and SK-N-BE mammalian cells. Moreover, repression assays in yeast and protein–protein binding experiments demonstrate that Rox repression properties are mediated by a key interaction with the PAH2 domain of the Sin3 co-repressor. The N-terminus of Rox shows homology with the SID of Mad1, Mxi1, Mad3 and Mad4, indicating that Rox exerts repression through a common mechanism shared by the Myc antagonists (Ayer *et al.*, 1995; Hurlin *et al.*, 1995b; Schreiber-Agus *et al.*, 1995). Interestingly, the Rox SID appears to be bipartite, with an 18 amino acid residue inserted in the midst of the SID consensus (Hurlin *et al.*, 1995b).

Major differences emerge when one compares the overall structure and amino acid composition of Rox with those of previously identified Myc antagonists. The Rox amino acid sequence is considerably longer (582 amino acids) compared with that of Mad, Mxi1, Mad3 and Mad4 (209–228 amino acids). Furthermore, the regions both preceding and following the bHLHZip domain have a proline content which has not been reported in any other members of the Mad family. Proline-rich regions are found in proteins showing transcription activation properties, such as CTF/NF-1 (Mermod *et al.*, 1989) and WT-1 (Gessler *et al.*, 1990), and in transcriptional repressors, such as FS1 (Han and Manley, 1993) and RGM1 (Estruch, 1991). Although proline usually plays only a structural role, its restricted mobility results in an increased ability of proline-rich regions to be involved in protein–protein interactions.

Another major difference between Rox and previously identified Max-interacting proteins is the DNA binding specificity. We demonstrated that the Rox–Max heterodimer recognizes the E box consensus CACGTG, however, it has a higher affinity for the non-canonical CACGCG binding site. Previous studies described the basic region as essential in determining DNA binding specificity. Moreover, crystallization of four bHLH proteins (Ferre-D'Amare *et al.*, 1993, 1994; Ellenberger *et al.*, 1994; Ma *et al.*, 1994) showed that the conserved residues in this

region make either base or phosphate contacts with the E box. Rox has a unique basic domain among bHLH proteins, with a negatively-charged residue (Glu223), a hydrophobic residue (Val224) and a positively-charged residue (Lys227) flanking the His–Asn bHLHZip conserved residues at positions 225–226 (Figure 4D). It has been shown that multiple alanine substitutions in the basic region increase the DNA binding affinity (Fisher *et al.*, 1993; Takemoto and Fisher, 1995), suggesting that specific amino acid side chains positioned along one face of an α-helical backbone are important for sequence-specific and high affinity DNA recognition. The differences between the Rox basic domain and that of the other members of the family is consistent with the different DNA binding specificity observed for the Rox–Max heterodimer, suggesting that basic region non-conserved residues affect the binding specificity. Taken together, these observations further support the notion that proteins in the bHLH family have evolved to bind DNA with different affinity, mainly through their different structures and sequence recognition.

MYC family members are activated in several neoplasias (Battey *et al.*, 1983; Brodeur *et al.*, 1984; Nau *et al.*, 1985). On the other hand, Mad was found to inhibit proliferation and tumorigenicity of human astrocytoma (Chen *et al.*, 1995) and *MXI1* was found to show loss of heterozygosity (LOH) and mutations in prostate cancer (Eagle *et al.*, 1995). Moreover, *MAD3* and *MAD4* map to regions that show LOH in acute myeologenous leukemia, acute non-lymphocytic leukemia, myelodysplastic syndrome and bladder carcinoma (Hurlin *et al.*, 1995b). Tantalizing enough, *ROX* maps to 17p13.3, a human genomic region which frequently undergoes LOH in several malignancies. Markers YNZ22 and 144D6, mapping ~200 kb and 3 Mb distal to *ROX* respectively are deleted in 50–75% of sporadic breast cancers (Stack *et al.*, 1995). In addition, LOH for this chromosomal region has also been demonstrated in ovarian cancer (Phillips *et al.*, 1993), astrocytoma (Saxena *et al.*, 1992), bladder cancer (Williamson *et al.*, 1994), medulloblastoma (McDonald *et al.*, 1994), neuroectodermal cancer (Biegel *et al.*, 1992) and osteosarcoma (Andreassen *et al.*, 1993). LOH for this chromosomal region has also been demonstrated in familial breast cancer (Lindblom *et al.*, 1993).

In conclusion, the ability of Rox to heterodimerize with Max, to act as a repressor and its expression pattern throughout the cell cycle and in differentiating cells, together with its mapping to a chromosomal region frequently undergoing LOH in human cancer, strongly suggest that we may have identified a novel tumor suppressor gene. An intriguing hypothesis is that Rox may act as a Myc antagonist by regulating different genes, representing a member of a network controlling cell growth and differentiation parallel to the Myc/Mad/Max network.

Materials and methods

cDNA isolation and sequencing

Two human fetal brain libraries (Stratagene and Clontech) were initially screened using the cosmid clone LL132 (Ledbetter *et al.*, 1990). Additional rounds of cDNA library screening were performed using *ROX* cDNA clones in order to isolate the entire coding region. Phage library plating and screening were performed using standard methods (Sambrook *et al.*, 1989) and recovery of cDNA in plasmids was performed using the λZAP plasmid rescue procedure according to the manufacturer's specifications. The genomic sequence corresponding to the 5′-end of the cDNA contig was obtained from cosmids reported elsewhere (Ledbetter *et al.*, 1992). Mouse *rox* cDNA clones were isolated from a mouse embryo 11.5 days p.c. cDNA library (Clontech) screened using two independent human cDNA clones. Sequence reactions were performed both manually using a Sequenase Version 2.0 7-deaza-dGTP Sequencing Kit (US Biochemical) or automatically using a Perkin Elmer ABI 377 DNA sequencer. Assembly of DNA sequences was performed using the AutoAssembler software (PE Applied Biosystem, version 1.4). Nucleotide and amino acid sequences were compared with the non-redundant sequence databases available at TigemNet (http://www.tigem.it/) using the BLAST algorithm (Altschul *et al.*, 1990). BlastX and BlastP homology searches were performed using both the Seg (Wootton and Federhen, 1993) and Xnu (Claverie and States, 1993) filters in order to mask low complexity sequence regions and short repeats in proteins. Sequence alignments were performed using the Wisconsin Sequence Analysis Package programs (Genetics Computer Group, version 8.0).

Plasmids

ROX full-length cDNA was cloned in different expression vectors. HA-*ROX* was constructed by placing *ROX* cDNA 3′ in-frame with the HA epitope into plasmid pCDNAI (Invitrogen). *ROX* cDNA was also cloned into an RSV promoted vector (pCDL; Takebe *et al.*, 1988).

The reporter plasmid used in transactivation experiments was constructed by placing four Myc–Max binding sites (CACCCGGTCACGTGGCCTACAC) upstream of the –81 to +52 thymidine kinase minimal promoter in the luciferase plasmid pT81luc (Nordeen, 1988)

Antibodies, immunoblot and immunofluorescence

To generate anti-Rox serum, two rabbits were immunized with an *Escherichia coli* expressed and purified GST–Rox (amino acids 96–219) fusion protein. The antiserum was protein A purified and the anti-GST antibodies were absorbed on a GST column. Immunoblotting was performed with the anti-Rox primary antibody at 1:1000 dilution and HA mAb (12CA5; Boehringer Mannheim) at 4 μg/μl. Visualization of antibody binding was carried out with ECL (Amersham) according to the manufacturer's instructions. Indirect immunostaining was performed on paraformaldehyde-fixed transfected HeLa cells. Cells were permeabilized with Triton X-100, blocked with porcine serum and incubated with anti-Rox (1:400 dilution) or affinity purified anti-HA mAb (1 μg/μl). Staining was obtained after incubation with secondary FITC-conjugated isotype-specific antibodies. Nuclear staining was obtained after 15 min incubation with 0.5 μg/ml Hoechst 33258.

Yeast manipulations

Interaction mating assays were performed as previously described (Reymond and Brent, 1995).

The β-galactosidase assays were performed as described in Guarente and Mason (1983) using the EGY48 or the ARY01 yeast strain (Estojak *et al.*, 1995; this work). The repression reporters are derivatives of pLGD312S (Guarente and Mason, 1983) containing one or four *lexA* operators (pCK26 and pJK1621; reviewed in Keleher *et al.*, 1992). The ARY01 strain was constructed by integrating the *LEU2* gene into the *SIN3* ORF of the EGY42 strain (Estojak *et al.*, 1995). This construct deletes the four PAH domains of Sin3. Correct single gene replacement was tested by polymerase chain reaction and Southern blotting.

Expression and purification of proteins in E.coli

His-tagged and GST fusion proteins were isolated as described (Reymond and Brent, 1995). Briefly, *E.coli* BL21 were grown to an OD_{600} of 0.5 at 37°C, IPTG was added to the medium to reach a final concentration of 0.33 mM and the culture was grown for 10 h at 20°C. Cells were harvested by centrifugation, frozen and thawed twice by immersing the tubes in dry ice and a 37°C water bath and resuspended in 1/50 of the starting culture volume of phosphate-buffered saline (PBS), pH 7.2, 5 mM EDTA, 1 mM dithiothreitol (DTT), 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mg/ml lysozyme. After 30 min on ice, Triton X-100 was added to a final concentration of 1% and the cells were sonicated. The supernatant was collected by centrifugation and the proteins were bound to a glutathione–Sepharose resin (Pharmacia) column. The column was washed with 1× PBS, 1% Triton X-100, 5 mM EDTA, 1 mM DTT, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatinA and 1 mM PMSF, with the same solution containing 250 mM KCl and finally equilibrated in 50 mM Tris–HCl, pH 8.0, 25% glycerol. GST fusion proteins were eluted in 50 mM Tris–HCl, pH 8.0, 10 mM glutathione

and 25% glycerol, then aliquoted and stored at –80°C. Protein quantitation was done by the Bradford Coomassie dye binding method according to the manufacturer's instructions (Bio-Rad).

Co-immunoprecipitation

Rox full-length or truncated proteins, HA-Max and LexA–c-Myc were synthesized *in vitro* using the TNT coupled transcription/translation system and L-[^{35}S]methionine, according to the manufacturer's instructions (Promega). Samples were incubated for 30 min at 30°C. Aliquots of 100 ng purified GST–c-Myc, GST–Max, GST–Mxi, GST–Rox or His-Max or 400 ng GST were added to 30 µl IVT products and incubated for 30 min at 30°C. The volume was then adjusted to 500 µl with ice-cold 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.25% Nonidet P-40 (Sigma). After centrifugation, the supernatant was immunoprecipitated with 5 µg rabbit anti-GST, anti-Max C-17 or C-124 antibodies (Santa Cruz Biotechnology). The immune complexes were collected with protein A–Sepharose (Pharmacia) as described (Reymond and Brent, 1995), subjected to SDS–PAGE, dried and autoradiographed.

For *in vivo* co-immunoprecipitation, 1×10^7 HA-*ROX*-transfected HEK293 cells were lysed in PBS containing 1% NP-40, 0.2 mM PMSF, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 5 mg/ml aprotinin and 10 mM NaF, sonicated and clarified by centrifugation. The supernatant was immunoprecipitated with 3 µg anti-Max C-17, 10 µg anti-HA or 3 µg of an unrelated antibody for 2 h at 4°C and the immune complexes collected with protein A– or protein G–Sepharose beads. The complexes were subjected to 12% SDS–PAGE and immunoblotting performed as described.

Band shift assays

The binding reaction was performed in a volume of 40 µl consisting of 59.25 mM potassium acetate, 0.375 mM magnesium acetate, 6.25% glycerol, 15 µM hemin, 7.5 mM creatine phosphate, 37.5 µg/ml creatine phosphokinase, 1.5 mM DTT, 37.5 µg/ml calf liver tRNA, 50 ng non-specific oligonucleotide (AR015, 5′-ACGCGGATCCTACTAGGGCGG-ACAGAAGTCGGA-3′) and 2 µg poly(dI·dC) (Boehringer Mannheim). A mock or a *ROX*-containing transcription/translation reaction was performed without labeling according to the manufacturer's instructions (Promega). After this incubation the glycerol, the non-specific competitors and ~1 ng end-labeled probe (5′-GGAAGCAGACCACGTGGT-CTGCTTCC-3′ or 5′-GGAAGCAGACCACGCGGTCTGCTTCC-3′ where stated) were added to the reaction and a further 15 min incubation at room temperature was performed to allow the DNA–protein complex to form. These complexes were analyzed by electrophoresis through a 5% acrylamide (37.5:1)–0.5× TBE non-denaturing gel at 8 V/cm at 4°C. When specified, 1 µl unlabeled probe or specific competitor (quantities specified in figure legends) was added at the same time as the labeled probe. The sequences of the competitor were as follows: CATGTG AR146/AR147, 5′-GGAAGCAGACCATGTGGTCTGCT-TCC-3′; CATGCG AR148/AR149, 5′-GGAAGCAGACCATGCGGT-CTGCTTCC-3′; CACGCG AR150/AR151, 5′-GGAAGCAGAC-CACGCGGTCTGCTTCC-3′; CAGCTG AR152/AR153, 5′-GGA-AGCAGACCAGCTGGTCTGCTTCC-3′; CACGAG AR154/AR155, 5′-GGAAGCAGACCACGAGGTCTGCTTCC-3′; CGCGCG AR159/AR159, 5′-GGAAGCAGACCGCGCGGTCTGCTTCC-3′; CGCGTG AR160/AR161, 5′-GGAAGCAGACCGCGTGGTCTGCTTCC-3′; CAC-GGA AR138/AR139,5′-GGAAGCAGACCACGGAGTCTGCTTCC-3′.

For supershift experiments, 1 µg purified anti-Max C-17, anti-Max C-124 or unrelated antibody (Santa Cruz Biotechnologies) and 3 µg purified anti-Rox or 2 µg anti-HA antibodies were added to the reaction mix after protein–DNA complex formation. Following the addition of antibodies, the incubation was continued for an additional 5 min at room temperature.

mRNA analysis

Northern blot filters from human adult and mouse tissues (Clontech) were hybridized with a human *ROX* cDNA probe corresponding to positions 495–1610 of the human cDNA consensus. Washing conditions were 0.2× SSC, 0.1% SDS at 65°C for both human and mouse filters. Total RNA was prepared from 5×10^7 U937 or WI38 cells for each treatment using the guanidine isothiocyanate/phenol procedure (Chomczynsky and Sacchi, 1987). RNase protection assays were performed using the RPA II kit (Ambion) according to the manufacturer's instructions. Hybridization was carried out using 25 µg total RNA with 3×10^5 c.p.m. [^{32}P]UTP-labeled riboprobe for each sample. *ROX* antisense riboprobe transcript was generated from a 250 bp cDNA fragment cloned in pBS (Stratagene) using T3 polymerase. SP6 polymerase was used to generate *MYC* and *MAD* antisense transcripts from a *MYC* exon 2-containing genomic fragment (Lombardi *et al.*, 1987) and from a 212 bp *MAD* fragment, both cloned in pGEM3 (Promega).

Cell culture and transfections

The HEK293, SK-N-BE and U2OS cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). The U937 cell line was cultured in RPMI with 10% FCS and differentiation was induced with 5 nM TPA. WI38 human embryo lung fibroblasts were cultured at early passages in DMEM supplemented with 10% FCS and arrested in G_0 by incubation for 3 days in medium with 5% bovine calf serum. They were stimulated to re-enter the cell cycle by adding 20% FCS. Transfections were performed by the calcium phosphate method (Graham and Van der Eb, 1973). For the transactivation experiments, 1.5×10^5 cells/35 mm plate were transfected with 1 µg luciferase reporter plasmid and 0.5–3 µg expression vector. Total cell extracts were prepared 48 h after transfection with three cycles of freezing and thawing. Luciferase activity was measured using a luminometer (Lumat LB9501).

Acknowledgements

We thank Pierre Colas, Pier Giuseppe Pelicci, Alessandro Bulfone, Orsetta Zuffardi and Alessandro Guffanti for their helpful suggestions and for critical reading of the manuscript. We thank P.J.Hurlin and R.N.Eisenman for communicating unpublished results and D.E.Ayer, B.A.Cohen, M.D.Cole, M.Carlson, C.V.Dang, R.A.DePinho, A.D. Johnson, D.Kadosh, E.V.Prochownik, U.Schmidt, N.Schreiber-Agus, G.Sprague, A.S.Zervos and F.Grignani for plasmids and reagents. We would like to thank Ms Melissa Smith for help in preparation of the manuscript. This work was supported by the Italian Telethon Foundation, by Associazione Italiana per la Ricerca sul Cancro and by a Swiss Cancer League Fellowship and a Swiss National Science Foundation Fellowship to A.R.

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Received on November 15, 1996; revised on February 5, 1997

Note added in proof

During the revision of this manuscript, Hurlin *et al.* independently reported the isolation and functional characterization of the murine rox gene. [Hurlin,P.J., Queva,C. and Eisenman,R.N. (1997) Mnt, a novel Max-interacting protein is coexpressed with Myc in proliferating cells and mediates repression at Myc binding sites. *Genes Dev.*, **11**, 44–58.]