c-Myb Protein Interacts with Rcd-1, a Component of the CCR4 Transcription Mediator Complex[†]

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ABSTRACT: Transcriptional initiation of eukaryotic genes depends on the cooperative interaction of various transcription factors. Using the yeast two-hybrid assay, we have identified the murine Rcd-1 protein as a cofactor of the c-myb proto-oncogene product. Rcd-1 is evolutionarily conserved among many species, and moreover the yeast homologue CAF40 is part of the carbon catabolite repressor protein transcriptional mediator thought to be involved in the negative regulation of genes transcribed by RNA polymerase II. Rcd-1 is located mainly in the nucleus, and it interacts with c-Myb both in vitro and in vivo. The activation of the myeloid c-myb-specific mim-1 promoter is repressed by Rcd-1. Interestingly, rcd-1 is an erythropoietin regulated gene, which also represses the action of the AP-1 transcription factor on its target genes.

The products of the retroviral oncogene v-myb (for a review, see ref *1*) and its cellular progenitor, the proto-oncogene c-myb, are nuclear DNA-binding proteins, which interact specifically with the DNA sequence (C/T)AAC(G/T)G (*2*, *3*) Members of the Myb protein family have been identified in all kingdoms, namely animals (*4*), plants (*5*), and fungi (*6*). Such Myb proteins can act as transcription factors and play an essential role during cell proliferation and differentiation. Their activity is regulated by phosphorylation during the cell cycle (*7*).

The role of c-Myb is best characterized during the differentiation of hematopoietic cells, when several proteins interact cooperatively with c-Myb. Among these proteins are members of the c/EBP family (*8*−*10*) and the p300/CBP family (*11*−*13*). Moreover, Ets family proteins (*14*, *15*) or the GABP protein can interact with c-Myb (*16*). The p100 protein binds to the so-called EVES motif in the c-Myb C terminus (*17*) and cooperates with the Pim-1 kinase in the cytokine-regulated control of hematopoietic cell diffentiation, growth, and apoptosis (*18*).

We have used the *yeast two-hybrid* system to detect further cofactors interacting with c-Myb. Because mice with a disabled c-myb gene die at E15 (*37*), we hypothesized that an additional cofactor might play important roles prior to this stage. Therefore, we screened a murine c-DNA library derived from E11 embryos using the c-Myb C terminus as bait. Among others, we isolated the murine rcd-1 gene.

Rcd-1 is a component of the CCR4−NOT[1] complex (for a review, see ref *60*), a transcription mediator of RNA polymerase II. CCR4−NOT is evolutionarily conserved and consists of yeast from the proteins CCR4, NOT1−5, and several CCR4 associated factors (CAFs), which were characterized recently (*19*−*21*). Depending on its composition, the regulator occurs as a 1.0 or 1.9 MDa complex in vivo.

The complex influences the transcription initiation positively as well as negatively (*20*, *22*) and interacts with several proteins involved in transcription, e.g., ADA2 (*23*), a part of the multicomponent SAGA complex that possesses histone acetyltransferase activity, RNA helicase Dhh1p (*24*, *25*), or the putative tumor suppressor BTG1 (*26*). Furthermore, it regulates the Msn2p-dependent transcription via the Ras/cAmp signaling pathway (*27*). The CCR4−NOT regulator complex shows ubiquitin-protein ligase activity (*28*), and the CCR4 protein is the catalytic subunit of the cellular mRNA deadenylase (*29*−*32*).

CCR4-associated proteins are also present in other species. For example, sequences homologous to the CAF40 factor can be found in the databases from 22 different genera. In *Saccharomyces pombe*, a CAF40 homologue inhibits sexual differentiation induced upon nitrogen starvation. Hence, the gene was termed rcd-1 (required for cell differentiation) (*33*). It was shown recently (*59*) that Rcd-1 is required for the retinoic-acid-induced differentiation of F9 cells. In this paper, Rcd-1 was identified as a component of the DRF transcriptional complex, which consists of at least p300/CBP, ATF-2, and the retinoic acid receptor RAR. Furthermore, in mice, Rcd-1 plays a role in the branching morphogenesis of the

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[1] Abbreviations: CCR4, carbon catabolite repressor protein; CAF40, CCR4 accessory factor; EPO, erythropoietin; LB, Luria−Bertani medium; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; GST, glutathione-*S*-transferase; GFP, green fluorescent protein; TBP, TATA-binding protein; GTF, general transcription factor; TAF, transcription accessory factor; PIC, preinitiation complex; SRBP, suppressors of RNApol B; HAT, histone acetylase; HDAC, histone deacetylase.

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embryonic lung. The murine rcd-1/CAF40 homologue (*34*) is an erythropoietin responsive gene thought to be involved in hematopoietic cell development. Moreover, we demonstrate that the c-Myb and Rcd-1 proteins physically interact with each other and that the Myb-specific mim-1 promoter is down-regulated by Rcd-1 in a dose-dependent manner.

MATERIALS AND METHODS

Molecular Biology Procedures. All synthetic oligodeoxynucleotides were ordered from MWG (85560 Ebersberg, Germany). The constructs and plasmids isolated from the cDNA library were sequenced by GATC (78467 Konstanz, Germany). The matchmaker two-hybrid system, mouse E11 embryo matchmaker cDNA library, and dropout supplements were purchased from BD Biosciences Clontech GmbH (69126 Heidelberg, Germany) and used according to the directions of the manufacturer.

The oligodeoxynucleotides CCGGAATTCGAATCGGATGAATCTGGA, CGCGGATCCGCATG GCTGCAAGGGAC, and *Pfu*-DNA polymerase (Boehringer, 55216 Ingelheim, Germany) were used to amplify the sequence coding for amino acids 501−600 of the c-Myb protein. The PCR product was trimmed with *Eco*RI and *Bam*HI and ligated into the bait vector pAS2−1 (BD Biosciences Clontech) digested with the same enzymes. To express a GST-Myb fusion protein in *Escherichia coli*, the same fragment was cloned into the pGEX4T vector (Amersham-Biosciences, 79111 Freiburg, Germany), yielding GST-Myb. The rcd-1 fragment was generated by *Eco*RI digestion of the original pGAD10 clone obtained in the two-hybrid screen and was ligated into the pGEX4T plasmid (Amersham-Biosciences), yielding pGEX4Trcd-1. For eucaryotic expression pGEX4Trcd-1 was digested by *Not*I and *Pae*I. The resulting 1.2-kbp fragment was cloned into the pcDNA1.1/AMP vector (Invitrogen) and trimmed with the same enzymes, yielding pcDNArcd1. For expression in bacteria, the rcd-1 coding region was isolated from pGEX4Trcd-1 by *Nsp*I digestion. The sequence was prepared as above and ligated into a *Sph*I-digested pQE32 plasmid (Qiagen, 40724 Hilden, Germany), which then expressed a His_6-tagged Rcd-1. For eukaryotic expression, the pGEX4Trcd-1 plasmid was digested with *Not*I and *Pae*I, and the rcd-1 fragment was prepared as above and ligated into the plasmid pcDNA1.1/Amp (Invitrogen, 76131 Karlsruhe, Germany), which had previously been digested with *Not*I and *Pae*I. To obtain the Rcd-1-EGFP expression vector, the rcd-1 sequence was isolated by *Eco*RI digestion from the original pGAD10 clone and cloned into the pEGFPC2 plasmid (BD Biosciences Clontech), yielding pEGFPrcd-1.

Expression of the Rcd-1 Protein. E. coli LK111(λ) cells were transformed with the His_6-tag-rcd-1 plasmid and grown overnight. A total of 500 mL of LB medium was inoculated with the overnight culture and grown to an $OD_{600} = 0.7$. The culture was induced with 2 mM IPTG for 2 h. The cells were pelleted by centrifugation, suspended in 50 mL of PBS buffer, and lysed with a Branson sonifier. Cell debris was removed by centrifugation, and the supernatant was applied to a column filled with 1 mL of Ni-chelate agarose. The column was washed with 50 mL of PBS supplemented with 30 mM imidazole. The protein eluted with a step gradient of imidazole in PBS ranging from 75 to 200 mM. The fractions were analyzed on a 17% SDS PAGE gel, pooled, and dialyzed against PBS to remove the imidazole.

RT PCR and Expression Pattern. A six-month-old white New Zealand rabbit was sacrificed. The organs were collected and immediately frozen in liquid nitrogen. The RNeasy mini kit (Qiagen) was used to isolate RNA from different organs of a white New Zealand rabbit. c-DNA synthesis was carried out using an Advantage RT-PCR kit (BD Biosciences Clontech) according to the protocol of the manufacturer. PCR amplification was performed using the oligodeoxynucleotides ATG GCC CGG AGA CCC CG (sense) and GTA GCC TCC CTG TTC CAC CT (anti-sense) [c-myb] and act CAA GTG GAC AGA GAG AAG ATC TAT (sense) and TTG CCA GGT TCT TCA CCA GTT G (anti-sense) [rcd-1] as primers. The primers TCG AAT TCT AAT GCC GAT GAT GAT ATC GCC GCG (sense) and GGG GAT CCG TCT AGA AGC ATT TGC GGT GGA C (anti-sense) were used to amplify the β-actin gene as an internal control. A total of 20 cycles were performed with an annealing temperature of 48 °C. Elongation at 72 °C was allowed to proceed for 2 min. The products were separated on 10% (w/v) acrylamide gels and analyzed with an Easy video system, using the Wineasy software for quantification.

Transient Transfections. The plasmids pMim-1Luc (reporter), pEQ176 (monitor), and pEQ176P2Myb (Myb expression vector) were a kind gift of B. Lüscher and have been described previously (*12*). HEK293 or COS7 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Invitrogen, 76131 Karlsruhe, Germany), supplemented with 10% (v/v) fetal calf serum (FCS; Gibco), 0.45% (w/v) glucose, 0.45% (w/v) sodium pyruvate, 0.5 unit/mL penicillin, and 5 μg/mL streptomycin. For a transient transfection assay, 1×10^6 cells were seeded in a 100-mm Petri dish at 1 day prior to transfection. Effectene (QIAGEN) was used as a transfection reagent according to the instructions of the manufacturer. A total of 2 μg of DNA was transfected per dish. After incubation for 2−3 days, the cells were harvested with 500 μL of extraction buffer (25 mM Tris-HCl at pH 7.8, 2 mM EDTA, 10 mM DTT, 10% (v/v) glycerol, and 1% (v/v) Triton X-100) and frozen at −80 °C immediately. β-galactosidase and luciferase assays were carried out as described (*12*). All experiments were performed in duplicate, and each experiment was repeated at least 3-fold.

Pulldown Assay. The GST-tagged c-Myb protein was expressed in *E. coli* and purified with Glutathione−Sepharose from which it was eluted by 10 mM glutathione/PBS. The product was dialyzed against PBS and was more than 95% pure as judged from SDS−PAGE analysis. A total of 100 μg of the purified His_6-tagged Rcd-1 was coupled to CNBr-activated Sepharose (total bed volume = 100 μL) according to the protocol of the manufacturer. As a control, *E. coli* single-strand binding protein (SSB) was immobilized on another aliquot of Sepharose. The Sepharose beads were incubated with GST-Myb, washed three times with PBS, and applied directly to SDS−PAGE analysis.

Phage Display. The phage-display technique exploits the unique features of the bacteriophage life cycle: genes can be cloned into a phagemid, which will then be expressed as heterologous fusion proteins with the g3p protein on the surface of the phage. A bait protein was immobilized on

microtiter wells and was incubated with the phages. After extensive washes, the phages were rescued with *E. coli* cells and the number of retained clones was determined as described previously (*13*). A total of 2 μg of purified Rcd-1 was immobilized in each well of a microtiter plate and incubated at 4 °C overnight. The supernatant was discarded, and the wells were filled with 300 μL of blocking buffer [1% (w/v) BSA and 0.1% (v/v) Triton X-100] for 30 min at room temperature. Subsequently, the wells were incubated with 2×10^8 phages presenting amino acids 501−600 of the c-Myb protein. After incubation for 4 h at 4 °C, the wells were washed 20 times with phage-display washing buffer (0.1% (v/v) Triton X-100 buffered with PBS). To rescue the bound phages, 200 μL of a TG1 bacterial culture in Luria−Bertani medium [$OD_{600} = 0.5$] was added for 30 min at 37 °C. The solution was plated on LB^{Amp} dishes and incubated overnight. On the next day, the number of colonies was determined. BSA was used as a negative control.

Immunofluorescence Microscopy. Transfected COS7 cells were grown on sterile 20 × 20 mm coverslips for 2 days and fixed with a solution of 3% *p*-formaldehyde in PBS (w/v) for 20 min at room temperature (*58*). The cells were rinsed with PBS, neutralized with 50 mM NH_4Cl/PBS, and permeabilized with 0.1% Triton X-100 (v/v) in PBS for exactly 5 min at room temperature. The samples were blocked with 5% (v/v) goat serum in PBS, rinsed with PBS, and incubated with Mab414 (Berkeley Antibody Company, Denver, PA 17517), a mouse monoclonal antibody directed against nuclear pore proteins. A Lissamine-Rhodamine goat anti-mouse conjugate (Dianova, 20148 Hamburg, Germany) served as a secondary antibody. The cells were embedded in Mowiol and analyzed using an Olympus CK40 fluorescence microscope.

Erythropoietin Stimulation and Nuclear Extracts. Subconfluent HEL cells were incubated with 25 units/mL erythropoietin (EPO) for 90 min at 37 °C. Subsequently, the medium was removed, the cells were washed with PBS, and nuclear extracts were prepared. A total of 1×10^7 cells were washed twice with PBS, suspended in 400 μL of lysis buffer (500 mM HEPES at pH 7.9, 500 mM KCl, 500 mM EDTA, 500 mM EGTA, 500 mM $MgCl_2$, 100 mM DTT, and 10.5 mM leupeptin), and incubated on ice for 30 min. The cells were lysed using a Branson sonifier, and complete lysis was monitored by trypan blue staining of an aliquot. Nuclei were sedimented for 10 min in an Eppendorf microfuge at 13 000 rpm. The supernatant was discarded, whereas the pellet was washed twice with lysis buffer. The nuclei were lysed be resuspending them in 40 mL of wash buffer (500 mM HEPES at pH 7.9, 2.5 M NaCl, 500 mM EDTA, 500 mM EGTA, 100 mM DTT, 100 mM PMSF, and 10.5 mM leupeptin). The samples were loaded onto SDS−PAGE gels. After the electrophoresis, the proteins were electroblotted on a PVDF membrane. The Western blot was incubated with the anti-Rcd-1 antibody and stained with a goat anti-rabbit IgG antibody coupled to horseradish peroxidase. The bands were scanned with the Easy video system and quantified with the Wineasy software. RNA amounts were determined with the Advantage RT-PCR kit (BD Biosciences Clontech) as described under RT PCR. The primers GATCCACTGATAGATCTTCTCTCTGTC (sense) and CTCCAAGGCTGGTGAGCCG (anti-sense) were used in the PCR reaction.

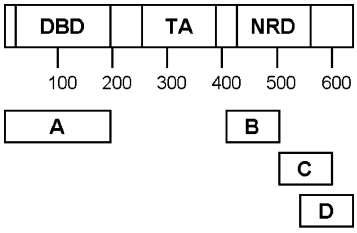

FIGURE 1: Schematic map of the baits used in the *two-hybrid* assay. The c-myb protein contains a N-terminal DNA-binding domain (DBD), a transactivating (TA) domain, and a negative regulatory (NRD) domain. Bait A covers the DBD and baits B, C, and D are located at the C-terminal domain. Only bait C identified positive clones in the *two-hybrid* assay.

RESULTS

We constructed four baits for a *yeast two-hybrid* screen: one covering the entire N-terminal DNA-binding domain (A) and three baits of 100 amino acids each at positions 400−500 (B), 500−600 (C), and 530−636 (D), respectively (Figure 1). The fact that mice with a disrupted c-myb gene die as embryos around day 15 (*37*), lead us to suspect that cofactors of c-myb might play important roles prior to this gestation stage. Therefore, we used a murine embryonic library of E11 embryos for the screen. Whereas baits A and B produced a large background of positive clones, no clones could be obtained with bait D. However, bait C resulted in positive clones in the screening of the embryonic library. A total of 4.4×10^6 clones were examined, and 34 positive clones were further analyzed. Sequencing revealed that 14 clones contained at least one stop codon in each reading frame, and two clones covered regions located downstream of the coding sequences of known genes. The majority of the remaining clones covered either frequently expressed genes (e.g., embryonic ζ-globin, ubiquitin, and collagen) or sequences of unknown function; however, all of them had at least one counterpart in the mouse EST database. One clone was identical to the murine rcd-1 gene, which was published recently (see ref *34*; accession number AF221849), including 74 bases upstream of the start codon and 363 bases downstream of the stop codon.

The rcd-1 gene encodes a protein of 299 amino acids and a molecular mass of 33.6 kD, which could be expressed as a His_6-tagged protein in *E. coli*. Using Ni-chelate affinity chromatography, we purified the native protein. A database analysis identified 22 species with homologous sequences. Table 1 shows a partial alignment of Rcd-1 proteins from vertebrates, insects, helminths, protozoa, yeasts, and various plants. The murine protein is identical to its human counterpart except for amino acid 13, where an alanine has been substituted by a threonine.

The protein is rich in leucines (15%), has a theoretical pI of 8.23, and is in agreement to the prediction of a high α-helical content (47%; data from circular dichroism spectroscopy not shown). Database mining did not reveal any conserved protein motif or signature. Although little is known about the physiological function of this protein, the high degree of homology between different species suggests an important function that was evolutionarily conserved.

Semiquantitative RT PCR demonstrated that rcd-1 RNA levels were high in lung, spleen, liver, kidney, and bone marrow (Figure 2). A similar pattern could be determined for the c-myb gene (Figure 2). C-myb is expressed predomi-

Table 1: Partial Alignment of Rcd-1 Sequences from Different Species[a]

	140	150	160	170	180	190
hsapi	KTRPFEYLRLTSLGVIGALVKTDEQE	....	VINFLLTTEIIPLCLRIMES	..	GSELSKTVATFILQKIL	
musmu	KTRPFEYLRLTSLGVIGALVKTDEQE	....	VINFLLTTEIIPLCLRIMES	..	GSELSKTVATFILQKIL	
ratno	KTRPFEYLRLTSLGVIGALVKTDEQE	....	VINFLLTTEIIPLCLRIMES	..	GSELSKTVATFILQKIL	
xenpu	KTRPFEYLRLTSLGVIGALVKTDEQE	....	VINFLLTTEIIPLCLRIMETES		GSELSKTVATFILQKIL	
zebrf	ITMPFEYLQLTSLGVIGALVNTDEQE	(EST-sequence, ends here)				
drome	KTRPFEYLRLTSLGVIGALVKTDEQE	....	VINFLLTTEIVPLCLRIMDS	..	GSELSKTVATFIIQKIL	
celeg	VSRSFEYLRLTSLGVIGALVKTDDKEQLLI		VINFLLSTEIIPLCLRIMEQ	..	GTELSKTVATFILQKIL	
trimu	TARPFEYLRLTSLGVIGALVKTDEHD	....	VINFLLNTEIIPLCLKIMES	..	GSELSKTVATFILQKIL	
schjp	RTRPFEYLRLTSLGVIGALVKTDEPE	...	EVINFLLGSEIIPLCLVIMES	..	GSETEQNCSYFHHAEAL	
trybr	TGNVTEGLRLTSLGVIGALVKTDDND	....	VMQYLLSTEIFPLCLKIMES	..	GLELSRTLATFIVQKIL	
chrei	KTRPFEYLRLTSLGVIGALVKVDDQD	....	VINFLLSTEIIPLCLRIMEI	..	GTELSKTVATFILQKIL	
leish	AERS.EYLRLTSLGVIGALVKADDQA	....	IISYLLNTEIFPLCLRIMEX	...	AIISKXISTFIXQKLL	
spomb	KSKPFEYLRLTSLGVIGALVKNDSPE	....	VINFLLSTEIIPLCLRIMEN	..	GSELSKTVAIFIVQKFL	
scerv	RQRTFEYLRLTSLGVIGALVKNDEQE	....	VITFLLRTDIIPLCLRIMES	..	SSELSKTVAIFILQKIL	
arabt	KTRPFEYLRLTSLGVIGALVKTDEQE	....	VINFLLTTEIIPLCLRIMES	..	GSELSKTVATFILQKIL	
coton	KSRPFEYLRLTSLGVIGALVKVDDTE	....	VISFLLSTEIIPLCLRIMEM	..	GSELSKTVATFILQKIL	
horde	KAQPFEYLRLTTLGVIGALVKNEDTE	....	VINYLLESEIISLCLKIMEI	..	GNEPSKTVSTFILLKLL	
lotus	KSRPFEYLRLTSLGVIGALVKVDDTE	....	VISFLLSTEIIPLCLRTMEM	..	GSELSKTVATFIVQKIL	
tomat	KSRPFEYLRLTSLGVIGALVKVDDTE	....	VISFLLSTEIIPLCLRTMEM	..	GSELSKTVATFIVQKIL	
metru	KSRPFEYLRLTSLGVIGALVKVDDTE	....	VISFLLSTEIIPLCLRTMEM	..	GSELSKTVATFIVQKIL	
soybe	KSRPFEYLRLTSLGVIGALVKVDDTE	....	VISFLLSTEIIPLCLRTMEM	..	GSELSKTVATFIVQKIL	
sotub	KSRPFEYLRLTSLGVIGALVKVDDTE	....	VISFLLSTEIIPLCLRTMEM	..	GSELSKTVATFIVQK	..

[a] Numbers in the first line of the table indicate the amino acid positions of the human and murine Rcd-1 proteins. A full alignment was not possible, because several sequences are only available as partial EST sequences. Abreviations and accession numbers: hsapi, *Homo sapiens* (NP_005435); musmu, *Mus musculus* (AF221849); ratno, *Rattus norvegicus* (AI731762); xenpu, *Xenopus laevis* (BC044281); zebrf, zebrafish (AI384537); drome, *Drosophila melanogaster* (AC009391); celeg, *Caenorhabdidis elegans* (U13875); trimu, *Trichuris muris* (AW288446); schjp, *Schistosoma japonicum* (AI891433); trybr, *Trypanosoma brucei* (AQ645259); chrei, *Chlamydomonas reinhardtii* (AV391570); leish, *Leishmania major* (AQ851005); spomb, *Saccharomyces pombe* (Q92368); scerv, *Saccharomyces cerevisiae* (P5329); arabt, *Arabidopsis thaliana* (AI995030); coton, *Gossypium hirsutum* (AI731770); horde, *Hordeum vulgare* (BE195586); lotus, *Lotus japanicus* (AW719555); tomat, *Lycopersicon esculento* (AI896651); metru, *Medicago trunculata* (AW560190); soybe, *Glycine max* (AW132481); sotub, *Solanum tuberosum* (AW907378).

nantly in hematopoietic organs (*36*) including embryonic liver (*37*). The high amount of c-myb RNA in the lung can be explained by the fact that c-myb is also expressed in vascular smooth muscle cells (VSMC) (*38*−*41*), which are present in large amounts in the lung.

To determine the subcellular localization of the protein, we expressed a Rcd-1 green fluorescent protein (GFP) fusion protein in COS7 cells. The GFP-Rcd-1 fusion protein is primarily localized in the nuclei, which were labeled with an anti-nuclear pore antibody (Figure 3).

Because the nuclear transcription factor c-Myb is localized in the same cellular compartment, we asked whether both proteins interact directly with each other in vivo or in vitro. We first used a GST pull-down assay. Purified Rcd-1 protein was immobilized on BrCN-activated Sepharose and incubated with the GST-Myb protein. GST-Myb was retained on the Rcd-1−Sepharose but not on the control Sepharose, thus suggesting an in vitro interaction (Figure 4).

We confirmed these data by a phage-display experiment, in which Rcd-1 was immobilized on microtiter plates and probed with phages expressing the C-terminal c-Myb domain. Whereas only few phages were bound to the control protein, Rcd-1 retained more than 20 times as many phages (Figure 5).

We next asked whether this interaction has any functional consequences for the cell. Therefore, we performed reporter gene assays in which the firefly luciferase gene was under the control of the c-Myb-specific mim-1 promoter. When HEK293 cells were transfected with the pmimLuc reporter construct and the eukaryotic c-Myb expression vector, the luciferase signal was drastically increased (Figure 6). However, when c-myb and rcd-1 were cotransfected, the signal decreased in a dose-dependent manner. The Rcd-1 protein has no DNA-binding activity by itself, because it does not bind to DNA−Sepharose (data not shown). Thus, the negative effect of Rcd-1 on the c-Myb driven transactivation of the mim-1 promoter must have been due to the protein−protein interaction between Rcd-1 and c-Myb.

We wanted to know whether rcd-1 can regulate other transcriptional pathways in the absence of c-myb; therefore, we used the Mercury-Pathway Profiling System (BD Clontech). This is a reporter system of different plasmids in which the luciferase gene is under the control of distinct cis-acting elements characteristic of known signal transduction pathways. The system can scrutinize the regulation of a responsive element by any factor in a given genetic background. To identify further potential targets of Rcd-1, the jun/fos-, cAMP-, Interferon-, E2F-, and serum-responsive elements were probed for regulation by Rcd-1 (Figure 7). COS7 cells were grown in a serum-free medium and transfected as described in the Materials and Methods. The cells grew slowly and did not give rise to high luciferase output. The signals of the interferon- and serum-responsive elements remained unchanged under the influence of Rcd-1 alone. In contrast, the luciferase signal of the AP1 (jun/fos) responsive element was drastically reduced when Rcd-1 was transfected into cells. Moreover, an almost 3-fold downregulation was observed for the CRE pathway and a clear upregulation in

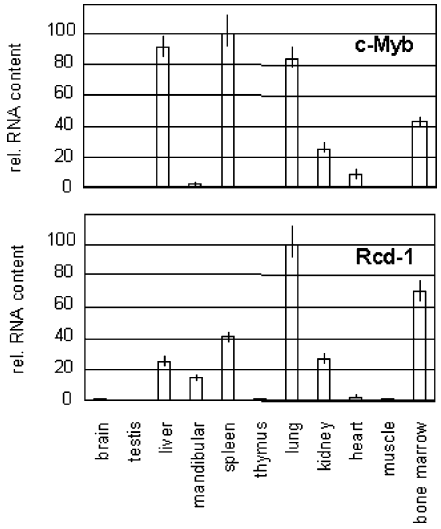

FIGURE 2: Expression pattern of c-Myb and Rcd-1 in different organs. RNA was prepared from the organs of a sacrificed rabbit. Quantitative RT PCR was performed on equal amounts of RNA with the specific primers for c-myb and rcd-1 as described in the Materials and Methods. RT PCR on the β-actin gene was performed as an internal control for the standardization of the different samples. The c-myb and rcd-1 RNA content of each organ was normalized with the respective β-actin amount, and the highest signal was set to 100. The figure thus compares the relative abundance of these RNAs in different organs. To eliminate errors caused by net weight or yields of the RT reaction, the experiments were repeated three times. Quantitative PCR products were run on 10% (w/v) acrylamide gels and analyzed with the Easy video documentation system using the Wineasy software for quantification. The numbers indicate relative amounts of the PCR product.

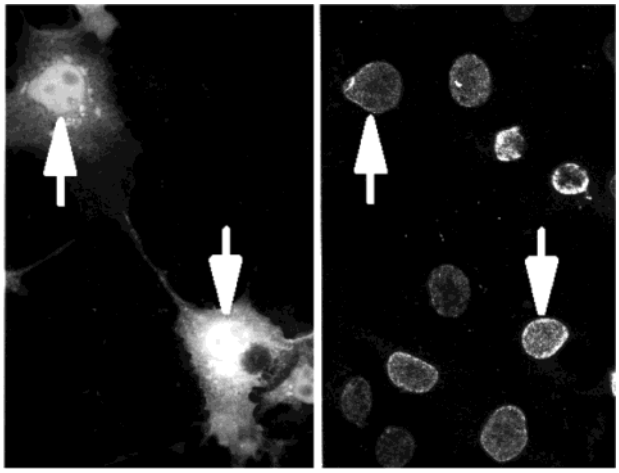

FIGURE 3: Subcellular localization of Rcd-1. Rcd-1 was expressed transiently as a EGFP-fusion protein in COS7 cells, and the cells were fixed after 2 days (left panel). Nuclei were labeled with a mouse monoclonal antibody directed against nuclear pore proteins. A Lissamine-Rhodamine goat anti-mouse conjugate served as a secondary antibody (right panel). The Rcd-1 EGFP-fusion protein was detected by its endogenous fluorescence (right panel). The arrows indicate identical cells.

the E2F pathway. Although the latter two were not as prominent as in the case of AP1, these data raise the possibility that Rcd-1 is targeting other pathways besides AP1.

Because c-Myb is involved in hematopoietic differentiation and because the AP1 pathway is also induced by erythropoietin (EPO) (*57*), we asked whether the rcd-1 gene is stimulated by EPO in HEL cells that are known to express

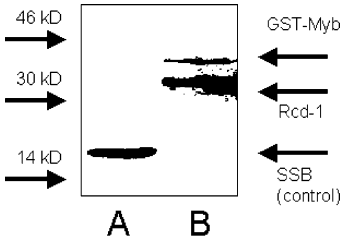

FIGURE 4: In vitro interaction of Rcd-1 and c-myb. SSB from *E. coli* as a control (A) and Rcd-1 (B) were immobilized on CNBr-activated Sepharose. The resins were incubated with GST-Myb. After washing, GST-Myb was retained on the column containing Rcd-1−Sepharose but not on the one with SSB.

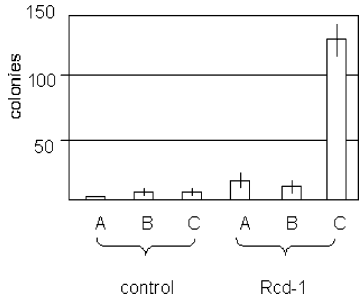

FIGURE 5: Phage-display experiment with Rcd-1 and c-myb. Rcd-1 or the control protein were immobilized on microtiter dishes. The proteins were incubated with phages expressing amino acids 501−600 of c-Myb. The wells were washed 20 times. To rescue bound phages, *E. coli* TG1 cells were added, plated on LB^{Amp} dishes, and incubated overnight. On the next day, the number of colonies was determined. Columns A correspond to 15 washing steps, columns B correspond to 20 washing steps, and columns C represent the final phage number. Whereas only a few phages were bound to the control protein, more than 20-fold more phages remained in the Rcd-1-coated wells.

the EPO receptor. The cells were challenged with EPO, harvested, and analyzed by semiquantitative PCR and Western blot. The results indicate that the transcription and translation of the rcd-1 gene was stimulated by EPO (Figure 8).

DISCUSSION

During development, multipotent progenitors differentiate via specific lineages into several different specialized cell types. For example, hematopoietic cells mature into erythroid, lymphoid, and myeloid lineages. This process is mediated by a variety of hematopoietic transcription factors that are involved in multiple protein−protein and protein−DNA interactions (*42*). The c-myb proto-oncogene is required for the generation of monocytes from a myeloid progenitor (reviewed in ref *43*). The promyelocyte-specific mim-1 gene, identified first by ref *44*, and myeloid genes such as NF/M (*9*), tom-1 (*45*), and neutrophil elastase (*46*) are all regulated by c-Myb. Moreover, further specific transcription factors, cofactors, and transcription mediators cooperate with c-Myb in the activation of corresponding promoters (see ref *47* for a review).

According to the current model of transcription initiation, RNA polymerase II associates with the TATA-binding protein (TBP), general transcription factors (GTFs) (for a review, see ref *48*), and transcription accessory factors (TAFs) to form the preinitiation complex (PIC) at the promoter. The formation of the RNA polymerase holoenzyme is completed by the addition of mediator and suppres-

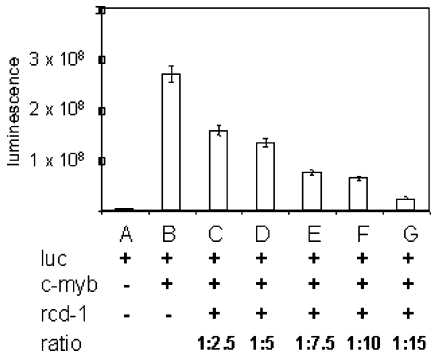

FIGURE 6: Reporter gene assay with c-myb and rcd-1. The plasmid pMim-1Luc with the firefly luciferase under the control of the mim-1 promoter was used as the reporter. As an internal control, the vector pEQ176, which expresses the bacterial β-galactosidase, was cotransfected to monitor the transfection efficiency. The experiment was carried out in duplicate as described in the Materials and Methods, and it was repeated three times. Because the monitor plasmid pEQ176 shares the genetic background of the c-myb effector vector EQ176P2Myb, including the promoter, we could test that cotransfection of pEQ176 and the eucaryotic Rcd-1 expression vector had no effect on the β-galactosidase activity (data not shown). Therefore, it is not likely that the effect of Rcd-1 coexpression is due to repressive effects on the c-Myb expression vector. Column A shows the signal of the luciferase construct and column B, the cotransfection of the reporter and c-myb plasmid. In the following columns, the amount of c-myb was kept constant and the rcd-1 expression plasmid was added. The ratios of c-myb/rcd-1 are indicated in the last line.

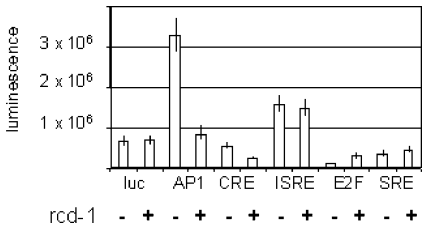

FIGURE 7: Pathway profiling of Rcd-1 on various promoters in the absence of c-myb. COS7 cells were grown in serum-free medium and were transfected with luciferase reporter vectors under the control of various promoter elements. Rcd-1 dramatically decreases the reporter signal of the AP1 element, and it also seems to decrease the signal of the CRE element. A slight increase of the E2F promoter is also detectable, suggesting that rcd-1 may act via various pathways. Cotransfection of rcd-1 did not change the luciferase signal of the ISRE or SRE elements.

sor (SRBP) proteins, and further completed by specific transcription factors and bridging proteins, which assist by binding cooperatively to remote DNA sequences. The chromatin structure is remodeled by the activity of histone acetyltransferases (HATs), histone deacetylases (HDACs), and transcription mediators (for a review, see refs *49*−*51*). The entire differentiation and development of an organism is thus mediated by varying the subunit composition of such multiprotein complexes.

The yeast CCR4 complex is a transcription mediator. Its human orthologs (*52*) differ clearly from other regulatory complexes such as SAGA, SWI/SNF, or TFIID (for a recent review, see ref *53*). The human ortholog is in vivo in either a 1.0 or 1.9 MDa complex and has been implicated in repression of RNA polymerase II transcription. The complex contains ubiquitin ligase (*28*) and mRNA deadenylase activities (*29*, *30*, *32*) and is associated with TAFs (*54*) and SRB proteins (*55*) of polymerase II. CAF40 is an integral component of the CCR4−NOT complex. In *Saccharomyces*

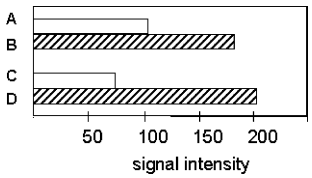

FIGURE 8: EPO stimulation of the rcd-1 gene. HEL cells were stimulated with erythropoietin, and the relative amount of rcd-1 RNA before (A) and after (B) stimulation was determined by semiquantitative RT PCR with the Advantage RT-PCR kit (BD Biosciences Clontech). The PCR products were separated on 10% PAGE gels and analyzed by the Easy video system using the Wineasy software for quantification. For this reason, we could record only the relative increase of the bands. The Rcd-1 protein of uninduced and challenged HEL cells was analyzed on Western blots, and the bands were quantified using the video system as mentioned above. The Rcd-1 protein was detectable in HEL cells (C) and was increased after EPO stimulation (D).

pombe, the gene inhibits sporulation induced by nitrogen starvation; therefore, it was termed Rcd-1 (*33*).

The murine rcd-1/CAF40 homologue was isolated as an erythropoietin-responsive gene thought to be involved in hematopoietic cell development (*34*). We could demonstrate that rcd-1 gene transcription is stimulated by EPO. Rcd-1 consists of 299 amino acids that are evolutionarily conserved. However, no significant protein motifs were identified, using several search algorithms. The protein shares a similar expression pattern with c-myb in liver, spleen, lung, kidney, and bone marrow. The expression pattern matches basically the results of a recent paper (*59*), in which Rcd-1 protein was quantified in a Western blot. This paper also reports that Rcd-1 is required in the branching morphogenesis of the embryonic lung of the mouse, which explains the high level of Rcd-1 in this organ. Our studies demonstrate that the protein is mainly localized in the nucleus and that Rcd-1 interacts directly with c-Myb.

Cotransfection of c-myb and rcd-1 downregulates the c-Myb-specific mim-1 promoter in a dose-dependent manner. Furthermore, the activity of the AP1 transcription factor on its target genes is reduced in the presence of Rcd-1 protein.

Recently, ref *59* described Rcd-1 as a novel transcriptional cofactor, which mediates retinoic-acid-induced differentiation of F9 cells. Rcd-1 is a component of the DRF transcriptional complex, which consists of at least CBP, ATF-2, and the retinoic acid receptor RAR. It is known that c-Myb can also associate with histone acetyltransferases; e.g., it cooperates with the C/EBP family member NF-M and the histone acetyltransferase CBP at the mim-1 promoter (*12*). Furthermore, c-Myb recruits the SWI/SNF chromatin-remodeling complex via a C/EBP β isoform, which in turn stimulates mim-1 transcription (*56*). Thus, it is tempting to speculate that chromatin remodeling is involved in the CCR4−NOT-complex-dependent repression of polymerase II activity.

When taken together, Rcd-1 is an important protein of the CCR4 complex involved in the repression of RNA polymerase II transcription. Rcd-1 interacts with c-Myb and depresses the transactivation of c-Myb and AP on their target genes. It is conceivable that other proteins of the CCR4 complex are involved in this cross-talk network. Further research will reveal whether the cross talk between other modules of the CCR4 transcription mediator and c-Myb will have an effect on the transcription of hematopoietic genes.

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