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c-*myb* Transactivates *cdc2* Expression via Myb Binding Sites in the 5′-Flanking Region of the Human *cdc2* Gene*

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The c-*myb* protooncogene is preferentially expressed in hematopoietic cells and is required for cell cycle progression at the G_1/S boundary. Because c-*myb* encodes a transcriptional activator that functions via DNA binding, it is likely that c-*myb* exerts its biological activity by regulating the transcription of genes required for DNA synthesis and cell cycle progression.

One such gene, *cdc2*, encodes a 34-kDa serine-threonine kinase that appears to be required for G_1/S transition in normal human T-lymphocytes. To determine whether c-*myb* is a transcriptional regulator of *cdc2* expression, we subcloned a segment of a *cdc2* human genomic clone containing extensive 5′-flanking sequences and part of the first exon. Sequence analysis revealed the presence of two closely spaced Myb binding sites that interact with bacterially synthesized Myb protein within a region extending from nucleotides −410 to −392 upstream of the transcription initiation site. A 465-base pair segment of 5′-flanking sequence containing these sites was linked to the CAT gene and had promoter activity in rodent fibroblasts. Cotransfection of this construct with a full-length human c-*myb* cDNA driven by the early simian virus 40 promoter resulted in a 6–8-fold enhancement of CAT activity that was abrogated by mutations in the Myb binding sites. These data suggest that c-*myb* participates in the regulation of cell cycle progression by activating the expression of the *cdc2* gene.

The c-*myb* protooncogene is the cellular homologue of the transforming v-*myb* gene of the avian myeloblastosis and the avian leukemia (E26) viruses (1, 2). Although c-*myb* expression has been detected in non-hematopoietic cell types (3–5), the predominant pattern of c-*myb* expression in immature hematopoietic cells has long suggested that c-*myb* plays an important role in the regulation of proliferation and/or differentiation of these cells (6, 7).

Myb proteins have several properties characteristic of transcriptional regulators, including nuclear localization (8, 9), DNA binding ability *in vitro* (10, 11), and transcriptional activation and repression functions (12), suggesting that the regulatory effect is exerted at the transcriptional level. The DNA binding domain recognizes the consensus sequence YAAC(G/T)G (where "Y" is a pyrimidine nucleotide; Ref. 13). Several studies have demonstrated Myb binding to variants of this consensus sequence (14–16), although the YAAC sequence appears to be the major element needed for high affinity Myb binding. In transient expression assays, Myb binding sites have clearly been shown to enhance the expression of the reporter gene regardless of the orientation of the binding sites (16–18). Only a few promoters have been shown to contain functional Myb binding sites (14–17, 19, 20). These promoters contain three or four closely spaced Myb binding sites, a feature that might be significant in c-*myb*-regulated promoters.

Exposure of mitogen- or antigen-stimulated peripheral blood mononuclear cells to c-*myb* antisense oligodeoxynucleotides prevents T-lymphocyte proliferation by blocking these cells at the G_1/S boundary (21, 22). In one of these investigations (22), it was also shown that c-*myb* antisense oligodeoxynucleotide treatment prevented the accumulation of the *cdc2*-encoded product (p34 serine-threonine kinase), which is implicated in the regulation of mammalian cell proliferation (23, 24).

Because *cdc2* antisense oligodeoxynucleotide treatment also prevented G_1/S transition of normal T-lymphocytes (20), we investigated the possibility that c-*myb* expression is functionally linked to that of *cdc2*. In light of the function of Myb proteins as transcriptional regulators, we asked whether they are directly involved in transcriptional regulation of the *cdc2* gene by interacting with Myb binding sites in the *cdc2* 5′-flanking region.

MATERIALS AND METHODS

Introduction of a Constitutively Expressed c-myb cDNA into CTLL-2 Cells—CTLL-2 is a murine interleukin-2 (IL-2)[1] dependent cell line (25). The plasmids used for transfection were pMbmI (26), which contains a human c-*myb* full-length cDNA driven by the SV40 early promoter, and pLHL4 (27), which contains the gene encoding hygromycin resistance. 2.5–5.0 × 10^6 CTLL-2 cells were washed twice in ice-cold magnesium- and calcium chloride-free phosphate-buffered

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L06298.

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[1] The abbreviations used are: IL-2, interleukin-2; CAT, choloramphenicol acetyltransferase; PCR, polymerase chain reaction; nt, nucleotide(s); SV, simian virus.

saline, resuspended in 0.8 ml of phosphate-buffered saline, and placed in 0.4-cm electrode gap Gene Pulser cuvettes (Bio-Rad), containing 5 μg of linearized pLHL4 and 50 μg of linearized pMbmI. Cuvettes were incubated on ice for 10 min, pulsed at 300 mV and 960 microfahrads (Gene Pulser apparatus, Bio-Rad), and incubated on ice an additional 10 min. Cells were kept in 10 ml of RPMI 1640, 10% heat-inactivated fetal calf serum, 15 mM sodium pyruvate, 10 mM L-glutamine for 12 h, washed, and placed in fresh medium. After 48 h, cells were exposed to 550 μg/ml hygromycin B and washed, and medium was replaced every 4 days until hygromycin-resistant clones appeared at days 15–20.

RNA Blots—Total RNA was extracted by the method of Chomczynski and Sacchi (28) and analyzed on RNA blots by the method of Thomas (29).

Hybridization probes were the human c-*myb* cDNA insert derived from plasmid pMbmI (26), the human *cdc2* cDNA insert derived from plasmid pcdc2 HS.1 (the kind gift of Dr. David Beach), and the human β-actin cDNA insert (30).

Isolation of the cdc2 5′-Flanking Region—A human liver genomic library (Promega, Madison, WI) was screened as described (30) with a full-length cDNA insert from the plasmid pcdc2 HS.1. Four clones were isolated after tertiary screening, purified, and subjected to restriction enzyme analysis. Two of the clones appeared to represent the full-length gene and were characterized in greater detail. A 5.0-kilobase *Sac*I-*Not*I human genomic fragment, which hybridized to a synthetic oligomer corresponding to the most 5′ segment of the human *cdc2* cDNA, was subcloned into the *Sac*I-*Not*I restriction sites of the Bluescript SK vector (Stratagene, La Jolla, CA) and was named pcdc2SN.

Sequence Analysis—The pcdc2SN insert was sequenced by the dideoxy sequencing method using the Sequenase enzyme from United States Biochemical Corp, or *Taq* Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems). Synthetic oligonucleotide primers were synthesized on an Applied Biosystems apparatus.

Plasmid Construction—Reporter constructs were made by linking different segments of the human *cdc2* 5′-flanking region to the bacterial choloramphenicol acetyltransferase (CAT) gene of the pUCCAT plasmid (Promega, Madison, WI) as reporter. The pUCCAT reporter vector contains a polylinker upstream of the CAT gene followed by the SV40 polyadenylation signal. All plasmids were constructed from pcdc2SN by ligation of fragments generated by restriction enzyme digestion (*cdc2-Pst*I, *cdc2-Pvu*II, *cdc2-Ssp*I) or by polymerase chain reaction (PCR) amplification (*cdc2*-11, *cdc2*-7, *cdc2*-12) to the pUCCAT polylinker.

For production of Myb protein in bacteria, the c-*myb* plasmid pc-myb/Flag6A was constructed as follows. A blunt-ended *Nco*I-*Nde*I fragment derived from plasmid pMbmI (26) and extending from nucleotide (nt) 112 (centered on the initiation codon) to nt 2440 (in the 3′-untranslated region), was ligated to a blunt-ended *Hin*dIII site of the pFlag-1 expression vector (IBI, New Haven, CT). Sequence analysis confirmed that the c-*myb* coding sequence was in the sense orientation downstream of the bacterial *tac* promoter.

Mutagenesis of Myb Binding Sites in pcdc2-7 Plasmid—Mutagenesis of the Myb binding sites was carried out by PCR as described (16), using a mutated 5′ oligonucleotide primer, that corresponds to nt −416 to −385 and changes the wild-type sequence containing two closely spaced Myb binding sites (5′-AAAAACATAACTATA-CACTCCTAACCCTAAGT-3′) to (5′-AAAAACAGGGCTATA-CACTCCGGGCCCTAAGT-3′), and a 3′ primer (5′-TCTA-GAGTCGACCTGCCAGGC-3′) corresponding to a segment of the pUCCAT polylinker downstream of the *Sal*I restriction site. This plasmid is named pcdc2-7 MUT.

Gel Retardation Assay—HB101 cells containing the parental pFlag (IBI, New Haven, CT) expression vector or HB101 cells containing the pc-MYB/Flag vector were incubated for 4 h in the presence of 1.5 mM IPTG to an OD = 0.5 ($A_{600\,nm}$). Bacterial lysates were quantitated by the Bradford method. Myb expression was determined by Western blot using an anti-Myb-specific antibody (Cambridge Research). Gel retardation assays were performed as described (16) except that probe (10^4 cpm) was incubated with or without 1.0 μg of poly(dI·dC) and reactions were run on a 5% polyacrylamide gel electrophoresis in 0.25 × TBE.

Oligodeoxynucleotide probes were made by synthesizing the oligomers (sense and antisense strands) using an automated DNA synthesizer (Applied Biosystems) and combining them in equal molar amounts to form double-stranded fragments. The wild-type probe (*cdc2*-7 MYB) contains two Myb binding sites and extends from nt −416 to −385 of the human *cdc2* 5′-flanking region; the mutant probe (*cdc2*-7 MUT) corresponds to the same fragment with three nucleotide substitution in each Myb binding site. The sequences of the probes are as follows.

cdc2-7 MYB 5′-AAAAACATAACTATACACTCCTAACCCTAAGT-3′
TTTTGTATTGATATGTGAGGATTGGGATTCAT

SEQUENCE 1

cdc2-7 MUT 5′-AAAAACAGGGCTATACACTCCGGGCCCTAAGT-3′
TTTTGTCCCGATATGTGAGGCCCGGGATTCAT

SEQUENCE 2

Transient CAT Analysis—CAT assays were performed as described (31). Briefly, 2 μg of CAT reporter plasmid was transfected with or without 10 μg of effector plasmid plus 1 μg of pSV-B-gal, which contains the bacterial β-galactosidase gene driven by the SV40 promoter as an internal control of transfection efficiency into Balb/c/3T3 and Tk-ts13 Syrian hamster fibroblasts (32) using the calcium-phosphate precipitation method (33). At 48 h after transfection, cells were harvested and proteins were extracted by freeze-thawing and normalized for transfection efficiency by β-galactosidase assay as described by the manufacturer (Promega). For each assay, cellular lysate was incubated with [^{14}C]choloramphenicol and acetyl CoA for 1 h at 37 °C. Transactivation of reporter constructs was assayed by measuring the amount of acetylated [^{14}C]choloramphenicol by thin-layer chromatography followed by autoradiography and Cerenkov counting.

RESULTS

Expression of cdc2 mRNA in Nontransfected and SV-myb (bMbmI)-transfected CTLL-2 Cells—CTLL-2 cells were transfected by electroporation with a human c-*myb* cDNA driven by the early SV40 promoter (pMbmI) and the plasmid containing the gene encoding hygromycin resistance (pLHL4). After selection, a hygromycin-resistant mixed cell population was expanded and analyzed for expression of the exogenous human c-*myb*. High stringency hybridization with a human specific probe derived from the 3′-untranslated region of the human c-*myb* cDNA, revealed c-*myb* expression in SV-myb-transfected CTLL-2 cells but not in nontransfected cells (not shown).

cdc2 mRNA levels were then measured in parental and SV-myb-transfected CTLL-2 cells; because CTLL-2 cell proliferation is strictly dependent on the presence of IL-2 in the medium, CTLL-2 cells (nontransfected and SV-myb-transfected) were washed and cultured in the absence of IL-2 for 2, 4, and 8 h before total RNA extraction and analysis of *cdc2* mRNA levels. *cdc2* mRNA levels were lower in parental than in SV-myb-transfected CTLL-2 cells. Such levels were readily down-regulated in nontransfected CTLL-2 cells, but remained essentially constant in SV-myb-transfected CTLL-2 cells (Fig. 1), suggesting the existence of a functional link between c-*myb* and *cdc2* genes.

Isolation of the Genomic Clone Containing the Human cdc2 5′-Flanking Region—Using a full-length human *cdc2* cDNA insert, we screened a human genomic DNA library for clones carrying the genomic counterpart of the *cdc2* cDNA and 5′-

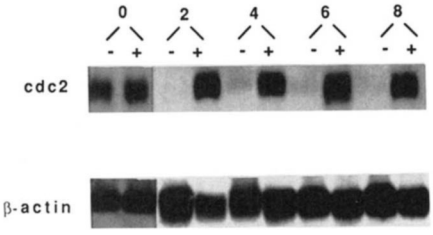

FIG. 1. ***cdc2* mRNA levels in parental and SV-myb-transfected CTLL-2 cells.** Total RNA was isolated from CTLL-2 cells and hybridized with a human *cdc2* or a human β-actin cDNA insert. *0*, *2*, *4*, *6*, and *8* indicate hours of growth in the absence of IL-2 in parental (−) or SV-myb-transfected (+) CTLL-2 cells.

flanking sequences. Of four clones isolated, two were found to be identical by restriction enzyme digestion. A 5.0-kilobase *Sac*I-*Not*I fragment hybridizing to the most 5′ region of the human *cdc2* cDNA was subcloned into the SK vector and sequenced. Sequence analysis of subclone pcdc2SN revealed that 50 base pairs were identical to the 5′ region of the published sequence of the human *cdc2* cDNA (34).

The CAP site of *cdc2* mRNA was determined using both S1 nuclease assay and primer extension analysis. Both methods identified two initiation sites: one major site located at +1 of the sequence and a minor site (Fig. 2, *asterisk*), in agreement with the findings recently published by Dalton (35).

Functional Analysis of the Human cdc2 5′-Flanking Region in Fibroblasts—To delineate the sequences in the 5′-flanking region that are important in regulating human *cdc2* gene expression, a functional analysis was carried out using hybrid genes in which segments of various lengths of the *cdc2* 5′-flanking region were linked to the bacterial CAT gene. The sequences cloned upstream of the bacterial CAT gene included the 5′-flanking sequence of *cdc2* gene from residue -1034 (*cdc2*-11 in Fig. 2) to residue +64 (*Not*I restriction site) in the 5′-untranslated region of *cdc2* cDNA. Progressive deletions were carried out using unique restriction sites in the 5′-flanking region or by PCR amplification using as 5′ primers the oligomers *underlined* in Fig. 2. These deletions are summarized in Fig. 3*A*. After transfection of exponentially growing TK-ts13 cells (31) with the chimeric plasmids, promoter activity was determined by assaying CAT activity 48 h later. With the exception of the *cdc2*-12 deletion, all of the constructs had promoter activity (Fig. 3*B*).

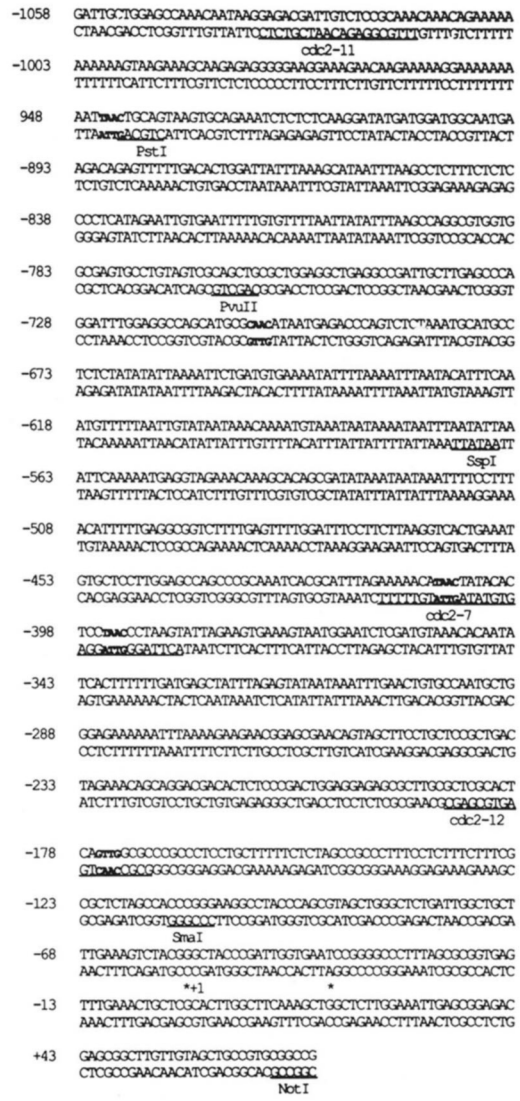

FIG. 2. **Partial sequence of pcdc2SN genomic subclone insert.** Nucleotides are *numbered* from the major initation site (+1).

Binding of Myb Protein to Putative Myb Binding Sites in the cdc2 5′-Flanking Region—Because sequence analysis revealed the presence of two closely spaced putative Myb binding sites in the *cdc2*-7 deletion mutant, we used this mutant in analyses to determine whether Myb protein binds to these sites and transactivates *cdc2* gene expression. Gel retardation assays in which a bacterial lysate containing Myb protein was reacted with a ^{32}P-labeled DNA oligomer containing the putative Myb binding sites (nt −416 to −383) revealed two complexes with retarded mobility (Fig. 4*A*, *lane 3*), whereas no complex was seen with a bacterial lysate that lacked Myb protein (Fig. 4*A*, *lane 2*). The specificity of Myb protein binding to this fragment was demonstrated by the almost complete disappearance of complex formation when a radiolabeled synthetic oligomer with nucleotide substitutions in the Myb binding sites (*cdc2*-7 MUT) was used as probe (Fig. 4*A*, *lane 6*). The formation of two complexes is most likely due to interaction of two Myb molecules with distinct binding sites in the fragment because gel retardation assays with synthetic oligomers containing each of the two binding sites eliminates only one complex (Fig. 4*B*, *lanes 1* and *2*).

Transactivation by c-myb of CAT Gene Expression Driven by the Human cdc2 5′-Flanking Region Containing Myb Binding Sites—The ability of c-*myb* to transactivate a CAT reporter construct, containing a 465-base pair segment of the human *cdc2* 5′-flanking region with two Myb binding sites able to interact with bacterially produced Myb protein, was assessed in transient expression assays. In Balb/c/3T3 cells transfected at a 5:1 effector-to-reporter ratio and assayed after 48 h, the SV40 c-*myb* effector plasmid induced a 6-fold in-

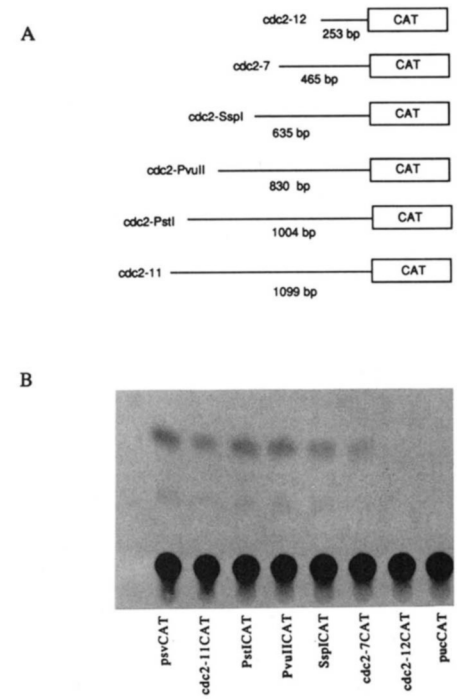

FIG. 3. **Promoter activity of *cdc2* 5′-flanking region.** *A*, reporter constructs in which different segments of the human *cdc2* 5′-flanking region drive the bacterial CAT gene. *B*, autoradiograms of CAT assays.

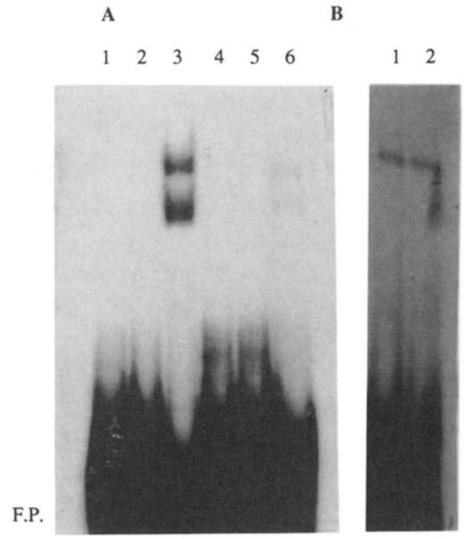

FIG. 4. **Gel retardation assays using bacterially synthesized Myb protein.** *Panel A*: *lanes 1* and *4*, free probe (*F.P.*) only; *lanes 2* and *5*, probe plus 1 μg of parental bacterial lysate; *lanes 3* and *6*, probe plus 1 μg of bacterial lysate containing human Myb protein. In *lanes 1–3*, the probe used is *cdc2*-7 MYB; in *lanes 4–6*, the probe used is *cdc2*-7 MUT. *Panel B*: *lane 1*, synthetic 21-mer including the 5′ Myb binding site (from nt −416 to −396); *lane 2*, synthetic 19-mer including the 3′ Myb binding site (from nt −403 to −385).

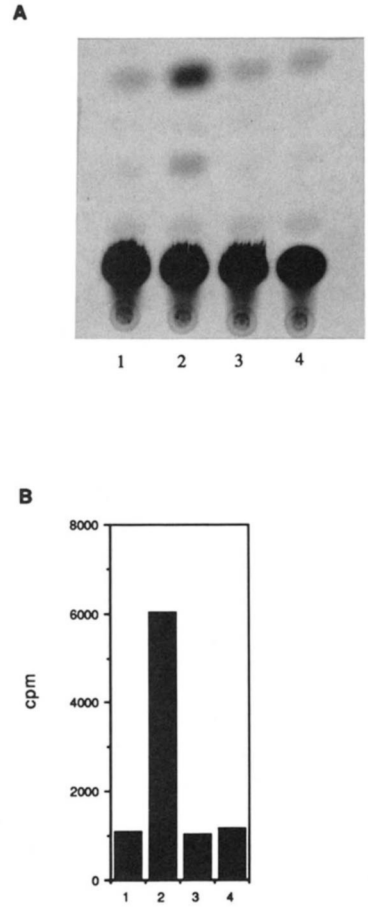

FIG. 5. ***cdc2* promoter transactivation by c-*myb* in Balb/c/3T3 fibroblasts.** *A*, autoradiogram shows CAT activity in lysates of cells transfected with: *lane 1*, pcdc2–7 CAT only; *lane 2*, pcdc2–7 CAT and pMbmI; *lane 3*, pcdc2–7MUTCAT only; *lane 4*, pcdc2–7MUTCAT and pMbmI. *B*, Cerenkov counting of acetylated [^{14}C] choloramphenicol. *Lanes* correspond to those in *A*.

crease in CAT expression driven by the wild-type *cdc2*-7 fragment (Fig. 5, *A* and *B*, *lane 2*), whereas such transactivation was essentially abolished in Balb/c/3T3 cells transfected with the construct pcdc2–7 MUT in which the two Myb binding sites were mutated (Fig. 5, *A* and *B*, *lane 4*). To demonstrate that the transactivating activity of c-*myb* on the *cdc2* promoter was not restricted to Balb/c/3T3 cells, parental Syrian hamster TK-ts13 cells, and TK-ts13 cells, which constitutively express an exogenous human c-*myb* cDNA (33), were transfected with the wild-type or the mutated pcdc2–7 construct and assayed for CAT activity 48 h later. An 8-fold increase in CAT expression driven by the wild-type pcdc2–7 fragment (Fig. 6, *A* and *B*, *lane 2*) was detected in SV-myb TK-ts13 as compared to parental TK-ts13 cells (Fig. 6, *A* and *B*, *lane 1*) and such transactivation was nearly abolished in SV-myb TK-ts13 cells transfected with pcdc-7MUT (Fig. 6, *A* and *B*, *lane 4*).

DISCUSSION

The importance of c-*myb* in cell cycle progression has been recently supported by experiments demonstrating that inhibition of Myb protein synthesis prevents G_1/S transition of normal and leukemic hematopoietic cells (21, 36–38). Because the c-*myb* gene encodes a transcriptional regulator, it appeared likely that the transactivating property was essential for Myb function and that genes directly involved in DNA synthesis and cell cycle progression are targets of c-*myb* regulation.

The *cdc2* gene was first shown to regulate the G_1/S and G_2/M transition of the cell cycle in the fission yeast *Schizosaccaromyces pombe* (39). The gene encodes a 34-kDa (p34) serine-threonine kinase essential for initiation of DNA replication and entry into mitosis (39). A human homologue of the *cdc2* gene, cloned by functional complementation in yeast (34) and encoding a serine-threonine kinase (p34cdc2) with 63% amino acid identity to fission yeast p34, has been implicated in the regulation of mitotic initiation in mammalian cells (23, 24), and perhaps at the G_1/S transition (22).

Although post-translational regulation has been thought as the primary mechanism in the control of p34cdc2 kinase activity in the cell cycle of eukaryotes (40), there is evidence that p34cdc2 levels in mammalian cells are also regulated by growth factors that induce an increase in *cdc2* mRNA levels. For example, *cdc2* mRNA is undetectable in quiescent T-lymphocytes, but is abundantly expressed at the G_1/S transition in T-lymphocytes exposed to phytohemagglutinin or IL-2 (22). In addition, transcriptional control of *cdc2* expression in mammalian cells has been recently demonstrated (35).

The observations that *cdc2* mRNA expression is induced after that of c-*myb* in phytohemagglutinin-stimulated T-lymphocytes, and that antisense oligodeoxynucleotides to *cdc2* mRNA prevent G_1/S transition in these cells (22) prompted us to investigate whether c-*myb* participates directly in the regulation of *cdc2* expression. A genomic *cdc2* clone containing extensive 5′-flanking sequences was identified, and various segments of the 5′-flanking region were linked to the reporter bacterial CAT gene and assayed for promoter activity. The reporter construct pcdc2–7CAT containing 465 base pairs of 5′-flanking sequences had promoter activity, as revealed by transient CAT expression analysis, and contained two closely spaced Myb binding sites (Fig. 2). Upon cotransfection with a full-length human cDNA driven by the SV40 early promoter, CAT activity of this reporter construct was transactivated 6–8-fold in rodent fibroblasts. Mutation of the Myb binding sites abolished this effect almost completely.

The extent of Myb transactivation is modest but consistent

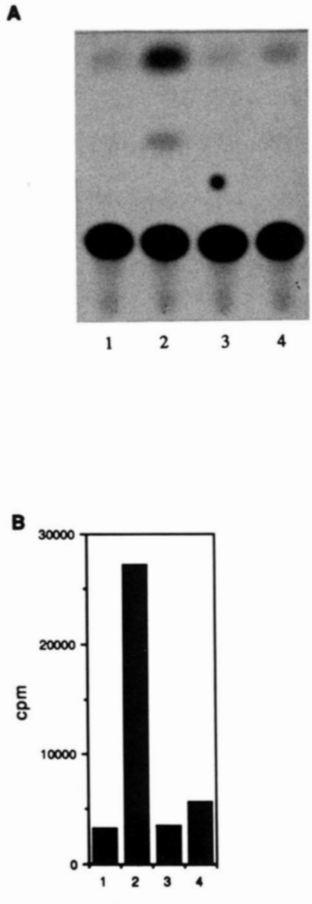

FIG. 6. ***cdc2* promoter transactivation in Syrian hamster SV-myb TK-ts13 cells.** *A*, autoradiogram shows CAT activity in lysates of: *lane 1*, parental TK-ts13 cells transfected with pcdc2–7CAT; *lane 2*, SV-myb TK-ts13 cells transfected with pcdc2–7CAT; *lane 3*, parental TK-ts13 cells transfected with pcdc2–7MUTCAT; *lane 4*, SV-myb TK-ts13 cells transfected with pcdc2–7MUT CAT. *B*, Cerenkov counting of acetylated [^{14}C]choloramphenicol.

with previous observations indicating that c-*myb* is a low activity transactivator (16, 41). Because Myb has been shown to interact synergistically with the Epstein-Barr virus BZLF1 transactivator in lymphoid cells (42), with *ets2* gene to transactivate the mim-1 promoter (41), and with Jun family members to transactivate the c-*myb* promoter itself (43), we suggest that c-*myb* exerts its action in cooperation with other transactivators. Because we have been unable to demonstrate cooperation between c-*myb* and c-*jun* or *ets2* in transactivating *cdc2* expression, other partners might be involved in the regulation of *cdc2* expression. The identification of such partners would allow a better understanding of mechanism(s) of action of c-*myb* in cell cycle progression.

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