Regulatory domains of the A-Myb transcription factor and its interaction with the CBP/p300 adaptor molecules

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The A-Myb transcription factor belongs to the Myb family of oncoproteins and is likely to be involved in the regulation of proliferation and/or differentiation of normal B cells and Burkitt's lymphoma cells. To characterize in detail the domains of A-Myb that regulate its function, we have generated a series of deletion mutants and have investigated their trans-activation potential as well as their DNA-binding activity. Our results have allowed us to delineate the trans-activation domain as well as two separate regulatory regions. The boundaries of the trans-activation domain (amino acid residues 218–319) are centred on a sequence rich in charged amino acids (residues 259–281). A region (residues 320–482) localized immediately downstream of the trans-activation domain and containing a newly identified conserved stretch of 48 residues markedly inhibits specific DNA binding. Finally the last 110 residues of A-Myb (residues 643–752), which include a sequence conserved in all mammalian *myb* genes (region III), negatively regulate the maximal trans-activation potential of A-Myb. We have also investigated the functional interaction between A-Myb and the nuclear adaptor molecule CBP [cAMP response element-binding protein (CREB)-binding protein]. We demonstrate that CBP synergizes with A-Myb in a dose-dependent fashion, and that this co-operative effect can be inhibited by E1A and can also be observed with the CBP homologue p300. We show that this functional synergism requires the presence of the A-Myb charged sequence and that it involves physical interaction between A-Myb and the CREB-binding domain of CBP.

INTRODUCTION

Transcription factors control crucial cellular processes such as cell growth and differentiation, and also link extracellular signals to cellular responses. Over the past few years many transcription factors have been shown to contain regulatory domains, which can increase or decrease their transcriptional and/or DNA-binding activity [1–6]. The mechanisms by which this regulation takes place frequently involve phosphorylation, dimer formation or interaction with negative or positive cofactors [2,4,5,7]. A complete understanding of the function of transcription factors and of their possible deregulation in disease requires a careful analysis of the functional role of their different domains.

The Myb family of transcription factors includes the product of the v-*myb* oncogene, which transforms cells of haemopoietic origin, that of its normal cellular equivalent c-*myb* and those of the two structurally related genes *A-myb* and *B-myb* [5,8,9]. All members of the family are transcription factors that recognize the same DNA sequence (YAACG/TG) [10–14]. Furthermore all three members are involved in the regulation of proliferation and differentiation of different cells [5,15]. In particular, c-Myb is known to be an important regulator of both the proliferation and the differentiation of haemopoietic cells [16,17]. B-Myb is required for proliferation of many cell types [18,19]. A-Myb expression, in contrast, is restricted to a narrow window of differentiation in spermatocytes, some neural cells and B lymphocytes [20,21]. Of particular interest is the finding that A-Myb expression is particularly high in a small subset of human neoplastic B-cells, and in particular in Burkitt's lymphoma cells [22]. A-Myb is thus likely to play a role in the biology or transformation of these cells.

Although it is generally accepted that the oncogenic activation of c-Myb involves a deregulation of its transcriptional activity [5], the role of the different domains of c-Myb in such deregulation and in oncogenesis has not yet been completely unravelled. Different domains of c-Myb and v-Myb have been characterized: the N-terminal domains consist of three imperfect repeats of DNA, two of which (R2 and R3) form a helix–turn–helix structure and are sufficient for specific binding to the DNA [23–25]. The trans-activation domain of c-Myb and v-Myb is centred on a highly charged stretch of amino acids near the centre of the molecule [26–29]. The C-terminal region of c-Myb, including a potential leucine zipper, seems to regulate negatively both trans-activation and perhaps DNA binding, functions that are lost by the oncogenic form of the protein (v-Myb) [5,27,30–32].

The A-Myb transcription factor shares four regions of sequence homology with c-Myb [9,14]. The most highly conserved includes the three N-terminal repeats required for DNA binding (region I). The second and third regions are localized in the C-terminal half of the molecule (labelled II and III respectively in Figure 1) [5,9]. Finally a 23-residue charged sequence located within the c-Myb trans-activation domain is also conserved in A-Myb but not in B-Myb, whereas the potential leucine zipper is not conserved in either protein [14].

CBP (CREB-binding protein) was first identified as a specific interaction partner of CREB, the cAMP response element-binding protein [33]. More recently, CBP and the related p300 molecules have been shown to interact with a variety of different transcription factors, including c-Myb, c-Fos and nuclear hormone receptors, as well as with components of the basal transcription machinery [34–36]. Thus CBP has been proposed as a

Abbreviations used: AMBS, A-Myb-binding site; CAT, chloramphenicol acetyltransferase; CBP, CREB-binding protein; CREB, cAMP response element-binding protein; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; GST, glutathione S-transferase; MBS, Myb-binding site.

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bridging protein, bringing together proteins of the Pol II-dependent basal transcription complex with specific transcription factors [36]. The interaction of CBP or p300 with the histone acetylase enzyme P/CAF might further promote transcription through nucleosome destabilization [37]. Finally the interaction of CBP and p300 with the viral E1A oncoprotein seems to be crucial for the oncogenicity of E1A [37–39].

Thus CBP is a central regulator of gene transcription. Recent data indicate that mutations of CBP might cause Rubinstein–Taybi syndrome [40], indicating that CBP is a limiting component in cells, perhaps responsible for cross-talk between different signalling pathways through competition for CBP [34,36]. Furthermore alterations in the CBP locus might occur in some myeloid leukaemias, suggesting an important role for this factor in haemopoietic differentiation [41].

Previously we characterized the DNA-binding and transcriptional activity of the human A-Myb protein [14,42]. In view of the potential role of A-Myb in Burkitt's lymphoma [22], we have set out to define the regulatory domains of the A-Myb protein. In addition we have investigated whether the co-activator CBP and its homologue p300 could interact functionally and physically with A-Myb.

MATERIALS AND METHODS

Cell culture

NIH-3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 4.5 g/l glucose, 2 mM glutamine (Gibco), 10 % (v/v) fetal calf serum (Hyclone) and 50 μg/ml gentamicin.

Plasmid construction

The reporter plasmid used was the KHK–chloramphenicol acetyltransferase (CAT) construct [43], a gift from Professor J. Lipsick (Department of Microbiology, SUNY, Stony Brook, NY). The complete A-Myb cDNA cloned in the pSG5 and pECE vectors has been described previously [14]; the clones were called pSA and pLL1 respectively.

Mutant 1 was generated by deletion of the *Spe*I–*Spe*I DNA fragment (nt 1056–1982) [9] from pSA. This created a frame shift, resulting in the addition of Leu-Arg at residue position 319 [9] followed by a stop codon. To generate mutant 2, the *Spe*I–*Pst*I fragment of pSA (nt 1056–2693) was replaced by the *Spe*I–*Pst*I fragment from the A-Myb in pGem3 plasmid [44]. This introduced a His residue at residue position 482 followed by a stop codon. Mutant 3 was constructed by exchanging the *Nde*I–*Eco*RV (nt 754–2310) DNA fragment of pLL1 with the *Nde*I–*Hin*cII (nt 754–2029) DNA fragment from pSA plasmid. This caused a frame shift at residue position 642, adding eight residues (SEYLHSYQ) followed by a stop codon. Mutant 4 was made by deletion of the *Eco*RV–*Pst*I (nt 2320–2693) fragment of pSA. This resulted in the replacement of the last 17 residues of A-Myb with six irrelevant residues (LLFCYR) followed by a stop codon. To construct mutant 8 a *Bam*HI site was introduced at nt 884 by PCR amplification. The resulting plasmid was then cut with *Bam*HI and *Pvu*II, blunted with Klenow enzyme and religated. Mutant 8 thus lacks residues 263 to 278 (DKEKKIKELEMLLMSA) at the centre of the highly conserved stretch of charged amino acids [14]. Mutant 6 was constructed by introducing the *Bam*HI–*Pvu*II (nt -85 to $+936$) fragment from the complete A-Myb cDNA into the pSG5 vector cut with *Bam*HI–*Bgl*II and blunted. This introduced three residues (GSY) at position 277 followed by a stop codon. Mutant 7 has the *Nde*I–*Pvu*II (nt 757–937, 60 amino acid residues) deleted.

All mutants were verified by DNA sequencing.

The pCMV-CBP expression plasmid has been described previously [34]. The preparation and partial purification of the glutathione S-transferase (GST)–CBP fragments were performed as described previously [34]. The expression vector for p300 was obtained from R. Eckner and D.M Livingston [45] and that for E1A and NTdl814, an N-terminal E1A deletion mutant, from P. Whyte [34].

DNA transfection and CAT assays

NIH-3T3 fibroblasts were transfected by the calcium phosphate precipitation method [46]. Effector plasmid (0.1–4 μg) was co-transfected with 5 μg of KHK–CAT reporter. The empty pECE or pSG5 vector was used to bring the total amount of DNA to 20 μg for each plate. Cells were left in contact with DNA for 16 h and lysed after 48 h. Transfections were done in duplicate or triplicate plates. The transfection efficiencies were measured for each lysate by a dot–blot technique and densitometric scanning as described previously [14]. CAT activity was measured as described [14].

Nuclear extracts

Transfected cells were left to grow for 48 h. To prepare nuclear extracts the cells were washed in Tris-buffered saline and pelleted by centrifugation. The cell pellets were resuspended in cold buffer A [10 mM Hepes (pH 7.9)/10 mM KCl/0.1 mM EDTA/0.1 mM EGTA/1 mM dithiothreitol (DTT)/0.5 mM PMSF/0.5 mM benzamidine/5 μg/ml leupeptin/5 μg/ml pepstatin] by gentle pipetting. The cells were left on ice for 15 min, after which 25 μl of 10 % (v/v) NP40 was added and the solution was vortex-mixed for 10 s and centrifuged at 11000 ***g*** for 30 s. The nuclear pellets were resuspended in 50 μl of cold buffer B (consisting of 20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and inhibitors of proteases as in buffer A) and frozen in aliquots at -70 °C. The amount of total protein in the extract was determined by the Bio-Rad assay. In some experiments COS7 cells were transfected and total cell extracts were prepared by lysing cells in 400 μl of buffer F [10 mM Tris/HCl (pH 7.0)/50 mM NaCl/30 mM sodium pyrophosphate/50 mM NaF/5 μM $ZnCl_2$/100 μM Na_3VO_4/1 % (v/v) Triton X-100/1 mM PMSF/5 i.u./ml α-macroglobulin/2.5 units/ml pepstatin/2.5 units/ml leupeptin/0.15 mM benzamidine/2.8 μg/ml aprotinin), as described [47].

Western blot

For Western blots, 30 μg of nuclear extract from each transfected sample was subjected to SDS/PAGE [10 % (w/v) gel], blotted on nitrocellulose for 5 h at 60 V and assayed for A-Myb with a pre-adsorbed rabbit antiserum raised against a bacterial N-terminal fragment of human A-Myb protein, spanning residues 1–1546 (p1500) [48] and the ECL detection system (Amersham). The levels of A-Myb were quantified by densitometric scanning (GS300 densitometric Scanner; Hoefer Scientific Instrumeents, San Francisco, CA, U.S.A.) and a Macintosh Plus computer.

Electrophoretic mobility-shift assay (EMSA)

The MRE-A (equivalent to *mim-A* [14] and derived from the *mim*-1 *A* site [11]) and mutated MRE-Am oligonucleotides were as detailed previously [47]. The annealed oligonucleotides were labelled by filling with [α-^{32}P]CTP and Klenow enzyme and purified on a non-denaturing 5 % (w/v) polyacrylamide gel.

Binding reactions (10–20 μl) were done by incubating for 40 min at 4 °C 0.2 ng of labelled oligonucleotide with 3–10 μg of nuclear extract in a buffer containing 10 mM Tris/HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 4 % (w/v) Ficoll, 5 mM DTT, 1 mM PMSF and 1–3 μg of poly(dI-dC). Complexes were separated from free oligonucleotides in a non-denaturating 5 % (w/v) polyacrylamide gel, dried and autoradiographed. Competition experiments were done by mixing the appropriate competitor DNA (in a 500–1000-fold molar excess over the labelled probe) to the binding reaction before adding the nuclear extract. Lysates were normalized for total protein and A-Myb protein contents by adding, when necessary, an aliquot of an identical lysate prepared from mock-transfected cells. For supershift assays, fusion proteins with GST (1 mg/ml) were purified on glutathione–agarose. Total cell lysates from COS7 transfected cells were used and EMSAs were performed as described [47].

Computer-based sequence analysis

The computer-aided calculations and visualizations were done with an Indigo XS Silicon Graphics workstation. The amino acid sequences of the different Myb proteins were obtained from the SwissProt DataBank (version 31.0) and from the GenBank Database (release 89.0). These sequences were aligned with the Match-box interface [49] as implemented in the computer program Proexplore (Oxford Molecular Limited, Oxford, U.K.). The genetic mutation probability matrix [50] was used as score matrix. A window size of 7 was used in the alignment.

RESULTS

Analysis of conserved regions and construction of A-Myb deletion mutants

To identify regions of potential functional importance, we have aligned all four A-Myb protein sequences cloned since 1988 (from *Xenopus*, chicken, mouse and man) [9,20,42,51]. The results show that the A-Myb sequence can be divided into six regions of higher conservation, with percentage amino acid identity ranging from 58 to 81 % within these regions (bold numbers in Figure 1, upper panel), interspersed with regions of lower conservation (from 19 to 42 % identity) (Figure 1, upper panel). The three regions homologous with c-Myb and B-Myb (marked I, II and III in Figure 1, upper panel) [9] are also highly conserved within the different A-Myb proteins, suggesting that they play an important functional role. In addition the stretch of 23 charged amino acid resdues (previously identified as the acidic region [14]) also present in c-Myb is conserved within A-Myb proteins. Finally we have identified two other small regions, of 15 and 48 residues, located on either side of the charged sequence and showing 80 % and 58 % amino acid identity within A-Myb respectively (Figure 1, upper panel).

To characterize the function of the different regions of A-Myb, we set out to construct a series of deletion mutants of A-Myb. The most conserved region I, the DNA-binding domain, has been characterized previously (Figure 1) [14]. We therefore concentrated our study on the rest of the protein. As shown in Figure 1, the C-terminus of A-Myb was progressively deleted, removing part or all of homology region III (mutants 4 and 3 respectively), homology region II (mutant 2) and the region further upstream including the 58 % conserved sequence (mutant 1). In addition, to localize more precisely the trans-activation domain and determine whether the highly conserved stretch of 23 mostly charged amino acid residues is indeed required for trans-activation [26–29], we constructed deletion mutants around this region (mutants 6 and 7) as well as one that removes only 16 of the 23 conserved residues (mutant 8).

We then verified that all constructs were functional and could direct the synthesis of the protein of the expected length and with a nuclear localization like the wild-type A-Myb protein. As shown in Figure 1 (lower panel), Western blot analysis of nuclear extracts of NIH-3T3 cells transiently transfected with the constructs showed that all proteins were efficiently synthesized and localized in the nucleus.

Trans-activation is inhibited by the A-Myb C-terminus but requires the region centred on the charged stretch of amino acids

We next assayed the transcriptional activity of the different mutants in standard co-transfection assays in NIH-3T3 cells using the KHK–CAT reporter construct that carries eight Myb-binding sites (MBSs) upstream of the CAT gene [43]. Equimolar concentrations of the different constructs were used in parallel with wild-type A-Myb. The amounts of effector plasmid used were those that give maximal activity of A-Myb yet do not lead to significant squelching effects ([14], and results not shown). The results are presented as the percentage activity relative to the wild type (Figure 2). We found that removal of the last 110 residues of A-Myb (mutant 3) led to a strong (5–6-fold) increase in trancriptional activity of A-Myb, whereas removal of only the last 16 residues (which cover half of homology region III) (mutant 4) had no significant effect on trans-activation potential (Figure 2). Further deletion of homology region II (mutant 2) or the region upstream from it (mutant 1) did not lead to a significantly higher increase in trans-activation potential relative to mutant 3 (Figure 2). Dose–response experiments confirmed that the maximal activities of mutants 1, 2 and 3 were comparable (results not shown). Furthermore Western blot analysis of the same samples showed that the levels of expression of the different constructs were similar to each other (results not shown). C-terminal deletion further upstream from residue 319 (mutant 6) led, in contrast, to a 67 % loss of activity compared with wild-type protein and of more than 90 % relative to mutant 1. In addition mutants intact at the C-terminus but lacking residues 218–278 (mutant 7) or only residues 262–278 (mutant 8) showed 94 % and 85 % decreases in activity relative to wild type respectively (Figure 2). These results demonstrate that the C-terminal half of the molecule, up to residue 319, is dispensable for trans-activation, that in contrast the last 110 residues strongly inhibit trans-activation, whereas the 100-residue region centred on the charged stretch of residues is necessary for the full transcriptional activity of A-Myb. We hypothesize that this charged region is part of the trans-activation domain of A-Myb.

Functional regulation of the DNA-binding activity

To investigate whether differences in transcriptional activity of the mutants reflected differences in DNA binding, we performed electrophoretic mobility shift assays with nuclear extracts from transfected NIH-3T3 cells. Representative results are shown in Figures 3(A) and 3(B). Strong DNA binding was reproducibly detected with the shorter mutants 1 and 6 but not with the longer mutants or wild-type A-Myb. That the same extracts expressed equivalent amounts of A-Myb wild-type or mutant proteins was verified in Western blots (results not shown). Furthermore because a decrease in transcriptional activity is observed when transfecting over 10 μg of wild-type-expressing plasmid [14], we have also tried to detect the binding of wild-type protein by using relatively small amounts of plasmid. As expected, an increased binding of mutant 1 was detected with 15 μg rather than 2 μg of plasmid, whereas no binding of wild-type A-Myb could be

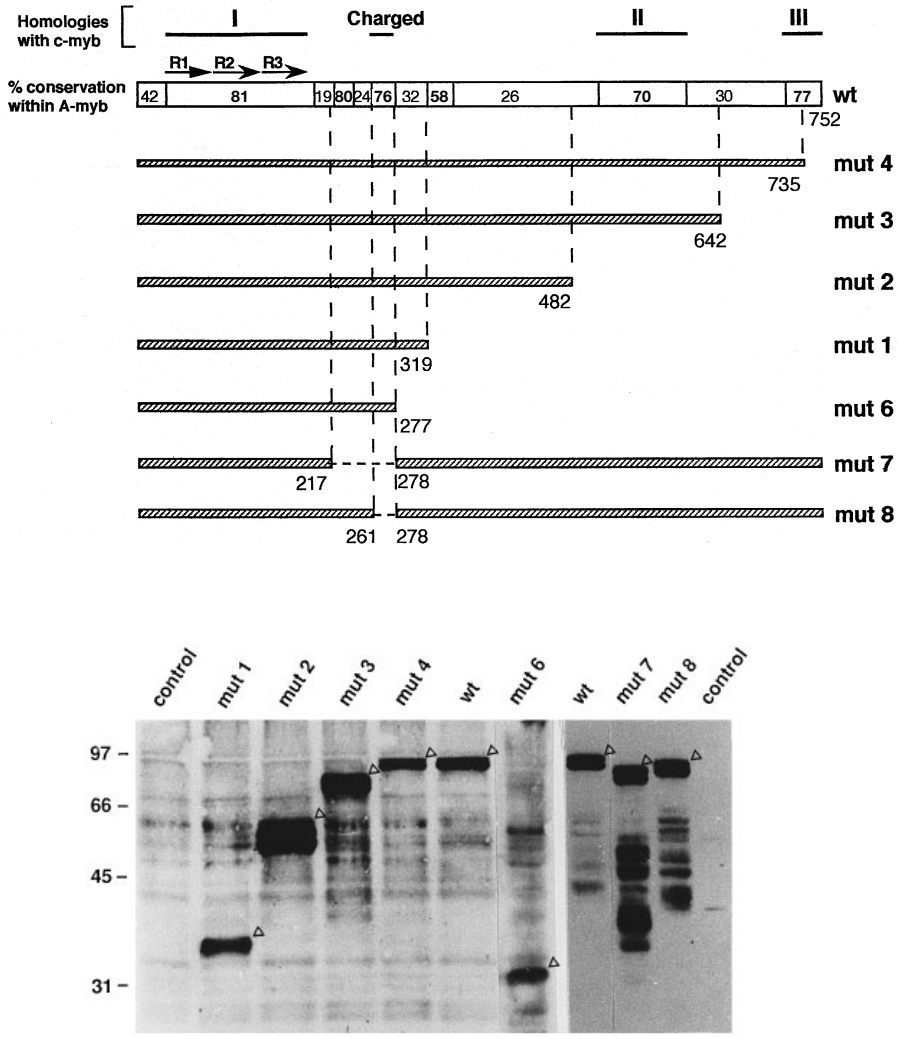

Figure 1 Structure and expression of A-Myb wild-type and deletion mutants

Upper panel: scheme of A-Myb and mutants. The 752-residue A-Myb wild-type (wt) protein is shown schematically in the upper part. The percentage residues identity between A-Myb proteins from *Xenopus*, chicken, mouse and human A-Myb proteins for the different A-Myb domains is shown in the boxes, with highly conserved (more than 50% identity) in bold numbers (81, 80, 76, 58, 70, 77). The localization of the regions conserved with c-Myb is shown above the diagram (regions I, II, III and charged). The predicted proteins encoded by the deletion constructs are drawn schematically below the scheme of the wild-type A-Myb protein. The mutant numbers (mut *n*) are indicated at the right. The numbers below each protein indicate the exact amino acid positions of the deletions. Lower panel: expression of wild-type (wt) and mutant (mut *n*) constructs. NIH-3T3 cells were transfected with 20 μg of each construct and lysed after 48 h. Nuclear extracts were prepared and analysed in Western blots. The positions of molecular mass markers (in kDa) are indicated at the left. The A-Myb proteins are indicated by arrows. The control lanes contained extracts from empty vector-transfected cells.

detected in either condition (Figure 3A). Finally, binding was shown to be specific because it was competed by unlabelled specific oligonucleotide and was not observed with a labelled oligonucleotide carrying a mutation within the MBS (Figure 3B). Binding of mutants longer than mutant 1 was never observed with standard concentrations of MRE-A oligonucleotide (the *mim-1 A* site [11]), nor with an oligonucleotide containing a double A-Myb-binding site (2 × AMBS, derived from the KHK sequence [14]), nor with the whole labelled KHK fragment containing eight MBSs, under a variety of binding conditions, with high and low salt concentrations (results not shown). We also tested the binding of wild-type A-Myb in total cellular extracts from COS7-transfected cells, in conditions where binding of wild-type c-Myb protein has been reported [52]. We did indeed observe binding of c-Myb, whereas wild-type A-Myb showed only a very faint band detectable on long exposure of the gel (results not shown). Thus the binding of mutant 1 was at least 100-fold more efficient than the binding of wild-type A-Myb.

Interestingly, mutant 6 bound DNA to a similar extent to mutant 1 (Figure 3A), confirming that the deletion of residues 277–319 removes part of the trans-activation domain without altering DNA binding.

We conclude that the C-terminal half of A-Myb strongly inhibits specific binding to DNA. In addition, we suggest that the region spanning residues 320–482 is mainly responsible for this inhibition.

CBP co-operates functionally with A-Myb

CBP has recently been described as a transcriptional co-activator of c-Myb [34,35] and shown to bind directly to the region containing the c-Myb transcriptional activation domain [35]. In

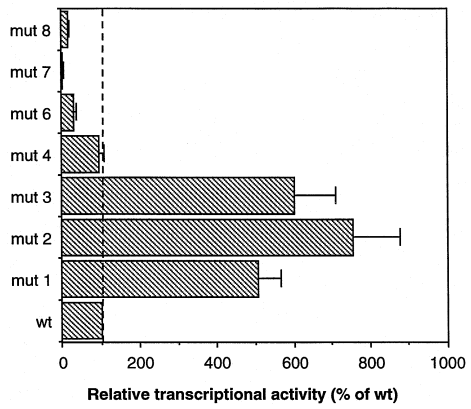

Figure 2 Trans-activation activity by the wild-type and mutant proteins

NIH-3T3 cells were transfected with 5 μg of KHK–CAT reporter construct and equimolar amounts of the wild-type or mutant plasmids (1.0–1.5 pmol). The efficiency of transfection in the pooled extracts was measured and the CAT assays were performed after normalization for transfection efficiencies. The results are shown as activities of the mutants relative to that of the wild-type, the latter being set arbitrarily at 100% (indicated by the broken line) and are the means $\pm$ S.D. for three separate evaluations. Several dilutions of the samples were assayed for CAT activity to obtain less than 90% acetylation for all samples.

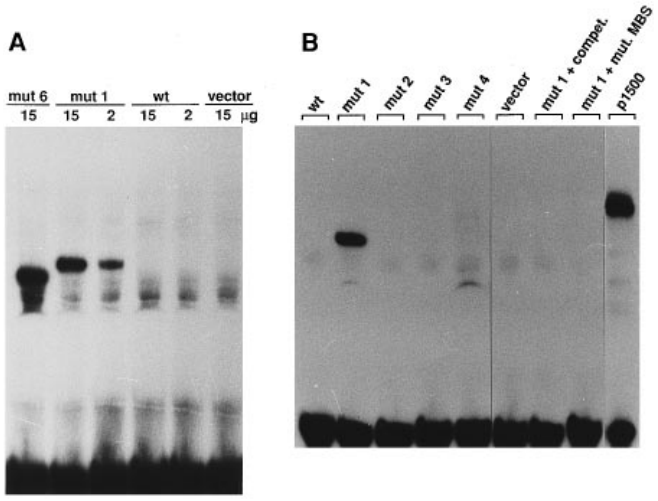

Figure 3 EMSA of wild-type (wt) and mutant (mut *n*) A-Myb proteins from nuclear extracts

NIH-3T3 cells were transfected with 2 or 15 μg (**A**) or with 20 μg (**B**) of effector plasmid and lysed after 48 h. Nuclear extracts (8 μg) and 0.2 ng of labelled MRE-A oligonucleotide were used in the EMSA. Cells transfected with the empty vector were used as negative controls to check for non-specific binding (vector). The positive controls were done with the bacterially expressed p1500 protein (**B**) [14]. In some cases a 1000-fold excess of unlabelled MRE-A was added as a competitor for the binding reaction (**B**, +compet.) or ^{32}P-labelled mutated MRE-A oligonucleotide was used as probe (**B**, +mut. MBS).

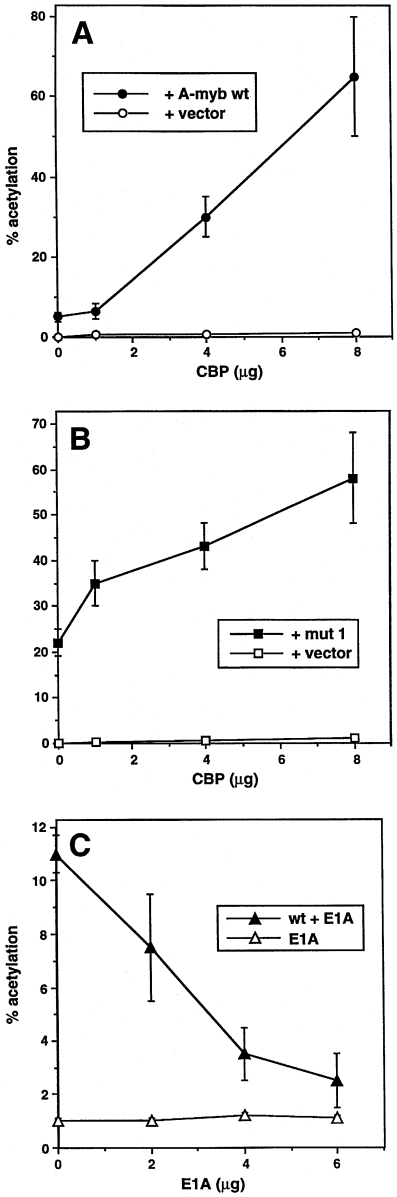

Figure 4 Co-operation of A-Myb with CBP and inhibition by E1A

NIH-3T3 cells were transfected with increasing concentrations of CBP (**A**, **B**) or E1A plasmids (**C**) together with 50 ng of A-Myb (**A**, **C**) or 40 ng of mutant 1 (mut 1) plasmids (approx. 0.01 pmol) or with empty vector plasmid (pSG5). Transfection efficiency and CAT activities were measured 48 h after transfection. The results are shown as the mean percentages of acetylation and ranges for duplicate samples. Abbreviation: wt, wild-type.

view of the structural and functional conservation between A-Myb and c-Myb, we investigated whether CBP could also be a functional partner of A-Myb. Wild-type or mutant 1 A-Myb, that is the full-length and the minimal active A-Myb proteins, were co-transfected at suboptimal concentrations with increasing doses of CBP. As shown in Figures 4(A) and 4(B), CBP synergized with both wild-type and mutant 1 A-Myb in a dose-dependent manner. Usually, a 2–4-fold increase in transcriptional activity was observed with 8 μg of CBP, in agreement with previous results obtained with c-Myb, but occasionally even stronger effects could be seen (Figure 4A).

E1A is known to interact with CBP and inhibit its activity [37,38,53,54]. We therefore investigated whether E1A can diminish A-Myb transcriptional activity, through interference with CBP. An E1A expression plasmid was co-transfected at different doses with a suboptimal dose of wild-type A-Myb. CBP is expressed constitutively in all cells, including fibroblasts [33,36]. Therefore the activity of A-Myb alone is expected to be due to synergism between transfected A-Myb and the endogenous CBP. As expected, adding increasing concentrations of E1A to the cells

Table 1 Synergism of CBP/p300 with wild-type A-myb and mutants

Effector	Co-activator	Relative increase (±S.D.) in transactivation (% of control)
Wild type	CBP	280±10
Mutant 1	CBP	264±31
Mutant 6	CBP	323±32
Mutant 8	CBP	103±18
Wild type	p300	440±30

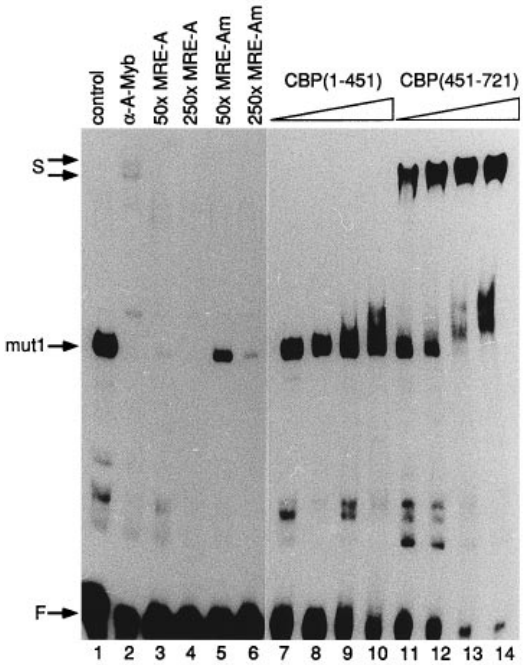

Figure 5 Direct interaction of CBP with A-Myb mutant 1

Whole-cell lysates were prepared from mutant 1-transfected COS7 cells. Lysate (0.1 μl) was incubated with 0.1 ng of ^{32}P-labelled MRE-A oligonucleotide; the protein–DNA complexes were analysed on a non-denaturing gel. Lane 1, no further addition; lane 2, 1 μl of anti-A-Myb (α-A-Myb) serum; lanes 3–6, unlabelled MRE-A or mutated MRE-Am [47] as indicated; lanes 7–10 and 11–14, increasing amounts (50, 100, 200 or 400 ng) of partly purified GST–CBP(1–451) and GST–CBP(451–721) respectively. Abbreviations: mut1, specific protein–DNA complex; S, supershifted complexes; F, free probe.

did lead to a dose-dependent decrease in transcription (Figure 4C). A similar dose-dependent inhibition of A-Myb transcriptional activity was also observed when co-transfecting E1A with A-Myb and CBP (results not shown). In contrast, a deletion mutant of E1A that does not bind to CBP/p300 did not inhibit A-Myb activity in the presence or absence of CBP (results not shown).

The co-operation between CBP and mutant 1 (Figure 4B) had already demonstrated that the A-Myb domain responsible for co-operation with CBP was localized within the first half of the molecule. To define this domain more closely we tested the A-Myb trans-activation mutants that, although weak trans-activators, still showed measurable residual activity. All constructs were used at suboptimal concentrations and tested in the absence or presence of 8 μg of CBP. As shown in Table 1, whereas CBP synergized with mutants 1 or 6 to the same degree as with wild-type protein, it did not synergize with mutant 8. Similar results were obtained in at least three separate experiments. Thus our results show that whereas the whole C-terminal half of A-Myb, downstream from residue 277, is dispensable for co-operation with CBP, the stretch of 16 charged residues missing from mutant 8 is absolutely required for this function.

A close relative of CBP is the nuclear factor p300, originally identified as an E1A-associated protein [45,53,54]. Because both CBP and p300 have been shown to mediate similar co-activator functions, we also tested whether p300 could affect A-Myb activity. Indeed p300 was found to be at least as active as CBP in co-operating with A-Myb (Table 1).

CBP interacts directly with A-Myb

c-Myb is known to interact physically with CBP region 451–721 [34,35]. To demonstrate that A-Myb can bind to the same region of CBP, we performed supershift assays with extracts from COS7 cells transfected with mutant 1. We observed a strong supershift of the mutant 1 specific band with the GST–CBP(451–721) fragment but not with GST–CBP(1–451) or with all the other CBP fragments covering the entire CBP molecule (Figure 5, and results not shown). Increasing doses of CBP region 451–721 led to an increase in the higher supershifted complex and a parallel decrease in the lower mutant 1 complex (Figure 5, lanes 11–14), indicating that the supershift was dose-dependent. No CBP supershift was observed in vector-transfected cells (results not shown). These results strongly suggest that mutant 1 can interact physically and efficiently with CBP region 451–721.

DISCUSSION

In this study we have characterized in detail the role of different regions of the A-Myb transcription factor in the regulation of the DNA-binding and transcriptional activities and have demonstrated for the first time the functional and physical interaction between A-Myb and the co-activators CBP and p300. The results of the analysis of a series of deletion mutants of A-Myb presented here show that, whereas the DNA-binding domain together with a region of approx. 100 residues centred on the 23-residue charged sequence are sufficient for maximal trans-activation, the C-terminal half of the molecule plays a negative regulatory role, which involves two separate regions. A more central portion of the protein (residues 320–482) can strongly inhibit DNA binding. In contrast, the removal of only the last 110 residues of A-Myb, including the conserved homology region III, increases the maximal trans-activation potential of A-Myb by 5–6-fold. Finally, CBP was shown to synergize with A-Myb in a dose-dependent fashion; this co-operative effect could be inhibited by E1A but not by an inactive E1A mutant, and could also be observed with the CBP homologue p300. This functional synergism requires the presence of the A-Myb charged sequence. We further showed that, like c-Myb, A-Myb can physically interact with CBP region 451–721. The deduced functional domains of A-Myb are shown schematically in Figure 6.

The results presented here demonstrate that the C-terminal half of A-Myb, spanning 433 residues, markedly regulates the activity of A-Myb in a negative manner. In particular the removal of the last 110 residues was sufficient for a large increase in activity, by 5–6-fold relative to the wild-type protein. This region contains the homology region III also present in c-Myb [9,14]. c-Myb has also been shown to contain a negative regulatory domain localized within the C-terminal half of the protein [12,27,55]. The precise location of the inhibitory domain seems, however, to vary with the experimental system and cell

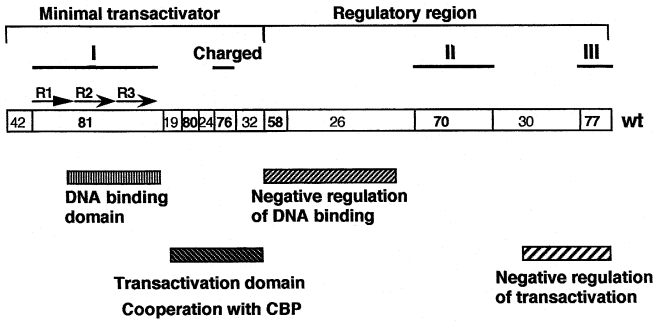

Figure 6 Scheme of the functional domains of A-Myb

The localization of the different functional domains of A-Myb, deduced from the trans-activation and DNA binding assays with wild-type and mutant A-Myb proteins, is shown below the scheme of the structural homology regions of the protein.

types used, and some authors found a positive rather than a negative effect of the same c-Myb region [5]. We have shown here that at least as far as A-Myb is concerned, a major inhibitory role on trans-activation is played by the extreme C-terminus of the protein. These conclusions are somewhat different from those of Takahashi et al. [56], who constructed a different set of A-Myb deletion mutants. Those authors do not report an increased activity of the mutants lacking the C-terminus half of the protein. The discrepancy with our results might reflect the different reporter constructs and cell lines used in the two studies.

To determine whether the changes in trans-activation potential reflect a change in DNA-binding ability, as reported by some investigators for c-Myb [31,57], we studied the DNA-binding capacity of all our transcriptionally active mutants, with nuclear extracts from transiently transfected cells, thus using conditions as close as possible as those used to study transcriptional activity. We observed strong DNA binding of the shorter mutants that carried a still-intact DNA-binding domain (mutants 1 and 6), as expected. However, specific DNA binding could not be observed with longer mutants or the wild-type protein. This was true also when different binding sites were used (MRE-A derived from *mim-1* or AMBS derived from the KHK sequence [14]), when a double MBS or a fragment carrying multiple MBSs was tested. Only very weak binding (at most 1/100 of that of mutant 1) was observed in COS7-transfected cells in conditions where binding of c-Myb was clearly detected (V. Facchinetti, unpublished work) [34]. Similarly, Takahashi et al. [56] were unable to detect DNA binding of mutants longer than residue 396. Thus the addition of the region immediately downstream of the trans-activation domain (residues 320–482, present in mutant 2 but not mutant 1) is alone sufficient to cause a marked decrease in specific DNA binding. It is, however, clear that full-length A-Myb does bind to the MBS in the intact cells (because trans-activation has been shown to be dependent on DNA binding [14]). One possible explanation is that A-Myb binding *in vivo* requires an activation step or cofactor: only a portion of A-Myb molecules are in an active form so that their levels are too low to be detected in our EMSA assay. An inhibitory function has also been described for the C-terminus of c-Myb protein expressed *in vitro* or bacterially [31,57], again suggesting an analogy between the behaviour of these two proteins, although similar findings were not observed with overexpressed c-Myb purified from eukaryotic cells [52]. A similar regulation of DNA binding by regulatory domains or by interaction with inhibitory proteins has been described for other transcription factors such as p53 [4,58] and E2A [59].

We therefore suggest that the C-terminal half of A-Myb contains at least two separate regulatory functions, one region affecting maximal transcriptional activity and the other affecting DNA binding. Interestingly, homology region II does not seem to play a major role in either of these functions (Figure 6).

In this study we have also delineated the boundaries of the trans-activation domain of A-Myb and demonstrated the importance of the stretch of charged amino acids also conserved in c-Myb and v-Myb. Although the v-Myb/c-Myb trans-activation domain had been originally localized around the equivalent stretch of charged amino acid residues [26,27], conflicting reports about the functional requirement for this sequence have been published more recently [28,29]. Our results on A-Myb, where deletion of only 16 of these residues was shown to decrease drastically the transcriptional activity, are in accordance with the results suggesting an important functional role for this charged sequence [26,29]. This region, although atypical in structure, might act as the acidic domains present in many other transcription factors [25,60]. More recently we have confirmed that residues 217–319 can function autonomously as a trans-activation domain in conjunction with the GAL4 DNA binding domain (G.-G. Ying and J. Golay, unpublished work).

Recently CBP has been shown by a number of workers to act as an adaptor molecule linking several transcription factors, including CREB, c-Myb, c-Fos, c-Jun, nuclear hormone receptors, to the basal transcription machinery and to a newly identified molecule P/CAF with histone acetylase activity [34–37]. These properties of CBP are probably crucial for the activation of transcription by bringing the RNA polymerase II complex to the site of transcription initiation and by allowing changes in the chromosomal structure [36,37]. Finally, the multiple targets of CBP allow cross-talk between different classes of transcription factors, thus 'integrating' the relative transcriptional responses of specific target genes after the activation of multiple cellular signalling pathways [34,36]. We therefore investigated whether CBP could also synergize with A-Myb. CBP was found to synergize with both A-Myb and with the shortest fully active mutant 1, demonstrating that the sequence responsible for co-operation with A-Myb was present within the N-terminal half of the molecule. Synergism was dose-dependent; the CBP inhibitor E1A, as expected, decreased A-Myb transcriptional activity [37,38,53]. E1A was inhibitory either when co-transfected with A-Myb and CBP together or with A-Myb alone, suggesting that the basal activity of A-Myb is already up-regulated by the endogenous CBP protein present in fibroblasts [33,36]. As expected, an E1A mutant that does not bind to CBP/p300 had no inhibitory effect on A-Myb activity. Further study of the effect of CBP on the activity of trans-activation mutants showed that the charged stretch of residues is required for co-operation with CBP because mutant 8, which lacked only this sequence and maintained a minimal transcriptional activity, did not synergize with CBP. Supershift assays showed that physical interaction between CBP and A-Myb mutant 1 takes place. A-Myb binding was specific for the residues 451–721 of CBP. In addition, wild-type A-Myb specifically bound, albeit weakly, to GST–CBP (451–721) in pull-down assays (results not shown). Thus A-Myb binds to the same region of CBP as c-Myb but does not bind to the CBP sequences involved in interactions with other transcription factors such as c-Fos or the nuclear hormone receptors [34,35]. We suggest that the transcriptional activity of A-Myb is regulated through its binding to the adaptor/integrator molecules CBP and p300.

The functionally important regions defined in this report will

be useful tools for further investigations of the potential modifications of A-Myb, such as phosphorylation and interaction with other proteins such as CBP, which will explain the complex regulation of this transcription factor, particularly in the context of normal B-cells and Burkitt's lymphoma cells.

We thank F. and V. De Ceglie for the photographic work. This work was supported by the Italian Association for Cancer Research (AIRC) and the National Research Council (C.N.R., Rome, project A.C.R.O.), and by the Deutsche Forschungsgemeinshaft to B. L. This work was performed with the help of a collaborative grant from the NATO (ref. CRG.941147). J.G. is supported by the Istituto Superiore di Sanità, Rome.

REFERENCES

1 Kowenz-Leutz, E., Twamley, G., Ansieau, S. and Leutz, A. (1994) Genes Dev. **8**, 2781–2791
2 Dörfler, P. and Busslinger, M. (1996) EMBO J. **15**, 1971–1982
3 Baichwal, V. R., Park, A. and Tjian, R. (1992) Genes Dev. **6**, 1493–1502
4 Hupp, T. R., Meek, D. W., Midgley, C. A. and Lane, D. P. (1992) Cell **71**, 875–886
5 Introna, M., Luchetti, M., Castellano, M., Arsura, M. and Golay, J. (1994) Semin. Cancer Biol. **5**, 113–124
6 Hanna-Rose, W. and Hansen, U. (1996) Trends Genet. **12**, 229–234
7 Meek, D. W. and Street, A. J. (1992) Biochem. J. **287**, 1–15
8 Beug, H., Hayman, M. J. and Graf, T. (1982) EMBO J. **1**(9), 1069–1073
9 Nomura, N., Takahashi, M., Matsui, M., Ishii, S., Date, T., Sasamoto, S. and Ishizaki, R. (1988) Nucleic Acids. Res. **16**, 11075–11089
10 Biedenkapp, H., Borgmeyer, U., Sippel, A. E. and Klempnauer, K. H. (1988) Nature (London) **335**, 835–837
11 Ness, S. A., Marknell, A. and Graf, T. (1989) Cell **59**, 1115–1125
12 Sakura, H., Kanei Ishii, C., Nagase, T., Nakagoshi, H., Gonda, T. J. and Ishii, S. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 5758–5762
13 Mizuguchi, G., Nakagoshi, H., Nagase, T., Nomura, N., Date, T., Ueno, Y. and Ishii, S. (1990) J. Biol. Chem. **265**, 9280–9284
14 Golay, J., Loffarelli, L., Luppi, M., Castellano, M. and Introna, M. (1994) Oncogene **9**, 2469–2479
15 Golay, J., Basilico, L., Loffarelli, L., Songia, S., Broccoli, V. and Introna, M. (1996) Int. J. Clin. Lab. Res. **26**, 24–32
16 Gewirtz, A. M. and Calabretta, B. (1988) Science **242**, 1303–1306
17 Mucenski, M. L., McLain, K., Kier, A. B., Swerdlow, S. H., Schreiner, C. M., Miller, T. A., Pietryga, D. W., Scott, W. J. J. and Potter, S. S. (1991) Cell **65**, 677–689
18 Arsura, M., Introna, M., Passerini, F., Mantovani, A. and Golay, J. (1992) Blood **79**, 2708–2716
19 Reiss, K., Travali, S., Calabretta, B. and Baserga, R. (1991) J. Cell. Physiol. **148**, 338–343
20 Trauth, K., Mutschler, B., Jenkins, N. A., Gilbert, D. J., Copeland, N. G. and Klempnauer, K. H. (1994) EMBO J. **13**, 5994–6005
21 Golay, J., Erba, E., Bernasconi, S., Peri, G. and Introna, M. (1994) J. Immunol. **153**, 543–553
22 Golay, J., Luppi, M., Songia, S., Palvarini, C., Lombardi, L., Aiello, A., Delia, D., Lam, K., Crawford, D. H., Biondi, A. et al. (1996) Blood **87**, 1900–1911
23 Ogata, K., Morikawa, S., Nakamura, H., Sekikawa, A., Inoue, T., Kanai, H., Sarai, A., Ishii, S. and Nishimura, Y. (1994) Cell **79**, 639–648
24 Myrset, A. H., Bostad, A., Jamin, N., Lirsac, P. N., Toma, F. and Gabrielsen, O. S. (1993) EMBO J. **12**, 4625–4633
25 Lipsick, J. S. (1996) Oncogene **13**, 223–235
26 Weston, K. and Bishop, J. M. (1989) Cell **58**, 85–93
27 Kalkbrenner, F., Guehmann, S. and Moelling, K. (1990) Oncogene **5**, 657–661
28 Chen, R. H., Fields, S. and Lipsick, J. S. (1995) Oncogene **11**, 1771–1779
29 Frampton, J., Kouzarides, T., Doederlein, G., Graf, T. and Weston, K. (1993) EMBO J. **12**, 1333–1341
30 Kanei Ishii, C., MacMillan, E. M., Nomura, T., Sarai, A., Ramsay, R. G., Aimoto, S., Ishii, S. and Gonda, T. J. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 3088–3092
31 Ramsay, R. G., Ishii, S. and Gonda, T. J. (1991) Oncogene **6**, 1875–1879
32 Dash, A. B., Orrico, F. C. and Ness, S. A. (1996) Genes Dev. **10**, 1858–1869
33 Chrivia, J. C., Kwok, R. P. S., Lamb, N., Hagiwara, M., Montminy, M. R. and Goodman, R. H. (1993) Nature (London) **365**, 855–859
34 Oelgeschlaeger, M., Janknecht, R., Krieg, J., Schreek, S. and Luescher, B. (1996) EMBO J. **15**, 2771–2780
35 Dai, P., Akimaru, H., Tanaka, Y., Hou, D. X., Yasukawa, T., Kanei-Ishii, C., Takahashi, T. and Ishii, S. (1996) Genes Dev. **10**, 528–540
36 Janknecht, R. and Hunter, T. (1996) Nature (London) **383**, 22–23
37 Yang, X. J., Ogryzko, V., Nishikawa, J. I., Howard, B. H. and Nakatani, Y. (1996) Nature (London) **382**, 319–324
38 Arany, Z., Newsome, D., Oldread, E., Livingston, D. M. and Eckner, R. (1995) Nature (London) **374**, 81–84
39 Moran, E. (1993) Curr. Opin. Genet. Dev. **3**, 63–70
40 Petrij, F., Giles, R. H., Dauwerse, H. G., Saris, J. J., Hennekam, R. C. M., Masuno, M., Tommerup, N., van Ommen, G. J. B., Goodman, R. H., Peters, D. J. M. and Breuning, M. H. (1995) Nature (London) **376**, 348–351
41 Wessels, J. W., Mollevanger, P., Dauwerse, J. G., Cluitmans, F. H. M., Breuning, M. H. and Beverstock, G. C. (1991) Blood **77**, 1555–1559
42 Foos, G., Grimm, S. and Klempnauer, K. H. (1994) Oncogene **9**, 2481–2488
43 Grasser, F. A., LaMontagne, K., Whittaker, L., Stohr, S. and Lipsick, J. S. (1992) Oncogene **7**, 1005–1009
44 Golay, J., Capucci, A., Arsura, M., Castellano, M., Rizzo, V. and Introna, M. (1991) Blood **77**, 149–158
45 Eckner, R., Ewen, M. E., Newsome, D., Gerdes, M., DeCaprio, J. A., Bentley-Lawrence, J. and Livingston, D. M. (1994) Genes Dev. **8**, 869–884
46 Graham, F. L. and van der Eb, A. J. (1973) Virology **52**, 456–467
47 Oelgeschläger, M., Krieg, J., Lüscher-Firzlaff, J. M. and Lüscher, B. (1995) Mol. Cell. Biol. **15**, 5966–5974
48 Golay, J., Introna, M. and Graf, T. (1988) Cell **55**, 1147–1158
49 Depiereux, E. and Feytmans, E. (1991) Protein Eng. **4**, 603–613
50 Dayhoff, M. O., Barker, W. C. and Hunt, L. C. (1983) Methods Enzymol. **91**, 524–545
51 Sleeman, J. P. (1993) Oncogene **8**, 1931–1941
52 Krieg, J., Oelgeschläger, M., Janknecht, R. and Lüscher, B. (1994) Oncogene **10**, 2221–2228
53 Lee, J. S., Galvin, K. M., See, R. H., Eckner, R., Livingston, D. M., Moran, E. and Shi, Y. (1995) Genes Dev. **9**, 1188–1198
54 Lundblad, J. R., Kwok, R. P. S., Laurence, M. E., Harter, M. L. and Goodman, R. H. (1995) Nature (London) **374**, 85–88
55 Nakagoshi, H., Nagase, T., Kanei Ishii, C., Ueno, Y. and Ishii, S. (1990) J. Biol. Chem. **265**, 3479–3483
56 Takahashi, T., Nakagoshi, H., Sarai, A., Nomura, N., Yamamoto, T. and Ishii, S. (1995) FEBS Lett. **358**, 89–96
57 Ramsay, R. G., Ishii, S. and Gonda, T. J. (1992) J. Biol. Chem. **267**, 5656–5662
58 Srinivasan, R. and Maxwell, S. A. (1996) Oncogene **12**, 193–200
59 Kadesh, T. (1992) Immunol. Today **13**, 31–35
60 Zawel, L. and Reinberg, D. (1995) Annu. Rev. Biochem. **64**, 533–561

Received 4 December 1996/7 February 1997; accepted 20 February 1997